Age and Sex Related Behavioral Changes in Mice Congenitally Infected with *Toxoplasma gondii*: Role of dopamine and other neurotransmitters in the genesis of behavioral changes due to congenital infection and attempted amelioration with interferon gamma

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ABSTRACT

Evidence suggests that the neurotropic parasite *Toxoplasma gondii* may play a role in the development of cognitive impairments. My hypothesis was that congenital exposure to *T. gondii* would lead to detectable age and sex related differences in behavior and neurotransmitter levels in mice. The neurotransmitter dopamine and commonly used anti-schizophrenic agents were evaluated against *T. gondii* in human fibroblast cells. Dopamine caused a significant increase in tachyzoite numbers at 250 nM but not 100 nM and the drugs valproic acid, fluphenazine, thioridazine and trifluoperazine inhibited *T. gondii* development. The effects *T. gondii* infection had on behavior were examined using a congenital mouse model. Previous work demonstrated maternal immune stimulation (MIS) with interferon gamma (INF-g) resulted in decreased fetal mortality from congenital *T. gondii* infections; therefore I examined the effects of INF- g treatment of mothers to determine if protection from the behavioral effects of *T. gondii* occurred in their offspring. No differences in concentrations of neurotransmitters in the brains of congenitally infected mice were observed. I found that mice infected with *T. gondii* developed adult onset behavior impairments with decreased rate of learning, increased activity and decreased memory, indicating cognitive impairment for male mice and not female mice. My findings support the evidence *T. gondii* is a factor in the development of cognitive impairments. My results for *T. gondii* exposed male mice are consistent with the convention that males have more cognitive impairments in the prodromal stage of schizophrenia. MIS with IFN-g had a minimal effect on behavior post sexual maturity but had a greater effect on pre sexual maturity female mice which exhibited difficulties with spatial memory, coordination and the ability to process stimuli. The results indicate the behavior alterations from IFN- g are transient. When MIS is given prior to congenital infection with *T. gondii*, we detected no behavior deficits in any group of mice, including male mice post sexual maturity. Based on the results of my study, I must reject the hypothesis that neurotransmitter levels are influenced by congenital toxoplasmosis and accept the hypothesis that congenital *T. gondii* infection caused cognitive impairments in male mice post sexual maturity.

Dedication

This dissertation is dedicated to my wife Stephanie K Goodwin for her undying support and my parent Bruce and Jennifer Goodwin for the opportunity of education they bestowed upon me.

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1.1. History of *Toxoplasma gondii*

Toxoplasma gondii is an obligate intracellular organism discovered just over 100 years ago by Manceaux and Nicolle, at the Pasteur institute in Tunisia. *T. gondii* has a complex lifecycle which requires two hosts (definitive and intermediate) for completion of its life cycle. The only definitive hosts for *T. gondii* are felids. Sexual reproduction of the parasite can only occur in the intestine of feline definitive hosts and results in an unsporulated oocysts being shed into the environment. In the environment, it takes approximately 48 hours for the oocyst to mature and sporulate. After sporulation, the oocyst can infect an intermediate host by ingestion of contaminated food or water. In the intermediate host, sporozoites are released from the oocysts and differentiate into tachyzoites that will disseminate the infection thought the body, eventually undergoing stage transformation to become bradyzoites inside tissue cysts. Tissue cysts are most commonly located in neural tissue, skeletal and cardiac muscle. For the life cycle of *T. gondii* to be completed, a felid has to ingest infected tissue containing a tissue cyst. Felids are also intermediate hosts and contain tissue cysts in their extra-intestinal tissues. Transmission of *T. gondii* from mother to fetus can also occur resulting in congenital infections.

T. gondii can also be transmitted horizontally from intermediate host to intermediate host. This can be done by ingestion of the tissue cyst stage containing bradyzoites from raw or undercooked meat of an infected animal. In humans, transmission can occur by less natural means, by blood transfusions and organ transplants, from an infected individual to an uninfected individual. Reports indicate that vegetables that have come in contact with feline feces containing oocysts are also capable of causing infection (Yaneza and Kumari 1994).

Unidentified changes in the functioning of the central nervous system due to the presence of latent tissue cysts are believed to be associated with the development of schizophrenia. The tissue cysts occupy space in the brain and can potentially cause compression of neighboring cells. The presence of tissue cysts in the brain can potentially alter the structural functioning of the brain and lead to changes in neurotransmitters resulting in schizophrenia or other neurological disorders.

1.2. Schizophrenia background

Schizophrenia was first recognized in 1887 as a mental illness. Prior to 1887, schizophrenia was thought to be an early form of dementia/Alzheimer's disease and was classified accordingly. Dr. Emile Kraepelin separated it from dementia, naming it dementia praecox. It was renamed in 1911, because too many people were confusing it with dementia because the names were similar. The new name was schizophrenia, coined by Swiss psychiatrist Dr. Bleuler. The word schizophrenia is derived from Greek; schizo, meaning split and phrene meaning mind, also known as scatted thoughts.

Schizophrenia occurs in about 1% of the world's population and the illness is evenly distributed regardless of race, socioeconomic or geographic location (Torrey and Yolken 2003). The definition of schizophrenia as a mental disorder can vary from country to country. Some countries do not recognize schizophrenia as a mental disorder (Peralta and Cuesta 2000) and the criteria for diagnosis of schizophrenia often varies greatly from country to country. In the past 30 years, the diagnosis of schizophrenia has become more standardized by the use of the Diagnostic and Statistical Manual of Mental Disorders, Fourth Edition (DSM-IV). Today, schizophrenia is considered an aggregate of three major symptoms: positive symptoms, negative symptoms and cognitive impairment (see below for definition of symptoms). The onset of schizophrenia usually begins at late adolescents or early adulthood. Males typically are diagnosed with schizophrenia at a younger age then females and males are usually affected more severally (Nicole et al. 1992). Females normally present with less severe symptoms but develop schizophrenia from early adulthood to their mid-40's where the prevalence of schizophrenia in males and females proportion equal (Sham et al. 1994).

Genetics and environment are the two major factors which place people at risk for developing schizophrenia. Inheritable genes that predispose an individual to developing schizophrenia are one factor. Mutations in the immune system resulting in altered neurotransmitter levels (Muller and Schwarz 2006) are a mechanism proposed for the development of schizophrenia. Environmental factors, including exposure to pathogens, psychical stress, and starvation during *in utero* development, leave offspring at an increased risk of developing schizophrenia (Yolken et al. 2001; Susser and Lin 1992). Experiments examining identical twins, found if one twin has schizophrenia the

other twin has a 50% chance of being diagnosed with schizophrenia. For fraternal twins and other siblings, there is a 10% chance of developing schizophrenia. If a person has an aunt, uncle, cousin or grandparent with schizophrenia the chance of being diagnosed drops to 3%. Exposure to pathogens, influenza, cytomegalovirus, and *T. gondii in utero* have, been linked to an increased prevalence in schizophrenia (Leweke et al. 2004). Schizophrenia can present in 3 major ways, as positive symptoms, negative symptoms, and as cognitive impairment. The positive symptoms (psychosis) of schizophrenia can present in several ways. Auditory hallucinations are common. The paranoia associated with schizophrenia is caused by delusions where one constructs a reality that is not real. A similar but different symptom is illusions, when schizophrenics perceive something different from reality. All three of these positive symptoms are caused by excessive dopamine in the mesolimbic region of the brain (Abi-Dargham and Moore 2003). The result is a loss of cognitive functions that affect the prefrontal cortex that is ultimately responsible for cognitive function. Negative symptoms include social incompetence, when a person becomes reclusive and withdraws from society, and loss of initiative, where a person loses motivation for daily activities. Negative symptoms are linked to a decrease in dopamine function in the mesocortical region of the brain (Abi-Dargham and Moore 2003), resulting in the frontal cortex not working properly. A decrease in volume of the frontal cortex is also observed in some schizophrenics (Molina et al. 2004). Negative symptoms do not respond well to medications (Chertkow et al. 2009). The third major classification of symptoms is cognitive impairments. Cognitive impairments include decreased IQ, verbal communication and problem solving. People living with schizophrenia tend to perform poorly in such learning tests as prepulse

inhibition, Wisconsin card sorting and Stroop test (Goldberg and Weinerberger 1988; Abramczyk et al. 1983). People with cognitive impairments: tend to have a loss in thalamic gray matter (Ananth et al. 2002) and tend to have increased dopamine 1, receptors in the frontal cortex (Abi-Dargham and Moore 2003). Some but not all of the anti-psychotic drugs have a D1 receptor antagonist component to them, indicating that other neurotransmitters are affected.

Diagnosis of schizophrenia is eventually made by a psychiatrist using the DSM-IV criteria, partially developed by Bleuler. The DSM-IV test consists of 6 parts. The first part deals with the characteristic symptoms broken into 5 categories, delusions, hallucinations, disordered speech, grossly disorganized/catatonic behavior, and negative symptoms. The second section of the DSM-IV evaluates social and occupational dysfunction. The third section looks at duration of symptoms lasting longer than 6 months. Sections 4, 5 and 6 are exclusion sections of the DSM-IV. Section 4 deals with schizo-effective and/or mood disorders containing at least 1 of the 5 symptoms from section 1. Presenting clinically with 1 symptom is not enough for a diagnosis of schizophrenia. Section 5, hallucinations and or delusions caused from drug abuse cannot be used towards a diagnosis. Section 6, a pre-existing developmental disorder, autism or other disorder predisposing people for schizophrenia-like symptoms disqualifies them from a diagnosis of schizophrenia.

Friends, co-workers, and family often report the signs and symptoms of many people who are eventually diagnosed with schizophrenia. The people who associate with schizophrenics often recognize there is a psychological problem before the schizophrenic does. The psychotic episodes are often intermittent and the symptoms

have to be observed over an extended period of time, approximately a month for a diagnosis of schizophrenia to be considered. Another factor complicating the diagnosis of schizophrenia is that different people rarely exhibit the same symptoms. People usually are required to present with 2 or more of the 5 characteristic symptoms in section 1 from the DSM-IV test to be diagnosed as having schizophrenia. If delusions and or the hallucinations are considered severe enough then only 1 of them is required for diagnosis.

The spectrum of schizophrenic episodes varies from case to case. Some people experience complete resolution after an initial episode with no residual effects while others have increased psychological impairment over time with no recovery. The sooner the initial psychosis is treated the better the prognosis for a positive outcome (Shoval et al. 2011).

Schizophrenia is among the most devastating psychiatric diseases in America. People diagnosed with schizophrenia not only suffer mental illness, they have a life expectancy that is 20% shorter than the average (Newman and Bland 1991). The suicide rate within the schizophrenic population can exceed 10 times that of the contemporary population (Dutta et al. 2010). Teens living with schizophrenia have a 50% chance of committing or attempting suicide (Shoval et al. 2011). It currently is the tenth most costly disease in the U.S. just ahead of bipolar disorder and behind Alzheimer's disease. It is estimated that $62.7 billion is spent each year on schizophrenia. Of the $62.7 billion, $22.7 billion is spent on direct health care costs, cost of living is $7.6 billion and excess cost, including unemployment (time lost) is $32.4 billion dollars (Wu. et al. 2005).

1.3. Mental health and *Toxoplasma gondii*

Milestones

- 1953 Burkinshaw et al. First to examine association between toxoplasmosis and mental health.
- 1994 Felgr and Hrdy Described subtle personality qualities associated with chronic toxoplasmosis
- 2001 Yolken et al. Demonstrate individuals experiencing their first schizophrenic psychotic episode have increased levels of IgG, IgA and IgM to *Toxoplasma gondii*.
- 2001 Buka et al. Demonstrate link between maternal antibodies to *T. gondii* in infants at birth and development of schizophrenia as adults
- Schwarz and Hunter 2007. Immune response to intracellular pathogens varies, predisposition to improperly handling infection can be a contributing factor to neurotransmitter imbalance

The association between *T*. gondii and mental health disorders was first proposed in 1953 by Burkinshaw et al. in England. Little progress was made in this area for nearly 40 years. Between the mid 50's and the mid 90's several papers were published noting a causal relationship between occurrence of schizophrenia, and presents of antibodies to *T. gondii.* Unfortunately, there was no continuity in the designs or goals of those papers, the techniques used by the research groups or even in the classification of the mental health disorders that would tie together the theory that *T. gondii* was a contributing factor to schizophrenia. Many of the papers reporting a relationship between the occurrence of schizophrenia and exposure to *T. gondii* were case reports or studies with a small number of patients.

During this period, numerous studies linked exposure to *T. gondii* with behavior deficits. In 1985, Stibbs examined the potential effect acute and chronic toxoplasmosis

had on neurotransmitters in mice. The results were that acute toxoplasmosis increased the turnover rate of dopamine by 40%, measured by an increase in homovanillic acid, a dopamine metabolite, and chronic toxoplasmosis increased overall dopamine concentrations by 14%. Serotonin concentrations remained stable for both acute and chronic infections. While these findings were interesting, the utility of the study is diminished because the dose and strain/type of *T. gondii* given to the mice was not specified. For *T. gondii* infections both dose and strain/genotype play a large role in the amount of tissue damage caused during asexual replication. The pathogenicity of *T. gondii* infections is highly dependent on strain and dose. While the Stibbs' study had some faults, it was the first publication that bridged the gap between observational studies correlating behavioral changes to neurotransmitter alterations induced by *T. gondii* infection. Until 1985, the only studies that existed extrapolated observed behavior changes as they were related to neurotransmitter concentrations, i.e. increased dopamine equals decreased exploration, and decreased prepulse inhibition. Stibbs quantitatively measured neurotransmitter concentrations in acutely and chronically infected mice. The finding of altered neurotransmitters in the mouse brain was similar to contemporary findings in patients with schizophrenia. The report provides evidence that there is a link between schizophrenia and chronic toxoplasmosis.

Flegr, working at the University of Prague in Czechoslovakia, has published numerous studies dating back to the mid 90's, linking exposure to *T. gondii* to behavior alterations. Felgr and Hrdy (1994) observed subtle behavior changes correlated with the presents of antibodies to *T. gondii*, in a sample population from Charles University.

When the students were analyzed using Cattell's 16s personality questionnaire (Felgr and Hrdy, 1994) which detects subtle personality alterations, a divergence in 4 personality traits was observed for chronically infected males compared to non-infected males. Males seropositive for *T. gondii* were found to be more reserved ($P<0.05$), followed urges more ($P<0.05$), disregarded rules more ($P<0.01$), and were more ($P<0.01$) jealous than their *T. gondii* antibody negative counter parts. Flegr et al. (1996) published a follow up paper, this time finding statistical differences amongst women. Women seropositive for *T. gondii* were shown to be warm hearted (P<0.01), self-assured, and preferred their own decisions ($P<0.05$) when compared to *T. gondii* negative counter parts. Additional studies done investigating young adult women, demonstrated that seropositivity for *T. gondii* was associated with increased intelligence and decreased guilt proneness compared to *T. gondii* negative women (Flegr and Havlicek 1999). Interestingly, similar behavioral alterations were associated with chronic toxoplasmosis when Cloninger's Temperament and Character Inventory (Cloninger's TCI) was used for personality analysis (Flegr et al., 2003). Both *T. gondii* infected males and females have decreased levels of novelty seeking and tend to be slower and more reserved than individuals who were negative to *T. gondii* antibodies. Males seropositive for *T. gondii* appeared to be more reserved, detached, disregarded rules, and isolate from groups settings when compared to negative controls (Lindova et al., 2006). These personality observations would hypothetically distance *T. gondii* seropositive males from society and away from the gene pool. Females, seropositive for *T. gondii* conversely were more likely to have increased self-control, be more conscientious, tidy and warm hearted than seronegative females (Lindova et al., 2006).

While these behaviors do not increase the transmission rate today in humans, it does suggest infection with *T. gondii* is capable of changing human personality.

Kocazeybek *et al.* (2009) claimed that chronic toxoplasmosis causes increased concentrations of dopamine, which in turn decreased reaction time in both drivers of automobiles and pedestrians, resulting in an increased rate of accidents amongst people with chronic toxoplasmosis. Individuals seropositive for *T. gondii* demonstrated an increased reaction time in a computerized test compared to seronegative individuals (Havlicek et al., 2001). Both reports implicated chronic *T. gondii* infections with a negative impact on reaction time. A mechanism by which infection with chronic *T. gondii* decreases reaction time has not been proposed to date. Chronic infection was implicated in causing decreased memory and rates of learning in some rodent models (Hutchenson et al., 1980, Hay et al., 1984), lending support for infection with *T. gondii* as it causes similar changes in humans.

The proposed link between schizophrenia and chronic *T. gondii* infection is still a relatively new theory. It was first proposed in a 2001 paper by Yolken et al. (2001) with a study correlating schizophrenic's experiencing their first psychotic episode with high *T. gondii* antibody titers. The theory has been expanded to include congenital infections with *T. gondii* as a potential agent of adult onset mental health disorders (Buka et al., 2001). *In utero* exposure to other neurotropic intracellular pathogens was investigated as environmental factors contributing to schizophrenia. Several pathogens appear to be potential factors in schizophrenia but infection with *T. gondii* was considered a major factor because it is preventable (Brown et al., 2005). The theories that *T. gondii* is a

factor in causing schizophrenia was further supported by evidence that chronic toxoplasmosis in mice caused an increase in dopamine levels (Stibbs, 1985).

Several studies linked the onset of an individual's first psychotic episode with titers to *T. gondii* (Yolken et al., 2001; Leweke et al., 2004). While the mechanism causing these psychotic episodes was not known, it is of interest that 42% of those people experiencing psychotic episodes had antibody titers to *T. gondii* (Yolken et al., 2001) compared to 10% of the U.S. population with titers to *T. gondii*. Enzyme linked immunosorbent assay and western blot antibody tests for IgM and IgG antibodies, demonstrated that the infections associated with psychotic episodes were acute infections. The odds ratio linking the two events together ranges from 2.5-5.5 (Wang et al*.,* 2006). Leweke et al*. (*2004) looked at serointensity (increased titer) levels, as an indicator of mental illness instead of exposure. Higher titers were indicative of a recent infection. The result was that persons experiencing psychotic episodes have high titers to *T. gondii* indicating a recent exposure or a recrudescent infection. The pitfalls of some of these experiments were that not all the serological tests were done the same way, inducing variability in titer values. Test procedures included were done with the Sabin-Feldman dye test, the skin test, commercial enzyme linked immunosorbent assay (ELISA/RIA) tests and Immunofluorescent assays (IFA). Because not all the techniques share the same sensitivity, comparing results is difficult. Further confounding these results is schizophrenia is not a well defined disorder. The standard for classifying schizophrenics varies greatly between countries. While an association appears to exist it is difficult to determine the strength of the connection between *T. gondii* exposure and the development of schizophrenia.

Two retrospective studies examined individuals currently experiencing schizophrenia and determining the *T. gondii* status of maternal blood that was archived at the time of their birth (Buka et al*.,* 2001; Brown et al*.,* 2005). Mothers of current day schizophrenics where found to have antibody titer levels higher to *T. gondii* than mothers who were seropositive and had offspring with no mental health problems. These finding indicated an acute infection, not just previous exposure, may play a role in fetal outcome. These long term retrospective studies give us the opportunity to compare naturally occurring infections to real mental health disorders; however there are many unaccountable variables that are not considered.

Toxoplasma gondii is an intracellular organism that causes a chronic for the life of an individual. Similar to *T. gondii,* many viral infections become chronic after acute infections. For this reason researchers have looked at *Cytomegalovirus (CMV)*, Influenza virus, Epstein Barr virus, *Herpes simplex* viruses I, II, 6, and *Varicella zoster* virus to see if exposure was associated with the development of adverse mental health conditions. The findings of these studies show a correlation between *T. gondii, CMV, Herpes simplex* 6 and untreated people experiencing mental health problems (Leweke et al*.,* 2004). The link indicates that infectious agents besides *T. gondii* may be contributing factors to mental illness in individuals that are seropositive for *T. gondii*.

Toxoplasmosis and schizophrenia are associated with increased dopamine levels or dopamine receptor concentrations in the brains of affected individuals. One of the early prevailing theories about schizophrenia is the dopamine theory (Wise and Stein 1973). Dopamine is a potent neurotransmitter that is essential for cognitive function. People living with schizophrenia have altered dopamine levels. On post

mortem examination, dopamine 1 receptor concentrations in the frontal cortex are elevated compared to those of uninfected individuals (Kenable et al., 1996). This finding indicates desensitization of the receptors from prolonged increased dopamine concentrations. Although the frontal cortex is not the region of the brain with the highest dopamine concentrations, it does have concentrations of dopamine that are easily measured by HPLC or visualized by in situ hybridization. The frontal cortex is the region of the brain responsible for cognitive function. Decreased size of the prefrontal cortex has been observed with those people living with schizophrenia experiencing psychotic episodes (Brugger et al., 2010). No reports exist in the literature of altered frontal cortex dopamine concentrations in mice or human with toxoplasmosis, only elevated receptor concentration.

Many factors are believed to play a role in mental health disorders, genetic, environmental, exposure to infectious agents, and chemical imbalances. The mechanism of how chronic toxoplasmosis causes changes in neurotransmitter concentration is not well understood. It is clear that *T. gondii* is not the causative agent of mental health disorders, but *T. gondii* appears to be an environmental factor. It has been proposed that infection with *T. gondii* causes an immune response in a subpopulation of people that is handled by incorrect immune cell types (Schwarcz and Hunter, 2007). If the immune response is handled inappropriately then a cascade of events may follow resulting in neurotransmitter imbalances. Typically, intracellular brain pathogens would attract glial cells to the site of infection. The glial cells will usually bind up all the tryptophan and bring the pathogen's replication to a halt. For some people who have a genetic predisposition, the body mounts an inappropriate immune

response, resulting in astrocytes activating and binding tryptophan instead of the glial cells (Schwacz and Hunter, 2007). Astrocytes cannot metabolize tryptophan as efficiently as glial cells. When tryptophan is metabolized, the by-product is Kynurenic acid (KYNA), which is reduced further by enzymes to Indoleamine 2,3-Dioxyganase (IDO) or Tryptophan 2,3-Dioxygenase (TDO2). The major difference between the two enzymes is IDO directs the immune system to a Th1 response, ideal for eliminating intracellular pathogens, while TDO2 is more of a Th2 response used for eliminating extracellular pathogens. Astrocytes lack the enzyme IDO and are low in concentrations of TDO2 available. The result is a buildup of KYNA in the brain. Elevated KYNA even in low quantities will inhibit NMDA and nicotinic acetylcholine by blocking presynaptic receptors. Both neurotransmitters are responsible for cognitive processes. Deregulation of KYNA levels also can result in an imbalance of catecholamine and indolamines, precursor molecules of dopamine and serotonin, respectively (Schwarz and Hunter 2007). Ultimately altered dopamine concentrations are one of the hallmark characteristics of schizophrenic psychotic episodes. Some people appear to have a genetic predisposition to increased TDO2 activity, and/or astrocyte activation (Schwarz and Hunter, 2007). It is plausible that an increase in TDO2 and/or astrocyte activation brought about by *T. gondii* infections could be a contributing environmental factor to a neurochemical imbalance leading to a psychotic episode.

1.4. Anti-psychotic drugs and *Toxoplasma gondii*

Anti-psychotic and mood stabilizing drugs have been used since the late 1950's. The drugs revolutionized how people suffering from mental illness were treated. Prior to the advent of anti-psychotic drugs, people suffering from mental illness were institutionalized. Typical anti-psychotic drugs allowed people suffering from mental illness to return to society. The first generation of drugs included typical antipsychotics, a class of phenothiazines. Phenothiazines include, fluphenazine, thioridazine, and trifluoroperazine. Haloperidol is a typical antipsychotic drug from the butyrophenone drug class. Atypical anti-psychotic drug first came to market in the early 1970. Atypical drugs were first classified as antipsychotic drugs that did not cause extrapyramidal effects. This drug class includes but is not limited to clozapine, risperidone, and olanzapine. The mood stabilizing drug valproic acid was first used clinically in the early 1960's. It is mostly used for the treatment of epilepsy, bipolar disorder, and schizophrenia, to a lesser extent.

The mechanism for many of the anti-psychotic and mood stabilizing drugs used for treatment is yet to be fully described. Many of the traditional anti-psychotic drugs have more of a duel target mechanism. The target of these drugs is the dopamine system and calmodulin. These drugs work as Dopamine 2 receptor antagonists. The calmodulin mechanism works by inhibiting the release of neurotransmitters into the synaptic cleft. A major side effect of these drugs can be observed as extrapyramidal symptoms; including dry mouth, tremors, muscle stiffness and more severe side effects of tardive dyskinesia. Unfortunately some of these side effects can mimic the negative symptoms of schizophrenia. The traditional anti-psychotic drugs are still used for

treating the more persistent schizophrenia cases that are not responsive to atypical drugs.

Newer, atypical antipsychotic drugs have a multi target mechanism. These drugs have specific dopamine receptor targets but also appear to cross react with other neurotransmitters and receptors, specifically: serotonin, histamine, nicotine and other neurotransmitters to a much lesser extent. The major difference between these newer antipsychotics and traditional drugs is the extrapyramidal effects are not present, unless excessively high does are administered for a long period of time. The lack of these side effects makes them a better choice for the patients.

The mechanism for the mood stabilizing drug, valproic acid, is still unknown, after 50 years of clinical use. Valproic acid, unlike typical and atypical anti-psychotic drugs, is hypothesized to work on the neurotransmitter GABA by increasing its concentrations in the brain. Valproic acid is also shown to be a histone deacetylase inhibitor and is effective against *T. gondii* tachyzoites in vitro *(*Strobl et al., 2007*)*. The mechanism by which valproic acid works on *T. gondii* is currently unknown.

Antipsychotic drug activity against infectious agents has been known for many years (Jones-Brando et al*.,* 2003). The idea that antipsychotic drugs have a static or cidal effect on *T. gondii* was first examined because of the association between schizophrenia and *T. gondii*. It was observed that people experiencing psychotic episodes were more likely to be infected with *T. gondii* (Yolken et al*.*, 2001). People experiencing their first psychotic episode, meaning they were not on anti-psychotic medication prior to their episode, have higher titers to *T. gondii* when compared to patients who were experiencing mental health problems and were already on

antipsychotic medication (Leweke et al., 2004). These findings suggest that antipsychotic medication was suppressing replication of *T. gondii*, evident by lower titers to *T. gondii*. Several research groups have determined that replication of tachyzoites, the fast replicating form of *T. gondii*, can be inhibited by antipsychotic drugs (Jones-Brando et al., 2003 and Goodwin et al., 2011).

In vitro drug screens of several anti-psychotic drugs indicate they have activity against tachyzoites. The assays performed by Jones-Brando et al. (2003) indicate that haloperidol inhibited tachyzoite growth at a concentration of 15uM. A follow up experiment by Goodwin et al. (2011) yielded different findings. Goodwin et al. found that haloperidol had no effect on tachyzoite growth at concentrations up to 10 uM. Some drugs have in vitro activity, but in vivo they fail to produce the desired results. Valproic acid was one of these drugs. While valproic acid caused rodents to regain their fear of cat urine (Webster et al., 2006) it did not decrease the pathogenicity of *T. gondii* in mice (Goodwin et al., 2008).

Vyas et al. (2007) discovered that rodents chronically infected with *T. gondii* lose their innate fear of cat urine, suggesting that *T.gondii* exerts selection pressures on rodents to complete its life cycle. The theory being rodent predation by cats, the definitive host, permits the completion of the life cycle of *T. gondii*. Webster et al. (2006) hypothesized that treating rodents, chronically infected with *T. gondii,* with antipsychotic and/or anti-toxoplasmic drugs would negate the negative effects chronic toxoplasmosis exerts on innate rodent behavior. The drugs used in Webster's et al. (2006) study were haloperidol (anti-psychotic), dapsone/pyrimethamine (anti-toxoplasmic), and valproic acid (mood stabilizing drug). The findings suggest the drugs

increase the rodent's natural fear of feline urine to near normal levels. It remains unclear whether these drugs are working on the tissue cysts or the neurotransmitters.

Goodwin et al. (2008) examined valproic acid extensively for in vivo activity against *T. gondii*. However, in their experiments no behavior tests were performed. The focus of the experiments was to determine if valproic acid decreased the pathogenicity of *T. gondii* infections in vivo. It was concluded by survival experiments that valproic acid did not increase the life expectancy of mice when compared to control mice receiving no treatment. It was determined that valproic acid did not affect *T. gondii* pathogenicity in vivo, examined by histology and gross necropsy.

A drugs activity against *T. gondii* replication in vitro may not equal activity in vivo. For example, valoproic acid inhibited *T. gondii* tachyzoite replication at a dose of 10 mM in vitro, but this did not translate in vivo (Goodwin et al., 2008). At this time, more anti-psychotic drugs have been tested for anti *T. gondii* activity in vitro then drugs tested in vivo. Fluphenazine, thioridazine, trifloroperazine and other drugs that have in vitro activity against *T. gondii* should be examined in vivo.

1.5. Dopamine and *Toxoplasma gondii*

Stibbs (1985) measured the concentrations of dopamine in the brains of mice acutely and chronically infected with *T. gondii.* The concentrations were determined using the whole brain and HPLC to measure dopamine, homovanillic acid, norepinephrine, serotonin and 5-hydroxyindoleacetic acid. Acutely infected mice had a higher turnover of dopamine, more homovanillic acid, a metabolite of dopamine, but no

change in total dopamine compared to controls. Chronically infected mice had elevated dopamine concentrations and no change in homovanillic acid concentrations compared to controls. Serotonin, 5-hydroxyindoleacetic acid and norepinephrine levels remained unchanged for both acute and chronic *T. gondii* infections in these mice.

The effect dopamine had on exploratory behavior in chronically *T*. gondii infected mice was examined by use of a dopamine reuptake inhibitor GBR 12909 (Skallova et al*.,* 2006). Mice infected with *T. gondii* were shown to poses increased hole board activity compared to control mice. When GBR 12909 was administered to the mice it caused decreased hole board activity in infected females and increased hole board activity in controls. The results indicated behavior phenotypes of infected mice could be reversed with GBR 12909. This finding suggested *T. gondii* played a role in behavior via dopamine.

Herriquez et al. (2009) discovered that *T. gondii* contains 2 aromatic amino acid hydroxylase enzymes. These enzymes are essential for the synthesis of dopamine and serotonin precursors in humans. The production of these enzymes potentially permitted the parasite to synthesize neurotransmitter precursors. These precursors in turn could be used by the host to synthesize dopamine and serotonin causing an increase in neurotransmitter concentrations. *T. gondii* has two genes encoding these 2 enzymes (Gaskell et al*.,* 2009). It was determined these genes are turned on when *T. gondii* stage converts from tachyzoite to bradyzoite. Bradyzoites were the only stage to express these enzymes, lending further support that tissue cysts promote the over production of neurotransmitters.

Fleger et al. (2003) reported that subtle behavior changes in humans chronically infected with *T. gondii* could be attributed to elevated dopamine concentrations. Dopamine concentrations were not tested, but were correlated to behavior changes often associated with altered dopamine concentrations. Flegr used Cloninger's Temperament and Character Inventory to determine a correlation between exposure to *T. gondii* and decrease in novelty seeking. Novotna et al. (2005) also looked at novelty seeking in humans as a measure of dopamine concentrations, and considered a decreased novelty seeking as indicative of increased dopamine concentrations. They found that patients positive for the neurotropic chronic infections, cytomegalovirus or *T. gondii*, demonstrated a decrease in novelty seeking and they attributed this to an increase in dopamine concentrations. Skallova et al. (2006) further expanded upon the research of novelty seeking and chronic *T. gondii* infection. They looked at size of residence (domicile) as a possible factor for novelty seeking. When size of residence was factored in and corrected, males infected with *T. gondii* had decreased novelty seeking in a population of male military conscripts. The same results were found in male and female blood donors (Skallova et al., 2005).

Yolken et al. (2009) reviewed the current literature correlating chronic toxoplasmosis and schizophrenia on 6 different levels. The paper looked at prevalence of *T. gondii* antibodies within the schizophrenic population, *T. gondii* induced behavioral changes, existence of epidemiological similarities between *T. gondii* and schizophrenia, antipsychotic drug's ability to inhibit the proliferation of *T. gondii (*Jones-brando et al. 2003*)*, how experimental *T. gondii* infection increases dopamine in rodents, and higher exposure of schizophrenics to felids as children. Yolken et al. (2009) indicated that

there were many other factors not accounted for in many of these studies, which include timing of infection, pathogenicity of *T. gondii* strain, and the patient human genetics.

Most studies did not measure dopamine concentrations in subjects. Most studies simply cite the Stibbs (1985) article demonstrating elevated dopamine concentrations in mice with chronic toxoplasmosis. Many of the authors go a step further and say dopamine concentrations cause the observed behavior changes which were similar to behavior changes associated with chronic toxoplasmosis in mice. The conclusions have been that infection with chronic *T. gondii* changed behavior via alterations in dopamine concentrations. The problem in these studies was most of them cited Stibbs' study that does not specify the strain of *T. gondii* used or the concentration used to infect the mice. Nor did Stibbs perform behavioral experiments on the mice used in his experiment. There appears to be a missing link in the literature that would tie toxoplasmosis infection in mice, elevated dopamine concentrations, and behavior changes together in one experiment.

1.6. Behavior in rodents and *Toxoplasma gondii*

Until recently, chronic infections were thought to be asymptomatic and of little to no clinical importance, except for recrudescent infections in immunocompermised individual (McAllister 2005). Chronic infections resulting in tissue cyst formation in the central nervous system and brain are thought to be responsible for the behavior and personality alterations observed in infected rodents. The mechanism for how tissue cysts cause problems is not known however several hypotheses exist. The first is that

the slow metabolism of the intracellular tissue cyst releases toxic metabolites causing cellular damage (Dubey et al., 1998). The second theory is that the tissue cysts cause a state of chronic inflammation and the resulting damage is more from auto immunity than from the actual tissue cysts (Hermes et al., 2008). The result from either theory is an altered neurological behavior profile in infected rodents.

