Interactions of *Toxoplasma gondii* with the central nervous system and neurological dysfunction

Mohammad Abdulaziz Alsaad

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Research Institute of Integrative and Comparative Biology

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The candidate confirms that the work submitted is his own, except where work which has formed part of jointly authored publications has been included. The contribution of the candidate and the other authors to this work has been explicitly indicated below. The candidate confirms that appropriate credit has been given within the thesis where reference has been made to the work of others.

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Abstract

Pathogens, including viruses and parasites, manipulate the host's system to enhance their ability to cause infection. One such parasite is *Toxoplasma gondii,* a protozoan parasite that has felines as the only definitive host. Studies of infected mice, the parasite's intermediate host, have shown behavioural changes in the host during infection, resulting in easy predation of the intermediate host by the definitive host. It has been shown that *T. gondii* modulates dopamine and glutamine in the infected host, while the location and distribution of *T. gondii* cysts in the brain may mediate changes in behaviour. *T. gondii* has been associated with schizophrenia and bipolar disorder and may play a role in other neural disorders, such as Parkinson's disease (PD) that affects dopaminergic neurones. During this dissertation, a possible correlation between *T. gondii* and PD was investigated with anti-*Toxoplasma gondii* IgG detection in PD (n=206) and control (n=176) samples. Anti-*T. gondii* IgG was detected in both PD and controls with almost identical seropositivity. Secondly, to understand the involvement of *T. gondii* in neurological disorders, behavioural studies and neurotransmitter analysis performed on *T. gondii*-infected mice that had been bred for a heterozygous mutation in the neurexin-1α gene, which has been linked to schizophrenia. *T. gondii* infected mice showed less anxiolytic behaviours and sociability, but no obvious combined effect of *T. gondii* and neurexin-1α mutation was detected. Finally, rat brain cysts were located and quantitated in the brains of 109 infected rats. *T. gondii* cysts showed some tropism toward the cerebellum, cerebral cortex, colliculus and thalamus, although encysted parasites were found in all brain regions. This study increases our understanding of the potential mechanisms this parasite has developed to achieve specific manipulation of the intermediate host to increase transmission success to the feline definitive host; this has implications for the impact of infection on the health and behaviour of infected mammals, including humans.

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Abbreviations

AIDS	Acquired immunodeficiency syndrome
Anti-BAG1	Anti-bradyzoite surface antigen
AADC	Aromatic amino acid decarboxylase
ASD	Autistic spectrum disorder
CNS	Central nervous system
CHCHD2	Coiled-coil-helix-coiled-coil helix domain containing 2
Ctrl	Control
CNVs	Copy number variants
DA	Dopamine
DAT	Dopamine transporter
DMEM	Dulbecco’s modified Eagle’s medium
EPM	