Mouse behavior

Mice experimentally infected with *T. gondii* have been shown to have altered behavior characteristics when compared to uninfected controls. Uninfected mice are very apprehensive to novel stimuli and new environments. Typically mice seek small dark enclosed areas and are fearful of open bright areas (Hay et al., 1984). Hutchinson et al. (1980) examined the effect of chronic *T. gondii* infection on mouse activity, specifically rearing and digging. Rearing and digging are both natural behaviors for mice in a new area. Rearing is used to look around and smell new surroundings. Rodents are naturally borrowing animals so digging is a natural behavior for them. Rodents found that infected mice were more active than uninfected mice. The activity measured was non-specific movement (i.e. movement that is not rearing, digging or grooming), infected mice also had more frequent and longer times where they were immobile in an open field. Further activity testing carried out by Hay et al. (1984) examined activity over a 24 day time period, longer than any experiment previously done. They found that congenitally infected mice exhibited hyperactivity compared to uninfected mice at the initial observation. The hyperactivity remained at a higher level over the next five observations periods. Previous experiments only examined one or

two time points; however experiment of Hay et al. (1984) reinforced the finding that the activity observed was permanent and not a transient change caused by introduction to a novel environment. Congenitally infected mice also displayed increased activity when a wheel was installed in their cage (Hay et al*.,* 1985) over a period of 24 days. These reports in congenitally infected mice were also previously observed in mice experimentally infected after birth with chronic infection (Hutchinson et al., 1980). Social behavior of congenitally infected male mice has also been examined (Arnott et al*.,* 1990). Arnott et al. (1990) found that the breeding success of congenitally infected mice was not affected. Congenitally infected males were more aggressive over territory and more inquisitive of potential mates (Amott et al., 1990). An increase in non-specific activity has been suggested to make chronically infected mice easier targets for cats (Webster 2007).

Rat Behavior

Webster et al*.* (1994) examined behavior in wild captured and purpose bred lab rats. *T. gondii* is naturally present in rat populations and is typically maintained at a relatively constant rate by horizontal transmission (Webster 2001). In a series of behavior experiments with wild and wild lab crossed hybrid rats, *T. gondii* infected rats demonstrate a decrease in neophobia, fear of a new environment. Naturally *T. gondii* infected rats encountering novel stimuli of food, odor and open areas are shown to have a lower aversion to these stimuli when compared to uninfected rats (Vayas et al*.,* 2007). Webster (1994) examined activity of chronically infected wild and experimentally infected rats. Activity was measured for a period of 6 nights for 10 hr. Webster (1994)

found that chronically *T. gondii* infected rats have increased activity as do chronically infected mice. Similar to mice, the increased activity did not have an effect on breeding success (Webster 1994). Research has demonstrated that rodents with chronic *T. gondii* infection preferentially lose their fear of predators (Berdoy et al*.,* 2000). Vyas et al*.* (2007) demonstrated that chronically infected mice maintain their apprehension towards the urine odor of foreign animals while losing their aversion to the odor of cat urine. Typically non-infected rodents have a strong aversion to cat urine because cats typically possess the largest threat for predation. The authors suggested that the tissue cyst alters the rodent's behavior, aiding in the completion of the parasites lifecycle (Webster 2007). Gonzalez et al. (2007) proposed an alternative theory. He suggested that chronic *T. gondii* infection does not necessarily alter the rat's aversion to cat odor but acts as an anti-anxiety agent. Under this hypothesis, the rats were less anxious therefore they explored foreign odor and new surroundings more often because they were less stressed.

Motor and memory function in mice and rats

Motor and memory function in both mice and rats chronically infected with *T. gondii* was decreased compared to uninfected controls. Hutchinson et al. (1980) reported a decrease in motor function of chronically infected mice. In these experiments Hutchinson et al*.* (1980) looked at the ability of infected and non-infected mice to stay on a wooden rod. They found that infected mice had less coordination and were less capable of staying on a wooden rod compared to uninfected mice. Hay et al*.* (1983) examined whether the route of *T. gondii* infection played a role in loss of

coordination. They demonstrated that both chronically and congenitally infected mice had decreased motor functions compared to uninfected mice. There was no statistical difference between the two *T. gondii* infected groups: however, congenitally infected mice appeared to have more motor involvement when trying to stay on a wooden rod.

Piekarski (1981) reported that mice and rats chronically infected with *T. gondii* performed poorly in memory testing. Using a maze test, he found chronically infected rats and mice were unresponsive to novel stimuli. He hypothesized that the imbalance of chronically infected *T. gondii* mice and rats inability to recognize novel stimuli manifested itself as poor memory. Hodkova et al. (2007) reported that recognition of a geographic location was impaired in chronically *T. gondii* infected mice. It was the rodent's inability to recognize the environment that impaired the mouse's memory. The Hodkova et al. (2007) study used mice that had been infected with *T. gondii* for 10 weeks prior to the behavioral experiments, providing time for the establishment of chronic infection with tissue cysts in the brain. An alternative explanation is presented by Hydr et al. (2000) who suggested pathological changes that occurred in the brain gave rise to behavioral changes.

Critical comments by David G. Goodwin

Many of these early studies use an unrealistically high dose of parasites. With a high infectious dose the animal will display different behavior based on general malaise and not parasitic manipulation. Furthermore, many of the early papers do not give the stain of *T. gondii* being used. *T. gondii* comes in 3 major genotypes that display a wide array of pathogenicity. A low dose of highly pathogenic *T. gondii* genotype is capable of

producing a worse infection than a high dose of non-virulent *T. gondii* genotype. Because of this lack of information on dose or strain type comparison between studies is difficult.

It is suggested these behavioral changes could aid in increased transmission rates from rodents to cats. Rodents who lose their innate fear of novel environments, stimuli and odors are easy targets for any predator. Predation by any animal will not complete the life cycle of *T. gondii*. Ingestion by a felid is the only way for the completion of the life cycle. While some of the evidence points towards rodents preferentially losing their aversion to felids (Webster 2007;Vayas et al., 2007) other researchers conclude that infected mice simply lose their natural apprehension of a novel environment (Hrda et al., 2000). The hypothesis that *T. gondii* changes behavior in rodents to aid in the completion of the life cycle in cats has yet to be fully tested. Until this hypothesis is fully tested these statements regarding increased transmission rate need to be further examined.

1.7. Maternal Immune Simulation

Pregnancy presents a balancing act for the immune system. While components of the immune system need to be depressed to maintain the pregnancy, it needs to be robust enough to ward off infection of the mother. Early in pregnancy the immune system is slightly depressed but as the pregnancy progresses the immune system becomes increasingly more impaired and susceptible to infection (Reinhard et al., 1998). If the immune system does not stay depressed, fetal resorbtion or spontaneous

abortion may occur. Specific chemicals and pathogens have been found to cause birth defects if the fetus is exposed to them during pregnancy. In a rodent model, maternal immune stimulation (MIS) with a specific immune stimulant has been shown to decrease birth defects when given in conjunction or as a prophylactic with a known teratogen. Unfortunately, some of these MIS rodent models used to emulate infections were shown to have behavior deficits in the offspring of treated mothers. The negative behavior effects associated with these MIS disease models do not appear until late adolescence/early adulthood (Shi et al., 2003; Zuckerman and Weiner 2003), indicating that the changes in behavior observed were not from a pathogen but were a product of the maternal immune system (Shi et al., 2003) or a combination of the immune system plus pathogens.

MIS with interferon gamma (IFN-γ) is shown to decrease birth defects associated with administration of a known teratogen (Sharova et al., 2003; Hrubec et al., 2006). Birth defects include craniofacial deformities, such as changes in maxillary and mandibular lengths, neural tube defects, limb defects and cleft palats caused by a chemical teratogen. The effects on behavior of offspring after MIS with IFN-γ are not well defined. What is better understood and more clearly defined in a mouse model is MIS with polyriboinosinic-polyribocytidilic acid (Poly (I:C)) and the subsequent effects on behavior. Poly (I:C) mimics a viral infection, stimulating the Th1 immune response via a Toll like-3 receptor (Alexopoulou et al., 2001). Viral infections during pregnancy have been implicated in schizophrenia (Kendell and Kemp 1989). Schizophrenia is of interest because it is not recognized until late adolescents/early adulthood. Behavior testing of offspring born to dams that were MIS with Poly (I:C) exhibited a delayed behavioral

deficit, similar to what is observed in humans with schizophrenia (Shi et al., 2003). Immune stimulation during pregnancy yields varying behavioral results and is highly dependent on timing and dose of the immune stimulant.

Shi et al. (2003) reported adult onset behavior deficits in mice born to dams that were immune stimulated with either human influenza virus A/NWS/33CHINI or Poly (I:C) on day 9.5 of gestation. Offspring receiving poly (I:C) exhibited decreased prepulse inhibition (PPI). The Poly (I:C) treatment was given in 4 different doses, 2.5, 5, 10 and 20 mg/kg. Only the highest dose of 20 mg/kg had significant differences in PPI. Indicating that at day 9.5 of gestation, a high dose of immune stimulant is required to precipitate a decrease in prepulse inhibition (PPI).

The exact mechanism of how MIS with poly (I:C) causes behavior alterations is unknown. Ozawa et al. (2006) found dopamine hyper function in only adult offspring of dams that had been chronically immune stimulated while pregnant. Poly (I:C) had been administered to the dams at a dosage of 5 mg/kg from day 12 to day 17 of gestation. This stimulation regime with Poly (I:C) also caused increased methamphetamine induced hyperactivity, and a decrease in all of the following: novel object recognition, thigmotaxis, PPI and memory retention in adult offspring. All of these tests were done before the mice reached sexual maturity and again after sexual maturity. Before sexual maturity, the mice displayed no differences between treatment groups. After sexual maturity, only the mice exposed to Poly (I:C*) in utero* had impairments. Dopamine turnover (homovanillic acid) and receptor binding was also examined (Ozawa et al*.,* 2006). Increased dopamine turnover in the striatum, hippocampus, and frontal cortex was determined by HPLC. In the striatum, dopamine's ability to bind D2-like receptors

was shown to decrease while D1-like receptors had no change in binding affinity. This indicates that the maternal immune system plays a role in the development of pathways of adult offspring without the presence of a true infection.

A slightly different result occurs with MIS using a single 5 mg/kg dose of poly (I:C) on day 9 of gestation. The result is a change in the number of dopamine neurotransmitters and D1-like receptors of the offspring (Meyer et al., 2008a). Immune stimulation can also change the fetal transcription rate of multiple dopamine neuron developmental genes, sonic hedgehog, fibroblast growth factor 8, and transcription factors Nurr1 and Pitx3 (Meyer et al., 2008b). Maternal immune stimulation on day 9 of gestation caused an increase in dopamine concentrations in the prefrontal cortex and a decrease of serotonin and serotonin metabolites in the hippocampus (Winter et al., 2009). The delayed onset of these neuro-developmental behavior clusters indicates not only short term but long term changes in gene transcription rates for *in utero* exposure to immune stimulants.

Timing of MIS was evaluated to determine if a particular stage of pregnancy was more sensitive for inducing long term behavior deficits. Immune stimulation with Poly (I:C) during early pregnancy had a different effect than stimulation late in pregnancy Meyers et al. (2006) examined the effect of MIS on days 6, 9, 13 and 17 on neuro-development of adult offspring. Neuro-development was examined using latent inhibition (LI) and unconditioned stimulus pre-exposure effect (USPEE). LI is a measure of conditioned learning. It is the ability of an individual or animal to make associations with stimuli and to filter out background stimuli. USPEE measures rate of learning. Specifically, it measures the time it takes for conditioning, to a stimulus, to

occur. On days 6, 9 and 13 differences between Poly (I:C) treatment and controls were observed for both LI and USPEE. On day 17 of treatment, differences between controls and Poly (I:C) pups of dams treated were observed in the USPEE test only and not for the LI test. The poly (I:C) groups decreased learning according to the USPEE test for all 4 time points compared to controls, including day 17. These results indicate MIS decreases the offspring's ability to process stimuli properly if administered between days 6-13 during pregnancy and also decreased learning if administered days 6 and 17 of pregnancy.

The immune response by the dam has been shown to play a critical role in the neuro-development of offspring in a mouse model (Meyer et al., 2006). Several studies show that immune stimulation and not the infectious organisms are responsible for the behavioral sequelae (Shi et al., 2003; Zukerman and Weiner, 2005). Meyers et al. (2006) looked at the IL-10/TNF-alpha ratio in dams immune stimulated (Poly (I:C)) on day 9 and 17 of gestation. An increased ratio (more IL-10 to less TNF-alpha) was observed on day 17 of exposure but not on day 9 of exposure. The result is an increase in adult onset of behavioral differences using the LI test for day 9 MIS pups but not at day 17 MIS pups, indicating an increased ratio of IL-10 to TNF-alpha has a protective effect on long term behavior changes. If, however, IL-10 is increased in the absence of other pro-inflammatory cytokines, adult onset behaviors are still observed (Meyer et al., 2008c)

Day 9 and 17 of gestation in mice are significant in the stages of human development they represent. Day 9 is roughly equivalent to the end of the first trimester and day 17 represents mid to late of second trimester (Meyer et al. 2008b). When the

dam is exposed to Poly (I:C) on day 9 of gestation; sensorimotor gating, PPI and spatial exploration are decreased in off spring (Meyers et al*.,* 2006). On day 17 of gestation MIS with Poly (I:C) caused decreased working memory. Different behavior clusters are associated with certain critical time points for maternal immune stimulation. Clearly infection with specific agents during these crucial time points can have long lasting negative effects on offspring.

Smith et al. (2007) observed multiple behavior changes when MIS with murine IL-6 was administered on day 12 of gestation. They demonstrated a decrease in both PPI inhibition and LI in adult mice but not pre sexually mature mice indicating maternal IL-6 plays a role is adult onset behavior changes. Under typical conditions MIS with Poly (I:C) causes adult onset behavior changes, but when poly (I:C) is given in conjunction with antibodies to IL-6, behavior changes are not observed, indicating that cytokine IL-6 exposure *in utero* plays a major role in adult onset deficits for PPI and LI. These results are further supported a study using MIS with Poly (I:C) to IL-6 knockout (KO) mice. The IL-6 KO mice demonstrated no behavior deficits. These experiments indicate exposure to maternal IL-6 can cause permanent behavioral changes that are only present after sexual maturity.

Immune stimulation can occur by other means. IFN-γ is a cytokine with wide spread effect on the immune system. IFN-γ plays a key role in regulating the Th-1 and Th-2 T-cell immune response. Th-1 T-cells primarily target intracellular pathogens. Specifically, CD8+ T-cells play a larger role then CD4+ T-cells (Abou-Bacar et al., 2004) in preventing congenital infection of *T. gondii*. By immune stimulating with IFN-γ during pregnancy, the immune system is able to decrease birth defects caused by exposure to

known teratogens (Sharova et. al. 2000). Excessive immune stimulation can occur and rejection of the pregnancy results. Maternal immune stimulation with IFN-γ is timing and dose dependent. Known teratogens, urethane and methylnitrosourea are known to cause facial and cranial defects (Prater et al., 2004). Amelioration of these defects with IFN-γ can be done successfully. The mechanism for the effect IFN-γ is currently unknown, but 2 hypotheses exist. The first is that IFN-γ crosses the placenta to mediate the immune response, the second is that IFN-γ causes an up regulation and/or down regulation of specific immune genes (Sharova et al., 2003). If the dose and timing for administering the IFN-γ are done incorrectly termination of the pregnancy can result from an over robust Th-2 immune response. The half-life of exogenous IFN-γ in pregnant dams is not known. The duration of the effects of MIS with IFN-γ on the immune system of dam and offspring are currently unknown.

The exact mechanism for vertical transmission of *T. gondii* is still being worked out. The placenta is an immune privileged organ. While some cells and some cytokines are permitted to cross the placenta, not all normal cellular function and communication occur across the placenta (Barragan et al., 2003). *T. gondii* and select other microscopic organisms can cross the placenta. *T. gondii* possesses the ability to mechanically penetrate cells, replicate, and rupture out, causing lesions in an immune privileged placenta. Trophoblast cells are the last line of defense protecting the fetus from becoming infected *in utero*. After the fetal trophoblast layer is compromised, further damage to the fetus besides lesions on the placenta from *T. gondii*, can occur from inflammation (Abbasi et al., 2003). Inflammation is mediated by the cytokine IFN-γ and other immune cells.

Interferon gamma is a T-cell mediator. When administered exogenously it shifts the T-cell response toward a Th-2 T-cell profile (Sharova et al., 2003). Pregnancy naturally pushes the immune system towards a Th-2 T-cell response and away from a Th-1 T-cell response (Lim et al., 2000). *T. gondii* elicits a strong Th-1 T-cell response, under normal conditions (Ismael et al., 2006). If a strong Th-1 T-cell response happens early in pregnancy the pregnancy maybe aborted. Research indicates *T. gondii* infections in IFN-γ knockout mice increased the pathogenicity of the infection (Norose et al*.,* 2001) to infected rodents.

No correlations between MIS with IFN-γ and behavior changes have been reported in the literature. All of the MIS and behavior changes reported have been conducted using Poly (I:C). Although viral infections elicit a Th-1 T-cell response, the immune response to poly (I:C) may be more refined and controlled than administering IFN-γ, a non-discriminate Th-1/Th-2 T-cell immune regulator.

REFERENCES

Abbasi, M., K. Kowalewska-Grochowska, M.A. Bahar, R.T. Kilani, B. Winkler-Lowen, and L.J. Guilbert. 2003. Infection of placental trophoblasts by *Toxoplasma gondii*. Journal of Infectious Disease 188: 608-616.

Abi-Dargham, A., and H. Moore. 2003. Prefrontal DA transmission at D1 receptors and the pathology of schizophrenia. Neuroscientist 9: 404-416.

Abou-Bacar, A., A.W. Pfaff, V. Letscher-Bru, D. Filisetti, R. Rajapakse, E. Antoni, O. Villard, J.P. Klein, and E. Candolfi. 2004. Role of gamma interferon and T cells in congenital *Toxoplasma* transmission. Parasite Immunology 26: 315-318.

Abramczyk, R.R., D.E. Jordan, and M. Hegel. 1983. "Reverse" Stroop effect in the performance of schizophrenics. Perceptual & Motor Skills 56: 99-106.

Alexopoulou, L., A. C. Holt, R. Medzhitov, and R. A. Flavell. 2001. Recognition of double-stranded RNA and activation of NF-kappaB by Toll-like receptor 3. Nature 413: 732-738.

Ananth, H., I. Popescu, H.D. Critchley, C.D. Good, R.S. Frackowiak, and R.J. Dolan. 2002. Cortical and subcortical gray matter abnormalities in schizophrenia determined through structural magnetic resonance imaging with optimized volumetric voxel-based morphometry. American Journal of Psychiatry 159: 1497-1505.

Arnott, M.A., J.P. Cassella, P.P. Aitken, and J. Hay. 1990. Social interactions of mice with congenital *Toxoplasma* infection. Annals of Tropical Medicine and Parasitology 84: 149-156.

Barragan, A., and L.D. Sibley. 2003. Migration of *Toxoplasma gondii* across biological barriers. Trends in Microbiology 11: 426-430.

Berdoy M., J.P. Webster, and D.W. Macdonald. 2000. Fatal attraction in rats infected with *Toxoplasma gondii*. Proceedings of the Royal Society B: Biological Sciences 267: 1591-1594.

Brown, M., M.R. Lappin, J.L. Brown, B. Munkhtsog, and W.F. Swanson. 2005. *Exploring the ecologic basis for extreme susceptibility of Pallas' cats (Otocolobus manul*) to fatal toxoplasmosis. Journal of Wildlife Disease 41: 691-700.

Brugger, S., J.M. Davis JM, S. Leucht, and J.M. Stone. 2010. Proton Magnetic Resonance Spectroscopy and Illness Stage in Schizophrenia-A Systematic Review and Meta-Analysis. Biological Psychiatry. Epub ahead of print.

Buka, S.L., M.T. Tsuang, E.F. Torrey, M.A. Klebanoff, D. Bernstein, and R.H. Yolken. 2001. Maternal infections and subsequent psychosis among offspring. Archives of General Psychiatry 58: 1032-1037.

Burkinshaaw, J.,B.H. Kirman, and A. Sorsby. 1953. Toxoplasmosis in relation to mental deficiency. British Medical Journa 1: 702-704.

Chertkow, Y., O. Weinreb, M.B. Youdim, and H. Silver. 2009. Molecular mechanisms underlying synergistic effects of SSRI-antipsychotic augmentation in treatment of negative symptoms in schizophrenia. Journal of Neural Transmission 116: 1529-1541.

Dubey, J.P., D.S. Lindsay, and C.A. Speer. 1998. Structure of *Toxoplasma gondii* tachyzoites, bradyzoites, and sporozoites and biology and development of tissue cysts. Clinical Microbiology Review 11: 267-299.

Dutta, R., R.M. Murray, M. Hotopf, J. Allardyce,P.B. Jones, and J. Boydell. 2010. Reassessing the long-term risk of suicide after a first episode of psychosis. Archives of General Psychiatry 67: 1230-1237.

Flegr, J., and I. Hrdý. 1994. Influence of chronic toxoplasmosis on some human personality factors. Folia Parasitologica 41: 122-126.

Flegr, J., S. Zitková, P. Kodym, and D. Frynta. 1996. Induction of changes in human behaviour by the parasitic protozoan *Toxoplasma gondii*. Parasitology 113: 49-54.

Flegr, J., and J. Havlícek. 1999. Changes in the personality profile of young women with latent toxoplasmosis. Folia Parasitologica 46: 22-28.

Flegr, J., M. Preiss, J. Klose, J. Havlícek, M. Vitáková, and P. Kodym. 2003. Decreased level of psychobiological factor novelty seeking and lower intelligence in men latently infected with the protozoan parasite *Toxoplasma gondii* Dopamine, a missing link between schizophrenia and toxoplasmosis? Biological Psychology 63: 253-268.

Flegr, J. 2007. Effects of *Toxoplasma* on human behavior. Schizophrenia Bulletin 33: 757-760.

Gaskell, E.A., J.E. Smith, J.W. Pinney, D.R. Westhead, and G.A. McConkey. 2009. A unique dual activity amino acid hydroxylase in *Toxoplasma gondii*. PLoS One 4: e4801

Gonzalez, L.E., B. Rojnik, F. Urrea, H. Urdaneta, P. Petrosino, C. Colasante, S. Pino, and L. Hernandez. 2007. *Toxoplasma gondii* infection lower anxiety as measured in the plus-maze and social interaction tests in rats a behavioral analysis. Behavior Brain Research 177: 70-79.

Goldberg, T.E., D.R. Weinberger. 1988. Probing prefrontal function in schizophrenia with neuropsychological paradigms. Schizophrenia Bulletin 14: 179-183.

Goodwin, D.G., J.S. Stroble JS, and D.S. Lindsay. 2011. Evaluation of five antischizophrenic agents against *Toxoplasma gondii* in human cell. Journal of Parasitology 97: 148-151.

Goodwin, D.G., J. Strobl, S.M. Mitchell, A.M. Zajac, and D.S. Lindsay. 2008. Evaluation of the mood-stabilizing agent valproic acid as a preventative for toxoplasmosis in mice and activity against tissue cysts in mice. Journal of Parasitology 94: 555-557.

Havlícek, J., Z.G. Gasová, A.P. Smith, K. Zvára, and J. Flegr. 2001. Decrease of psychomotor performance in subjects with latent 'asymptomatic' toxoplasmosis. Parasitology 122: 515-520.

Hay, J., P.P. Aitken, and D.I. Graham. 1984. *Toxoplasma* infection and response to novelty in mice. Zeitschrift für Parasitenkunde 70: 575-588.

Hay J., P.P. Aitken, D.M. Hair, W.M. Hutchison, and D.I. Graham. 1984. The effect of congenital *Toxoplasma* infection on mouse activity and relative preference for exposed areas over a series of trials. Annals of Tropical Medicine and Parasitology 78: 611-618.

Hay, J., P.P. Aitken, and M.A. Arnott. 1985. The influence of congenital *Toxoplasma* infection on the spontaneous running activity of mice. Zeitschrift für Parasitenkunde 71: 459-462.

Henriquez, S.A., R. Brett, J. Alexander,J. Pratt, and C.W. Roberts. 2009. Neuropsychiatric disease and *Toxoplasma gondii* infection. Neuroimmunomodulation 16: 122-133.

Hermes, G., J.W. Ajioka, K.A. Kelly, E. Mui, F. Roberts,K. Kasza, T Mayr, M.J. Kirisits, R. Wollmann, D. J. Ferguson, C.W. Roberts, J.H. Hwang, T. Trendler, R.P. Kennan, Y. Suzuki, C. Reardon, W.F. Hickey, L. Chen, and R. McLeod. 2008. Neurological and behavioral abnormalities, ventricular dilatation, altered cellular functions, inflammation, and neuronal injury in brains of mice due to common, persistent, parasitic infection. Journal of Neuroinflammation 23: 48.

Hodkova, H., P. Kodym, and J. Flegr. 2007. Poorer results of mice with latent toxoplasmosis in learning tests: impaired learning processes or the novelty discrimination mechanism? Parasitology 134: 1329-37.

Hrdá, S., J. Votýpka, P. Kodym,J. Flegr. 2000. Transient nature of *Toxoplasma gondii*-induced behavioral changes in mice. Journal of Parasitology 86: 657-663.

Hrubec, T.C., M.R. Prater, K.A. Toops, and S.D. Holladay. 2006. Reduction in diabetes-induced craniofacial defects by maternal immune stimulation. Birth defects research. Part B, Developmental and reproductive toxicology 77: 1-9.

Hutchinson, W.M., M. Bradley, W.M. Cheyne, B.W. Wells, and J. Hay. 1980. Behavioral abnormalities in *Toxoplasma*-infected mice. Annals of Tropical Medicine and Parasitology 74: 337-345.

Hutchison, W.M., P.P. Aitken, B.W. Wells. 1980. Chronic *Toxoplasma* infections and motor performance in the mouse. Annals of Tropical Medicine and Parasitology 74: 507-510.

Hutchinson, W.M., M. Bradley, W.M. Cheyne, B.W. Wells, and J. Hay. 1980. Behavioral abnormalities in *Toxoplasma*-infected mice. Annals of Tropical Medicine and Parasitology 74: 337-345.

Jones-Brando, L., E.F. Torrey, and R. Yolken. 2003. Drugs used in the treatment of schizophrenia and bipolar disorder inhibit the replication of *Toxoplasma gondii*. Schizophrenia Research 62: 237-244.

Kendell, R.E., and I.W. Kemp. 1989. Maternal influenza in the etiology of schizophrenia. Archives of General Psychiatry 46: 878-882.

Knable, M.B., T.M. Hyde, A.M. Murray, M.M. Herman, and J.E. Kleinman. 1996. A postmortem study of frontal cortical dopamine D1 receptors in schizophrenics, psychiatric controls, and normal controls. Biological Psychiatry 40: 1191-1199.

Kocazeybek B., Y.A. Oner, R. Turksoy, C. Babur, H. Cakan, N. Sahip, A. Una, A. Ozaslan, S. Kilic, S. Saribas, M. Aslan, A. Taylan, S. Koc, A. Dirican, H.B. Uner, V. Oz, C. Ertekin, O. Kucukbasmaci, and M.M. Torun. 2009. Higher prevalence of toxoplasmosis in victims of traffic accidents suggest increased risk of traffic accident in *Toxoplasma*-infected inhabitants of Istanbul and its suburbs. Forensic Science International 187: 103-108.

Leweke, F.M., C.W. Gerth, D. Koethe, J. Klosterkötter, I. Ruslanova, B. Krivogorsky, E.F. Torrey, and R.H. Yolken. 2004. Antibodies to infectious agents in individuals with recent onset schizophrenia. European Archives of Psychiatry Clinical Neuroscience 254: 4-8.

Lim, K.J., O.A. Odukoya, R.A. Ajjan, T.C. Li, A.P. Weetman, and I.D. Cooke. 2000. The role of T-helper cytokines in human reproduction. Fertility and Sterility 73: 136-142.

Lindová, J., M. Novotná, J. Havlícek, E. Jozífková, A. Skallová, P. Kolbeková, Z. Hodný, P. Kodym, and J. Flegr. 2006. Gender differences in behavioural changes induced by latent toxoplasmosis. International Journal of Parasitology 36: 1485-1492.

McAllister, M.M. 2005. A decade of discoveries in veterinary protozoology changes our concept of "subclinical" toxoplasmosis. Veterinary Parasitology 132: 241-247.

Norose K., H.S. Mun, F. Aosai, M. Chen, H. Hata, Y. Tagawa,Y. Iwakura, and A. Yano. 2001. Organ infectivity of *Toxoplasma gondii* in interferon-gamma knockout mice. Journal of Parasitology 87: 447-452.

Meyer, U., M.Nyffeler, B.K. Yee, I. Knuesel, and J. Feldon. 2008a. Adult brain and behavioral pathological markers of prenatal immune challenge during early/middle and late fetal development in mice. Brain Behavior and Immunity 22: 469-486.

Meyer, U., A. Engler, L.Weber, M. Schedlowski, and J. Feldon. 2008b. Preliminary evidence for a modulation of fetal dopaminergic development by maternal immune activation during pregnancy. Neuroscience 154: 701-709.

Meyer, U., P.J. Murray, A. Urwyler, B.K. Yee, M. Schedlowski, and J. Feldon. 2008c. Adult behavioral and pharmacological dysfunctions following disruption of the fetal brain balance between pro-inflammatory and IL-10-mediated anti-inflammatory signaling. Molecular Psychiatry 13: 208-221.

Molina, V., J. Sanz, F. Sarramea, C. Benito, and T. Palomo. 2004. Lower prefrontal gray matter volume in schizophrenia in chronic but not in first episode schizophrenia patients. Psychiatry Research 131: 45-56.

Muller, N., and M. Schwarz. 2006. Schizophrenia as an inflammation-mediated dysbalance of glutamatergic neurotransmission. Neurotoxicity Research 10: 131-148.

Newman, S.C., and R.C. Bland. 1991. Mortality in a cohort of patients with schizophrenia: a record linkage study. Canadian Journal of Psychiatry 36: 239-245.

Nicole, L., A. Lesage, and P. Lalonde. 1992. Lower incidence and increased male:female ratio in schizophrenia. The British Journal of Psychiatry 161: 556-557.

Novotná, M., J. Hanusova, J. Klose, M. Preiss, J. Havlicek, K. Roubalová, and J. Flegr. 2005. Probable neuroimmunological link between *Toxoplasma* and cytomegalovirus infections and personality changes in the human host. BMC Infectious Disease 5: 54 http://www.biomedcentral.com/1471-2334/5/54

Ozawa, K., K. Hashimoto K, T. Kishimoto, E. Shimizu, H. Ishikura, and M. Iyo. 2006. Immune activation during pregnancy in mice leads to dopaminergic hyperfunction and cognitive impairment in the offspring: a neurodevelopmental animal model of schizophrenia. Biological Psychiatry 59: 546-54

Peralta, V. and M.J. Cuesta. 2000. Clinical models of schizophrenia: a critical approach to competing conceptions. Psychopathology 33: 252-258.

Piekarski, G. 1981. Behavioral alterations caused by parasitic infection in case of latent *Toxoplasma* infection. *Zentralbl Bakteriol Mikrobiol Hyg A* 250: 403-406.

Prater, M.R., K.L. Zimmerman, D.L. Ward, and S.D. Holladay. 2004. Reduced birth defects caused by maternal immune stimulation in methylnitrosourea-exposed mice: association with placental improvement. Birth defects research. Part A, Clinical and molecular teratology 70: 862-869.

Reinhard, G., A. Noll, H. Schlebusch, P. Mallmann,and A. V. Ruecker. 1998. Shifts in the TH1/TH2 balance during human pregnancy correlate with apoptotic changes. Biochemical and Biophysical Research Communication 245: 933-938.

Schwarcz, R., and C.A. Hunter. 2007. *Toxoplasma gondii* and schizophrenia: linkage through astrocyte-derived kynurenic acid? Schizophrenia Bulletin 33: 652-653.

Sham, P.C., C.J. MacLean, and K.S. Kendler. 1994. A typological model of schizophrenia based on age at onset, sex and familial morbidity. Acta Psychiatry Scandinavia. 89: 135-141.

Sharova, L., P. Sura, B.J. Smith, R.M. Gogal, A.A. Sharov, D.L. Ward, and S.D. Holladay. 2000. Nonspecific stimulation of the maternal immune system. II. Effects on gene expression in the fetus. Teratology 62: 420-428.

Sharova, L.V., A.A. Sharov, P. Sura, R.M. Gogal, B.J. Smith, and S.D. Holladay. 2003. Maternal immune stimulation reduces both placental morphologic damage and down-regulated placental growth-factor and cell cycle gene expression caused by urethane: are these events related to reduced teratogenesis? International Immunopharmacology 3: 945-955.

Shi L., S.H. Fatemi, R.W. Sidwell, and P.H. Patterson. 2003. Maternal influenza infection causes marked behavioral and pharmacological changes in the offspring. Journal of Neuroscience 23: 297-302.

Shoval, G., J. Feld-Olspanger, E. Nahshoni, D. Gothelf, S. Misgav, I. Manor, A. Apter, and G. Zalsman. 2011. Suicidal behavior and related traits among inpatient adolescents with first-episode schizophrenia. Comprehensive Psychiatry Mar 7 Epub ahead of print.

Skallová A., M. Novotná, P. Kolbeková, Z. Gasová, V. Veselý, M. Sechovská, and J, Flegr. 2005. Decreased level of novelty seeking in blood donors infected with Toxoplasma. Neuroendocrinology Letter 26: 480-486.

Skallová, A., P. Kodym, D. Frynta, and J. Flegr. 2006. The role of dopamine in Toxoplasma-induced behavioural alterations in mice: an ethological and ethopharmacological study. Parasitology 133: 525-535.

Smith, S.E., J. Li, K. Garbett, K Mirnics, and P.H. Patterson. 2007. Maternal immune activation alters fetal brain development through interleukin-6. Jouranl of Neuroscience 27: 10695-10702.

Stibbs HH. 1985. Changes in brain concentrations of catecholamines and indoleamines in *Toxoplasma gondii* infected mice. Annals of Tropical Medicine and Parasitology 79: 153-157.

Strobl, J.S., M. Cassell, S.M. Mitchell, C.M. Reilly, and D.S. Lindsay. 2007. Scriptaid and suberoylanilide hydroxamic acid are histone deacetylase inhibitors with potent anti-Toxoplasma gondii activity in vitro. Journal of Parasitology 93: 694-700.

Susser, E.S., and S.P. Lin. 1992. Schizophrenia after prenatal exposure to the Dutch Hunger Winter of 1944-1945. Archives of General Psychiatry 49: 983-988.

Torrey, E.F., R.H. Yolken. 2003. *Toxoplasma gondii* and schizophrenia. Emerging Infectious Diseases 9: 1375-1380.

Vyas, A., S.K. Kim, N. Giacomini, J.C. Boothroyd, and R.M. Sapolsky. 2007. Behavioral changes induced by *Toxoplasma* infection of rodents are highly specific to aversion of cat odors. Proceedings of the National Academy of Science U S A 104: 6442-6447.

Wang, H.L., A.Y. Bao, G.H. Wang, M.S. Jiang, Z.C. Liu, H.F. Dong HF, and Y. Guo. 2006. Effect of chronic *Toxoplasm*a infection on the spatial learning and memory capability in mice. Chines journal of parasitology and parasitic disease 24: 114-118.

Webster, J.P. 1994. The effect of *Toxoplasma gondii* and other parasites on activity levels in wild and hybrid *Rattus norvegicus*. Parasitology 109: 583-589.

Webster, J.P., C.F. Brunton, and D.W. MacDonald. 1994. Effect of *Toxoplasma gondii* upon neophobic behavior in wild brown rats, *Rattus norvegicus*. Parasitology 109: 37-43.

Webster, J.P. 2001. Rats, cats, people and parasites: the impact of latent toxoplasmosis on behavior 3:1037-1045.

Webster J.P., P.H. Lamberton, C.A. Donnelly, and E.F. Torrey. 2006. Parasites as causative agents of human affective disorders? The impact of anti-psychotic, mood-stabilizer and anti-parasite medication on *Toxoplasma gondii*'s ability to alter host behaviour. Proceedings of the Royal Society B: Biological Sciences 273: 1023-1030.

Webster, J.P. 2007. The effect of *Toxoplasma gondii* on animal behavior: playing cat and mouse. Schizophrenia Bulletin 33: 752-756.

Winter, C., A. Djodari-Irani, R. Sohr, R. Morgenstern, J. Feldon, G. Juckel, and U. Meyer. 2009. Prenatal immune activation leads to multiple changes in basal neurotransmitter levels in the adult brain: implications for brain disorders of neurodevelopmental origin such as schizophrenia. International Journal of Neuropsychopharmacology 12: 513-524.

Wise, C.D., and L. Stein. 1973. Dopamine-beta-hydroxylase deficits in the brains of schizophrenic patients. Science 181: 344-347.

Wu, E.Q., H.G. Birnbaum, L. Shi, D.E. Ball, R.C. Kessler, M. Moulis, and J. Aggarwal. 2005. The economic burden of schizophrenia in the United States in 2002. Journal of Clinical Psychiatry 66:1122-1129.

Yaneza, A. and P. Kumari. 1994. Prevalence of *Toxoplasma* antibodies in blood donors in Al-Hassa. Annals Saudi Medicine 14 : 230-232.

Yolken, R.H., S. Bachmann, I. Ruslanova, E. Lillehoj, G. Ford, E.F. Torrey, and J. Schroeder. 2001. Antibodies to *Toxoplasma gondii* in individuals with first-episode schizophrenia. Clinical Infectious Disease 32: 842-844.

Yolken, R.H., F.B. Dickerson, and and F.E. Torrey. 2009. *Toxoplasma* and schizophrenia. Parasite Immunology 31: 706-715.

Zuckerman, L., and I. Weiner. 2003. Post-pubertal emergence of disrupted latent inhibition following prenatal immune activation. Psychopharmacology 169: 308-313.

Zuckerman, L., and I. Weiner. 2005. Maternal immune activation leads to behavioral and pharmacological changes in the adult offspring. Journal of Psychiatric Research 39: 311-323.

Chapter 2

RH: RESEARCH NOTE

DOPAMINE STIMULATES PROLIFERATION OF *TOXOPLASMA GONDII* TACHYZOITES IN HUMAN FIBROBLAST CELL CULTURES

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ABSTRACT

Toxoplasma gondii is an obligate intracellular parasite often found in the brain of humans. Research has shown a correlation between prevalence of antibody titers to *T. gondii* and psychological illness in humans. Recent studies indicate that individuals seropositive for *T. gondii* antibodies are more likely to develop psychotic disorders including schizophrenia. Schizophrenia is associated with changes in the dopamine neurotransmitter system. Dopamine in the brain may play a role in proliferation, chemoattraction, or stage conversion of *T. gondii* in the brain. Because tachyzoites are the first developmental stage to reach the brain, the present study was conducted to determine the effects of dopamine on their development in vitro in human host cells. Dopamine was examined at either 100 nM or 250 nM in cell culture media and the numbers of tachyzoites produced at 2-3 days were determined and compared to vehicle treated controls. An increase of tachyzoite numbers and increased destruction in cell monolayer was observed at both concentrations of dopamine. Dopamine used at 100 nM was not significantly different from controls but dopamine used at 250 nM caused a significant ($P < 0.05$) difference in tachyzoites counts compared to controls. The role this increase in tachyzoite replication under the stimulus of dopamine plays in the genesis or modulation of schizophrenia or other mental illnesses awaits further studies.

Introduction

Toxoplasma gondii is an intracellular parasite often found in the central nervous system of humans as latent tissue cysts. Tissue cysts are essential for the maintenance of the *T. gondii* life cycle and they have historically been considered as simply a latent stage awaiting ingestion by a warm-blooded intermediate host or a feline definitive host. Because of their location in the brain, there has been increasing interest in the association of chronic *T. gondii* infection and alterations in intermediate host behavior. Hutchinson et al. (1980) and Hay et al. (1984) determined that mice chronically infected with *T. gondii* demonstrated increased open field activity, decreased coordination and diminished capacity to learn a maze. Others have demonstrated altered behavior in chronically *T. gondii* infected mice and rats (reviewed by Webster, 2007). It is possible that these alterations in behavior are due to *T. gondii* induced alteration in brain neurochemistry in infected rodents and humans (reviewed Fekadu, et al., 2010).

Schizophrenia is an important neurological disorder affecting approximately 1% of the American population (Regier et al., 1993). Several research groups from different countries have shown that there is an increase in antibodies against *T. gondii* in individuals with schizophrenia compared to individuals without the disease in the same populations (reviewed by Yolken et al., 2009). It has also been demonstrated that children born to mothers with antibodies to *T. gondii* are at an increased risk of developing schizophrenia at the onset of maturity (Yolken et al., 2009).

Humans with schizophrenia have increased dopamine levels or dopamine

function leading to the dopamine hypothesis of schizophrenia which has dominated thinking about the pathophysiology of schizophrenia since the early sixties (Van Rossum, 1967). Stibbs (1985) found both acute and chronic *T. gondii* infections can cause an increase in dopamine levels or dopamine turnover in the brains of experimentally infected mice. Henriquez et al. (2009) discovered that *T. gondii* has encoded in its genome 2 aromatic amino acid hydroxylase enzymes capable of synthesizing dopamine and serotonin precursors to within one final step. Gaskell et al. (2009) demonstrated that these genes are turned on when *T. gondii* stage converts from tachyzoites to bradyzoites. Host genes are also probably involved in the complex interaction between parasite and neurochemical functioning (Carter, 2009). In order to begin elucidating the parasite-neurochemical interactions, we examined the effect of 100 nM and 250 nM dopamine on the replication of tachyzoites of *T. gondii* growing in human fibroblast cells.

MATERIAL AND METHODS

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RESULTS

Human fibroblast cells (Hs68, American Type Culture Collection, Manassas, Virginia) were maintained in RPMI 1640 medium (Lonza Walkersville, Inc., Walkersville, Maryland) supplemented with 10% (v/v) fetal bovine serum (FBS, Atlanta Biologicals, Inc., Atlanta, Georgia), 100 U/mL penicillin, 100 ug/ml streptomycin (Lonza Walkersville Walkersville, Maryland), and 1 mM sodium pyruvate (Lonza Walkersville, Walkersville, Maryland). Bovine monocytes (BM) cells were maintained in this same culture medium. Both cell lines were cultured in a humidified incubator set at 37^{o}C with a 5% CO_2 atmosphere. The RH strain of *T.* gondii was propagated in BM cells. For experiments, tachyzoites were harvested from infected cell cultures as previously described (Strobl et al., 2007) and used immediately to infect monolayers of Hs68 cells in 48-well dishes. Each well of Hs68 cells was inoculated with 1-2 x 10^5 tachyzoites and they were allowed to penetrate for 2-3 hr. Non-infecting tachyzoites were removed by a single medium exchange and 1 mL of fresh medium composed of RPMI 1640 plus 2% (v/v) FBS with dopamine at 100 or 250 nM or PBS solvent control. The infected Hs68 cells were cultured for an additional 48-72 hours, then tachyzoites in the medium were collected by centrifugation (3,000 x g, 15 min) and counted using a haemocytometer

Dopamine (Sigma St. Louis Missouri) was dissolved in PBS and diluted to make 100 nM and 250 nM concentrations in 2% cell culture media. Dopamine was made fresh prior to administration to cell monolayers. Experiments were replicated 4 times

Statistical analyses were performed using PrismV5.02 (GraphPad, Inc., LaJolla, California). Triplicate determinations were averaged and concentration-response curves prepared using a curve-fit to a log model. Comparisons between groups was done using student T-test.

Dopamine stimulated tachyzoite proliferation at both concentrations used (Figure 1). Tachyzoites numbers were increased in Hs68 cells treated with 100 nm dopamine but the numbers were not significantly different ($P<0.05$). Treatment of *T. gondii* infected Hs68 cells with 250 nM dopamine produced a statistical ($P<0.05$) increase in tachyzoite numbers.

Discussion

Altered dopamine concentrations are a characteristic of schizophrenia. Altered dopamine concentrations were once thought to be the primary cause of schizophrenics. This has been disproven by demonstration of alternate neurotransmitters and neurochemical pathway associated with schizophrenia. Altered dopamine concentrations and dopamine receptor dysfunctions still are believed to be a major contributor to schizophrenia. Hermes et al. (2008) determined that tissue cysts are more frequently found in the diencephalon and cortex than other regions of the brain. The discovery supports the idea tissue cysts are in close proximity of regions of the brain with higher than average dopamine levels. The striatum, the region of the brain with the highest dopamine concentration, is located in very close proximity to the diencephalon. The frontal cortex has the second highest concentration of dopamine in the cortex.

Increased dopamine concentrations was suggested to be a potential explanation of why more males than females seropositive for *T. gondii* had an altered personality profile according to Cattell's 16 factor questionnaire (Flegr and Hrdy, 1994). The personality changes observed were an increase in rule breaking and in jealousy/suspicious behaviors for seropositive males. It was suggested these behavior changes in men are associated with chronic *T. gondii* infection (Flegr and Hrdy, 1994). However, actual dopamine concentrations were not evaluated in their subjects. The behavior profiles observed are similar to known behavior changes associated with

altered dopamine concentrations. Novotna et al. (2005) examined novelty seeking in humans and attempted to correlate dopamine concentrations with this activity in *T. gondii* seropositive individuals because decreased novelty seeking is correlated with increased dopamine concentrations. They found that *T. gondii* seropositive individuals had a decrease in novelty seeking which suggested that increased dopamine concentrations were present. Flegr et al. (2003) expanded upon this area of novelty seeking by evaluating the size of an individual's residence as a possible factor for novelty seeking in recent male military conscripts. When size of residence was factored in and corrected, males seropositive for *T. gondii* demonstrated decreased novelty seeking compared to seronegative uninfected males. In another study, age-matched male and female blood donors were examined using the same test. *Toxoplasma gondii* seropositive males and females both exhibited decreased novelty seeking compared to age matched seronegative males and females (Skallova et al. 2005).

Personality changes discovered in these tests are not as drastic as those found in people living with schizophrenia. The personality changes associated with *T. gondii* infection indicates chronic infection is capable of causing behavior alterations. Since dopamine concentrations were not measured in these studies the amount of dopamine change is unknown and cannot be compared to fluctuations in dopamine found in people with schizophrenia. However evidence supports changes in dopamine concentrations as a cause of behavioral changes.

Certain behavior profiles are associated with increased dopamine concentrations and chronic *T. gondii* infections. It is argued the behavior alterations associated with chronic *T. gondii* infections helps increase transmission of *T. gondii* to its definitive host

(Webster, 2007). The subtle and not so subtle schizophrenic behavior profiles in humans could be a side effect of *T. gondii* cysts in the neural tissue of warm-blooded intermediate hosts. Not all individuals infected with *T. gondii* have mental illnesses but infection with *T. gondii* might be a factor in initiating the expression of mental illness in susceptible individuals.

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REFERENCE

Carter, C. J. 2009. Schizophrenia susceptibility genes directly implicated in the life cycles of pathogens: cytomegalovirus, influenza, herpes simplex, rubella, and *Toxoplasma gondii*. Schizophrenia Bulletin **35:** 1163-1182.

Fekadu, A., T. Shibre, and A. J. Cleare. 2010. Toxoplasmosis as a cause for behaviour disorders--overview of evidence and mechanisms. Folia Parasitologica (Praha) **57:** 105-113.

Flegr, J., and I. Hrdy, 1994. Influence of chronic toxoplasmosis on some human personality factors. Folia Parasitologica (Praha). 41: 122-126.

Flegr, J., M. Preiss, J. Klose, J. Havlícek, M. Vitáková, and P. Kodym. 1993. Decreased level of psychobiological factor novelty seeking and lower intelligence in men latently infected with the protozoan parasite *Toxoplasma gondii* dopamine, a missing link between schizophrenia and toxoplasmosis? Biological Psychology **63:** 253-268.

Gaskell, E. A., J. E. Smith, J. W. Pinney, D. R. Westhead, and G. A. McConkey. 2009. A unique dual activity amino acid hydroxylase in *Toxoplasma gondii*. PLoS One. 4:e4801

Hay, J., P.P. Aitken, and D.I. Graham. 1984. *Toxoplasma* infection and response to novelty in mice. Zeitschrift für Parasitenkunde **70**: 575-588.

Henriquez, S. A., R. Brett, J. Alexander, J. Pratt, and C. W. Roberts. 2009. Neuropsychiatric disease and *Toxoplasma gondii* infection. Neuroimmunomodulation. **16:** 122-133.

Hermes, G., J. W. Ajioka, K. A. Kelly, E. Mui, F. Roberts, K. Kasza, T. Mayr, M. J. Kirisits, R. Wollmann, D. J. Ferguson, C. W. Roberts, J. H. Hwang, T. Trendler, R. P.

Kennan, Y. Suzuki, C. Reardon, W. F. Hickey, L. Chen, and R. McLeod. 2008. Neurological and behavioral abnormalities, ventricular dilatation, altered cellular functions, inflammation, and neuronal injury in brains of mice due to common, persistent, parasitic infection. Journal of Neuroinflammation **5**: 48.

Hutchinson, W.M., M. Bradley, W. M. Cheyne, B. W. Wells, and J. Hay. 1980. Behavioural abnormalities in *Toxoplasma*-infected mice. Annals of Tropical Medicine and Parasitology **74**: 337-345.

Novotná, M., J. Hanusova, J. Klose, M. Preiss, J. Havlicek, K. Roubalová, and J. Flegr. 2005. Probable neuroimmunological link between *Toxoplasma* and cytomegalovirus infections and personality changes in the human host. BMC Infectious Diseases **5:** 54. http://www.biomedcentral.com/1471-2334/5/54

Regier, D. A., W. E. Narrow, D. S. Rae, R. W. Manderscheid, B. Z. Locke, and F. K. Goodwin. 1993. The de facto US mental and addictive disorders service system. Epidemiologic catchment area prospective 1-year prevalence rates of disorders and services. Archives of General Psychiatry **50:** 85-94.

Skallová, A., M. Novotná, P. Kolbeková, Z. Gasová, V. Vesely, M. Sechovská, and J. Flegr. 2005. Decreased level of novelty seeking in blood donors infected with *Toxoplasma*. Neurological and Endocrinological Letters **26:** 480-486.

Stibbs, H. H. 1985. Changes in brain concentrations of catecholamines and indoleamines in *Toxoplasma gondii* infected mice. Ann Trop Med Parasitol. **79:** 153-157.

Strobl, J. S., M. Cassell, S. M. Mitchell, C. M. Reilly, and D. S. Lindsay. 2007. Scriptaid and suberoylanilidehydroxamic acid are histonedeacetylase inhibitors with potent anti-*Toxoplasma gondii* activity in vitro. Journal of Parasitology **93**: 694-700.

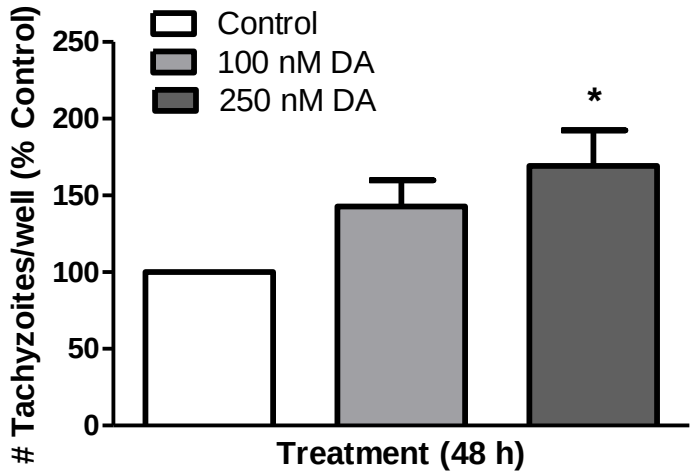
Van Rossum, J. 1967. The significance of dopamine receptor blockade for the action of neuroleptic drugs. In: Brill H, Cole J, Deniker P, Hippius H, Bradley P, editors. Proceedings of the 5th CINP, Washington DC. Amsterdam: Excerpta Medica Foundation; 1967. p. 321-9.

Webster, J., P. 2007. The effect of *Toxoplasma gondii* on animal behavior: playing cat and mouse. Schizophrenia Bulletin **33**: 752-756.

Yolken, R. H., F. B. Dickerson, E. F. Torrey. 2009. *Toxoplasma* and schizophrenia. Parasite Immunology **31**: 706-715.

FIGURES

Figure 1. Graph of dopamine and *T. gondii*. Bar graph demonstrating an increase in numbers of *Toxoplasma gondii* tachyzoites in human fibroblast cells 48 hour after treatment with 100 nM or 250 nM* dopamine (DA) compared to infected cells treated with vehicle only. * Indicates significantly different ($P<0.05$) from control.



RH: RESEARCH NOTE

EVALUATION OF FIVE ANTISCHIZOPHRENIA AGENTS AGAINST *TOXOPLASMA GONDII* IN HUMAN CELL CULTURES

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ABSTRACT

An increasing interest in the association of the presence of antibodies to *Toxoplasma gondii* and the development of schizophrenia in patients has been generated over the last several yr. Some anti-schizophrenia agents have been shown to have activity against *T. gondii* in cell culture assays and to ameliorate behavioral changes associated with chronic *T. gondii* infection in rats. In the present study, we examined the effects of commonly used antipsychotic and mood stabilizing agents (haloperidol, clozapine, fluphenazine, trifluoperazine, and thioridazine) for activity against developing tachyzoites of the RH strain of *T. gondii* in human fibroblast cell cultures. Haloperidol, and clozapine had no measurable activity. Fluphenazine had an IC_{50} of 1.7 µM, thioridazine had an IC_{50} of 1.2 µM, and trifluoperazine had an IC_{50} of 3.8 µM. Our study demonstrates that some agents used to treat schizophrenia have the ability to inhibit *T. gondii* proliferation in cell culture.

INTRODUCTION

It has been estimated that the prevalence of schizophrenia is approximately 1% in the American population (Regier et al., 1993). Several research groups from different countries have shown that there is an increase in antibodies against the zoonotic protozoan *Toxoplasma gondii* in individuals with schizophrenia compared to individuals without the disease in the same populations (Alvarado-Esquivelet al., 2006; Wang et al., 2006; Cetinkaya et al., 2007; Niebuhr et al., 2008). Researchers have also shown that children born to mothers with antibodies to *T. gondii* are at an increased risk of developing schizophrenia (Brown et al., 2005; Mortensen, Nørgaard-Pedersen, Waltoft, Sørensen, Hougaard et al., 2007; Mortensen, Nørgaard-Pedersen, Waltoft, Sørensen, Hougaard, Torrey et al., 2007). One study demonstrated that patients with schizophrenia and positive for antibodies to *T. gondii* had a significantly increased risk of dying from natural causes compared to patients with schizophrenia and no antibodies to *T. gondii* (Dickerson et al., 2007).

It has recently been proposed that schizophrenia susceptibility genes are directly implicated in life cycle of *T. gondii* and other neuropathogens in the human brain (Carter, 2009). These observations suggest that there is a pathobiological relationship between *T. gondii* infection and the occurrence of schizophrenia. Researchers have demonstrated that some agents used to treat schizophrenia and other psychological disorders have activity against *T. gondii* in cell culture systems (Jones-Brando et al., 2003; Goodwin et al., 2008). In the present study, we examined the effects of commonly

used antipsychotic and mood stabilizing agents (haloperidol, clozapine, fluphenazine, trifluoperazine, and thioridazine) for activity against developing tachyzoites of the RH strain of *T. gondii* in human fibroblast cell cultures.

MATERIALS AND METHODS

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RESULTS

Haloperidol, clozapine, and trifluoperazine were purchased from Sigma Chemical Company (St. Louis, Missouri). Fluphenazine, and thioridazine were gifts from E. R. Squib and Sons (Princeton, New Jersey), and Smith Kline Beecham (King of Prussia, Pennsylvania, respectively. Drugs were prepared as 1,000 x stock solutions in dimethylsulfoxide (DMSO), and diluted into culture medium to the desired drug concentration and a final concentration of 0.1% DMSO. Both water and 0.1% DMSO controls were also tested.

Human foreskin fibroblast cells (Hs68, American Type Culture Collection, Manassas, Virginia) were maintained in RPMI 1640 medium (Lonza Walkersville, Inc., Walkersville, Maryland) supplemented with 10% fetal bovine serum (Atlanta Biologicals, Inc**.,** Atlanta, Georgia), penicillin-streptomycin (100 U/ml - 100 mg/ml each, Lonza Walkersville), and 1 mM sodium pyruvate (Lonza Walkersville) were cultured in a humidified, 37 C incubator under a 5% CO_2 atmosphere. The RH strain (Sabin**,** 1941) of *T. gondii* was used for all experiments.

Tachyzoites were harvested from infected cell cultures as previously described (Strobl et al., 2009) and used immediately to infect monolayers of Hs68 cells in 48-well culture plates. Each culture well of Hs68 cells on the 48-well culture plate was

inoculated with 1 x 10^5 tachyzoites and they were allowed to penetrate for 2-3 hr. Non-infecting tachyzoites were removed by a single medium exchange and 1 mL of fresh medium composed of RPMI 1640 + 2% FBS and the test drugs or solvent control. The cells were cultured for an additional 48 hr. The activity of each agent was determined using the SYBR green assay as previously described (Strobl et al., 2009). Briefly, the supernatant from the culture plates was centrifuged, aspirated, and the pellet was used to determine the proliferation of the tachyzoites. The DNA-binding fluorescent probe SYBR green (Molecular Probes, Carlsbad, California) was utilized at 1:10,000 dilution prepared in lysis buffer, containing 0.01% saponin and 0.1% Triton X- 100, and added to the parasite pellets (Bennett et al., 2004). The pellets were incubated at 37 C for 30 min to allow complete lysis of tachyzoites before being read on a fluorescent plate reader with excitation at 460 nm and emission at 530 nm. The monolayers on the 48 welled plates were fixed and stained using 0.5% crystal violet–5% formaldehyde–50% ethanol–0.85% NaCl solution (Strobl et al., 2009) to qualitative compare to the results obtained using the SYBR green assay and to compare monolayer destruction among treatments.

Statistical analyses were performed using PrismV5.02 (GraphPad Inc. LaJolla, California). Triplicate determinations were averaged and concentration response curves prepared using a curve-fit to a log inhibitor-response model. Drug IC_{50} values were estimated from the curve-fits.

DISCUSSION

Clozapine and haloperidol did not have inhibitory activity in the assay when examined at 1, 5**,** or 10 µM; therefore, no IC_{50} could be calculated (data not shown). The mean and SEM from 2 independent experiments performed in triplicates was used to construct the graphs of activity for fluphenazine, thioridazine and trifluoperazine (Fig. 1). Fluphenazine had an IC_{50} of 1.7 ± 0.1 µM and thioridazine had an IC_{50} of 1.2 ± 0.1 µM. Trifluoperazine had slight stimulatory activity at lower levels (2.0 µM) and inhibitory activity at higher levels (IC_{50} 3.8 ± 0.1 µM). Results observed in the 48 welled plate studies were similar to those seen using the SYBR green assay (Figs. 2).

Jones-Brando et al. (2003) examined 12 neuroleptic agents against the RH strain of *T. gondii* growing in human fibroblast cells using an ELISA based assay to determine activity. They reported that the IC_{50} of haloperidol was 5.6 µg/mL (15 µM). Haloperidol was not active in the present study at any of the concentrations examined (1-10 µM). We demonstrated activity of fluphenazine, thioridazine, and trifluoperazine against developing tachyzoites of *T. gondii* in the present study.

Schizophrenia usually occurs in women around the ages of 25 to 35 yr., which corresponds to peak reproductive years, leading to a use of these agents in pregnant women. Fortunately, no study has demonstrated an association between the use of antipsychotic agents during pregnancy and an increased risk for birth defects or other adverse outcomes (see Einarson and Boskovic, 2009). However, studies in humans with schizophrenia that were treated with the anti-*T. gondii* agents trimethoprim (Shibre

et al., 2009) or azithromycin (Dickerson et al., 2009) failed to demonstrate significant improvement in clinical symptoms. Because anti-schizophrenia drugs are designed to act on the brain, it is likely that they may have more activity in the brain than other anti-*T. gondii* agents. Studies using rats have reported that haloperidol or pyrimethamine plus dapsone are effective in ameliorating behavioral changes associated with chronic *T. gondii* infection (Webster et al., 2006). A study in mice using valproic acid did not demonstrate an effect on acute toxoplasmosis and there was no effect on *T. gondii* tissue cyst numbers in chronically infected mice (Goodwin et al., 2008).

Fluphenazine showed good activity against *T. gondii* in the present study (an IC_{50} of 1.7 µM). It is a promising drug for further investigation because fluphenazine has a highly acceptable adverse effect profile and is considered to be among the best tolerated anti-psychotic drugs available for patient use. Therapeutic plasma levels of fluphenazine are approximately 5 nM, well below the concentrations of drug that inhibited *T. gondii* proliferation in the present cell culture tests. Nevertheless, it is likely that levels of fluphenazine in the brain exceed those in plasma, and fluphenazine could exert some anti-*T. gondii* actions within the central nervous system. The safety of fluphenazine for use in pregnant women has not been established (see Einarson and Boskovic, 2009); nevertheless, a case report showed a normal pregnancy and birth occurred in a woman who had taken fluphenazine continuously through her pregnancy (Cleary 1977). In addition, there is a slow-release form of fluphenazine available that provides a convenient, sustained delivery of drug to enhance patient compliance. Thioridazine has also been administered to a pregnant woman without adverse effects to the baby (Yaris et al., 2004). We found thioridazine is slightly more potent than

fluphenazine against *T. gondii* in cell culture, and because therapeutic plasma levels of thioridazine are in the range of 5 µM (Kirchherr and Kuhn-Velten, 2006), drug levels in patients undergoing treatment with thioridazine are more than sufficient to exert an anti-*T. gondii* activity.

Several laboratories including our own have found that anti-psychotic drugs and mood-stabilizing agents exert fairly potent effects on *T. gondii* tachyzoites (Pezzella et al., 1997; Jones-Brando et al., 2003; Strobl et al., 2007). However, defining a mechanism of action in *T. gondii* has proven to be more elusive. A confounding factor in elucidating the biological effects for these drugs in *T. gondii* is the existence of multiple pharmacological targets. For example, there are two reports that valproic acid, a drug approved for use as an anti-epileptic and mood-stabilizer, reduces tachyzoite proliferation (Jones-Brando et al., 2003; Strobl et al., 2007). The anti-epileptic properties of valproic acid are generally attributed to its ability to raise levels of the neurotransmitter gamma-amino butyric acid (GABA) in the brain as well as to inhibit certain sodium channels and T-type calcium channels (Golan et al., 2008). There are also numerous reports that valproic acid is a histone deacetylase inhibitor and exerts broad biological activity by causing changes in protein acetylation (Monti et al., 2009). Deciphering which of these mechanisms drive the valproic acid response in tachyzoites is difficult, and though we have suggested that histone deacetylase is a key target of valproic acid in *T. gondii* based upon the anti-proliferative actions of other chemical classes of histone deacetylase inhibitors in *T. gondii* (Strobl et al., 2007), there is no evidence that the regulation of ion channels, GABA metabolism or other signaling pathways by valproic acid does not exert effects in *T. gondii*.

An equally complex picture emerges when examining the pharmacologic targets of the anti-psychotic drugs. The typical anti-psychotic drugs are chemically related and share the phenothiazine ring structure. This class of antagonists includes chlorpromazine, trifluoperazine, fluphenazine, and thioridazine, and their 2 primary pharmacological targets are the dopamine D2 receptor and calmodulin (Prozialeck and Weiss, 1982; Miyamoto et al., 2005). Haloperidol is a typical anti-psychotic, a highly potent D2 dopamine receptor antagonist and calmodulin inhibitor, but has a butyrophenone ring structure which is chemically distinct from the phenothiazine group. In addition to these main drug targets, chlorpromazine, trifluoperazine, fluphenazine, and haloperidol have been reported to inhibit several types of potassium channels, and to mediate intracellular calcium-release through actions on the ryanodine receptor (Naiazawa et al., 1995; Qin et al., 2009; Suessbrich et al., 1997; Wagner et al, 2004). Second-generation anti-psychotic drugs target the dopamine D2 receptor as well as the serotonin receptor,5-HT-2A, and as a class bind less tightly to the dopamine receptor than typical anti-psychotics (Seeman 2002; Miyamoto et al., 2005). Clozapine is the prototype agent in this class which also includes olanzapine, risperidone, and quetiapine (Miyamoto et al., 2005). Unlike the typical antipsychotics, the second generation anti-psychotics are not calmodulin antagonists, and even some cellular actions of clozapine show dependence upon calmodulin-activated kinase activity (Ninan et al., 2003; Shin et al., 2006). Of note, in our work, clozapine (5 µM) was ineffective in protecting HS68 host cell death through proliferation of tachyzoites and cell lysis; although an earlier publication showed clozapine (ID_{50} 18 µM) inhibited *T. gondii* replication in host cells, this same paper showed the other second generation

antipsychotics tested, risperidone, quetiapine and olanzapine, were inactive (Jones-Brando et al., 2003). We suggest that the differential response of *T. gondii* to typical and second generation anti-psychotics implicates a role for calmodulin in the protection of RH *T. gondii* –infected HS68 cells by trifluoperazine, fluphenazine, thioridazine and haloperidol reported here. We recognize that there is no reason to exclude the idea that actions of these drugs on ion channels also contribute to inhibition of tachyzoite propagation in host cells. In fact, multiple mechanisms may be at work here.

Transient rises in intracellular calcium within the tachyzoites serve as a trigger for parasite egress from host cells and participate in the cell gliding motility and host cell invasion processes critical to *T. gondii* survival (Hoff and Carruthers, 2002; Chandramohanadas et al., 2009; Nagamune et al., 2008; Lourido et al., 2010). Calcium binding activates calmodulin and downstream calmodulin-dependent protein kinases have been implicated in life cycle and erythrocyte invasion of the apicomplexan, *P. falciparum*, (Vaid et al., 2008; Wurtz et al., 2009) but these pathways are not well understood in *T. gondii*. In our experiments, tachyzoites were observed within parasitophoros vesicles in host cells suggesting that anti-psychotics might blunt signals for *T. gondii* egress. This might involve calmodulin inhibition or, alternatively, the actions of anti-psychotics on in fluxes could act to repress the calcium signal for egress. Atypical antipsychotics stimulate excess calcium release from intracellular stores by opening ryanodine receptors (Wagner et al, 2004; Qin et al, 2009), and these drugs could thereby exert a direct cytotoxic effect on tachyzoites by amplification of the normal calcium release signals to initiate egress (Lovett and Sibley, 2003). Furthermore, there is good evidence that the actomyosin motor directing cell motility operates

independently of calmodulin (Herm-Gotz et al., 2002), yet calmodulin is nevertheless, intimately associated with the actomyosin complex in the apical complex of the invading tachyzoite (Pezzella-D'Alessandro et al., 2001). Calmodulin may exert its role in invasion by mediating calcium-dependent conoid extrusion and through calcium-stimulated secretion of lytic enzymes at the adhesion site on the host cell membrane which serves to fluidize the host cell membrane and facilitate parasite entry. In these ways, calmodulin inhibition would suppress tachyzoite invasion and protect host cell monolayers.

The importance of dopamine receptor antagonism in the inhibition of *T. gondii* by anti-psychotics is enigmatic. Clearly, the more potent D2 antagonists of the typical anti-psychotic class are more active inhibitors of *T. gondii* than the less potent second generation anti-psychotics, but at this time, there is no good evidence for expression of D2 receptors on *T. gondii*. Fibroblast cells do not express detectable levels of dopamine receptors making it unlikely that the actions are indirectly mediated through altered biology of the host cell (Tang et al., 1994).

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REFERENCES

Alvarado-Esquivel, C., O-P.Alanis-Quiñones, M-Á. Arreola-Valenzuela, A. Rodríguez-Briones, L-J. Piedra-Nevarez, E. Duran-Morales, S. Estrada-Martínez, S-A. Martínez-García, and O. Liesenfeld. 2006. Seroepidemiology of *Toxoplasma gondii* infection in psychiatric inpatients in a northern Mexican city. BMC Infectious Diseases **6**: 178.

Bennett, T. N., M. Paguio, B. Gligorijevic, C. Seudieu, A. D. Kosar, E. Davidson, and P. D. Roepe. 2004. Novel, rapid, and inexpensive cell-based quantification of antimalarial drug efficacy. Antimicrobial Agents and Chemotherapy **48:** 1807– 1810.

Berdoy, M., J. P. Webster, and D. W. Macdonald.1995. Parasite-altered behaviour: Is the effect of *Toxoplasma gondii* on *Rattus norvegicus* specific? Parasitology **111**: 403-409.

Berdoy, M., J. P. Webster, and D. W. Macdonald. 2000. Fatal attraction in rats infected with *Toxoplasma gondii*. Proceedings of the Royal Society B: Biological Science **267**: 1591-1594.

Brown, S. A., C. A. Schaefer, C. P. Quesenberry, Jr., L. Liu, V. P. Babulas, and E. S. Susser. 2005. Maternal exposure to toxoplasmosis and risk of schizophrenia in adult offspring. American Journal of Psychiatry **162**: 767–773.

Carter, C. J. 2009. Schizophrenia susceptibility genes directly implicated in the life cycles of pathogens: Cytomegalovirus, influenza, *Herpes simplex*, rubella, and *Toxoplasma gondii*. Schizophrenia Bulletin **35**: 1163–1182. Chandramohanadas, R., P. H. Davis, D. P. Beiting, M. B. Harbut, C. Darling, G. Velmourougane , M. Y. Lee, P. A.

Greer, D. S. Roos, and D. C. Greenbaum. 2009. Apicomplexan parasites co-opt host calpains to facilitate their escape from infected cells. Science **324**: 794-797.

Cetinkaya, Z., S. Yazar, O. Gecici, and M. N. Namli. 2007. Anti-*Toxoplasma gondii* antibodies in patients with schizophrenia—preliminary findings in a Turkish sample. Schizophrenia Bulletin **33**: 789–791.

Cleary, M. F. 1977. Fluphenazine decanoate during pregnancy. American Journal of Psychology **134**: 815-816.

Dickerson, F. B., J.J. Boronow, C. R. Stallings, A. E, Origoni, and R. H. Yolken. 2007. *Toxoplasma gondii* in individuals with schizophrenia: association with clinical and demographic factors and with mortality. Schizophrenia Bulletin **33**: 737–740.

Dickerson, F. B., C. R. Stallings, J. J. Boronow, A. E. Origoni, and R. H. Yolken. 2009. A double-blind trial of adjunctive azithromycin in individuals with schizophrenia who are seropositive for *Toxoplasma gondii*. Schizophrenia Research **112**: 198–199.

Einarson, A., and R. Boskovic. 2009. Use and safety of antipsychotic drugs during pregnancy. Journal of Psychiatric Practice **15:** 183-192.

Golan, D.E., A.H. Tashjian, Jr. E. J. Armstrong, and A. W. Armstrong. 2008). Principles of Pharmacology. The Pathophysiologic Basis of Drug Therapy, Second Edition, Lippincott, Williams & Williams, Philadelphia, p.234.

Goodwin, D. G., J. Strobl, S. M. Mitchell, A. M. Zajac, and D. S. Lindsay. 2008. Evaluation of the mood-stabilizing agent valproic acid as a preventative for

toxoplasmosis in mice and activity against tissue cysts in mice. Journal of Parasitology **94:** 555-557.

Herm-Gotz, A., S. Weiss, R. Stratman, S. Fujita-Becker, C. Ruff, E. Meyhofer, T. Soldati, D. J. Manstein, M. A. Geeves, and D. Soldati D. 2002. *Toxoplasma gondii* myosin A and its light-chian: a fast, single-headed, plus-end-directed motor. The EMBO Journal **21**: 2149-2158.

Hoff, E. F., and V. B. Carruthers. 2002. Is *Toxoplasma* egress the first step in invasion? Trends in Parasitology **18**: 251-255.

Jones-Brando, L., E. F. Torrey, and R. Yolken. 2003. Drugs used in the treatment of schizophrenia and bipolar disorder inhibit the replication of *Toxoplasma gondii*. Schizophrenia Research **62:** 237-244.

Kirchherr, H., and W. N. Kuhn-Velten. 2006. Quantitative determination of forty- eight antidepressant and antipsychotics in human plasma by HPLC tandem mass

spectrometry: A multi-level sample approach. Journal of Chromatography B **843**: 100-113.

Lourido, S., J. Shuman, C. Zhang, M. Shokat, R. Hiu and L. D. Sibley. 2010. Calcium-dependent protein kinase 1 is an essential regulator of exocytosis in Toxoplasma. Nature **465**: 359-362.

Lovett, J. L., and L. D. Sibley. 2003. Intracellular calcium stores in *Toxoplasma gondii* govern invasion of host cells. Journal of Cell Science **116**: 3009-3016.

Mortensen, P. B., B. Nørgaard-Pedersen, B. L. Waltoft, T. L. Sørensen, D. Hougaard, and R. H. Yolken. 2007. Early infections of *Toxoplasma gondii* and the later development of schizophrenia. Schizophrenia Bulletin **33**: 741–744.

Mortensen, P. B., B. Nørgaard-Pedersen, B. L. Waltoft, T. L. Sørensen, E. F. Torrey, and R. H. Yolken. 2007b. *Toxoplasma gondii* as a risk factor for early-onset schizophrenia: analysis of filter paper blood samples obtained at birth. Biological Psychiatry **61**: 688-693.

Monti, B., E. Polazzi, and A. Contestabile. 2009. Biochemical, molecular and epigenetic mechanisms of valproic acid neuroprotection. Current Molecular Pharmacology **2**: 95-109.

Nagamune, K., L. M. Hicks, B. Fux, F. Brossier, E. N. Chini, and L. D. Sibley. 2008. Abscisic acid controls calcium-dependent egress and development in *Toxoplasma gondii*. Nature **45**: 207-210.

Niebuhr, D. W., A. M. Millikan, D. N. Cowan, R. Yolken, Y. Li, and N. S. Weber. 2008. Selected infectious agents and risk of schizophrenia among U.S. military personnel. American Journal of Psychiatry **165**: 99–106.

Pezzella, N., A. Bouchot, A. Bonhomme, L. Pingret, C. Klein, H. Burlet, G. Balosser, P. Bonhomme, and J. M. Pinon. 1997. Involvement of calcium and calmodulin in *Toxoplasma gondii* tachyzoite invasion. European Journal of Cell Biology **74**: 92-101.

Prozialeck, W. C., and B. Weiss. 1982. Inhibition of calmodulin by phenothiazines and related drugs: structure activity relationships. Journal of Pharmacology and Experimental Therapeutics **222**: 509-516.

Qin, J., A. V. Zima, M. Porta, L. A. Blatter, and M. Fill. 2009. Trifluoperazine a ryanodine receptor agaonist. Pflügers Archiv - European Journal of Physiology **458**: 643-651.

Regier, D. A., W. E. Narrow, D. S. Rae, R. W. Manderscheid, B. Z. Locke, and E. K. Goodwin. 1993. The de facto US mental and addictive disorders service system. Epidemiologic catchment area prospective 1-year prevalence rates of disorders and services. Archives of General Psychiatry **50**: 85-94.

Sabin, A. B.1941. Toxoplasmic encephalitis in children. Journal of the American Medical Association **116**: 801-807.

Shibre T, A. Alem, A. Abdulahi, M. Araya, T. Beyero, G. Medhin, N. Deyassa, A. Negash, A. Nigatu, D. Kebede, and A. Fekadu. 2009. Trimethoprim as adjuvant treatment in schizophrenia: A double-blind, randomized, placebo-controlled clinical trial. Schizophrenia Bulletin PMID: 19193743.

Strobl, J. S., M. Cassell, S. M. Mitchell, C. M. Reilly, and D. S. Lindsay. 2007. Scriptaid and suberoylanilidehydroxamic acid are histonedeacetylase inhibitors with potent anti-*Toxoplasma gondii* activity in vitro. Journal of Parasitology **93**: 694-700.

Strobl, C. W. Seibert, Y. Li, R. Nagarkatti, S. M. Mitchell, A. C. Rosypal, D. Rathore, and D. S. Lindsay. 2009. Inhibition of *Toxoplasma gondii* and *Plasmodium falciparum* infections in vitro byNSC3852, a redox active antiproliferative and tumor cell differentiation agent. Journal of Parasitology **95**: 215- 223.

Tang, L., R. D. Todd, A. Heller, and K. L. O'Malley. 1994. Pharmacological and functional characterization of D2, D3 and D4 dopamine receptors in fibroblast and dopaminergic cell lines Journal of Pharmacology and. Experimental Therapeutics **268**:

495-502.

Vaid, A., D. C. Thomas, and P. Sharma. 2008. Role of Ca^{+2} /calmodulin-*Pf*PKB signaling pathway in erythrocyte invasion by *Plasmodium falciparum*. Journal of Biological Chemistry **283**: 5589-5597.

Wagner, R., R. H. Fink, and D. G. Stephenson. 2004. Effects of chlorpromazine on excitation-coupling events in fast-twitch skeletal muscle fibers of the rat. British Journal of Pharmacology **141**: 624-633.

Wang, H. L., G. H. Wang, Q. Y. Li, C. Shu, M. S. Jiang, and Y. Guo. 2006. Prevalence of *Toxoplasma* infection in first-episode schizophrenia and comparison between *Toxoplasma*-seropositive and *Toxoplasma*-seronegative schizophrenia. Acta Psychiatria Scandanavia **114**: 40-48.

Webster, J. P., P. H. Lamberton, C. A. Donnelly, and E. F. Torrey. 2006. Parasites as causative agents of human affective disorders? The impact of antipsychotic, mood-stabilizer and anti-parasite medication on *Toxoplasma gondii*'s ability to alter host behaviour. Proceedings of the Royal Society B: Biological Science **273**: 1023-1030.

Wurtz, N, J. Desplans, and D. Parzy. 2009. Phenotypic and transcriptomic analysis of *Plasmodium falciparum* protein kinase A catalytic subunit inhibition. Parasitol. Research **105**: 1691-1699.

Yaris, F., E.Yaris, M. Kadioglu, C. Ulku, M. Kesim, and N. I. Kalyoncu. 2004. Use of polypharmacotherapy in pregnancy: a prospective outcome in a case. Progress in Neuropsychopharmacology and Biological Psychiatry **28**: 603– 605.

FIGURES

Fig 1. Graph of SYBRGreen vs. Drug Concentration. Graphs of the SYBRGreen intensity vs the concentration of drug. Figure (a) is of Fluphenazine with a calculated IC50 of 1.7 μM. Figure (b) is of Thioridazine with a calculated IC50 of 1.2 μM. Figure (c) is of Trifluoperazine with a calculated IC50 of 3.8 μM.

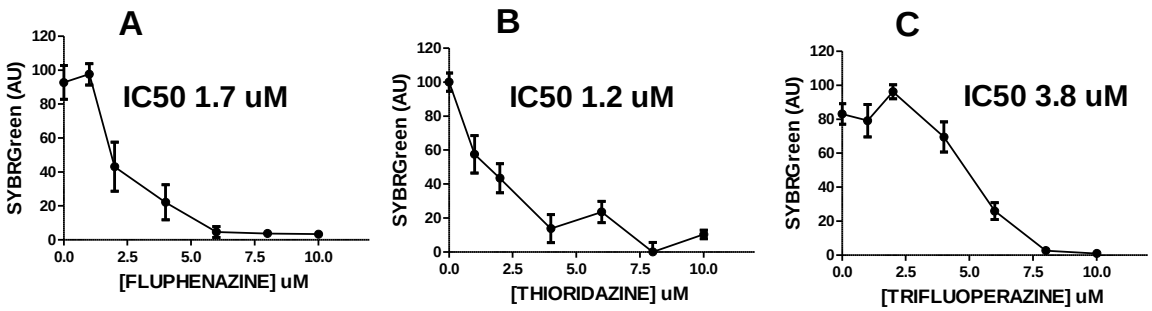
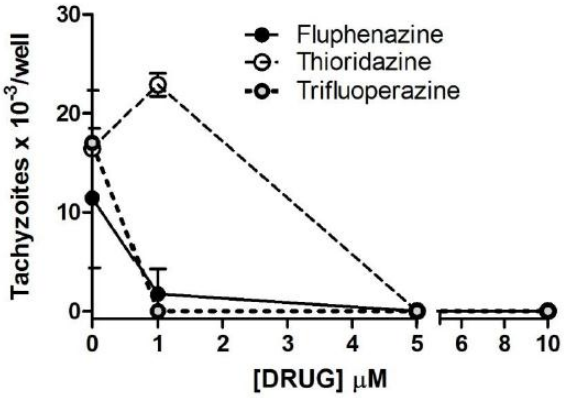


Fig. 2. Graph of T. gondii counts vs. Drug Councentrations. Graphs of the tachyzoite counts vs the concentration of drug.



Chapter 4

RH: RESEARCH NOTE

Evaluation of the Mood-Stabilizing Agent Valproic Acid as a Preventative for Toxoplasmosis In Mice and Activity Against Tissue Cysts in Mice

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ABSTRACT

Toxoplasma gondii is a common intracellular protozoan infection of humans worldwide. Severe disease can occur in immunocompromised individuals and the in the fetuses of non-immune pregnant women. Chronic infection is associated with vision and hearing problems, and functional mental alterations, including schizophrenia. The mood-stabilizing agent valproic acid has been shown to inhibit the development of *T. gondii* in vitro at dosages that are normally achieved in the serum and cerebral spinal fluid of human patients and to have positive effects on the behavior of rats chronically infected with *T. gondii*. The present study was done to examine the in vivo activity of valproic acid against acute toxoplasmosis in mice. Two studies were done with valproic acid given in the drinking water at concentrations of 1.5 mg/ml (Experiment 1) or 3.0 mg/ml (Experiment 2). In a third experiment (Experiment 3), valproic acid was injected intraperitoneally (i.p.) at doses of 200 or 300 mg/kg every 12 hr. Valproic acid was not effective in preventing acute toxoplasmosis. All mice treated with valproic acid died or were killed and did not ($P \leq 0.05$) live significantly longer than the controls. Tachyzoites were demonstrated in the tissues of infected valproic-acid–treated mice. A fourth study was done to determine if valproic acid has activity against *T. gondii* tissue cysts in chronically infected mice. Mice were chronically infected with the ME- 49 strain of *T. gondii* for 8 wk. and then treated orally with valproic acid at approximately 6.6 mg/ml (800 mg/kg/day) in the drinking water for 10 wk. (amount was varied due to increasing mouse weights). No significant differences ($P \leq 0.05$) were present in tissue cyst

numbers in valproic-acid–treated *T. gondii* chronically infected mice and in mice chronically infected with *T. gondii* but not given valproic acid. Our results indicate that valproic acid, although effective in vitro against *T. gondii* tachyzoites, is not effective as a preventative in mice inoculated with *T. gondii* tachyzoites. Additionally, no activity against tissue cysts was observed in chronically *T. gondii*–infected valproic-acid–treated mice.

INTRODUCTION

Toxoplasma gondii is a protozoan parasite that infects humans and most other warm-blooded animals. Humans become infected by ingesting meat containing tissue cysts or by ingesting sporulated oocysts in the environment. Researchers indicate that there are 1,500,000 cases of toxoplasmosis in the United States each year, and about 15% of those infected have clinical toxoplasmosis (Jones, Kruszon-Moran et al., (2001). The importance of maternal infection and congenital toxoplasmosis has long been recognized (Jones, Lopez et al., 2001). The role of chronic *T. gondii* infection on human health was manifested in the AIDS epidemic, with the numerous cases of toxoplasmic encephalitis due to reactivated infection and clinical toxoplasmic encephalitis. The association of chronic *T. gondii* infection and behavioral changes has come to light over recent years and has been strengthened by many studies in humans, mice, and rats (Holliman, 1997; Webster, 2001). Most notably, the association of chronic *T. gondii* infection and schizophrenia has gained attention (Yolken et al., 2001; Ledgerwood et al., 2003; Torrey and Yolken, 2003; Bachmann et al., 2005; Brown et al., 2005; Wang et al., 2006).

Valproic acid is a mood stabilizer used in the treatment of mental illnesses including bipolar disorder and schizophrenia (see Bowden, 2007). It has also been shown to inhibit *T. gondii* reproduction in human fibroblast cell cultures (Jones-Brando et al., 2003; Strobl et al., 2007). The IC50 of valproic acid (sodium salt) in a tachyzoite production assay was 266 "g/ml (Strobl et al., 2007), whereas in an ELISA-based assay the IC50 of valproic acid (free acid) was 4.7 "g/ml and the IC50 of valproic acid (sodium

salt) was 4.1 "g/ml (Jones-Brando et al., 2003). These studies indicate that this drug is active against tachyzoites of *T. gondii*.

Rats chronically infected with *T. gondii* lose their innate fear of cat odor (Berdoy et al., 2000; Vyas et al., 2007). This makes them easier prey for cats and enhances the transmission of the parasite. The effects of valproic acid on the feline avoidance behavior of chronically infected rats was examined by Webster et al. (2006) and it was shown to help treated *T. gondii*–infected rats (40 mg/kg valproic acid/day orally) retain their innate avoidance of cat smell.

MATERIAL and METHODS

&

RESULTS

The present study was done to examine the anti–*T. gondii* activity of valproic acid in the prevention of acute toxoplasmosis in mice or activity against the tissue cyst stage in chronically infected mice. Female ICR mice were housed in groups of 5 mice per cage (Experiments 1–3) or 3–4 mice per cage (Experiment 4). Mice in Experiments 1–3 were inoculated subcutaneously with 5 # 103 tachyzoites of the RH strain of *T. gondii* on Day 0. Valproic acid was given in the drinking water 1 day prior to subcutaneous inoculation of mice in Experiments 1 and 2. Valproic-acid–containing water in lightproof water bottles was provided ad libitum for the remainder of the study. Fresh valproic-acid– containing water was provided every 2 days. For dosing considerations, we assumed that each mouse would drink 4 ml of water each day. Saccharin was added at 0.2% (w/v) to mask the flavor of the valproic acid–treated water.

Experiment 1 contained 10 mice treated with 1.5 mg/ml valproic acid (Groups 1 and 2) in the drinking water supplemented with 0.2% (w/v) saccharin and 10 mice not treated with valproic acid (Groups 3 and 4) (Table I). Saccharin was added at 0.2% (w/v) to 1 group of 5 of these mice (Group 3) given water without valproic acid. Experiment 2 contained 5 mice treated with 3 mg/ml valproic acid in the drinking water (Group 5) and 5 mice not treated with valproic acid (Group 6) (Table II). The mean weight of valproic-acid–treated mice in Experiment 1 was 24 g and in Experiment 2 the mean weight was 22 g. Valproic acid concentrations in the drinking water provided

doses of 250 mg/kg/ day (Experiment 1) and 545 mg/kg/day (Experiment 2). These dosages translate to 300 mg/kg and 600 mg/kg for a 20-g mouse, respectively.

Experiment 3 was done to evaluate valproic acid administered intraperitoneally (i.p.) at doses of 200 or 300 mg/kg every 12 hr. (Table III). The mean weight of valproic-acid–treated mice was 23 g. Valproic acid was dissolved in sterile saline (0.14 M NaCl solution) and i.p. injections were started 2 days prior to RH strain *T. gondii* infection with 5×10^3 tachyzoites s.c. There were 2 groups (Group 7 and 8) of 5 mice each that received the 400-mg/kg/day total dose and 2 groups (Group 9 and 10) of 5 mice each that received the 600-mg/kg/day total dose. A group of 5 mice (Group 11) were infected controls and treated every 12 hr. with i.p. sterile saline only.

Experiment 4 (Table IV) was conducted to determine if valproic acid has activity against *T. gondii* tissue cysts in vivo. Eight mice (4 mice in Group 12 and 4 mice in Group 13) were s.c. infected with 1 # 103 tachyzoites of the ME49 strain of *T. gondii* in 0.5 ml HBSS and left untreated for 8 wk. Three mice (Group 14) were s.c. inoculated with 0.5 ml HBSS and treated similarly. After 8 wk., mice in Groups 12 and 14 were provided drinking water containing 0.2% saccharin and approximately 6.6 mg/ml (amount varied because of mouse weights, which changed during the study) valproic acid for 10 wk. to deliver a daily dose of 800 mg/kg. Mice in Groups 12–14 were killed 10 wk. after valproic-acid treatment, and their brains were removed. The left half of the brain was homogenized in 2 ml HBSS for 2 min with the use of a stomacher machine (Seward Lab Blender Stomacher 80, London, England). The numbers of tissue cysts in a 50-"l sample of the homogenized brain was determined with the use of light microscopy. The right half of the brain was fixed in 10% neutral buffered formalin

solution and processed routinely for histological examination following staining with hematoxylin and eosin.

Impression smears were made from the livers or lungs of mice that died or were killed during the study. They were examined unstained for tachyzoites with the use of an Olympus BH60 microscope equipped with interference contrast optics.

Kaplan–Meier survival analysis was performed with the use of PrismGraphpad version 4.0 on mice in Experiments 1–3. The data were analyzed for statistical significance with the use of the chi-square and log-rank tests with a *P* value of 0.05. Tissue cyst counts from mice in Experiment 4 were examined with the use of a 1-way ANOVA and an unpaired *t*-test with a *P* value of 0.05.

DISCUSSION

Acute toxoplasmosis occurred in all mice given the RH strain (Tables I–III). Neither oral (Experiments 1 and 2) nor i.p. (Experiment 3) treatment with valproic acid was effective. Deaths occurred in treated mice from 8 to 13 days after inoculation with tachyzoites and in untreated mice 9–12 days after tachyzoite inoculation (Tables I–III). There was no significant positive effect of valproic-acid treatment on mouse survival ($P < 0.05$). Tachyzoites were seen in tissues of all mice given the RH strain of *T. gondii*.

None of the mice inoculated with tachyzoites of the ME49 strain of *T. gondii* died during the study (Groups 12 and 13). None of the mice given only valproic acid (Group 14) died during the study. Tissue cysts were structurally normal when viewed as fresh preparations with light microscopy. They were also normal when viewed in stained histological sections. The mean number of tissue cysts in 50 μL was 1.3 ± 1.3 (range, 0–3) for mice in Group 12 and 2.0 ± 1.8 (range, 0–4) for mice in Group 13. No tissue cysts were seen in the brains of mice in Group 14. There were no significant differences ($P < 0.05$) in tissue cyst counts in mice infected with the ME49 strain of *T. gondii* and treated with valproic acid (Group 12) and those infected but not treated with valproic acid (Group 13).

Valproic acid is active against tachyzoites of *T. gondii* in 2 different cell-culture–based assays (Jones-Brando et al., 2003; Strobl et al., 2007). The studies of Jones-Brando et al. (2003) indicate that valproic acid is active at lower concentrations than

reported by Strobl et al. (2007), but both indicate that it is active and the differences are probably due to different test systems used to determine activity.

The results of the present study indicate that valproic acid is not active in preventing acute toxoplasmosis in mice. The highest oral daily dose of 800 mg/kg/day we tested is approaching the oral LD50 dose of this agent in mice (1,098 mg/kg); the highest i.p. dose of valproic acid tested, 600 mg/kg/day, is greater than the LD50 in mice for this route of administration (470 mg/kg) (American Pharmaceutical Partners, Inc., Bedford Labs, Bedford, Ohio). Therefore, further increasing the dose of valproic acid is impractical. In contrast, Webster et al. (2006) demonstrated that oral treatment of chronically *T. gondii*–infected rats with 40 mg/kg/day of valproic acid were beneficial in their study system. Treated *T. gondii*–infected rats retained their innate avoidance of feline smell (cat urine). The mode of action of valproic acid, including which stage of *T. gondii* is affected by valproic acid in the rats, is not known. Because the rats were chronically infected, it is possible that valproic acid acted on the tissue cyst/bradyzoite stages of *T. gondii*. Valproic acid might also influence the bradyzoite-to-tachyzoite or tachyzoite to bradyzoite stage conversion. We did not demonstrate a significant ($P<0.05$) effect of valproic acid on the numbers of tissue cysts in treated mice versus controls. Additional study is needed to examine the activity of valproic acid and similar agents against the tissue cysts/bradyzoites of *T. gondii*.

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References

BACHMANN, S., J. SCHRODER, C. BOTTMER, E. F. TORREY, AND R. H. YOLKEN. 2005. Psychopathology in first-episode schizophrenia and antibodies to *Toxoplasma gondii*. Psychopathology **38**: 87–90.

BERDOY, M., J. P. WEBSTER, AND D. W. MACDONALD. 2000. Fatal attraction in rats infected with BOWDEN, C. L. 2007. Spectrum of effectiveness of valproate in neuropsychiatry. Expert Reviews in Neurotherapy **7**: 9–16.

BROWN, A. S., C. A. SCHAEFER, C. P. QUESENBERRY, JR., L. LIU, V. P. BABULAS, AND E. S. SUSSER. 2005. Maternal exposure to toxoplasmosis and risk of schizophrenia in adult offspring. American Journal of Psychiatry **162**: 767–773.

HOLLIMAN, R. E. 1997. Toxoplasmosis, behavior and personality. Journal of Infection **35**: 105–110.

JONES, J. L., A. LOPEZ, M. WILSON, J. SCHULKIN, AND R. GIBBS. 2001. Congenital toxoplasmosis: A review. Obstetrical and Gynecological Survey **56**: 296–305.

JONES, D. KRUSZON-MORAN, M. WILSON, G. MCQUILLAN, T. NAVIN, AND J. B. MCAULEY. 2001. *Toxoplasma gondii* infection in the United States: Seroprevalence and risk factors. American Journal of Epidemiology **154**: 357–365.

JONES-BRANDO, L., E. F. TORREY, AND R. H. YOLKEN. 2003. Drugs used in the treatment of schizophrenia and bipolar disorder inhibit the replication of *Toxoplasma gondii*. Schizophrenia Research **62**: 237– 244.

LEDGERWOOD, L. G., P. W. EWALD, AND G. M. COCHRAN. 2003. Genes, germs, and schizophrenia: An evolutionary perspective. Perspectives in Biological Medicine **46**: 17–348.

STROBL, J. S., M. CASSELL, S. M. MITCHELL, C. M. REILLY, AND D. S. LINDSAY. 2007. Scriptaid and suberoylanilide hydroxamic acid are histone deacetylase inhibitors with potent anti-*Toxoplasma* activity *in vitro.* Journal of Parasitology **93**: 694–700

TORREY, E. F., AND R. H. YOLKEN. 2003. *Toxoplasma gondii* and schizophrenia. Emerging Infectious Diseases **9:** 1375–1380.

VYAS, A., S. K. KIM, N. GIACOMINI, J. C. BOOTHROYD, AND R. M. SAPOLSKY. 2007. Behavioral changes induced by *Toxoplasma* infection of rodents are highly specific to aversion of cat odors. Proceedings of the National Academy of Sciences of the United States of America **104**: 6442–6447.

WANG, H. L., G. H. WANG, Q. Y. LI, C. SHU, M. S. JIANG, AND Y. GUO. 2006. Prevalence of *Toxoplasma* infection in first-episode schizophrenia and comparison between *Toxoplasma*-seropositive and *Toxoplasma*-seronegative schizophrenia. Acta Psychiatrica Scandinavica **114**: 40–48.

WEBSTER, J. P. 2001. Rats, cats, people and parasites: The impact of latent toxoplasmosis on behaviour. Microbes and Infection. **3**: 1037–1045.

WEBSTER, P. H. LAMBERTON, C. A. DONNELLY, AND E. F. TORREY. 2006. Parasites as causative agents of human affective disorders? The impact of anti-psychotic, mood-stabilizer and anti-parasite medication on *Toxoplasma gondii*'s ability to alter host behaviour. Proceedings of the Royal Society B **273**: 1023–1030.

YOLKEN, R. H., S. BACHMANN, I. RUSLANOVA, E. LILLEHOJ, G. FORD, E. F. TORREY, AND J. SCHROEDER. 2001. Antibodies to *Toxoplasma gondii* in individuals with first-episode schizophrenia. Clinical Infectious Diseases **32**: 842–844.

TABLES

Table I. Results of Experiment 1. Protocol and results of Experiment 1 on 1.5 mg/ml dose of valproic acid given in the drinking water on acute toxoplasmosis in mice.

Group	Mouse number	Dose of valproic acid*	Day post inoculation died/killed
1	1	1.5 mg/ml	Died 9
1	2	1.5 mg/ml	Died 10
1	3	1.5 mg/ml	Died 11
1	4	1.5 mg/ml	Died 11
1	5	1.5 mg/ml	Died 12
2	6	1.5 mg/ml	Died 10
2	7	1.5 mg/ml	Died 11
2	8	1.5 mg/ml	Died 12
2	9	1.5 mg/ml	Died 12
2	10	1.5 mg/ml	Died 13
3	11	None	Died 10
3	12	None	Died 10
3	13	None	Died 11
3	14	None	Died 11
3	15	None	Died 11
4	16	None	Died 10
4	17	None	Died 11
4	18	None	Died 11
4	19	None	Died 11
4	20	None	Died 12

* Provided continuously in water in lightproof drinking bottle to provide an estimated total dose of 250mg/kg/day**.**

Table II. Results of experiment 2**.** Protocol and results of Experiment 2 on 3-mg/ml dose of valproic acid given in the drinking water on acute toxoplasmosis in mice.

Group	Mouse number	Dose of valproic acid*	Day postinoculation died/killed
5	21	3.0 mg/ml	Died 11
5	22	3.0 mg/ml	Died 11
5	23	3.0 mg/ml	Died 12
5	24	3.0 mg/ml	Died 12
5	25	3.0 mg/ml	Died 12
6	26	None	Killed 10
6	27	None	Killed 10
6	28	None	Died 10
6	29	None	Killed 11
6	30	None	Killed 11

* Provided continuously in water in lightproof drinking bottle to provide an estimated total dose of 545mg/kg/day**.**

Table III. Results of experiment 3. Protocol and results of experiment 3 examining 2 doses of valproic acid given intraperitoneally every 12 hr on acute toxoplasmosis in mice.

Group	Mouse number	Total dose of valproic acid*	Day post inoculation died/killed
7	31	400 mg/kg	Died 9
7	32	400 mg/kg	Killed 9
7	33	400 mg/kg	Died 10
7	34	400 mg/kg	Killed 10
7	35	400 mg/kg	Killed 10
8	36	400 mg/kg	Died 8
8	37	400 mg/kg	Killed 9
8	38	400 mg/kg	Killed 10
8	39	400 mg/kg	Died 10
8	40	400 mg/kg	Killed 10
9	41	600 mg/kg	Died 9
9	42	600 mg/kg	Killed 10
9	43	600 mg/kg	Died 10
9	44	600 mg/kg	Killed 10
9	45	600 mg/kg	Killed 10
10	46	600 mg/kg	Killed 9
10	47	600 mg/kg	Killed 10
10	48	600 mg/kg	Killed 10
10	49	600 mg/kg	Killed 10
10	50	600 mg/kg	Killed 10
11	51	None†	Killed 9
11	52	None	Killed 10
11	53	None	Died 10
11	54	None	Died 10
11	55	None	Killed 11

* Given intraperitoneally as 200 or 300 mg/kg every 12 hr.

† Sterile saline given intraperitoneally every 12 hr.

Table IV. Results of experiment 4. Protocol and results of experiment 4 on 6.6mg/ml dose of valproic acid given in the drinking water* for 10 wk on chronic toxoplasmosis in mice

Group	Mouse number	Dose of valproic acid	Inoculated with *Toxoplasma gondii*	Number of tissue cysts
12	56	3.0 mg/ml	Yes	0
12	57	3.0 mg/ml	Yes	1
12	58	3.0 mg/ml	Yes	1
12	59	3.0 mg/ml	Yes	3
13	60	None	Yes	0
13	61	None	Yes	1
13	62	None	Yes	3
13	63	None	Yes	4
14	64	3.0 mg/ml	No	0
14	65	3.0 mg/ml	No	0
14	66	3.0 mg/ml	No	0

* Provided continuously in water in lightproof drinking bottle to provide an estimated total dose of 800mg/kg/day**.**

RH: CONGENITAL *T. GONDII* AND BEHAVIOR

INVESTIGATION OF AGE AND SEX RELATED CHANGES IN BEHAVIOR AND NEUROTRANSMITTER CONCENTRATIONS IN MICE CONGENITALLY INFECTED WITH *TOXOPLASMA GONDII*

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ABSTRACT

We examined the effect of maternal *Toxoplasma gondii* infection on the behavior and neurotransmitter concentrations of congenitally infected female and male CD-1 mice at 4 and 8 wk. of age. This is a time when latent tissue cysts would be present in their brains. Because of sex associated behavioral changes that develop during aging, infected female mice were compared to control female mice and infected male mice were compared with control male mice. Female and male congenitally infected mice showed minimal behavioral differences from uninfected mice at 4 wk. of age in 4 different behavioral tests used to measure diverse behaviors. At 8 wk. of age, congenitally infected female mice remained similar to uninfected female controls. Infected male mice at 8 wk. were significantly different for increased random search technique ($P \leq 0.077$), increased activity ($P \leq 0.080$) and displayed significantly decreased memory ($P \leq 0.005$) compared to sero-negative male mice. This indicates 8-wk-old male mice suffered adverse spatial memory and rate of learning defects from congenital toxoplasmosis. Neurotransmitters and their metabolites (dopamine, 3,4-dihydroxyphenylacetic acid, homovanillic acid, norepinephrine, epinephrine, 3-methoxy-4-hydroxyphenylglycol, serotonin, and 5-hydroxyindoleacetic acid) concentrations in the frontal cortex and striatum were not different ($P > 0.1$) between infected and control mice when measured at 8 wk. of age. Results of our study indicate that congenitally *T. gondii* infected male mice suffer adult onset behavioral deficits. The exact mechanism for the observed behavioral changes is not known and further investigation may help elucidate

the molecular mechanism associated with the proposed link between adult onset schizophrenia or other behavioral changes and *T. gondii* infection in humans.

INTRODUCTION

Toxoplasma gondii is an obligate intracellular protozoan parasite able to infect most warm-blooded animals. The parasite has a heteroxenous life cycle with felids serving as the only known definitive host. Rodents in particular have been studied extensively as intermediate hosts of *T. gondii* because they serve as a model for human infections and are commonly preyed upon by felids. Transmission of infection to the intermediate host can occur naturally in 1 of 3 ways, i.e., by ingestion of sporulated oocysts, by ingestion of tissue cysts, or by congenital transmission of tachyzoites. Congenital transmission can occur if a pregnant female acquires a primary *T. gondii* infection allowing widespread bodily dissemination and infection of the fetus.

Congenital *T. gondii* infections are manifest in several ways. Clinical outcomes of infection can range from spontaneous abortions to only very mild symptoms in the offspring that are not apparent until later in life (see Jones et al., 2001). Severe developmental defects, including intra-cerebral calcification, hydrocephalus, and retinocorditis, can result from congenital toxoplasmosis. This wide range of symptoms results from several factors. The most important aspect is when fetal infection occurs during pregnancy. If the fetus is infected early in development, the outcome is more severe (Jones et al., 2001). Fetal infection early in pregnancy occurs at a lower frequency than infection later in pregnancy. Conversely, fetal infection later in pregnancy usually results in less severe disease and has a more favorable outcome for the fetus.

Many mental illnesses associated with cognitive impairments have no known etiology. It is believed that genetic and environmental factors contribute to the development of several mental health disorders associated with cognitive impairments. Suspected environmental factors include exposure to infectious agents such as *T. gondii*. Chronic postnatal *T. gondii* infections have been correlated with human behavior changes (see Webster and McConkey, 2010). Rodents infected postnatally with *T. gondii* and tested after the infection has become chronic, exhibit increased open field activity, decreased exploration, and loss of neophobia (Hutchinson et al., 1980; Hay et al., 1984; Webster et al., 1994; Vyas et al., 2007). People living with mental health disorders, associated with cognitive dysfunction, have been shown to have a decreased intelligence quotient, decrease rate of learning, difficultly interacting with society, and may have memory impairment. The dopamine hypothesis as a potential cause of cognitive deficits proposes that hyperfunction of dopamine neurotransmission is involved in prefrontal cortex functions, such as rate of learning and memory. Chronic toxoplasmosis has been reported to increase levels of dopamine in mice, consistent with the dopamine hypothesis (Stibbs, 1985). Many of the behavior changes in mice are similar to the behavior changes observed in humans living with cognitive impairments.

In addition to genetic and environmental factors, stress experienced in utero (McClellan et al., 2006) is also currently being investigated as a potential cause of cognitive impairments. In 2 retrospective studies, (Buka et al., 2001; Brown et al., 2005) archived maternal blood samples revealed that a significant number of offspring that developed schizophrenia, often associated with cognitive impairment, had been exposed to *T. gondii* infection in utero. It has also been suggested that maternal

exposure to *T. gondii* infection is a risk factor for the development of psychoses other than schizophrenia (Brown et al., 2005; Mortensen et al., 2007; Xiao et al., 2009). Based on the aforementioned findings, we hypothesized that congenital *T. gondii* infection will result in exploratory, learning/memory, sensory, and motor behaviors that are significantly different between infected and uninfected mice and that dopamine levels will also be higher in infected mice. Since the onset of most mental illness is observed in late adolescence or early adulthood (Jablensky, 1997; McGorry et al., 2010), we examined congenitally *T. gondii* infected mice pre- and post-sexual maturity to determine if their behavior was different from uninfected age- and sex-matched controls.

MATERIALS AND METHODS

Congenital infections

A total of 80 female and 40 male 5 wk. old CD-1 mice were used to produce the congenitally infected offspring used in this study. Food and water were given ad libitum for the duration of the experiment. The Institutional Animal Care and Use Committee, Virginia Tech, approved the study. Because the experiment examined congenital transmission of *T. gondii* infections, surrogate dams were used to eliminate negative effects of *T. gondii* infection on maternal parenting and lactation. Mice were acclimated to cages for 9 days. Eighty female mice were randomly allocated (40/40) into 3 groups; 20 *T. gondii* infected pregnant, 20 non-infected pregnant (negative controls), and 40 surrogates. The surrogates were used for both *T. gondii* infected mice and non-infected negative control mice. Two females were co-housed with 1 male and the females examined every morning for the presences of a vaginal plug. Plug positive females were removed and this was considered day 0 of pregnancy for the dams.

Experimental groups contained 20 control (not infected with *T. gondii*) and 20 *T. gondii* infected pregnant dams. Control mice received 500 µl of Hanks' balanced salt solution (HBSS, Cellgro, Mediatech, Manassas, Virginia) orally on day 11 of pregnancy. *Toxoplasma gondii* infected mice received 30 VEG tissue cysts (*T. gondii* genotype III) orally, in 500 µl HBSS on day 11 of pregnancy. These cysts were collected from the brains of chronically infected CD-1 mice. All experimental dams (control and infected,

but not surrogates) were killed after parturition and their pups moved onto an age matched surrogate dam.

Pups were handled regularly pre- and post-weaning to acclimate them to handling. Pups were weaned at 3 wk. of age. Mice were bled and ear notched for identification at 3.5 wk. of age. Their sera were examined for *T. gondii* antibodies at a 1:50 dilution using the RH strain of *T. gondii* as antigen and immunofluorescent antibody (IFA) test procedures routinely employed in our laboratory (Lindsay et al., 1990). This established the infection status of pups and the percent transmission for pups born to infected females. This age was chosen because maternal IgG disappears in the pups after 3 wk. (Whitelaw and Urquhart, 1985). Five seropositive mice of the same sex born to each infected mother, and 5 seronegative mice of the same sex born to each HBSS treated mother were saved for behavior testing. All behavior assessments were made blind to treatment groups. Behaviors were examined at 4 and 8 wk. because *T. gondii* tissue cysts are present in the brain at that time (Dubey 1997).

At the end of the experiment, each mouse used for 8 wk. Barnes maze testing was killed by cervical dislocation for brain dissections and neurotransmitter measurements. The remaining mice were killed by CO_2 asphyxiation and the brain from 1 mouse in each group was used for frontal cortex brain smears to determine the presence of *T. gondii* tissue cysts.

Data analysis: Transmission rates were compared between males and females using logistic generalized estimating equations (GEE) (proc genmod). The linear model specified pup sero-status (positive vs. negative) as the outcome, sex as the predictor,

logit as the link function, and binomial as the distribution. To request GEE, dam ids were specified as the clusters with an exchangeable working correlation matrix. *P*-values were based on type 3 Wald statistics. Sex ratios were compared between treatment groups using the Wilcoxon rank sum test (proc npar1way).

Barnes maze apparatus and testing

The Barnes maze is a spatial memory and learning test. The maze is used to examine short-term memory, acquisition of tasks, open field activity, and exploratory activity (Bach et al., 1995). The Barnes maze apparatus consisted of a circular plexiglass platform 90 cm in diameter with 16 holes (each 5 cm in diameter) equally spaced around the perimeter, 5 cm from the edge, and 10.5 cm from each other (Fig. 1). The maze was mounted on a stand 91.5 cm from the floor. There were 2 locations, 180 degrees apart, for an escape box (l x w x h = 19 cm x 9 cm x 7.5 cm) to be mounted underneath a hole by a drawer mechanism. At the start of each trial, a mouse was placed in the center of the platform in a square start chamber (8.8 cm in diameter and 8.1 cm in height). Visual cues were set up approximately 20 cm above and 45-50 cm from the horizontal edge of the circular maze. Cues were positioned in such a way as to not be identifiable with a specific hole. Video imaging equipment was set up approximately 149 cm above and 12 cm from the horizontal edge of the circular maze. The maze was set in the corner of a room with a black curtain used for the other 2 walls. The corner of the room had white walls with black cues taped to the wall. The other 2 sides were black and had white cues attached to the curtain. The black curtain was positioned to block the experimenter from view. A 150 W light was suspended 106 cm, almost directly above the center of the circular platform and a fan (Massey 8” High

Velocity Personal Fan, Bentonville, Arkansas) was placed 68.5 cm above the maze pointed directly down at the center of the maze. The light and fan served as 2 forms of adverse stimuli.

Each mouse was randomly assigned, by a coin toss, to 1 of the 2 escape box locations, which remained the same throughout the testing period. Testing was conducted daily for 7 days. On day 1, each mouse was placed on the maze for 30 sec, without aversive stimuli and then physically placed in its designated goal box for 1 min. On day 1, the mice underwent training consisting of 2 test trials through the maze. On days 2-5, the testing consisted of 2 consecutive trials followed by a third trial after 3-4 hr. rest. On the 6th and 7th day, each mouse was evaluated twice and once, respectively, totaling 17 trials per mouse.

For each trial, the mouse was placed in the center of the platform in a start chamber (Fig. 1A). Once placed on the platform the fan and light were switched on, and the mouse was kept in the start chamber for 10 sec. After 10 sec, the start chamber was lifted and the trial began. The trial ended once the mouse entered the goal box (forelimbs only, or both forelimbs and hind limbs), located the goal box (4 consecutive nasal investigations), or after a trial time of 5 min without entry or location of the goal box. When the mouse entered the goal box, the trial was stopped and the fan and light were turned off. If the mouse had located the goal box but did not enter, the trial was stopped, and the mouse was then guided into the goal box, and the fan and light were turned off. If the mouse did not locate or enter the goal box after 5 min, the trial was stopped, the mouse was placed into the goal box, and the fan and light were turned off. After 30 sec in the goal box the mouse was returned to its home cage. Following a 3-5

min waiting period, the mouse was placed on the platform for the second daily trial. After the third trial, the mouse was returned to its home cage until the next day's session. The maze and goal box were cleaned with 70% ethanol after every trial.

Each trial was recorded digitally and then viewed at a later date for quantitative analysis. Video equipment consisted of a Sony-DCR-TRV530 digital 8 camcorder connected via a Firewire cable to a Mac G. Imovie was used to capture and save each trial. Quicktime ® media player was used to view the video files.

Barnes maze behavioral measures (Table I) were viewed and graded by 1 person (DGG) who was blind to the treatment status of the mice. The recorded videos were scored on inactive time, memory (distance to goal), distance, activity, random search strategy (center crossings), and latency.

Inactive time was the amount of time a mouse remained motionless in the center of the maze at the beginning of the trial. Inactive time is the amount of time required for a mouse to initially assess its environment from visual cues. Short-term memory was the distance between the goal box and the first hole investigated. For example, if the first hole was adjacent to the goal box then the distance to goal value was recorded as 1. Short-term memory is indicative of how well the mouse recognized it's surrounding and remembered where the goal box was positioned (Bach et al., 1995). Distance was measured as how far the mouse traveled either before finding the goal box or the distance traveled during the 5-min observation time if the mouse was not successful in finding the goal box. Errors were recorded as the visitation to a hole other than the goal hole (Fig. 1C). Activity was calculated by the number of holes investigated divided by

the active time (active time is overall time minus the inactive time) and defined as the amount of time spent investigating/exploring each hole. Random search strategy (center cross) (Fig. 1B) was defined as passing through the center of the maze, or as skipping more than 4 holes when traveling from hole to hole, or as walking into the center of the maze and back out again. Random search strategy is a precursory approach where the mouse randomly searches for the goal box without a well-defined strategy. Acquiring the task was defined by a direct route to the goal box with errors ≤ 2 in a trial. A mouse was considered to have acquired the task (learned the maze) if 3 of 4 trials on 2 consecutive days were successful at any point during the 7-day trial period (Fig. 1D). Latency was the time elapsed from trial start to finish and is not related to task acquisition, but does indicate rate of learning.

The Barnes maze test was used to test 6 seronegative female mice, 8 *T. gondii*-infected female mice, 7 seronegative male mice and 7 *T. gondii*-infected male mice at the 4-wk time point. At the 8-wk time point 5 seronegative female mice, 7 *T. gondii*-infected female mice, 6 seronegative male mice and 7 *T. gondii*-infected male mice were tested with the Barnes maze.

Data analysis: Scatter plots for outcome against trial number showed that Latency, Active time, Distance and Errors followed an exponential decay. Subsequently, half-lives were generated for each of the four outcomes (for each mouse). The half-lives were then compared between the treatment groups using the Wilcoxon rank sum test. For outcomes that were slightly skewed (inactive time, distance to goal, activity, and center crosses), the treatment groups were compared using linear GEE. The linear model specified behavioral measure as the outcome, treatment as a predictor, identity

as the link function, and normal as the distribution. To request GEE, mouse ids were specified as clusters with an exchangeable working correlation matrix. *P*-values were based on the type 3 Wald statistics.

Functional observation battery tests

The FOB tests are designed to detect physiological changes, coordination, and startle and menace responses in mice (Moser et al., 1988; King et al., 2003). The specific physiologic parameters evaluated are listed in Table II. All of these parameters were graded on a “yes” or “no” scale. The FOB tests also evaluate coordination, startle response, and menace. The coordination of the mice was measured using 2 tests. The first test was the righting reaction test. This righting reaction test determines the mouse’s ability to right itself when placed on its back. The second test was performed to see how well the mice hold onto a wooden rod. The startle response was elicited using a clicker. The clicker was clicked approximately 6-8 cm behind the mouse’s head and was scored as “Yes” if the click elicited a flinch or startle, or “No” if no response was elicited. The FOB also examined menace response and this was done by placing a pencil about 1-2 cm from the face of the mouse. If the mouse looked or turned away then the mouse was scored with a No, no menace response. If the mouse acted aggressively to the pencil end the mouse was scored as “Yes”, acts menacingly to an object.

The FOB test used 9 seronegative female mouse litters, 4 *T. gondii*-infected female mouse litters, 8 seronegative male mouse litters and 5 *T. gondii*-infected male

mouse litters for the 4-wk time point. At the 8-wk time point**,** 7 seronegative female mouse litters, 8 *T. gondii*-infected female mouse litters, 7 seronegative male mouse litters and 7 *T. gondii*-infected male mouse litters were used for FOB testing.

Data analysis: The treatment groups were compared using logistic generalized estimating equations (proc genmod). The linear model specified FOB test as the outcome, Treatment as a predictor, logit as the link function, and binomial as the distribution. To request GEE, dam ids within treatment were specified as the clusters with an exchangeable working correlation matrix. *P*-values were based on the type 3 Wald statistics.

Visual placement

Vision and coordination of mice was evaluated by the visual placement test (Fox et al., 1965). The test was performed by holding the mouse upside down by its tail, so that its head was 3.5 to 5 cm from the edge of the counter top and approx. 2.5 cm below the surface of the counter top. This encourages the mouse to reach up and out to the counter top to keep from dangling upside down. The mouse was then scored on a 3 point scale, i.e., (1) the mouse made no attempt to reach out, (2) the mouse attempted to reach out but could not grab, and (3) the mouse reached out and grabbed the edge of the table.

The visual placement test used 9 seronegative female mouse litters, 4 *T. gondii*-infected female mouse litters, 8 seronegative mouse male litters and 5 *T. gondii*-infected male mouse litters for the 4-wk time point. At the 8-wk time point**,** 7 seronegative female mouse litters, 8 *T. gondii*-infected female mouse litters, 7 seronegative male mouse

litters and 7 *T. gondii*-infected male mouse litters were used for visual placement testing.

Data analysis: Only scores 2 and 1 were observed and recorded. Accordingly, the treatment groups were compared using logistic generalized estimating equations (proc genmod). The linear model specified score as the outcome, Treatment as a predictor, logit as the link function, and binomial as the distribution. To request GEE, dam ids within treatment were specified as the clusters with an exchangeable working correlation matrix. *P*-values were based on the type 3 Wald statistics.

Virtual cliff

The virtual cliff (Adams et al., 2002) consisted of a box 38 x 38 x 38 cm. The top surface of the box a piece of plexiglass that was half clear and half opaque. The virtual cliff was positioned 38 cm off the bottom of the box. Mice were placed in the center of the cliff with 1 front and hind limb of 1 side of the body on the clear portion and the contralateral limbs on the opaque portion. Mice were tested 3 times and the initial position remained constant. Mice were scored depending on which side of the plexiglass they traveled to, a score of 1 for the clear side and a score of 2 for the opaque side.

The virtual cliff test used 9 seronegative female mouse litters, 4 *T. gondii*-infected female mouse litters, 8 seronegative male mouse litters**,** and 5 *T. gondii*-infected male mouse litters for the 4-wk time point. At the 8-wk time point, 7 seronegative female mouse litters, 8 *T. gondii*-infected female mouse litters, 7 seronegative male mouse litters**,** and 7 *T. gondii*-infected male mouse litters were used for virtual cliff testing.

Data analysis: The number of times a mouse went to the opaque side was used as the outcome for this section (score range 0 to 3). After scoring, the treatment groups were compared using linear generalized estimating equations (proc genmod). The linear model specified score as the outcome, Treatment as a predictor, identity as the link function, and normal as the distribution. To request GEE, dam id within treatment were specified as clusters with an exchangeable working correlation matrix. *P*-values were based on the type 3 Wald statistics.

Rearing and Open Field Activity

Rearing tests look specifically at exploration of novel environments (Hutchinson et al., 1980). Four mice from each litter were individually placed in a box (20 cm x 42 cm) for 5 min. The numbers of times a mouse reared or lifted its forelimbs off the ground in an exploratory manner were counted. The box was cleaned with 70% ethanol between each mouse. The rearing test was not conducted at 4 wk. The rearing test used 6 seronegative female mouse litters, 9 *T. gondii*-infected female mouse litters, 6 seronegative male mouse litters**,** and 7 *T. gondii*-infected male mouse litters for the 8-wk time point.

Open field activity testing was carried out using San Diego Instruments Photobeam Activity System and a clear 20 cm x 42 cm cage. The cage had a metal halo that surrounded the perimeter of the box, with 3 laser beams that were evenly spaced 10 cm apart dividing the box in to 4 evenly spaced sections 20 X 10 cm. When the mouse moved from 1 quadrant to another quadrant the laser beam was broken. A record of the beam breaks was recorded onto the computer. Mice were left in the open

field for 5 min, while beam breaks were recorded. The computer measured ambulations and beam breaks. The box was cleaned with 70% ethanol between every mouse tested. Ambulations were recorded as movement of a mouse through at least 3 of the 4 quadrants without back tracking. Beam breaks occurred when the mouse broke the beams in a non-successive order, indicating non-specific activity in the box.

The open field activity test was not conducted at 4 wk. The test used 7 seronegative female mouse litters, 7 *T. gondii*-infected female mouse litters, 7 seronegative male mouse litters**,** and 7 *T. gondii*-infected male mouse litters for the 8-wk observation time point.

Data analysis: Effect of treatment on rearing was assessed using mixed model ANOVA (proc mixed). The linear model included rearing as outcome, treatment as a fixed effect, and litter Id within treatment as the random effect. Ambulations and beam breaks were compared between treatments at each time point using mixed-model repeated-measures ANOVA (proc glimmix). The linear model included ambulation or beam breaks as outcome, treatment as a fixed effect, and mouse within treatment as a random effect (G side). For the R side of the model, an autoregressive 1 correlation matrix was specified. Denominator degrees of freedom were specified as KenwordRoger.

Neurotransmitter determinations

Mice that completed the 8-wk Barnes maze test were used for neurotransmitter isolation and determination. The whole brain was removed and placed on ice, then cut in half along the sagittal plane. The striatum from both the right and the left hemispheres

was removed and placed into a microfuge tube on ice. The frontal cortex from the right hemisphere was removed and placed in a microfuge tube on ice. The tissue was then weighed and 6 times the v/w of isoproterenol buffer (4.7 pH) was added to the tissue for preservation of neurotransmitters. The brain tissue was then homogenized on ice followed by micro-centrifugation at 4 C for 10 min at 15,000 g. The supernatants were stored at -80 C until analysis by high performance liquid chromatography (HPLC) was conducted. Striatal and cortical samples were analyzed for dopamine and its metabolites, 3,4-dihydroxyphenylacetic acid and homovanillic acid, in addition norepinephrine, epinephrine**,** and the metabolite 3-methoxy-4-hydroxyphenylglycol, and serotonin and its metabolite, 5-hydroxyindoleacetic acid, were also analyzed.

Samples were examined in a HPLC Agilent Technologies (Wilmington, DE) 1100 Series equipped with a degasser, a quaternary pump, a refrigerated auto sampler (set at 4 ºC) and connected to a Waters 2465 electrochemical detector (Milford, MA). A C18 column (Macherey Nagel Bethlehem, PA) (Pyramid EC 250 x 4 mm i.d., 3 µm) was used with a flow rate of 0.7 ml/min. Column temperature was maintained at 35 ºC. The mobile phase composition was 5% methanol in aqueous sodium acetate (50 mM), citric acid (12.5 µM), EDTA (134 µM), octane sulfonic acid (230 µM), sodium chloride (2 mM), pH 4.7. Isoproterenol (1 µM) was used as the internal standard, and calibration curves of each neurotransmitter and metabolite were prepared using solutions of 10, 50, 100, 250**,** and 500 nM.

Data analysis: Neurotransmitter concentrations were compared between treatment groups using one-way analysis of variance (proc mixed). The linear model specified neurotransmitter as outcome, treatment as a fixed effect, and residual as the

denominator degrees of freedom. While the effect of treatment on epi was assessed using Wicoxon rank sum test.

Brain smears

One mouse from each litter was examined for the presence of tissue cysts at the termination of the experiment. The cranial cavity of the mouse was opened and approximately 0.1 g of the frontal cortex was removed placed on a slide, crushed with a 22 x 22 mm^2 cover slip and examined at 20x using an Olympus BX60 microscope for the presence or absence of tissue cysts.

Statistical considerations for all sections

Data were analyzed using SAS version 9.2 (Cary, North Carolina). Because initial findings demonstrated sex associated differences in mouse behavior between 4 and 8 wk. of age, mice were examined as 4 groups consisting of 4-wk-old female mice, 8-wk-old female mice, 4-wk-old male mice, and 8-wk-old male mice. Statistical significance was set to $P<0.1$.

RESULTS

Congenital transmission

Fifteen of 20 *T. gondii* infected dams produced litters resulting in 8 liters of *T. gondii*-infected females and 7 liters of *T. gondii*-infected males. Twelve of 20 control dams produced litters resulting in 6 liters of female and 7 liters of males. Congenital transmission of *T. gondii* was detected by IFA testing for 14 of 15 litters, using our inoculation schedule. Of the 15 litters exposed to *T. gondii* in utero, there was a transmission success of 70% for males and 74% for females. No statistical differences (P>0.1) in transmission success were detected between males and females. Of the 12 HBSS-treated litters, none of the litters tested positive for *T. gondii.* The male to female pup ratio was 1.3 for *T. gondii*-infected litters and 1.0 for control litters (one sided P-value 0.09). .

Behavior

The results of Barnes maze behavioral testing are summarized in Table **I**.

Behavior of congenitally infected female mice compared to seronegative control females in the Barnes maze

At 4 wk. of age, *T. gondii*-infected female mice spent 16.8 sec sitting in the middle of the maze compared to 6.1 sec for the female controls (a difference of 10.7 sec with a standard error of 5.260) (P=0.041). Similarly *T. gondii*-infected female mice crossed the center of the maze on average 0.65 times per maze trial, while control mice

only crossed the center of the maze 0.22 times per maze trial (a difference of 0.43 with a standard error of 0.261) (*P*=0.101).

At 8 wk. of age, *T. gondii*-infected female mice had an average of 0.13 center crosses per trial, while control female mice had an average of 0.30 center crosses per trial (a difference of 0.17 with a standard error of 0.081) (*P*=0.033).

Behavior of congenitally infected male mice compared to seronegative control males in the Barnes maze

At 4 wks. the first holes investigated by *T. gondii*-infected male mice were on average a distance of 3.1 holes from the goal box**,** while control mice first investigated a hole that was on average 4.1 holes from the goal box (a difference of 1 hole with a standard error of 0.476)(*P*=0.054).

At 8 wk. of age**,** the first hole investigated by infected male mice was 4.1 holes from the goal box compared to 3.0 holes for control male mice (a difference of 1.1 holes with a standard error of .389) (*P*=0.005). *Toxoplasma gondii*-infected male mice had an overall effect of treatment for difference in time spent investigating each wrong holes (*P*=0.006). Similarly, infected male mice spent more time than controls investigating wrong holes (an average of 5.1 sec/hole vs. 11.4 sec/hole; and a difference of 6.3 sec/hole with a standard error of 3.624) (*P*=0.080). Finally infected males had on average twice as many center crosses, 0.37, compared to 0.18 center crosses /per trial for control mice (a difference of 0.19 and a standard error of 0.109) (*P*=0.077).

FOB

At 4 wks., *T. gondii*-infected female mice were 6 times more likely to respond to the click test compared to the control mice (odds ratio 6.9, 95% confidence interval 1.2 to 30.5) (*P*=0.03). The FOB tests showed that infected male mice 4-wk-old were 80% less likely than the controls to complete the righting test (odds ratio 0.2, 95% confidence interval 0.1 to 1.2) (*P*=0.077). At 8-wk-old, no differences (*P*>0.1) in behavior were detected for both female and male mice tested with the FOB.

Virtual cliff and Visual Placement

At 4 wks., *T. gondii*-infected males went to the opaque side of the virtual cliff an average of 1.9 times (out of three trials) compared to 1.6 times in control mice (a difference of 0.3 and a standard error of 0.17) (*P*=0.053). Conversely, infected female mice at 4-wk-old went an average of 1.4 times to the opaque compared to 1.8 times for control female mice (a difference of 0.4 with a standard error of 0.1) (*P*=0.0007). No statistical differences (*P*>0.1) were observed for male or female mice in the virtual cliff test conducted at 8 wk. of age.

No significant differences (*P*>0.1) between infected and control mice were found for male or female mice at either 4- or 8-wk time points, when visual placement was assessed.

Activity and rearing

No significant differences (*P*>0.1) were detected in any group of mice for activity (ambulations and beam breaks) or rearing.

Neurotransmitter concentrations

Neurotransmitters were only measured after the completion of the Barnes maze at the 8-wk time point. There were no significant differences (P>0.1) in neurotransmitter or metabolite concentrations for either *T. gondii*-infected females or males compared to controls, data not shown.

Brain smears

Twelve of the 15 mice representing each infected litter tested positive for *T. gondii* via IFA. Of these 12 positive mice**,** 11 tested positive for tissue cysts in the frontal cortex. None of the mice from control litters tested positive for *T. gondii* by IFA or brain smear.

DISCUSSION

CONGENITAL TRANMISSION RATE OF *Toxoplasma gondii*

Congenital *T. gondii* transmission rates vary during the course of pregnancy when exposure occurs (Ocampo and Duarte-Gandica et al., 2010). The highest rate of maternal to fetal transmission in mice occurs between days 10 to 12 of pregnancy for mice. A transmission success of 73% in the present study is comparable to 90% reported by others (Wang et al., 2011). Equal transmission to male and female mice indicates both sexes were equally susceptible to congenital transmission of *T. gondii*. While the transmission rate to both sexes remained consistent we found more male mice were born to dams infected with *T. gondii* during pregnancy. Our findings are consistent with a 2007 report where mice with late acute infection/early chronic infection gave birth to more male mice (Kankova et al., 2007). The effects of congenital *T. gondii* infection on mice pre- and post-sexual maturity have not been previously been studied and**,** while the rate of transmission to male and female mice was equal, our study indicates that the behavioral manifestations of congenital infection differ in male and female mice.

Toxoplasma gondii infection and pre sexual maturity

We chose the Barnes maze to evaluate mouse behavior because it evaluates many facets of learning at once. Results of the Barnes maze test in the present study indicated that the method of learning differed between infected and uninfected mice both pre- and post-sexual maturity. *Toxoplasma gondii*-infected female mice at 4 wk. of

age have an increased initial assessment of their environment (*P*=0.041) in conjunction with increased random search strategy that approached significance (*P*=0.101). Together**,** these results indicated that the mice show a diminished ability to recognize their environment and decreased learning. This is consistent with studies indicating that chronically infected mice spend more time in the open field than seronegative control mice. Impaired memory and learning was previously reported in mice chronically infected with *T. gondii* by use of an 8-arm radial maze (Hodkova et al., 2007) and Y maze test (Hay et al., 1984). A decreased rate of learning, similar to our findings in female mice, has also been documented in humans experiencing cognitive deficits (Rossi et al., 1997). While these sets of behavior changes were not permanent and dissipated between 4 and 8 wk. of age, further investigation should be carried out to characterize these impairments in environmental assessment and learning in congenitally infected mice.

Toxoplasma gondii infected 4-wk-old male mice performed significantly better (*P*=0.054) than control mice the short-term memory portion of the Barnes maze test. The finding did not indicate *T. gondii* infected mice solved the maze at a faster rate than uninfected controls (*P* >0.1). Our results suggest infections with *T. gondii* in female and male 4-wk-old mice may change behavior transiently, perhaps by causing a delay in neurodevelopment caused by infection or is sex dependent and changes with age. Similarly**,** people living with cognitive deficit disorders show a shift in predominant symptoms during the course of the disease.

Infected female mice had a significantly increased (*P*=0.033) startle response to stimuli at 4 wk. of age. An increased startle response has not previously been reported

for mice infected with *T. gondii*. It is well documented in humans experiencing cognitive impairments, frequently have difficulties processing stimuli as indicated by decreased prepulse inhibition (Hazlett et al., 2008). By 8 wk. of age, in females, there was no difference (P>0.1) between infected and seronegative mice for the startle response, however deafness frequently occurs in CD-1 mice later in life and this may have been a contributing factor in our findings at 8 wk. (Shone et al., 1991).

Cognitive impairments and adult onset behavior profiles in mice

The presence of detectable *T. gondii* tissue cysts in the frontal cortex of 92% (11 of 12 mice) of the congenitally infected mice sampled suggests tissue cysts maybe altering some neurochemical pathway. Traditionally, researchers believed tissue cysts were inert and problematic only in recrudescent infections. Gaskell et al. (2009) demonstrated that *T. gondii* has 2 genes coding aromatic amino acid hydroxylases. These 2 hydroxylase genes are capable of synthesizing the precursor molecules of the neurotransmitter dopamine and are expressed in encysted bradyzoites in brain tissue (G. A. McConkey, pers. comm.). Dopamine was originally believed to be involved because dopamine receptor antagonists blocking dopamine reuptake, i.e., haloperidol**,** were effective in people experiencing schizophrenic episodes. It is now thought that dopamine and other neurochemical pathways are contributing factors in psychoses. Research indicates that the presence of tissue cysts may elicit a stronger or even a slightly altered immune response in a sub population not currently well recognized (Hinze-Selch et al., 2007). Clearly**,** there is a gap between the 10% of the US population living with chronic toxoplasmosis (Jones et al., 2007) and the less than 1% of the population living with schizophrenia related cognitive deficits (Regier et al., 1993).

People experiencing psychotic episodes often have an altered perception of reality, perceiving stimuli as a threat when in reality there is no threat. An altered perception for danger of falling is suggested in *T. gondii*-infected mice evaluated using the virtual cliff. Four-wk.-old infected male mice stayed away from the cliff (stayed on the opaque side and away from the clear side) significantly (P=0.053) more often than seronegative control mice**,** suggesting an increased fear of falling. Four-wk.-old female mice demonstrated the opposite result using the same test (P=0.0007). The results indicated congenital *T. gondii*-infection caused a reversal of altered perception of danger between male and female mice at 4 wk. of age. At 8 wk. of age, there were no differences (P>0.1) when *T. gondii*-infected mice were compared to uninfected age and sex matched control mice. The differing results between 4 and 8 wk. indicate altered perception of fear that was age related and as the mice matured the sex related differences in fear dissipated. The difference between male and female mice at 4 wks. of age indicated perception of fear at a young age that was influenced by infection and sex. There are no reports in the literature evaluating mice infected with *T. gondii* using a virtual cliff so our findings are novel and should be explored further.

Infected female mice at 8 wks. of age used the random search strategy significantly (P=0.033) less than control female mice in the present study. Normally**,** decreased random search technique can be a measure of increased memory retention**,** but our data suggest that the decreased random search technique was a result of decreased activity (data not shown) (P>0.1) in congenitally infected female mice. The decrease in activity is not significant**,** but does lend support for an alternative explanation for decreased use of the random search strategy with no increase in overall

maze performance. These results taken together indicate congenitally infected female mice at 8 wks. of age are more sedentary, resulting in a decrease in novelty seeking compared to age matched seronegative controls. The decreased performance by female 8-wk-old congenitally infected mice in the Barnes maze demonstrated that they had decreased random search technique, but no improvement in overall maze performance. Decreased novelty seeking was documented in adult men and women seropositive for *T. gondii* using Cloninger's TCI (Temperament and Character Inventory) test (Skallova et al., 2005). These results were not seen in the male mice in our study. Sex related behavior differences between mice infected with *T. gondii* and those observed in humans with *T. gondii* are similar, but are not identical.

Congenitally infected male mice at 8 wks. of age performed significantly or approached significance worse than uninfected males in 3 measured areas, short-term memory (P=0.005), increased activity (P=0.080), and increased random search strategy (P=0.077). This is consistent with the literature, which indicates chronically *T. gondii*-infected mice have diminished spatial memory (Kannan et al., 2010). Infected male mice spent less time investigating each hole (P=0.080) compared to seronegative control male mice. These observations are similar to those of Hay et al. (1985) who reported that mice chronically infected with *T. gondii* had increased activity when compared to uninfected mice. Congenitally infected male mice used random search strategy twice as frequently as non-infected control male mice, indicating that infected mice were progressing through the stages of learning the maze at a slower rate than control mice (P=0.077). A combination of these 3 measurements provides evidence that congenital *T. gondii* infections cause adult onset cognitive deficits in 8-wk-old male

mice. The decrease in learning observed in the infected male mice is similar to what is reported in people living with cognitive impairments.

Men and women living with schizophrenia, a mental illness associated with cognitive defects, have slightly different symptoms (Cowell et al., 1996). Males tend to experience negative symptoms worse in their 20's**,** while females have milder negative symptoms during their 20's (Rosenfield et al., 2009). Choi et al. (2008) found males in the prodromal stage of schizophrenia have more negative and cognitive impairments than females. How and why the clusters of symptoms are associated with a single sex is unknown. Our model may not emulate the divergence of negative symptoms observed in humans**,** but it does support that cognitive impairments can be caused by infection, similar to cognitive impairments observed in males with prodromal symptoms of first episodes of schizophrenia (Choi et al., 2008).

Toxoplasma gondii **and neurotransmitters**

Little is known about chronic *T. gondii* infection of the brain and its influence on behavior as related to alterations in neurotransmitters. Neurotransmitter measurements for our mice demonstrated no statistical difference ($P>0.1$) between congenitally *T. gondii*-infected mice and seronegative controls for both males and females in cortex or striatum. Our findings conflict with the study of Stibbs (1985) who reported that dopamine was increased by 14% for chronically post-natally *T. gondii*-infected mice compared to seronegative controls. However, we examined specific regions of the brain (striatum and cortex)**,** while he examined the entire brain. This may indicate that regional differences in neurotransmitter levels occur in the brains of mice with chronic *T.*

gondii infection. We found no differences in serotonin levels**,** which is in agreement with Stibbs (1985) findings. Serotonin levels are unlikely to differ with course of infection. This is particularly relevant as the immune response to *T. gondii* infection induces interferon gamma that releases indoleamine 2, 3-dioxygenase degrading tryptophan (MacKenzie et al., 2007). This could decrease serotonin as tryptophan is a precursor to serotonin. We used a congenital model, whereas Stibbs (1985) used a post-natal chronic infection model for his 7-wk-old acutely and 12-wk-old chronically infected female mouse model. It is possible Stibbs (1985) used a higher dose or a more virulent genotype of *T. gondii* for his infections because he did not report the dose or strain of *T. gondii* used for his study. Results from Skallove et al. (2006) suggested that male mice may be more susceptible to subtle neurotransmitter changes than female mice; however they did not examine neurotransmitter concentration in the brains of mice used in their study. Numerous other mouse behavior studies cite Stibbs (1985) for his research demonstrating increased dopamine levels in the brains of mice chronically or acutely infected with *T. gondii.* However, Stibbs (1985) did not test behavior in his mice. The absence of neurochemical changes associated with our behavioral changes suggests that other transmitter systems than those measured may be involved, e.g., an amino acid or peptide, or other mechanisms that change transmitter content or turnover are responsible.

Elucidating a potential environmental factor causing cognitive impairments is an important piece for all mental illnesses. Our mouse model demonstrates that congenital infection with *T. gondii* can result in adult onset behavior deficits similar to those observed in humans with cognitive impairments. Behavior alterations observed prior to

sexual maturity indicate lifelong subtle alterations in brain neurochemistry. Our congenital model is a promising system to further our understanding of the complex relationship between *T. gondii* infection and the development of mental illnesses associated with cognitive impairments.

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REFERENCES

LITERATURE CITED

Adams B., T. Fitch, S. Chaney, and R. Gerlai. 2002. Altered performance characteristics in cognitive tasks: Comparison of the albino ICR and CD1 mouse strains. Behavioural Brain Research **133:** 351-361

Bach, M.E., R.D. Hawkins, M. Osman, E.R. Kandel, and M. Mayford. 1995. Impairment of spatial but not contextual memory in CaMKII mutant mice with a selective loss of hippocampal LTP in the range of the theta frequency. Cell **81:** 905-915.

Brown, A.S., C.A. Schaefer, C. P. Quesenberry, L. Liu, V.P. Babulas, and E.S. Susser. 2005. Maternal exposure to toxoplasmosis and risk of schizophrenia in adult offspring. American Journal Psychiatry **162**: 767-773.

Chen Y.R., A.C. Swann, and D.B. Burt. 1996. Stability of diagnosis in schizophrenia. American Journal of Psychiatry **153:** 682-686.

Cowell, P.E., D.J. Kostianovsky, R. C. Gur, B.I. Turetsky, and R.E. Gur. 1996. Sex differences in neuroanatomical and clinical correlations in schizophrenia. American Journal of Psychiatry **153:** 799-805.

Dubey, J. P. 1997. Tissue cyst tropism in *Toxoplasma gondii*: A comparison of tissue cyst formation in organs of cats and rodents fed oocysts. Parasitology **115:** 15–20.

Flegr J., S. Zitkova, P. Kodym, and D. Frynta. 1996. Induction of changes in human behaviour by the parasitic protozoan *Toxoplasma gondii*. Parasitology **113:** 49-53.

Fox, W. M. 1965. Reflex-ontogeny and behavioural development of the mouse. Animal Behavior **13:** 234-241.

Gaskell, E.A., J.E. Smith, J.W. Pinney, D.R. Westhead, and G.A. McConkey. 2009. A unique dual activity amino acid hydroxylase in *Toxoplasma gondii*. PLoS One **4:** e4801

Hay, J., P.P. Aitken, and D.I. Graham. 1984. *Toxoplasma* infection and response to novelty in mice. Zeitschrift für Parasitenkunde **70:** 575-588.

Hazlett E.A., M.S. Buchsbaum, J. Zhang, R.E. Newmark, C.F. Glanton, Y. Zelmanova, M.M. Haznedar, K.W. Chu, I. Nenadic, E.M. Kemether et al. 2008. Frontal-striatal-thalamic mediodorsal nucleus dysfunction in schizophrenia-spectrum patients during sensorimotor gating. Neuroimage **42:** 1164-1177.

Hinze-Selch, D., W. Daubener, L. Eggert, S. Erdag, R. Stoltenberg, and S. Wilms. 2007. A controlled perspective study of *Toxoplasma gondii* infection in individuals with schizophrenia: Beyond seroprevalence. Schizophrenia Bulletin **33:** 782-788.

Hodkova, H., P. Kodym, and J. Flegr. 2007. Poorer results of mice with latent toxoplasmosis in learning tests: Impaired learning processes or the novelty discrimination mechanism? Parasitology **134:** 1329-1337.

Hutchinson, W.M., M. Bradley, W.M. Cheyne, B.W. Wells, and J. Hay. 1980. Behavioural abnormalities in *Toxoplasma*-infected mice. Annals of Tropical Medicine and Parasitology **74:** 337-345.

Jablensky, A. 1997. The 100-year epidemiology of schizophrenia. Schizophrenia Research **28:** 111–125.

Jones, J. L., A. Lopez, M. Wilson, J. Schulkin, and R. Gibbs. 2001. Congenital toxoplasmosis: A review. Obstetrical & Gynecological *Survey* **56:** 296-305.

Jones, J. L., D. Kruszon-Moran, K. Sanders-Lewis, and M. Wilson. 2007. *Toxoplasma gondii* infection in the United States, 1999 2004, decline from the prior decade.

American Journal of Tropical Medicine and Hygiene **77:** 405-410.

Kanková, S., P. Kodym, D. Frynta, R. Vavrinova, A. Kubena, and J. Flegr. 2007. Influnece of latent toxoplasmosis on the secondary sex ration in mice. Parasitology **94:** 122-127.

Kannan, G., K. Moldovan, J. C. Xiao, R.H. Yolken, L. Jones-Brando, and M. V. Pletnikov. 2010. *Toxoplasma gondii* strain-dependent effects on mouse behaviour. *Folia* Parasitologica **57:** 151-155.

King, M. D., D. S. Lindsay, S. Holladay, and M. Ehrich. 2003. Neurotoxicity and immunotoxicity assessment in CBA/J Mice with chronic *Toxoplasma gondii* infection and single-dose exposure to methylmercury. International Journal of Toxicology **22:** 53–61.

Lindsay, D.S., B.L. Blagburn, and J.P. Dubey. 1990. Infection of mice with *Neospora caninum* (Protozoa: Apicomplexa) does not protect against challenge with *Toxoplasma gondii*. Infection and Immunity **58:** 2699-2700.

MacKenzie, C.R., K. Heseler, A. Müller, and W. Däubener. 2007. Role of indoleamine 2,3-dioxygenase in antimicrobial defence and immuno-regulation: Tryptophan depletion versus production of toxic kynurenines. Current Drug Metabolism **8:** 237-244.

McClellan, J.M., E. Susser, and M.C. King. 2006. Maternal famine, de novo mutations, and schizophrenia. Journal of the American Medical Association **296:** 582-584.

McGorry, P. D., B. Nelson, S. Goldstone, and A. R. Yung. 2010. Clinical staging: a heuristic and practical strategy for new research and better health and social outcomes for psychotic and related mood disorders. Canadian Journal of Psychiatry **55:** 486-497.

Mortensen, P. B., B. Norgaard-Pedersen, B.L. Waltoft, T.L. Sorensen, D. Hougaard, and R. H. Yolken. 2007. Early infections of *Toxoplasma gondii* and the later development of schizophrenia. Schizophrenia Bulletin **33:** 741-744.

Moser, V. C., J. P. McCormick, J. P. Creason, and R. C. MacPhail. 1988. Comparison of chlordimeform and carbaryl using a functional observational battery. Fundamental and Applied Toxicology **2:** 189–206

Ocampo, L. M., and I. Duarte-Gandica. 2010. A model of congenital toxoplasmosis transmission dynamics. *Revista* de *Salud Públic*a **12:** 317-326.

Regier, D. A., W. E. Narrow, D. S. Rae, R. W. Manderscheid, B. Z. Locke, and F. K. Goodwin. 1993. The de facto US mental and addictive disorders service system. Epidemiologic catchment area prospective 1-year prevalence rates of disorders and services. Archives of General Psychiatry **50:** 85-94.

Rosenfield, P.J., K. Kleinhaus, M. Opler, M. Perrin, N. Learned, R. Goetz, A. Stanford, J. Messinger, J. Harkavy-Friedman, and D. Malaspina. 2010. Later paternal age and sex differences in schizophrenia symptoms. Schizophrenia Research **116:** 191-195.

Rossi, A., E. Daneluzzo, P. Mattei, M. Bustini, M. Casacchia, and P. Stratta. 1997. Wisconsin card sorting test and Stroop test performance in schizophrenia: a shared construct. Neuroscience Letters **226:** 87-90.

Shone, G., Y. Raphael, and J. M. Miller. 1991. Hereditary deafness occurring in cd/1 mice. Hearing Research **57:** 153-156.

Skallová, A., P. Kodym, D. Frynta, and J. Flegr. 2006. The role of dopamine in *Toxoplasma*-induced behavioural alterations in mice: an ethological and ethopharmacological study. Parasitology **133:** 525-535.

Skallová, A., M. Novotná, P. Kolbeková, Z. Gasová, V. Veselý, M. Sechovská, and J. Flegr. 2005. Decreased level of novelty seeking in blood donors infected with *Toxoplasma*. Neuroendocrinology Letters **26:** 480-486.

Stein, L. 1975. Norepinephrine reward pathways: role of self-stimulation, memory consolidation, and schizophrenia. Nebraska Symposium on Motivation **22:** 113-159.

Stibbs, H. H. 1985. Changes in brain concentrations of catecholamines and indoleamines in *Toxoplasma gondii* infected mice. Annals of Tropical Medicine and Parasitology **79:** 153-157.

Tilson, H. A., and V. C. Moser. 1992. Comparison of screening approaches. Neurotoxicology **13:** 1-13.

Trullas, R., and P. Skolnick. 1993. Differences in fear motivated behaviors among inbred mouse strains. Psychopharmacology **111**: 323-331.

Vyas, A., S.K. Kim, N. Giacomini, J.C. Boothroyd, and R.M. Sapolsky. 2007. Behavioral changes induced by *Toxoplasma* infection of rodents are highly specific to aversion of cat odors. Proceedings of the National Academy of Sciences USA **104:** 6442-6447.

Wang, T., M. Liu, X.J. Gao, Z.J. Zhao, X.G. Chen, and Z.R. Lun. 2011. *Toxoplasma gondii*: The effects of infection at different stages of pregnancy on the offspring of mice. Experimental Parasitology **127:** 107-112.

Webster, J. P., and G. A. McConkey. 2010. *Toxoplasma gondii*-altered host behaviour: clues as to mechanism of action. Folia Parasitologica (Praha) **57:** 95-104.

Webster, J. P., C.F. Brunton, and D.W. MacDonald. 1994. Effect of *Toxoplasma gondii* upon neophobic behaviour in wild brown rats, *Rattus norvegicus*. Parasitology **109:** 37-43.

Whitelaw, D.D., and G.M. Urquhart. 1985. Maternally derived immunity in young mice to infection with *Trypanosoma brucei* and its potentiation by Berenil chemotherapy. Parasite Immunology **7:** 289-300.

Yolken, R.H., S. Bachmann, I. Ruslanova, E. Lillehoj, G. Ford, E.F. Torrey, and J. Schroeder. 2001. Antibodies to *Toxoplasma gondii* in individuals with first-episode schizophrenia. Clinical Infectious Diseases **32:** 842-844.

Xiao, J., S.L. Buka, T.D. Cannon, Y. Suzuki, R.P. Viscidi, E.F. Torrey, and R.H. Yolken. 2009. Serological pattern consistent with infection with type I *Toxoplasma gondii* in mothers and risk of psychosis among adult offspring. Microbes and Infection **11:** 1011-1018.

FIGURE AND TABLES

FIGURE 1. The Barnes maze apparatus. The Barnes maze apparatus (arrow = goal box) used to examine mouse behavior. (**A**) Beginning of the test with mouse in the start box in the center of the maze. (**B**) Mouse demonstrating inactive time. (**C**) Mouse investigating a wrong hole. (**D**) Mouse entering the goal box.

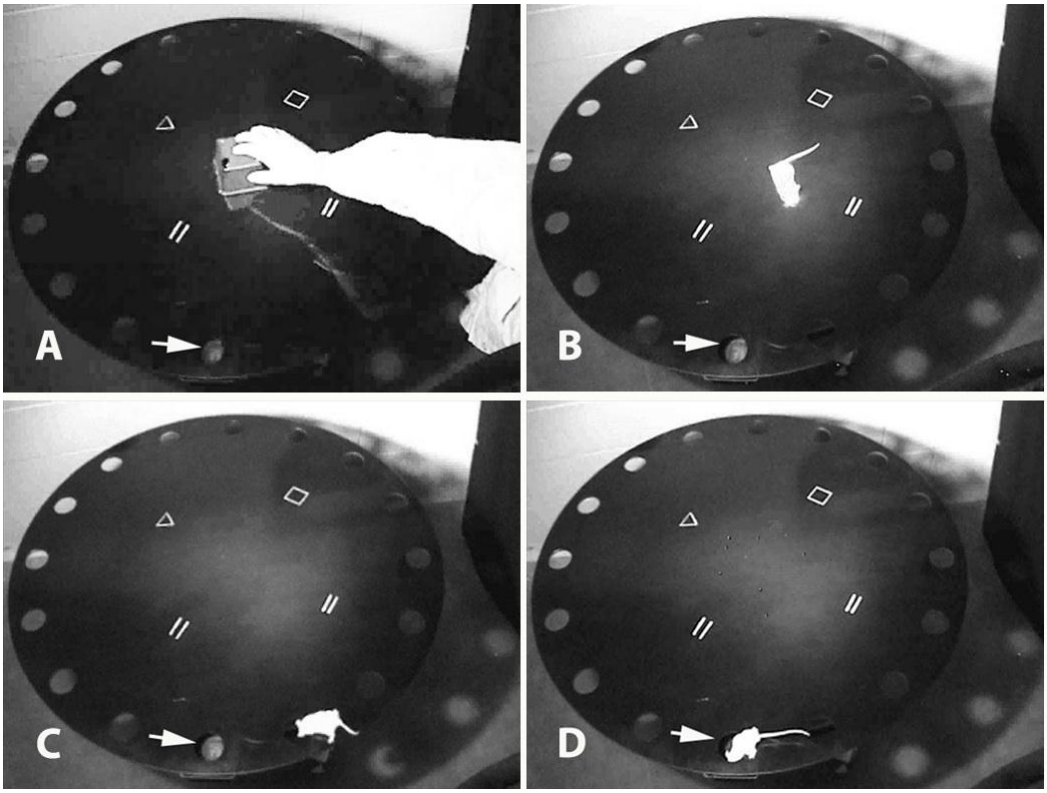

Table I. Barnes maze results summary. Behavioral parameters measured in and results* of the Barnes maze test for 4-wk-old and 8-wk-old female and male *Toxoplasma gondii* congenitally infected mice and non-infected controls.

Behavior	**Description**	**Result 4 wks.**	**Result 8 wks.**
Inactive time	Initial period of inactivity in maze center when trial starts	Females only*	None
Memory	Distance between goal box and 1st hole investigated	Males only*	Males only*
Distance	Distance traveled to goal or reached by expiry of observation period	None	None
Errors	Visitation to a hole other than the goal box hole	None	None
Activity	Number of holes visited/Active time (overall time minus inactive time)	None	None
Random search strategy	Passing through center of maze or skipping more than 4 holes	None	Females only*
Latency	Time elapsed from start to finish of trial	None	None
Task acquisition	Direct route to goal box with errors $\underline{\leq 2}$ in 3 of 4 trials on 2 consecutive days	None	None

*Results were significantly different ($P \leq 0.1$).

Table II. FOB negative results summary. Parameters evaluated in the Functional Observation Battery tests*.

Open field ataxia	Soft stool	Corneal bulging
Tremors/convulsions	Fecal stain	Partially closed eyes
Posture	Urine stain	Piloerection
Coat condition	Salivation	Dehydration
Tail Condition	Nasal discharge	Protruding penis
Respiration	Oral discharge	Cool to touch cyanosis
Vocalization	Lacrimation	Weight gain/loss
Diarrhea	Ocular discharge	Activity

*None of these measured parameters were significantly different ($P>0.1$).

Chapter 6

THE INFLUENCE OF MATERNAL IMMUNE STIMULATION WITH INTERFERON GAMMA OR INTERFERON GAMMA AND CONGENITAL INFECTION WITH *TOXOPLASMA GONDII* ON BEHAVIOR AND NEUROTRANSMITTERS IN MICE

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ABSTRACT

Maternal immune stimulation (MIS) with interferon gamma (INF-γ) is shown to decrease fetal mortality and increase fetal survival when given prophylactically to pregnant mice infected with the protozoan parasite *Toxoplasma gondii*. MIS however can be both beneficial and detrimental to developing fetuses. Research shows MIS with polyriboinosinic-polyribocytidilic acid (Poly (I:C)), a synthetic dsRNA, that mimics a viral infection can cause adult onset behavior deficits in a mouse model. The Poly (I:C) model supports the theory that exposure to viral pathogens *in utero* plays a role in adult onset behavior deficits in humans. Congenital infection with *Toxoplasma gondii* has been associated with adult onset behavioral changes in humans. Interferon gamma (INF-γ), a potent immune regulatory chemical, can decrease teratogenic birth defects, when administered at an appropriate time and dose. INF-γ is also a major mediator of the immune reaction towards *T. gondii*. Our hypothesis was that MIS of pregnant mice would be helpful in decreasing behavioral defects in congenitally *T. gondii* infected mice. We assessed behavior response of CD-1 mice born to mothers exposed to INF-γ, INF-γ/*T. gondii*, *T. gondii* only or no treatment mice were assessed with the Barnes maze and Function observational battery. Examinations were conducted pre sexual maturity at 4 wks. of age, and again at 8 wks. post sexual maturity post, by which time tissue cysts should be present and inciting an immune response in the brain. We demonstrated that infection with *T. gondii* decreased memory and learning in an age dependent fashion in our model. INF-γ treatment of dams also alters behavior of their offspring in a sex and age dependent manor. Immune stimulated female mice at 4 wks.

of age demonstrated alterations in special memory, coordination and processing of stimuli. Male mice demonstrated coordination difficulties at 4 wks. of age. By 8 wks. of age, post sexual maturity, all the deficits observed at 4 wks. of age, had disappeared for both sexes but male *T. gondii* infected mice experienced deficits involving spatial memory and rate of learning. INF-γ exposure in utero caused post sexual maturity changes in initial open field assessment in mice of both sexes. We found MIS with INF-γ eliminated negative effects of congenital *T. gondii* infection.

INTRODUCTION

Toxoplasma gondii is an obligate intracellular protozoan parasite. Natural infections of humans occur in one of three major ways: ingestion of sporulated oocysts, ingestion of tissue cysts from undercooked contaminated meat, or through transplacental congenital infection. *T. gondii* disseminates through the body of infected individuals before encysting in neural tissue, resulting in latent infection. Congenital *T. gondii* exposures, along with other in utero acquired central nervous system infections, are believed to play a role in schizophrenia, a mental illness with adult onset behavior changes (Yolken et al., 2001). Congenital *T. gondii* infections can cause severe problems, such as, hydrocephalus, intracranial calcification, blindness and severe mental retardation.

Stimulating the maternal immune system can have a profound effect on fetal development. Per conceptual administration of a number of immune stimulations has decreased both the incidence and severity of a wide variety of birth defects. Birth defects caused by a number of physical and chemical agents including x-rays, hypothermia, urethane, cyclophosphamide, hyperglycemia, lipopolysaccharides, and anticonvulsant therapeutics have been reduced or prevented. A variety of immune stimulants are effective in preventing fetal malformations including immune modulatory cytokines such as Interferon gamma (IFN-γ), granulocyte-macrophage colony-stimulating factor, Freud's complete adjuvant and bacille Calmette-Guérin inert particles such as pyran and even zenogenic lymphocytes. Maternal immune stimulation (MIS) can prevent malformation of the face eyes, palate, neural tube (brain and spinal cord)

limb digits and tails as well as reduce teratogen induced fetal death in mice (Reviewed by Hrubec et al 2005; Holladay et al 2002)

Schizophrenia has no known etiology. Genetic predisposition, environmental exposure to chemicals or pathogens and changes in biochemical pathways brought about by the physical stress of nutritional deficiencies during in utero development are three major areas of focus (Roth et al., 2009; Susser and Lin 1992). In the recent years, *in utero* exposure to pathogens is being explored as a contributing factor to mental illnesses with adult on set profile (Buka et al., 2001).

Pregnancy is a balancing act for the immune system. During pregnancy, maternal immunity is suppressed to maintain the pregnancy, leaving the fetus more susceptible to a variety of pathogens. Some infections do not cross the placenta; however there is evidence the maternal immune system may cause adult onset learning deficits on the fetus (Zukerman and Weiner 2005). The influence that the maternal immune response plays in fetal development is still undefined. Experimentally, MIS has been examined using polyriboinosinic-polyribocytidilic acid (Poly (I:C) in mice. Poly (I:C) mimics a viral infection, stimulating the maternal immune Th-1 immune response via toll-like receptor 3 (Kadowaki et al., 2001). The use of Poly (I:C) elicits a very specific defined immune response. Experimentally MIS with Poly (I:C) is shown to cause behavioral changes in adult, but not immature mice (Shi et al., 2003). Similar behavior profiles are observed in offspring of mice receiving MIS with IL-6 indicating specific maternal immune profiles may play a role in causing adult onset behavior alterations (Smith et al., 2007). These finding are of value because MIS due to a viral

infection, could be a factor in schizophrenia or other mental illnesses with adult onset behavior disorders. It is becoming increasing clear not all forms of MIS are beneficial.

The connection between MIS and adult onset behavior deficits in mice is of great importance. The literature indicates behavior changes in the offspring as adults occur when administration of poly (I:C) a viral mimic is used. The conclusion is maternal immune response to a viral mimic maybe causing the observed adult onset behavior deficits instead of an infectious agent as previously thought (Shi et al., 2003). The type of MIS given can dictate the type of behavior response observed. The majority of the research has employed MIS with Poly (I:C). The results are behavior deficits in adult offspring consisting of decreased memory retention, increased dopamine concentrations or turnover, measured by an increased in homovanillic acid a dopamine metabolite, decreased prepuls inhibition and decreased latent inhibition (Meyer et al., 2006; Ozawa et al., 2006; Shi et al., 2003). When IL-6 is used for MIS the behavior profile is nearly identical to poly (I:C) immune stimulation (Smith et al., 2007). IFN-γ has been used to successfully decrease birth defects when given prophylactically, but the mechanism is unknown at this time. IFN-γ is a potent immune stimulator and will shift the maternal immune system to up-regulate the gene coding for granulocyte-macrophage colony-stimulating factor a gene that is previously shown to regulate placental development and protect against birth defects induce by other teratogens. To our knowledge MIS with IFN-γ has not been examined for its effects on adult onset behavior alterations.

We used murine INF-γ to immune stimulated pregnant female mice 5 days prior to breeding and on day 10 of gestation followed by infection of pregnant mice with *T.*

gondii on day 11. Our goal is to ameliorate the adult onset behavior observed in congenitally infected mice with IFN-γ. We subjected the offspring to behavioral testing for assessment. We used the Barnes maze test and functional observational battery (FOB) at 4 wks. of age. At 8 wks. of age, the mice were again tested with the Barnes maze and FOB as well as assessing open field activity, and open field exploration. After behavioral testing was completed, we measured neurotransmitters, examined the frontal cortex for the presence of tissue cysts, and evaluated the brains histologically. We evaluated the offspring of IFN-γ treated, IFN-γ/*T. gondii* treated and *T. gondii* infected compared to non MIS/infected control mice; pre sexual maturity, 4 wks. old and post sexual maturity, 8 wks. old. Male and female were analyzed separately as responses in the sexes were different.

MATERIAL AND METHOD

MIS and fetal mortality

60 female CD-1 mice (Charles River Laboratories) were randomly split into 4 groups, 15 untreated, uninfected controls, 15 *T. gondii* infected, 15 INF-γ treated and 15 IFN-γ/*T. gondii* treated mice. Female mice in immune stimulated treatment groups were injected intraperitoneally (IP) with 1000 units of INF-γ (Pepro Tech, Inc. Rocky Hill, NJ) at 10 and 5 days before breeding. On day 3 of gestation, mice in the *T. gondii* infected groups were given orally 30 tissue cysts of the VEG strain of *T. gondii*. On day 17 of gestation, pregnant dams were killed and the uterus was removed. The fetuses were evaluated for malformations, developmental delay and fetal mortality. Fetuses were segregated into live, early fetal mortality and late fetal mortality. The percent of litters with viable fetuses was calculated and used as a measurement for mortality.

Congenital *T. gondii* infection

This study was conducted concurrently and was part of a study designed to examine the effects of congenital *T. gondii* infection on the behavior of mice pre and post sexual maturity (Goodwin et al., 2011 in review). The portion of the study devoted to the MIS effect of INF-γ and INF-γ/*T. gondii* used a total of 160 female and 80 male mice, 5 week-old CD-1 mice, used to produce the congenitally infected offspring used in this study. Food and water was given *ad libitum* for the duration of the experiment. The Institutional Animal Care and Use Committee, Virginia Tech, Blacksburg, Virginia approved the study. Because the experiment involved examining congenital

transmission of *T. gondii* infections, surrogate dams were used to negate any negative effects of *T. gondii* infection on the mother during lactation of the pups. Eighty of the 160 total female mice were used as aged matched surrogates for the experiment. Mice were permitted to acclimate to cages for 4 days. The female mice were then randomly split into 2 treatments, by use of a coin flip. Female mice in immune stimulated treatment groups were injected I.P. with 1000 units of INF-γ (Pepro Tech, Inc. Rocky Hill, NJ) 5 days prior to mating. Control mice received IP injections of Hanks balanced salt solution (HBSS, Cellgro, Mediatech, Manassas VA). Females were added to the male cages, 1 male per 2 females for both experimental and surrogate groups. The females were examined every morning for the presence of a vaginal plug. Plug positive females were removed daily and co-housed with a female from the same treatment group. The presence of a vaginal plug was indicative of mating and was considered day 0 of pregnancy for the dams.

Experimental groups contained 20 controls (untreated and uninfected), 20 IFN-γ stimulated mice, 20 *T. gondii* infected pregnant mice and 20 IFN-γ/*T. gondii* infected pregnant dams. Control and *T. gondii* infected only mice received HBSS IP on day -5 and day 10 of gestation. IFN-γ treated and IFN-γ/*T. gondii* mice received 1000 units of murine IFN-γ IP on day -5 and day 10 of gestation. Control mice received no *T. gondii* infected, only HBSS, acting as a negative control. The IFN-γ only mice received no tissue cysts. The *T. gondii* and IFN-γ/*T. gondii* treatments received 30 VEG tissue cysts (*T. gondii* genotype III) orally, in 500 μl HBSS on day 11 of pregnancy. The VEG tissue cysts were collected from chronically infected CD-1 mice. All dams were killed after parturition and their pups placed with an age matched non-infected surrogate dam.

Making this a blind test, random numbers produced from a number generator were assigned to each cage after dams gave birth. One person who had no part in the behavior testing or grading of the Barnes maze assigned the random numbers to the mice. The treatment groups were not revealed until all behavior testing was completed.

Pups were weaned at 3 weeks of age. Between 3 and 4 weeks of age mice were handled twice for 2-3 minutes to acclimatize them to handling. At 3.5 wks. mice were bled, and ear notched for identification. The sera were examined for *T. gondii* antibodies at a 1:50 dilution using the RH strain of *T. gondii* as antigen and immunofluorescent antibody (IFA) test. These procedures are routinely employed in our laboratory (Lindsay et al., 1998) to establish infection status of pups and percent rate of transmission for pups born to infected females. Three week old mice were chosen because maternal IgG disappears after 3 weeks in mice (Whitelaw and Urquhart 1985). Five seropositive mice of the same sex born to the same infected mother, and 5 seronegative mice of the same sex born to each HBSS and IFN-γ treated mothers were saved for behavior testing. Behaviors were examined at 4 and 8 wks. because *T. gondii* tissue cysts are present in the brain at that time (Dubey 1997).

At the end of the experiment, the mouse used for 8 week Barnes maze testing was killed, by cervical dislocation, for brain dissections and neurotransmitter measurements. The four remaining mice were killed by CO_2 asphyxiation. The brain from one of these four mice was used for frontal cortex brain smears. The remaining three mice were not used in post mortem experiments.

Data analysis: Transmission rates were compared between males and females using logistic generalized estimating equations (GEE) (proc genmod). The linear model

specified pup sero-status (positive vs. negative) as the outcome, sex as the predictor, logit as the link function, and binomial as the distribution. To request GEE, dam ids were specified as the clusters with an exchangeable working correlation matrix. *P*-values were based on type 3 Wald statistics. Sex ratios were compared between treatment groups using the Wilcoxon rank sum test (proc npar1way).

Barnes maze apparatus and testing

The Barnes maze is a spatial memory and learning test. The maze is used to examine short-term memory, acquisition of tasks, open field activity, and exploratory activity (Bach et al., 1995). The Barnes maze apparatus consisted of a circular plexiglass platform 90 cm in diameter with 16 holes (each 5 cm in diameter) equally spaced around the perimeter, 5 cm from the edge, and 10.5 cm from each other (Fig. 1). The maze was mounted on a stand 91.5 cm from the floor. There were 2 locations, 180 degrees apart, for an escape box (l x w x h = 19 cm x 9 cm x 7.5 cm) to be mounted underneath a hole by a drawer mechanism. At the start of each trial, a mouse was placed in the center of the platform in a square start chamber (8.8 cm in diameter and 8.1 cm in height). Visual cues were set up approximately 20 cm above and 45-50 cm from the horizontal edge of the circular maze. Cues were positioned in such a way as to not be identifiable with a specific hole. Video imaging equipment was set up approximately 149 cm above and 12 cm from the horizontal edge of the circular maze. The maze was set in the corner of a room with a black curtain used for the other 2 walls. The corner of the room had white walls with black cues taped to the wall. The other 2 sides were black and had white cues attached to the curtain. The black curtain was

positioned to block the experimenter from view. A 150 W light was suspended 106 cm, almost directly above the center of the circular platform and a fan (Massey 8” High Velocity Personal Fan, Bentonville, Arkansas) was placed 68.5 cm above the maze pointed directly down at the center of the maze. The light and fan served as 2 forms of adverse stimuli.

Each mouse was randomly assigned, by a coin toss, to 1 of the 2 escape box locations, which remained the same throughout the testing period. Testing was conducted daily for 7 days. On day 1, each mouse was placed on the maze for 30 sec, without aversive stimuli and then physically placed in its designated goal box for 1 min. On day 1, the mice underwent training consisting of 2 test trials through the maze. On days 2-5, the testing consisted of 2 consecutive trials followed by a third trial after 3-4 hrs. rest. On the 6th and 7th day, each mouse was evaluated twice and once, respectively, totaling 17 trials per mouse.

For each trial, the mouse was placed in the center of the platform in a start chamber (Fig. 1A). Once placed on the platform the fan and light were switched on, and the mouse was kept in the start chamber for 10 sec. After 10 sec, the start chamber was lifted and the trial began. The trial ended once the mouse entered the goal box (forelimbs only, or both forelimbs and hind limbs), located the goal box (4 consecutive nasal investigations), or after a trial time of 5 min without entry or location of the goal box. When the mouse entered the goal box, the trial was stopped and the fan and light were turned off. If the mouse had located the goal box but did not enter, the trial was stopped, and the mouse was then guided into the goal box, and the fan and light were turned off. If the mouse did not locate or enter the goal box after 5 min, the trial was

stopped, the mouse was placed into the goal box, and the fan and light were turned off. After 30 sec in the goal box the mouse was returned to its home cage. Following a 3-5 min waiting period, the mouse was placed on the platform for the second daily trial. After the third trial, the mouse was returned to its home cage until the next day's session. The maze and goal box were cleaned with 70% ethanol after every trial.

Each trial was recorded digitally and then viewed at a later date for quantitative analysis. Video equipment consisted of a Sony-DCR-TRV530 digital 8 camcorder connected via a Firewire cable to a Mac G. Imovie was used to capture and save each trial. Quicktime ® media player was used to view the video files.

Barnes maze behavioral measures (Table I) were viewed and graded by 1 person (DGG) who was blind to the treatment status of the mice. The recorded videos were scored on inactive time, memory (distance to goal), distance, errors, activity, random search strategy (center crossings), serial search strategy (perseverations) and latency.

Inactive time was the amount of time a mouse remained motionless in the center of the maze at the beginning of the trial. Inactive time is the amount of time required for a mouse to initially assess its environment from visual cues. Short-term memory was the distance between the goal box and the first hole investigated. For example, if the first hole was adjacent to the goal box then the distance to goal value was recorded as 1. Short-term memory is indicative of how well the mouse recognized it's surrounding and remembered where the goal box was positioned (Bach et al., 1995). Distance was measured as how far the mouse traveled either before finding the goal box or the

distance traveled during the 5-min observation time if the mouse was not successful in finding the goal box (Bach et al., 1995). Errors were recorded as the visitation to a hole other than the goal hole (Bach et al., 1995) (Fig. 1C). Activity was calculated by the number of holes investigated divided by the active time (active time is overall time minus the inactive time) and defined as the amount of time spent investigating/exploring each hole. Random search strategy (center cross) (Fig. 1B) was defined as passing through the center of the maze, or as skipping more than 4 holes when traveling from hole to hole, or as walking into the center of the maze and back out again. Random search strategy is a precursory approach where the mouse randomly searches for the goal box without a well-defined strategy (Bach et al., 1995). Serial search strategies are persistent entries into any hole that is not the goal box. They can also be an oscillation between two holes. Serial search strategy is used as a more advanced methodical search over the random search strategy (Bach et al., 1995). It also indicates a spatial learning and memory of the goal box. An increase in the rate of learning was defined as either a statistical decrease in the use of the random search strategy (injunction with an increase in the serial search strategy not statistically significant) or increase in the use of the serial search strategy (in conjunction with a decrease in the use of the random search strategy not statistically significant). Acquiring the task was defined by a direct route to the goal box with errors ≤ 2 in a trial (Bach et al., 1995). A mouse was considered to have acquired the task (learned the maze) if 3 of 4 trials on 2 consecutive days were successful at any point during the 7-day trial period (Fig. 1D). Latency was the time elapsed from trial start to finish and is not related to task acquisition, but does

indicate rate of learning (Bach et al., 1995). The numbers of mouse litters used are listed on Table II.

Data analysis: Scatter plots for outcome against trial number showed that Latency, Active time, Distance and Errors followed an exponential decay. Subsequently, half-lives were generated for each of the four outcomes (for each mouse). The half-lives were then compared between the treatment groups using the Wilcoxon rank sum test. For outcomes that were slightly skewed (inactive time, distance to goal, activity, and center crosses), the treatment groups were compared using linear GEE. The linear model specified behavioral measure as the outcome, treatment as a predictor, identity as the link function, and normal as the distribution. To request GEE, mouse ids were specified as clusters with an exchangeable working correlation matrix. *P*-values were based on the type 3 Wald statistics.

Functional observation battery

The FOB tests are designed to detect physiological changes, coordination, and startle and menace responses in mice (Moser et al., 1988; King et al., 2003). The specific physiologic parameters evaluated and displayed differences are listed in Table III. Parameters without differences are listed on Table IV. All of these parameters were graded on a “yes” or “no” scale. The FOB tests also evaluate coordination, startle response, and menace. The coordination of the mice was measured using 2 tests. The first test was the righting reaction test. This righting reaction test determines the mouse’s ability to right itself when placed on its back. The second test was performed to

see how well the mice hold onto a wooden rod. The startle response was elicited using a clicker. The clicker was clicked approximately 6-8 cm behind the mouse's head and was scored as "Yes" if the click elicited a flinch or startle, or "No" if no response was elicited. The FOB also examined menace response and this was done by placing a pencil about 1-2 cm from the face of the mouse. If the mouse looked or turned away then the mouse was scored with a No, no menace response. If the mouse acted aggressively to the pencil end the mouse was scored as "Yes", acts menacingly to an object. The numbers of mouse litters used are listed on Table II.

Data analysis: The treatment groups were compared using logistic generalized estimating equations (proc genmod). The linear model specified FOB test as the outcome, Treatment as a predictor, logit as the link function, and binomial as the distribution. To request GEE, dam ids within treatment were specified as the clusters with an exchangeable working correlation matrix. *P*-values were based on the type 3 Wald statistics.

Visual placement

Vision and coordination of mice was evaluated by the visual placement test (Fox et al., 1965). The test was performed by holding the mouse upside down by its tail, so that its head was 3.5 to 5 cm from the edge of the counter top and approx. 2.5 cm below the surface of the counter top. This encourages the mouse to reach up and out to the counter top to keep from dangling upside down. The mouse was then scored on a 3 point scale, i.e., (1) the mouse made no attempt to reach out, (2) the mouse attempted

to reach out but could not grab, and (3) the mouse reached out and grabbed the edge of the table. The numbers of mouse litters used are listed on Table II.

Data analysis: Only scores 2 and 1 were observed and recorded. Accordingly, the treatment groups were compared using logistic generalized estimating equations (proc genmod). The linear model specified score as the outcome, Treatment as a predictor, logit as the link function, and binomial as the distribution. To request GEE, dam ids within treatment were specified as the clusters with an exchangeable working correlation matrix. *P*-values were based on the type 3 Wald statistics.

Virtual cliff

The virtual cliff (Adams et al., 2002) consisted of a box 38 x 38 x 38 cm. The top surface of the box a piece of plexiglass that was half clear and half opaque. The virtual cliff was positioned 38 cm off the bottom of the box. Mice were placed in the center of the cliff with 1 front and hind limb of 1 side of the body on the clear portion and the contralateral limbs on the opaque portion. Mice were tested 3 times and the initial position remained constant. Mice were scored depending on which side of the plexiglass they traveled to, a score of 1 for the clear side and a score of 2 for the opaque side. The numbers of mouse litters used are listed on Table II.

Data analysis: The number of times a mouse went to the opaque side was used as the outcome for this section (score range 0 to 3). After scoring, the treatment groups were compared using linear generalized estimating equations (proc genmod). The linear model specified score as the outcome, Treatment as a predictor, identity as the link

function, and normal as the distribution. To request GEE, dam id within treatment were specified as clusters with an exchangeable working correlation matrix. *P*-values were based on the type 3 Wald statistics.

Rearing and Open Field Activity

Rearing tests look specifically at exploration of novel environments (Hutchinson et al., 1980). Four mice from each litter were individually placed in a box (20 cm x 42 cm) for 5 min. The numbers of times a mouse reared or lifted its forelimbs off the ground in an exploratory manner were counted. The box was cleaned with 70% ethanol between each mouse. The numbers of mouse litters used are listed on Table II. Open field activity testing was carried out using San Diego Instruments Photobeam Activity System and a clear 20 cm x 42 cm cage. The cage had a metal halo that surrounded the perimeter of the box, with 3 laser beams that were evenly spaced 10 cm apart dividing the box in to 4 evenly spaced sections 20 X 10 cm. When the mouse moved from 1 quadrant to another quadrant the laser beam was broken. A record of the beam breaks was recorded onto the computer. Mice were left in the open field for 5 min, while beam breaks were recorded. The computer measured ambulations (2 or more consecutive beam breaks indicating walking across the box) and beam breaks (nonconsecutive beam breaks indicating oscillations from quadrant to quadrant of nonspecific movement) . The box was cleaned with 70% ethanol between every mouse tested. Ambulations were recorded as movement of a mouse through at least 3 of the 4 quadrants without back tracking. Beam breaks occurred when the mouse broke the

beams in a non-successive order, indicating non-specific activity in the box. The numbers of mouse litters used are listed on Table II.

Data analysis: Effect of treatment on rearing was assessed using mixed model ANOVA (proc mixed). The linear model included rearing as outcome, treatment as a fixed effect, and litter Id within treatment as the random effect. Ambulations and beam breaks were compared between treatments at each time point using mixed-model repeated-measures ANOVA (proc glimmix). The linear model included ambulation or beam breaks as outcome, treatment as a fixed effect, and mouse within treatment as a random effect (G side). For the R side of the model, an autoregressive 1 correlation matrix was specified. Denominator degrees of freedom were specified as KenwordRoger.

Neurotransmitter determinations

Mice that completed the 8-wk Barnes maze test were used for neurotransmitter isolation and determination. The whole brain was removed and placed on ice, then cut in half along the sagittal plane. The striatum from both the right and the left hemispheres was removed and placed into a microfuge tube on ice. The frontal cortex from the right hemisphere was removed and placed in a microfuge tube on ice. The tissue was then weighed and 6 times the v/w of isoproterenol buffer (4.7 pH) was added to the tissue for preservation of neurotransmitters. The brain tissue was then homogenized on ice followed by micro-centrifugation at 4 C for 10 min at 15,000 g. The supernatants were stored at -80 C until analysis by high performance liquid chromatography (HPLC) was conducted. Striatal and cortical samples were analyzed for dopamine and its

metabolites, 3,4-dihydroxyphenylacetic acid and homovanillic acid, in addition norepinephrine, epinephrine**,** and the metabolite 3-methoxy-4-hydroxyphenylglycol, and serotonin and its metabolite, 5-hydroxyindoleacetic acid, were also analyzed.

Samples were examined in a HPLC Agilent Technologies (Wilmington, DE) 1100 Series equipped with a degasser, a quaternary pump, a refrigerated auto sampler (set at 4 ºC) and connected to a Waters 2465 electrochemical detector (Milford, MA). A C18 column (Macherey Nagel Bethlehem, PA) (Pyramid EC 250 x 4 mm i.d., 3 µm) was used with a flow rate of 0.7 ml/min. Column temperature was maintained at 35 ºC. The mobile phase composition was 5% methanol in aqueous sodium acetate (50 mM), citric acid (12.5 µM), EDTA (134 µM), octane sulfonic acid (230 µM), sodium chloride (2 mM), pH 4.7. Isoproterenol (1 µM) was used as the internal standard, and calibration curves of each neurotransmitter and metabolite were prepared using solutions of 10, 50, 100, 250**,** and 500 nM. The numbers of mouse litters used are listed on Table II.

Data analysis: Neurotransmitter concentrations were compared between treatment groups using one-way analysis of variance (proc mixed). The linear model specified neurotransmitter as outcome, treatment as a fixed effect, and residual as the denominator degrees of freedom. While the effect of treatment on epi was assessed using Wicoxon rank sum test.

Brain smears

One mouse from each litter was examined for the presence of tissue cysts at the termination of the experiment. The cranial cavity of the mouse was opened and

approximately 0.1 grams of the frontal cortex was removed placed on a slide, crushed with a 22 x 22 mm^2 cover slip and examined using an Olympus BX60 microscope for the presence or absence of tissue cysts. The numbers of mouse litters used are listed on Table II.

Histology

One mouse from each litter was examined for necrosis and inflammation around the tissue cysts. The brain was removed from the cranial vault and placed in 10% neutral buffered formalin (Fisher scientific). Following fixation the brains were sectioned at the following 4 transverse levels- head of caudate nucleus, mammillary body, midbrain, cerebellum and medulla oblongata and embedded in paraffin. Sections were cut at 7μM thickness and stained with hematoxylin and eosin and examined by B. Jortner, initially this was a blind study and was followed by an unblended assessment. Lesions were semi quantitatively scored as follows.

Meningitis and encephalitis 0) normal tissue,1) focal small lesions, 2) multifocal small or focal prominent lesions and 3) multifocal prominent lesions.

Statistical considerations for all sections

Data were analyzed using SAS version 9.2 (Cary, North Carolina). Because initial findings demonstrated sex associated differences in mouse behavior between 4 and 8 wks. of age, mice were examined as 4 groups consisting of 4-wk-old female mice, 8-wk-old female mice, 4-wk-old male mice, and 8-wk-old male mice. Statistical significance was set to $P<0.1$.

Results

IFN-γ decreased fetal mortality

12 of 15 control dams bred had 91.5% of their fetuses viable, 9 of 15 *T. gondii* dams bred had 23% of their fetuses viable, 13 of 15 IFN-γ dams bred had 92.9% of their fetuses viable and 9 of 15 IFN-γ/*T. gondii* dams bred had 57.7% of their fetuses viable. Control dams had 91.5% viable fetuses with 1.5% late fetal mortality/death and 7 % early fetal mortality. IFN-γ treated dams had 93% viable fetuses with 1% late fetal mortality/death and 6 % early fetal mortality; *Toxoplasma. gondii* infected dams had 23% viable fetuses, with 3% late fetal mortality/death and 74% early fetal mortality. IFN-γ/*T. gondii* treated dams had 58% viable fetuses with 20% late fetal mortality/death and 22% early fetal mortality. When litters are evaluated as the unit of measure, both control and INF-γ mice had 100% of the litters with developed fetuses. *T. gondii* infected litters had 22% with developed fetuses. IFN-γ/*T. gondii* treated litters had 77% with developed fetuses.

Congenital transmission

Twelve of 20 HBSS treated dams produced litters, fifteen of the 20 immune stimulated dams produced litters, fifteen of 20 *T. gondii* infected dams produced litters and thirteen of 20 IFN-γ/ *T. gondii* infected dams produced litters. The transmission rate of *T. gondii* was 66% for males and 77% for females. The difference in numbers of males and females infected was not statistically different ($P>0.1$). In the HBSS treated controls and INF-γ treated litters, no pups tested positive for *T. gondii* antibodies. The

male to female ratio for the litters infected vs. not infected were statistically different (*P*=0.090 one-sided *P* value) between infected and non-infected groups.

Behavior

The results of Barnes maze behavioral testing are summarized in Table **I**.

Transient 4-week old Barnes maze behavior for IFN-γ treated mice, T. gondii infected and IFN-γ*/T. gondii treated mice female and male mice*

Four wk. old IFN-γ treated female mice spent more time investigating each hole, 11.4 sec per trial compared to 7.4 sec per trial for control (a difference of 4 sec with a standard error of 2.22) (*P*=0.075). IFN-γ treated mice had an increase in center crosses with 0.7 crosses per trial vs. 0.22 per trial for the control (a difference of 0.48 crosses per trial with a standard error of 0.229) (*P*=0.036).

Four wk. old *T. gondii*-infected female mice spent 16.8 sec sitting in the middle of the maze compared to 6.1 sec for the female controls (a difference of 10.7 sec with a standard error of 5.260) (*P*=0.041). *T. gondii*-infected female mice crossed the center of the maze on average 0.65 times per maze trial, while control mice only crossed the center of the maze 0.22 times per maze trial (a difference of 0.43 with a standard error of 0.261) (*P*=0.101).

Four wk. old IFN-γ/*T. gondii* treated female group had an increase in serial search strategy from 1.42 per trial vs. 0.93 per trial for controls (a difference of 0.49 with a standard error of 0.300) (*P*=0.098). *IFN-γ/T. gondii* treated mice spent less time sitting

in the middle of the maze 6.1 sec per trial vs. 7.9 sec per trial for controls (a difference of 1.8 sec with a standard error of 1.07) (*P*=0.086).

Four wk. old IFN-γ treated male mice had no statistical differences (*P*>0.1) compared to control mice with the Barnes Maze.

Four wk. old *T. gondii*-infected male mice first investigated holes that were on average a distance of 3.1 holes from the goal box vs. 4.1 holes for control mice (a difference of 1 hole with a standard error of 0.476)(*P*=0.054).

Four wk. old IFN-γ/*T. gondii* treated male mice had a decrease in the number of trial 5.4 compared to controls 6.6 trials to decrease the time required to solve the maze by half (a difference of 1.2 trials, treated mice had a range of 2.0-20.2 and control mice had a range of 5.6-18.0)) (*P*=0.085). Four wk. old IFN-γ/*T. gondii* treated males had an increase in serial search strategy from 1.7 per trial vs. 1.2 per trial for controls (a difference of 0.5 with a standard error of 0.312) (*P*=0.079)

Adult onset 8 week old Barnes maze behavior for IFN-γ treated mice, T. gondii infected and IFN-γ/T. gondii treated mice female and male mice

Eight wk. old Female IFN-γ treated mice had a decrease in center crosses with 0.18 per trial vs. 0.30 per trial for controls (a difference of 0.12 with a standard error of 0.073) (*P*=0.082).

Eight wk. old *T. gondii*-infected female mice had an average of 0.13 center crosses per trial vs. 0.30 per trial for controls (a difference of 0.17 with a standard error of 0.081) (*P*=0.033).

Eight wk. old IFN-γ/*T. gondii* treated female mice had an increase in distance traveled 32.0 holes vs. 4.0 holes for controls (a difference of 28.0 holes and a standard error of 3.74) (*P*=0.067). IFN-γ/*T. gondii* treated female mice had a decrease in inactive time 10.4 sec per trial vs. 38.1 sec per trial for controls (a difference of 17.7 sec per trial and a standard error of 16.1) (*P*=0.086). IFN-γ/*T. gondii* treated female mice had a decrease in center crosses 0.16 per trial vs. 0.3 per trial for controls (a difference of 0.14 crosses per trial and a standard error of 0.060) (*P*= 0.015).

Eight wk. old IFN-γ treated male mice had a decrease in the number of trials 3.7 compared to controls 10.0 trials to decrease the time required to solve the maze by half (a difference of 6.3 trials with a standard error of 3.93) (*P*=0.062). IFN-γ treated male mice spent more time sitting in the middle of the maze 17.0 sec per trial vs. 9.4 sec per trial for the controls (a difference of 7.6 sec with a standard error of 4.27) (*P*=0.078).

Eight wk. old *T. gondii* infected male mice first investigated a hole that was on average 4.1 holes from the goal box vs. 3.0 holes for control male mice (a difference of 1.1 holes with a standard error of 0.389) (*P*=0.005). *T. gondii*-infected male mice spent more time investigating each wrong hole 11.4 sec per hole vs. 5.1 sec per hole for controls (a difference of 6.3 sec with a standard error of 3.624) (*P*=0.080). Finally infected males had on average twice as many center crosses, 0.37 vs. 0.18 center crosses per trial for control mice (a difference of 0.19 and a standard error of 0.109) (*P*=0.077).

Eight wk. old IFN-γ/*T. gondii* treated male mice spent on average more time sitting in the middle of the maze at the start of the test 13.3 sec per trial vs. 9.4 sec per trial for controls (a difference of 3.9 sec with a standard error of 2.02) (*P*=0.058).

FOB

IFN-γ, *T. gondii* and IFN-γ/*T. gondii* treated female mice at 4 wks. of age had an elevated response to the click stimuli compared to control mice. IFN-γ treated mice were 3 times more likely to respond to the click test compared to control mice (odds ratio of 2.7, 95% confidence interval 0.9 to7.9) (*P*=0.080). *T. gondii*-infected female mice were 7 times more likely to respond to the click test compared to the control mice (odds ratio 6.9, 95% confidence interval 1.2 to 30.5) (*P*=0.03). IFN-γ/*T. gondii* treated female mice were 5 times more likely to respond to the click test compared to control mice (odds ratio of 5.1, 95% confidence interval 1.8 to 14.5) (*P*=0.002). The FOB tests showed that infected male mice at 4-wk-old righted themselves 92% of the time compared to 80%for the control mice (odds ratio 0.2, 95% confidence interval 0.05 to 1.2) (*P*=0.077). At 8 wks. of age female and male mice had no differences (*P*>0.1) when the groups were compared to the control group for FOB testing.

Virtual cliff

Four wk. old female mice in the virtual cliff test had a significant difference between control mice and *T. gondii* infected mice, infected mice went an average of 1.4 times to the opaque compared to 1.8 times for control female mice (a difference of 0.4 with a standard error of 0.1) (*P*=0.0007). A difference between control mice and INF-γ/*T. gondii*, INF-g/*T. gondii* mice went on average of 1.2 times to the opaque side compared to 1.8 times for the control female mice (a difference of 0.6 with a standard

error of 0.162) (*P*=0.011). No differences (*P*>0.1) were found between control and IFN-γ female mice.

Four wk. old *T. gondii*-infected males went to the opaque side of the virtual cliff an average of 1.9 times (out of three trials) compared to 1.6 times in control mice (a difference of 0.3 and a standard error of 0.17) (*P*=0.053). No differences (*P*>0.1) were found between control vs. IFN-γ or control vs. IFN-γ/*T. gondii* male mice.

No differences (*P*>0.1) in virtual cliff were found between the male or female mouse treatment groups at the 8 wk. time points.

Visual placement

Four wk. old female mice in the visual placement test had a statistical differences (*P*=0.051) between IFN-γ mice, with a completion rate of 95.6%, vs. control mice, with a 71.5% completion rate (an odds ratio of 4.6 and a 95% confidence interval of 1.0-21.9). No differences (*P*>0.1) were found between control vs. IFN-γ and control vs. *T. gondii* infected female mice.

Four wk. old male mice in the visual placement test were not statistically (*P*>0.1) different between groups.

No differences (*P*>0.1) in visual placement were found between the male or female treatment groups at the 8 wk. time point.

Activity and Rearing

No significant differences (*P*>0.1) were detected in any group of mice for activity (ambulations and beam breaks) or rearing.

Neurotransmitter concentrations

Neurotransmitters were measured after the completion of the Barnes maze at the 8 week time point. Female and male mice for each group were analyzed separately. There was no statistical difference (P>0.1) in neurotransmitter concentrations in any group of mice (Table V).

Brain Smears

Each litter had one mouse chosen for brain smears. Twelve of the 15 mice representing each infected litter tested positive for *T. gondii* via IFA. Of these 12 positive mice 11 tested positive for tissue cysts in the frontal cortex. Nine of the 13 mice representing the IFN-γ /*T. gondii* treated litters tested positive for *T. gondii* via IFA. Of 9 positive mice 7 tested positive for tissue cysts in the frontal cortex. None of the HBSS and IFN-γ treated litters tested positive for *T. gondii* tissue cysts by brain smear.

Histology

Eight mice from the IFN-γ /*T. gondii* were evaluated for tissue cysts and necrosis and 11 mice from the T. gondii infected group were evaluated for tissue cysts and necrosis. The average score for the IFN-γ /*T. gondii* mice was 1.1 compared to 1.9 for the *T. gondii* infected mice.

DISCUSSION

MIS with IFN-γ

The mechanism for how exogenous administration of IFN-γ serves to protect a fetus from infectious agents is not well understood. Endogenous IFN- γ is required for modulation of Th-1 T-cell response, required for limiting *T. gondii* infections. A strong Th-1 T-cell response can also be detrimental to the embryo by causing rejection of the embryo. Optimizing dose and timing of the administration of IFN-γ must be done to achieve the desired results of protection of the embryo. Contrary to the expected outcome, of administration of exogenous IFN- γ, causes a shift towards a Th-2 T-cell immune response. Preliminary work has demonstrated exogenous IFN-γ is not detectable 24 hrs. after administration (data not shown) leading us to the conclusion that the protective effects observed by MIS mice, days 10 and 5 pre breeding prior to infections is the result a downstream cellular mechanisms.

General mouse behavior

Data from previous studies indicated MIS with INF- γ given prior to *T. gondii* infection decreased fetal mortality from congenital infections. The mechanism for how MIS with IFN- γ decreased mortality is unknown. It is suggested that prophylactic immune stimulation causes an up-regulation in the immune system to prevent*, in utero*, *T. gondii* infections from causing fetal mortality (provisional patent number VCOM-113-PRO).

When IFN-γ /*T. gondii* infected mice in our study were evaluated for tissue cysts by brain smear we looked at the frontal cortex. Seven of 9 IFA positive mice are positive for tissue cysts in the frontal cortex. According to Wang et al., (2011) the cortices were one of the two most likely regions of the brain for tissue cysts to be identified. The presence of tissue cysts in the frontal cortex indicated that a foreign agent was presence and potentially capable of causing cognitive impairments observed in the IFN-γ /*T. gondii* infected group of mice.

Congenital *T. gondii* transmission rate between males and females using our infection model was 70% and 74% respectively. When MIS was administered once prior mating and again the day before infection, with *T. gondii,* the transmission rate decreased for males and increased for females but was not statistically significant ($P>0.1$). Our experiment suggested that although mortality was decreased, the congenital transmission rate for *T. gondii* was not altered. The reason for the shift in infection rate between males and females was most likely caused by a subtle shift in male to female ratio between treatment groups caused by treatment of either IFN-γ or *T. gondii*. A shift in male to female ratio, in female mice with chronic toxoplasmosis, has previously been observed by Kankova et al., (2007). A shift for male to female ratio for the IFN-γ/*T. gondii* treated dams has not previously been reported.

Mice from the MIS treatment group had decreased inflammation and necrosis around the tissue cysts in the brain compared to controls. Our findings suggest an immune shift in mice receiving IFN-γ treatment *in utero* persisting as long as 9 wks. after birth. A finding of potentially lifelong attenuated immune response has not been indicted anywhere in the literature. Currently the literature suggests MIS can cause a

shift in the offspring immune response. To date, 28 days post parturition was the longest time for a shift in offspring immune system to persist as a result of MIS (Ponzio et al., 2007).

Our activity and rearing data revealed no difference between males or females in treatment groups for the first 5 minutes in a new environment. In the present study, it appears that IFN-γ, *T. gondii* and IFN-γ/*T. gondii* treatments do not negatively affect novel open field activity or exploration as has been previously reported with postnatal *T. gondii* infected mice (Hutchinson et al., 1980) and other models of MIS (Meyer et al., 2006).

Behavior response to the administration of MIS and congenital infection with *T. gondii* varied greatly with the age and sex of mice. For this reason male and female mice were analyzed separately. Typically male and female mouse behavior does not differ before sexual maturity. After 5-6 wks. of age, when mice become sexually mature divergent behavior changes are observed (Kvist and Selander 1987). Female mice become more sedentary. By staying in a smaller area, it permits mature male mice to seek them out for breeding purposes. Male mice, after 5-6 wks. of age, become more active and typically travel greater distance in search of female mice. To control for the divergence in behavior between male and females, treated mice were only compared to age and sex matched mice.

Transient FOB changes

The FOB is a compilation of 30 tests; we tested the mice using the virtual cliff (VC) and visual placement (VP) concurrently with FOB. It should be noted for many of

the aspects of the FOB, VC and VP, no differences between the groups existed indicating that the different treatments; infection, MIS or a combination of both, had no measurable effect on the mice. Furthermore all of the differences observed at 4 weeks of age in the FOB, VC and VP for females and males treatments dissipated with time and by 8 weeks of age no statistical (P>0.1) differences were found.

Female mice from the three treatment groups; IFN-γ, *T. gondii* and IFN-γ/*T. gondii* all experienced impairments in the click response compared to controls. Failure of all the experimental female mouse groups at 4 wk. old to respond to stimuli properly was a novel finding but was not unexpected, because mice receiving MIS with Poly (I:C) perform poorly in prepulse inhibition tests (Meyer et al., 2006). Prepulse inhibition measures conditioned response to startle. Although the tests were different, with the prepulse test measuring the processing of stimuli and click measuring response to stimuli, there was a trend of impaired response to stimuli. 4 wk. old male mice exhibited no differences (P>0.1) between treatment groups. The lack of a response to click at 8 wks. of age could be caused by deafness. Deafness frequently occurs in CD-1 mice later in life and this may have been a contributing factor in our findings at 8 wks. (Shone et al., 1991).

Congenital infection with *T. gondii* is the leading cause of retinochoriditis in the United States and can cause severe complications in vision (Jones and Holland 2010). Blindness is potentially caused by recrudescent infections in the eye, an immune privileged organ. As a result of the inflammatory response, lesions develop, resulting in vision impairments or blindness. Our VP test indicated 4 wk. old female mice were not negatively affected by treatment with either INF-γ or *T. gondii* when compared to control

mice. INF-γ and *T. gondii* treated female mice completed the VP test at a higher rate than control and IFN-γ/*T. gondii* mouse groups. The tests incorporated coordination and movement for completion so an increase in nonspecific movement and hyperactivity, previously described for other MIS and post natal infection with *T. gondii* models, could have aided in a higher completion rate over controls. It should be noted that age matched male mice did not experience differences in the completion of the visual placement test. By 8 wks. of age, no differences in completion rate for either sex or treatment groups were observed indicating MIS was not affecting vision. Furthermore, these results indicated the eyesight should not be considered a factor for performance in the Barnes maze. The virtual cliff tests fear avoidance, and at 4 weeks of age a divergence between sexes and treatment groups emerged. Female mice infected with *T. gondii*, regardless of MIS status exhibited decreased fear of falling, evident by walking onto the clear side of the virtual cliff more often than uninfected female mice. 4 week old male mice had a different result with *T. gondii* male mice traveling to the dark side of the virtual cliff more often than uninfected mice. *T. gondii* infected mice, regardless of sex, had an altered fear of falling is unknown. Our results indicated MIS did not correct for changes observed in the virtual cliff associated with *T. gondii* infections. By 8 wks., female and male mice had no statistical ($P>0.1$) differences between groups for the VC test, indicating decreased fear of falling observed in female mice at 4 wks. of age resolved with time.

Barnes maze 4 week old female

When only MIS was evaluated compared to control mice, the results varied greatly depending on age and sex. MIS, IFN-γ, 4 wk. old female mice when tested using the Barnes maze had a trend for increased, latency (*P*=0.101) and initial assessment of environment (*P*=0.104), while these values were not statistically significant they are of value for assessing behavior in the IFN-γ, treated female mice at 4 wks. of age. The IFN- 4 wk. old female mice also had a statistical increase random search strategy (*P*=0.036) and decreased time spent exploring each hole (*P*=0.075) compared to control female mice. Increased activity, decreases in memory retention and recognition of environment, were reported in mice receiving MIS with poly (I:C) as an adult (9-10 wks. old) onset behavior profile (Ozawa et al., 2006). We were observing similar behavior profiles reported in the literature from other MIS models, except our observations were for pre-sexual mature female mice only. In our study we found IFN-γ MIS mice had difficulty acquiring the task, manifested by more deficits in learning than control mice. Further testing at 8 wks. of age revealed that all of these specific behavior deficits dissipated with time indicating the changes observed were not lifelong.

We have proposed that MIS with IFN-γ would ameliorate the negative effects that congenital infection with *T. gondii* has on behavior. 4 wk. old female *T. gondii* and IFN-γ/*T. gondii* treated mice both had increased initial recognition of the environment (*P*=0.04 and *P*=0.088) respectively. A similar increase in time spent assessing the environment was observed for MIS, IFN-γ female mice; indicating both MIS and *T. gondii* infection caused similar outcomes. MIS and *T. gondii* female mice also shared a decrease in spatial learning evident by an increase in random search strategy, not observed for the IFN-γ/*T. gondii* treated group. The IFN-γ/*T. gondii* treated group did

have an increase in serial search strategy (*P*=0.079) but they also had an increase in random search strategy (increase not statistically significant at *P*>0.1) making it impossible to formulate a conclusion of increased rate of learning. Taken together 4 wk. old female mice were most negatively affected by MIS, followed by *T. gondii* and least effected by IFN-γ/*T. gondii*. The behavior patterns exhibited by MIS appear to cause more deficits when given alone rather in combination with *T. gondii* infection. MIS with INF-γ given prophylactically to *T. gondii* infected mice decreases some but not all of the behavior deficits associated with *T. gondii* alone

Barnes maze 4 week old male

Male mice 4 wks. of age receiving MIS with IFN-γ, unlike MIS IFN-γ female mice, showed no difference (*P*<0.1) in behavior compared to control male mice. The difference in response to IFN-γ MIS between sexes was unexpected. The divergent effects MIS had on behavior between females and males prior to sexual maturity has not been reported in the literature for any MIS regimes. We currently have no explanation for the differences between sexes. Further confounding our results was an unexpected decrease in latency (*P*=0.085) for IFN-γ /*T. gondii* infected male mice. Clearly MIS alone with male mice did not influence latency but when co-administered with *T. gondii* the result was a decrease in latency. Four wk. old IFN-γ/*T. gondii* male mice increased the use of serial search strategy (*P*=0.079) compared to control mice, while decreasing the use of random search strategy (decrease not statistically significant at *P*>0.1). Increased use of serial search strategy indicated increased rate of learning, resulting in the decrease maze latency observed for IFN-γ/*T. gondii* group of

mice. It was unclear why MIS with INF-γ and congenital infection with *T. gondii* would improve serial search strategy and latency over control mice. Four wk. old male mice unlike female mice were less affected from MIS or infection with *T. gondii* but like 4 wk. old female mice, the male mice receiving both IFN-γ and *T. gondii* performed as good if not better in the Barnes maze than mice receiving only MIS or *T. gondii*.

To date behavior assessments examining the effects of MIS with IFN-γ on post sexually mature mice have not been reported in the literature. Clearly MIS with Poly (I:C) has a clear expected effect on adult onset behavior profile (Ozawa et al., 2006). How MIS caused behavior profile alterations is not fully understood, but it appears MIS with Poly (I:C) causes a lifelong shift in the expression of some genes associated with neurotransmitter production (Meyer, Engler et al., 2008). An adult behavior profile has not been established in mice born to dams using MIS with INF-γ. We demonstrated some adult onset behavior shifts associated with MIS with IFN-γ treated mice. The behavior patterns observed were not as drastic as behaviors associated MIS with Poly (I:C) administered on a similar time schedule.

Barnes maze 8 week old male and female

Female mice post sexual maturation experienced minimal effects from MIS with IFN-γ, infection with *T. gondii* and IFN-γ/*T. gondii* treatment. All three treatment groups experience a decrease in random search strategy (P=0.082, 0.015, and 0.03 respectively) compared to control mice, concurrently IFN-γ and *T. gondii* infected mice had an increase in serial search strategy (P>0.1). While the increase was not statistically significant it should be considered to indicate an increase in rate of learning

when examined in conjunction with random search strategy. The increase in serial search strategy was not found for IFN-γ/*T. gondii* female mice. Our findings for IFN-γ and *T. gondii* treated groups were an increased rate of learning over control mice. The increase was minimal and ultimately had no effect in overall completion of the maze. IFN-γ/*T. gondii* treated mice had decreased initial assessment of environment and increased distance traveled compared to control mice. Changes in these two aspects of the Barnes maze did not negatively affect overall maze performance but should be noted because increased distance traveled/increased overall activity were previously described for mice infected with *T. gondii* and MIS with Poly (I:C) respectively (Hutchinson et al., 1980; Ozawa et al., 2006).

Male IFN-γ MIS mice, similar to their female, counterparts did not show signs of adult onset deficits in the Barnes maze. To the contrary, IFN-γ MIS male mice completed the maze faster than control mice. An interesting observation for both IFN-γ and IFN-γ /*T. gondii* groups of mice was they experienced an increase time required for initial assessment of environment (P=0.078 and 0.058 respectively). Increased initial assessment of environment in most cases did not negatively affect latency in the maze. It is possible for the IFN-γ treated mice, the increased time for initial assessment of environment, aided in completion of the maze faster, although this trend does not exist for other treatment groups with increased initial assessment of environment.

T. gondii infected male mice at 4 wks. of age had minimal behavior alterations but by 8 weeks of age male mice demonstrated what was considered adult onset behavior deficits consisting of increased activity (P=0.08), decreased memory (P=0.05) and rate of learning (P=0.08). A behavior profile that was not observed in the IFN-γ/*T.*

gondii treated mouse profile, indicating MIS had a protective effect for adult onset behavior profile caused by congenital infection with *T. gondii*.

At 8 wk. of age, the IFN-γ male mice similar to the 8 wk. IFN-γ female mice were not displaying the adult onset behavior deficits associated with other MIS mouse models. How the male and female mice responded to MIS with IFN-γ appeared to be sex related, but a mechanism for these results has yet to be proposed. Our IFN-γ MIS treatment regime differs from the other MIS regimes. We gave the IFN-γ pre-breeding and 24 hours prior to infection. The half–life of exogenous IFN-γ and its bioavailability to the fetuses in a mouse is currently not known. Many of our findings for mice 4 wks. old were similar to findings for adult onset behavior changes associated with poly (I:C) MIS models (Ozawa et al., 2006) and post natal infection with *T. gondii* models (Hutchinson et al 1980). But the behaviors dissipated by 8 wks. in our mice. Clearly our IFN-γ MIS model caused behavior alterations but in a much different pattern than what was reported with poly (I:C) MIS models.

The protection MIS with IFN-γ had when given in conjunction with *T. gondii* was not a simple correction of all *T. gondii* induced behavior changes to normal control levels, instead it corrected all behavior changes induced by *T. gondii*, while exhibiting most behavior changes caused by administration of IFN-γ alone and producing some behavior alterations not explained by either IFN-γ or *T. gondii* treatment. In most of these cases IFN-γ/*T. gondii* behavior profile were either an improvement over controls or caused no deficits. IFN-γ negatively affected female mice at 4 wks. of age more than any other group which was unexpected because age matched male mice receiving the same treatment had no effect. It should be noted when IFN-γ was given in conjunction

with *T. gondii* it provided a protective effect, warranting further investigation into timing, dose and concentration in relation to exposure to a known behavior altering teratogen.

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LITERATURE CITED

Adams, B., T. Fitch, S. Chaney, and R. Gerlai 2002. Altered performance characteristics in cognitive tasks: comparison of the albino ICR and CD1 mouse strains. Behavioural Brain Research **133**: 351-361

Buka, S.L., M.T. Tsuang, E.F. Torrey, M.A. Klebanoff, D. Bernstein, and R.H. Yolken. 2001 Maternal infections and subsequent psychosis among offspring. Archives of General Psychiatry **58**: 1032-1037.

Dubey, J.P. 1997. Tissue cyst tropism in *Toxoplasma gondii*: a comparison of tissue cyst formation in organs of cats and rodents fed oocysts. Parasitology **115**: 15–20.

Fox, W.M., 1965. Reflex-ontogeny and behavioural development of the mouse. Animal Behavior **13**: 234-241

Hay J., P.P. Aitken, W.M. Hutchison, and D.I. Graham. 1983. The effect of congenital and adult-acquired *Toxoplasma* infections on the motor performance of mice. Annals of Tropical Medicine and Parasitology **77**: 261-277.

Holladay, S.D., L.V. Sharova, K. Punareeewattana, T.C. Hrubec, R. M. Gogal Jr. M.R. Prater M.R., and A.A. Sharov. 2002. Maternal immune stimulation in mice decreases fetal malformations caused by teratogens. International Immunopharmacology **2**: 325-332.

Hrubec, T.C., M.R. Prater, K. Punareewattana, and S.D. Holladay. 2005. Reduction of teratogen induced birth defects in mice: role of maternal immune stimulation. Current Topics in Toxicology **2**: 33-40.

Hrubec, T.C., M.R. Prater, K.A. Toops, and S.D. Holladay. 2006. Reduction in diabetes-induced craniofacial defects by maternal immune stimulation. Birth Defects Research. Part B, Developmental and reproductive toxicology **77**: 1-9.

Hutchinson, W.M., M. Bradley, W.M. Cheyne, B.W. Wells, and J. Hay. 1980. Behavioural abnormalities in *Toxoplasma*-infected mice. Annals of Tropical Medicine and Parasitology **74**: 337-345.

Jones, J.L., and G.N. Holland. 2010. Annual burden of ocular toxoplasmosis in the US. American Journal of Tropical Medicine and Hygiene **82**: 464-465.

Kadowaki, N., S. Ho, S. Antonenko, R.W. Malefyt, R.A. Kastelein, F. Bazan, and Y.J. Liu. 2001. Subsets of human dendritic cell precursors express different toll-like receptors and respond to different microbial antigens. Journal of Experimental Medicine **194**: 863-869.

Kanková, S., P. Kodym, D. Frynta, R. Vavrinova, A. Kubena, and J. Flegr. 2007. Influnece of latent toxoplasmosis on the secondary sex ration in mice. Parasitology **94:** 122-127.

King, M.D., D. S. Lindsay, S. Holladay, and M. Ehrich. 2003. Neurotoxicity and immunotoxicity assessment in CBA/J Mice with chronic *Toxoplasma gondii* infection and single-dose exposure to methylmercury. International Journal of Toxicology **22:** 53–61.

Kvist, B., and R. Selander. 1987. Sex difference in open field activity after learning in mice. Scandinavian Journal of Psychology **28**: 88-91.

Meyer, U., J. Feldon, M. Schedlowski, and B.K. Yee. 2006. Immunological stress at the maternal-foetal interface: a link between neurodevelopment and adult psychopathology. Brain Behavior and Immunity **20**: 378-388.

Meyer, U., A. Engler, L.Weber, M. Schedlowski, and J. Feldon. 2008. Preliminary evidence for a modulation of fetal dopaminergic development by maternal immune activation during pregnancy. Neuroscience **154**:701-709.

Moser, V.C., J. P. McCormick, J. P. Creason, and R. C. MacPhail. 1988. Comparison of chlordimeform and carbaryl using a functional observational battery. Fundamental and Applied Toxicology **2:** 189–206

Ozawa, K., K. Hashimoto K, T. Kishimoto, E. Shimizu, H. Ishikura, and M. Iyo. 2006. Immune activation during pregnancy in mice leads to dopaminergic hyperfunction and cognitive impairment in the offspring: a neurodevelopmental animal model of schizophrenia. Biological Psychiatry **59**:546-54

Ponzio, N.M., R. Servatius, K. Beck, A. Marzouk, and T. Kreider. 2007. Cytokine levels during pregnancy influence immunological profiles and neurobehavioral patterns of the offspring. Annals of New York Academy of Science **1107:** 118-128.

Prater, M.R., K.L. Zimmerman, D.L. Ward, and S.D. Holladay. 2004. Reduced birth defects caused by maternal immune stimulation in methylnitrosourea-exposed mice: association with placental improvement. Birth Defects Research. Part A, Clinical and Molecular Teratology **70:** 862-869.

Punareewattana, K., L. V. Sharova, W. Li, D. L. Ward, and S.D. Holladay. 2003. Reduced birth defects caused by maternal immune stimulation may involve increased expression of growth promoting genes and cytokine GM-CSF in the spleen of diabetic ICR mice. International Immunopharmacology **3:** 1639-1655.

Roth, T.L., F.D. Lubin, M. Sodhi, and J.E. Kleinman. 2009. Epigenetic mechanisms in schizophrenia. Biochim Biophys Acta **1790:** 869-877.

Sharova, L.V., A.A. Sharov, P. Sura, R.M. Gogal, B.J. Smith, and S.D. Holladay. 2003. Maternal immune stimulation reduces both placental morphologic damage and down-regulated placental growth-factor and cell cycle gene expression caused by urethane: are these events related to reduced teratogenesis? International Immunopharmacology **3**:945-955.

Shi, L., S.H. Fatemi, R.W. Sidwell, and P.H. Patterson. 2003. Maternal influenza infection causes marked behavioral and pharmacological changes in the offspring. Journal of Neuroscience **23**: 297-302.

Smith, S.E., J. Li, K. Garbett, K Mirnics, and P.H. Patterson. 2007. Maternal immune activation alters fetal brain development through interleukin-6. Jouranl of Neuroscience **27**: 10695-10702.

Susser, E.S., and S. P. Lin. 1992. Schizophrenia after prenatal exposure to the Dutch Hunger Winter of 1944-1945. Archives of General Psychiatry **49:** 983-988.

Yolken, R.H., S. Bachmann, I. Ruslanova, E. Lillehoj, G. Ford, E.F. Torrey, and J. Schroeder. 2001. Antibodies to *Toxoplasma gondii* in individuals with first-episode schizophrenia. Clinical Infectious Disease **32**: 842-844.

Wang, T., M. Liu, X.J. Gao, Z.J. Zhao, X.G. Chen, and Z.R. Lun. 2011. *Toxoplasma gondii*: The effects of infection at different stages of pregnancy on the offspring of mice. Experimental Parastology **127**: 107-112.

Zuckerman, L., and I. Weiner. 2005. Maternal immune activation leads to behavioral and pharmacological changes in the adult offspring. Journal of Psychiatric Research. **39**:311-323.

FIGURE AND TABLES

FIGURE 1. The Barnes maze apparatus. The Barnes maze apparatus (arrow = goal box) used to examine mouse behavior. (**A**) Beginning of the test with mouse in the start box in the center of the maze. (**B**) Mouse demonstrating inactive time. (**C**) Mouse investigating a wrong hole. (**D**) Mouse entering the goal box.

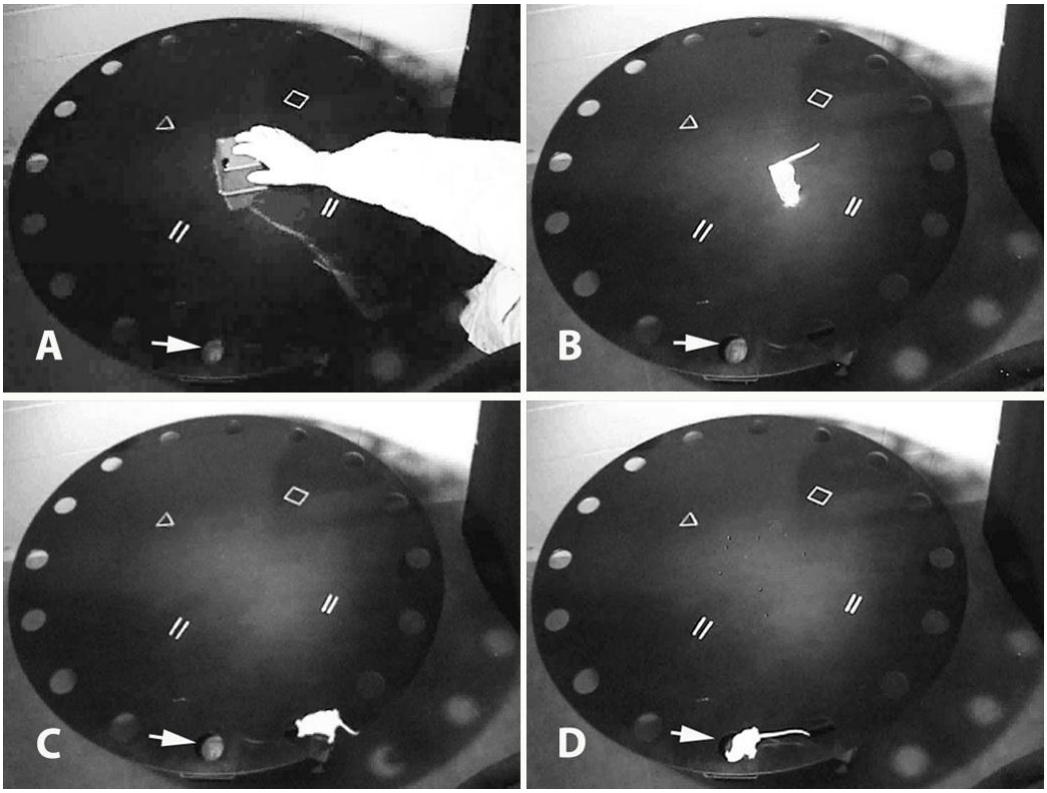

Table I. The Barnes maze results summary. Behavioral parameters measured in and results* of the Barnes maze test control vs. MIS with IFN-γ, control vs. *T. gondii* and control vs. MIS with IFN-γ/*Toxoplasma gondii* congenital infection for 4-wk-old and 8-wk-old female and male mice.

Behavior	**Description**	**Result 4 wk.**	**Result 8 wk.**
Inactive time	Initial period of inactivity in maze center when trial starts	T.g., and MIS/T. g. females*	MIS/T. g. females* MIS and MIS/T.g. males*
Memory	Distance between goal box and 1st hole investigated	T.g males*	T.g males*
Distance	Distance traveled to goal or reached by expiry of observation period	None	MIS/T.g females*
Errors	Visitation to a hole other than the goal box hole	None	None
Activity	Number of holes visited/Active time (overall time minus inactive time)	MIS females*	T.g. males*
Random search strategy	Passing through center of maze or skipping more than 4 holes	MIS and T.g. females*	MIS, T.g., and MIS/T. g. females* T.g. males*
Serial search strategy	Persistent sequential errors in holes besides the goal box	MIS/T.g. females* MIS/T.g. males*	None
Latency	Time elapsed from start to finish of trial	MIS males	MIS males*
Task acquisition	Direct route to goal box with errors $\underline{\leq 2}$ in 3 of 4 trials on 2 consecutive days	None	None

*Results were significantly different ($P \leq 0.1$).

Table II. Male and female mice used. Number of female (F) and male (M) litters used for each treatment group, control, MIS (IFN-γ), congenital *T. gondii* exposed (T.g.), and MIS and congenital *T. gondii* exposed (IFN-γ/T.g.).

4 week	Control	IFN-γ	T.g.	IFN-γ/T.g
BM	6F, 7M	7F, 8M	8F, 7M	6F 7M
FOB	9F, 8M	9F, 9M	4F, 5M	7F, 8M
VP	9F, 8M	9F, 9M	4F, 5M	7F, 8M
VC	9F, 8M	9F, 9M	4F, 5M	7F, 8M
8 week	Control	IFN-γ	T.g.	IFN-γ/T.g
BM	5F, 7M	5F, 8M	7F, 7M	5F, 5M
FOB	7F, 7M	7F, 8M	8F, 7M	6F, 7M
VP	7F, 7M	7F, 8M	8F, 7M	6F, 7M
VC	7F, 7M	7F, 8M	8F, 7M	6F, 7M
Activity	7F, 7M	7F, 8M	8F, 7M	6F, 7M
BS	4F, 5M	7F, 8M	9F, 6M	6F, 7M
NT	6F, 6M	6F, 9M	8F, 7M	6F, 7M

Barnes Maze (BM), Functional observational battery (FOB), Visual placement (VP), Virtual cliff (VC), Open field activity (Activity), Brain smear (BS), and neurotransmitter (NT).

Table III. FOB results. The results of behavioral testing of control vs. MIS with IFN-γ, control vs. *T. gondii* and control vs. MIS with IFN-□□/Toxoplasma gondii congenital infection, mice at 4 and 8 wk. of age using the functional observational battery test.

Behavior	**Description**	**Result 4 wk.**	**Result 8 wk.**
Virtual Cliff	Initial period of inactivity in maze center when trial starts	T.g, and MIS/T. g. females* T. g. males*	None
Righting	Distance between goal box and 1st hole investigated	T.g males*	None
Click	Distance traveled to goal or reached by expiry of observation period	MIS, T.g., and MIS/T.g. females	None
Visual placement	Visitation to a hole other than the goal box hole	MIS females	None

Table IV. FOB negative results summary. Parameters evaluated in the Functional Observation Battery tests*.

Open field ataxia	Soft stool	Corneal bulging
Tremors/convulsions	Fecal stain	Partially closed eyes
Posture	Urine stain	Piloerection
Coat condition	Salivation	Dehydration
Tail Condition	Nasal discharge	Protruding penis
Respiration	Oral discharge	Cool to touch cyanosis
Vocalization	Lacrimation	Weight gain/loss
Diarrhea	Ocular discharge	Activity

* None of these measured parameters were significantly different ($P>0.1$)

Table V. Neurrotransmitters measured. Mean concentrations of neurotransmitters and their metabolites in the brains of male (M) and female (F) mice; control, MIS (IFN-γ), congenital *T. gondii* exposed (T.g.), and MIS and congenital *T. gondii* exposed (IFN-γ/T.g.) examined at 8 weeks of age.

Sex	treatment	Number of mice	Neurotransmiter or metabolite	Location in brain cortex/striatum*
M	HBSS	6	dopamine	0.047/0.474 mM/mg
M	IFN-γ	9	dopamine	0.057/0.563 mM/mg
M	*T. g*.	7	dopamine	0.040/0.476 mM/mg
M	IFN-γ/*T. g*.	7	dopamine	0.050/0.407 mM/mg
F	HBSS	6	dopamine	0.049/0.608 mM/mg
F	IFN-γ	6	dopamine	0.027/0.367 mM/mg
F	*T. g* .	8	dopamine	0.052/0.447 mM/mg
F	IFN-γ/*T. g*.	6	dopamine	0.041/0.407 mM/mg
M	HBSS	6	3,4-dihydroxyphenylacetic acid	0.0062/0.050 μM/mg
M	IFN-γ	9	3,4-dihydroxyphenylacetic acid	0.0061/0.057 μM/mg
M	*T. g*.	7	3,4-dihydroxyphenylacetic acid	0.0062/0.046 μM/mg
M	IFN-γ/*T. g*.	7	3,4-dihydroxyphenylacetic acid	0.0065/0.041 μM/m
F	HBSS	6	3,4-dihydroxyphenylacetic acid	0.0066/0.070 μM/mg
F	IFN-γ	6	3,4-dihydroxyphenylacetic acid	0.0029/0.039 μM/mg
F	*T. g*.	8	3,4-dihydroxyphenylacetic acid	0.0082/0.060 μM/mg
F	IFN-γ/*T. g*.	6	3,4-dihydroxyphenylacetic acid	0.0065/0.050 μM/mg
M	HBSS	6	homovanillic acid	0.009/0.044 μM/mg
M	IFN-γ	9	homovanillic acid	0.010/0.053 μM/mg
M	*T. g*.	7	homovanillic acid	0.010/0.054 μM/mg
M	IFN-γ/*T. g*.	7	homovanillic acid	0.009/0.045 μM/mg
F	HBSS	6	homovanillic acid	0.008/0.057 μM/mg
F	IFN-γ	6	homovanillic acid	0.006/0.045 μM/mg
F	*T. g*.	8	homovanillic acid	0.008/0.046 μM/mg
F	IFN-γ/*T. g*.	6	homovanillic acid	0.010/0.051 μM/mg
M	HBSS	6	norepinephrine	0.010/0.017 μM/mg
M	IFN-γ	9	norepinephrine	0.012/0.020 μM/mg
M	*T. g*.	7	norepinephrine	0.013/0.014 μM/mg
M	IFN-γ/*T. g*.	7	norepinephrine	0.010/0.020 μM/mg
F	HBSS	6	norepinephrine	0.011/0.019 μM/mg
F	IFN-γ	6	norepinephrine	0.008/0.018 μM/mg
F	*T. g*.	8	norepinephrine	0.008/0.013 μM/mg

F	IFN-γ/*T. g*.	6	norepinephrine	0.011/0.017 μM/mg
M	HBSS	6	epinephrine	0.355/0.97 pM/mg
M	IFN-γ	9	epinephrine	0.452/0.67 pM/mg
M	*T. g*.	7	epinephrine	N/A
M	IFN-γ/*T. g*.	7	epinephrine	0.180/0.33 pM/mg
F	HBSS	6	epinephrine	0.386/1.53 pM/mg
F	IFN-γ	6	epinephrine	0.365/1.27 pM/mg
F	*T. g*.	8	epinephrine	N/A
F	IFN-γ/*T. g*.	6	epinephrine	0.183/0.58 pM/mg
M	HBSS	6	3-methoxy-4-hydroxyphenylglycol	2.12/2.74 pM/mg
M	IFN-γ	9	3-methoxy-4-hydroxyphenylglycol	2.03/3.21 pM/mg
M	*T. g*.	7	3-methoxy-4-hydroxyphenylglycol	2.72/1.8 pM/mg
M	IFN-γ/*T. g*.	7	3-methoxy-4-hydroxyphenylglycol	1.64/3.12 pM/mg
F	HBSS	6	3-methoxy-4-hydroxyphenylglycol	2.43/3.15 pM/mg
F	IFN-γ	6	3-methoxy-4-hydroxyphenylglycol	1.39/3.40 pM/mg
F	*T. g*.	8	3-methoxy-4-hydroxyphenylglyco	1.87/2.35 pM/mg
F	IFN-γ/*T. g*.	6	3-methoxy-4-hydroxyphenylglyco	2.18/2.20 pM/mg
M	HBSS	6	serotonin	0.011/0.035 μM/mg
M	IFN-γ	9	serotonin	0.011/0.036 μM/mg
M	*T. g*.	7	serotonin	0.011/0.031 μM/mg
M	IFN-γ/*T. g*.	7	serotonin	0.009/0.032 μM/mg
F	HBSS	6	serotonin	0.011/0.039 μM/mg
F	IFN-γ	6	serotonin	0.007/0.030 μM/mg
F	*T. g*.	8	serotonin	0.011/0.031 μM/mg
F	IFN-γ/*T. g*.	6	serotonin	0.011/0.032 μM/mg
M	HBSS	6	5-hydroxyindoleacetic acid	2.91/7.34 pM/mg
M	IFN-γ	9	5-hydroxyindoleacetic acid	3.03/7.97 pM/mg
M	*T. g*.	7	5-hydroxyindoleacetic acid	3.71/7.90 pM/mg
M	IFN-γ/*T. g*.	7	5-hydroxyindoleacetic acid	2.55/7.21 pM/mg
F	HBSS	6	5-hydroxyindoleacetic acid	3.65/9.35 pM/mg
F	IFN-γ	6	5-hydroxyindoleacetic acid	2.18/7.80 pM/mg
F	*T. g*.	8	5-hydroxyindoleacetic acid	3.59/8.31 pM/mg
F	IFN-γ/*T. g*.	6	5-hydroxyindoleacetic acid	3.85/9.17 pM/mg

* mean value for group

† ND = not determined

SUMMARY

Over the past decade, evidence supports that chronic infection with *T. gondii* can cause lifelong changes from subtle behavior alteration, learning and memory deficits, to more complex interactions including possible contributions to mental illness. The neurotropic nature of *T. gondii* infection makes it a likely candidate for causing neurologic changes. Additional evidence that *T. gondii* infection may contribute to mental illness is our findings that demonstrating phenothiazines, a class of typical antipsychotic drugs, inhibit *T. gondii* replication and that dopamine, a potent neurotransmitter, stimulates T*. gondii* replication. Maternal exposure and transmission of *T. gondii* to the fetus has long been recognized as the most detrimental time for infection. MIS with the immune stimulant IFN-γ has been shown to successfully decrease birth defects when administered prophylactically to administration of known teratogens. We determined that IFN-γ when given prior to *T. gondii* decreased the negative behavior effects associated with congenital *T. gondii* infection. Our findings support a theoretical mechanism for *T. gondii* infections and prophylactic treatment for amelioration of potential negative effects of a teratogen.

Previous research indicates a connection between an elevation in the neurotransmitter dopamine and infection with *T. gondii*. More recently increased numbers of *T. gondii* tissue cysts were found in the region of the brain with high dopamine levels. We found dopamine at levels as low as 250 nM have a pronounced

effect on the proliferative capacity of the rapidly dividing tachyzoite stage of *T. gondii*. How dopamine increases the proliferative capacity is currently unknown. We found no indication that *T. gondii* has within its genome a code for a human dopamine receptor that would bind dopamine and cause an increase in proliferation we found. The proliferative capacity of *T. gondii* experimentally is highly susceptible to free radicals. Dopamine has been found to possess strong antioxidant properties, indicating that the chemical structure of dopamine may play a role in increased proliferation. With this alternative explanation one can speculate an alternative mechanism for dopamine's effects on *T. gondii* proliferation is not direct but is caused by a microenvironment more suitable for *T. gondii* proliferation. The result is a potential for an increase in the number of tissue cysts in regions of the brain with high dopamine levels. The propensity for *T. gondii* to encyst lifelong in neural tissue of infected individuals makes it a probable candidate for causing behavior changes

Schizophrenia in the past decade has been linked to maternal exposure to *T. gondii* in utero decades earlier. We found that phenothiazines, a drug class used for the treatment of schizophrenia, inhibit the proliferation of *T. gondii* in vitro. Fluphenazine, a drug in the phenothiazine class showed the most promise with an IC_{50} of 1.7 ± 0.1 µM. Chronic infection with *T. gondii* results in slow growing relatively inert tissue cysts causing few overt clinical symptoms. These tissue cysts naturally rupture causing a recrudescent infection that result in the dissemination of the infection throughout the body in the form of tachyzoites. These tachyzoites are the stage of *T. gondii* that is susceptible to fluphenazine. Fluphenazine, because of its target, is already designed to cross the blood brain barrier giving it access to region of the body currently difficult to

treat with some drugs. Our finding suggests a theoretical way for antipsychotic drugs to act on chronic *T. gondii* infection.

We further tested the mood stabilizing drug, valproic acid using an in vivo mouse model to evaluate anti-parasitic activity. While fluphenazine had the lowest I.C. 50 concentration valproic acid was previously used by Webster et al. to return rat's innate fear of cat urine to normal levels when chronically infected with *T. gondii*. We tested valproic acid at varying concentration both prophylactically and post infection under different infection regimes and found valproic acid to have no effect on parasite burden. For these in vivo experiments, death and tissue cyst enumeration were used as parameters for efficacy. Mice, unlike rats, are more susceptible to *T. gondii* infection. The mechanism of action for valproic acid on *T. gondii* is currently unknown. However, it is suspected to be a folic acid inhibiter. These experiments found valproic acid had no anti-parasitic effect in vivo, on both acute and chronic *T. gondii* infection. No explanation for its diminished effect in vivo can be explained.

Maternal exposure and subsequent infection with *T. gondii* is implicated in mental illness and alterations in both rodent and human behavior. By using the Barnes maze, we were able to test for cognitive impairments. We determined congenital toxoplasmosis in a mouse model did alter the offspring rate of learning, perception of environment and memory. We also determined that the effects of infection with *T. gondii*, or treatment, with IFN-γ, affected male and female mouse offspring differently. We determined that MIS with IFN-γ did not decrease the transmission rate of congenital infection but it did decrease mortality when administered prophylactically before infection with *T. gondii*.

Congenital *T. gondii* infection of 4 week old female and male mice had only minor effects on behaviors tested by the Barnes maze. All the changes in behaviors observed at the 4 week time point disappeared by the 8 week time point, indicating the behavior patterns were not permanent. *T. gondii* infected female mice at 8 weeks of age similar to the mice 4 weeks of age, had few behavior modifications. Alternatively, congenitally *T. gondii* infected male mice 8 weeks of age had deficits in memory, a decrease in activity and decreased rate of learning. Our conclusion was male mice were more susceptible to cognitive impairments cause by congenital *T. gondii* infection.

When dams were MIS, without infection with *T. gondii*, 4 week old female mice had deficits in task acquisition, decreased activity, increased assessment of environment and random search strategy. Male mice at 4 weeks of age had no deficits when compared to seronegative control mice. Male and female 8 week old mice had minimal difference compared to seronegative control mice indicating the deficits in the 4 week old female mice were not permanent and would change over time.

MIS, when given prior to infections with *T. gondii,* caused no deficits for 4 week old male and female mice. Minimal improvements in rate of learning were observed for 4 week old male mice. Eight week old female mice had minimal impairments; conversely 8 week old male had increased assessment of environment and correction of all negative deficits caused by congenital infection. The results indicate MIS, when given prior to infection, ameliorated the negative cognitive deficits caused by infection for 8 week old male mice. The exact mechanism for how IFN-γ affects fetal outcome is not yet understood but we have evidence suggesting its affects can be both long term and transient.

The practical utility of these experiments explains many unanswered questions while opening several other questions. We can certainly say congenital infection with *T. gondii* may have subtle lifelong effects on the infected individual. Many of the negative effects can be corrected with the prophylactic administration with the immune stimulant IFN-γ. IFN-γ may cause transient behavior changes but our model does not indicate adult onset cognitive deficits as a result of MIS. We also discovered as with every other drug tested thus far neither MIS nor treatment with mood stabilizing drugs has any measurable effect on tissue cyst production or elimination. In an in vitro model we found that dopamine is a potent neurotransmitter thought to be at the center of several mental illnesses and a prerequisite for cognitive processes, can stimulate *T. gondii* replication. Conversely phenothiazines, potent dopamine antagonists, inhibit *T. gondii* replication in vitro. The use of phenothiazines to inhibit *T. gondii* growth is still unclear because drugs that mechanistically are similar but are structurally different to phenothiazines yielded much different results. Further research into increased proliferative capacity of *T. gondii* in and around regions of the brain with high dopamine levels is an area for future research. Similarly the efficacy of phenothaizines as an in vivo drug used for treatment of acute toxoplasmosis warrants further investigation.

Appendix A

Four and eight wk. Barnes Maze results

All females	tx	n	Exp Decay med	Overall time (sec)	Active time (sec)	Distance (holes)	Errors (holes)	GEE mean	Inactive time (sec)	Dis to goal (holes)	Activity (sec/hole)	Center cross	Persevera tions
4w													
All females	N	6		5.7 AB	4.8	7.6	7.2		6.1 ABC	3.3	7.4 A	0.22 A	0.93 A
All females	I	7		12.9 A	9.8 A	7.4	7.4		10.8 A	3.9	11.4 AB	0.7 AB	1.36
All females	T	8		5.1 B	3.7 AB	3.6	4.8 A		16.8 BD	3.5	6.5 B	0.65	0.75 B
All females	I&T	6		12.5	10.7 B	7.4	12.1 A		7.9 CD	3.3	8.4	0.59 B	1.42AB
8w													
All females	N	5		7.7	3.7	4.0 A	6.1		38.1A	4.0	8.5	0.3 ABC	1.05
All females	I	7		6.1	5.5	12.3	5.1		12.2 B	3.6	7.4 A	0.18 A	1.21
All females	T	7		13.8	7.8	7.8	6.2		39.4 BC	4.2	12.3 AB	0.13 B	1.2
All females	I&T	6		11.7	7.7	32.0 A	9.4		10.4 AC	3.8	6.9 B	0.16 C	0.84
All males	tx	n	Exp Decay med	Overall time (sec)	Active time (sec)	Distance (holes)	Errors (holes)	GEE mean	Inactive time (sec)	Dis to goal (holes)	Activity (sec/hole)	Center cross	Persevera tions
4w													
All males	N	7		6.6 A	7.3	6.2	6.8		9.4	4.1 A	7.7	0.63	1.2 A
All males	I	8		6.3	5.5	8.2	10.3		10	3.6	9.4	1.08 AB	1.4
All males	T	7		6.7	5.3	10.2	6.8		8.6	3.1A	7.7	0.35 B	1.0 B
All males	I&T	7		5.4 A	4.6	6.3	6.6		9.5	3.4	11.1	0.4 A	1.7 AB
8w													
All males	N	6		10.0 A	7.9	7.5	7.8		9.4 AB	3.0 A	11.4 A	0.18 A	1.3
All males	I	8		3.7 A	3.1	8.6	4.1		17.0 A	3.4 B	10.9 B	0.14 B	1.0
All males	T	7		7.9	6.1	7.4	6.8		10.9	4.1 AB	5.1 ABC	0.37 AB	0.7
All males	I&T	7		10.3	6	7.3	6.1		13.3 B	3.9	9.4 C	0.18	1.0

catgegoreis with the same letter are differenet from each other P<0.1

Appendix B

Four week old mice FOB test results

4w females	C	I	T	I&T
Dead	0	0	0	0
Ataxia	0	0	0	0
Tremors	0	0	0	0
Righting	12.7	6.1	10.7	18.5
Click	11ABC	18.5A	38.1B	33C
Menace	3.6	8.2	0	8.8
Rod	100	98.1	100	100
Posture	0	0	0	0
Coat	5.6	1.6	0	0
Tail	0	0	0	0
Respiration	0	0	0	0
Vocalization	0	1.2	0	0
Diarrhea	0	0	0	0
SStool	0	0	4.2	0
FStain	0	0	0	0
UStain	0	0	0	0
Salivation	0	0	0	0
NDischarge	0	0	0	0
ODischarge	0	0	0	0
Lacrimation	0	0	0	0
OcDischarge	0	0	0	0
Cornea	0	0	0	0
Eye	0	0	0	0
Piloerection	0	0	0	0
Dehydration	0	0	0	0
Penis	n/a	n/a	n/a	n/a
Touch	0	0	0	0
Cyanosis	0	0	0	0
Vision box	1.7AB	1.9	1.4A	1.2B
Visual Placement	71.5A	95.6B	96.4	80.1

4w males	C	I	T	I&T
Dead	0	0	0	0
Ataxia	0	0	0	0
Tremors	0	0	0	0
Righting	20.2A	6.5	8.1A	9.7
Click	18.5	19.7	22	20.5
Menace	0	2.2	8.7	5.9
Rod	92.9	98.4	98	86.6
Posture	0	0	0	0
Coat	0	0	0	1.9
Tail	0	0	0	0
Respiration	0	0	0	0
Vocalization	2.1	4.8	2	5.7
Diarrhea	0	0	0	0
SStool	4.9	5.5	0	1.4
FStain	0	0	0	0
UStain	0	0	0	0
Salivation	0	0	0	0
NDischarge	0	0	0	0
ODischarge	0	0	0	0
Lacrimation	0	0	0	0
OcDischarge	0	0	0	0
Cornea	0	0	0	0
Eye	0	0	0	0
Piloerection	0	0	0	0
Dehydration	0	0	0	0
Penis	0	0	0	0
Touch	0	0	0	0
Cyanosis	0	0	0	0
Vision box	1.6A	1.3	1.9A	1.8
Visual Placement	94.8	89.2	97.1	91.3

catgegoreis with the same letter are differenet from each other P<0.1

Appendix C

Eight week old mice FOB test results

8w females				
	C	I	T	I&T
Dead	0	0	0	0
Ataxia	0	0	0	0
Tremors	0	0	0	0
Righting	0	0	0	0
Click	0	0	0	0
Menace	2.9	0	0	0
Rod	100	100	100	100
Posture	0	0	0	0
Coat	0	8.6	0	20
Tail	0	0	0	0
Respiration	0	0	0	0
Vocalization	14.3	0	2.5	0
Diarrhea	0	0	0	0
SStool	0	0	0	0
FStain	0	0	0	0
UStain	0	0	0	0
Salivation	0	0	0	0
NDischarge	0	0	0	0
ODischarge	0	0	0	0
Lacrimation	0	0	0	0
OcDischarge	0	0	0	0
Cornea	0	0	0	0
Eye	0	0	0	0
Piloerection	0	0	0	0
Dehydration	0	0	0	0
Penis	n/a	n/a	n/a	n/a
Touch	0	0	0	0
Cyanosis	0	0	0	0
Vision box	1.4	1.6	1.4	1.6
Visual Placement	100	97.1	97.5	96.7

8w males				
	C	I	T	I&T
Dead	0	0	0	0
Ataxia	0	0	0	0
Tremors	0	0	0	0
Righting	0	0	0	0
Click	5.7	2.5	2.9	0
Menace	2.9	0	2.9	2.9
Rod	100	100	100	100
Posture	0	0	0	0
Coat	0	0	0	5.7
Tail	0	0	0	0
Respiration	0	0	0	0
Vocalization	0	0	2.9	0
Diarrhea	0	0	0	0
SStool	2.9	0	0	2.9
FStain	0	0	0	0
UStain	0	0	0	0
Salivation	0	0	0	0
NDischarge	0	0	0	0
ODischarge	0	0	0	0
Lacrimation	0	0	0	0
OcDischarge	0	0	0	0
Cornea	0	0	0	0
Eye	0	0	0	0
Piloerection	0	0	0	0
Dehydration	0	0	0	0
Penis	0	0	0	0
Touch	0	0	0	0
Cyanosis	0	0	0	0
Vision box	1.6	1.7	1.5	1.7
Visual Placement	100	100	97.1	100

catgegoreis with the same letter are differenet from each other P<0.1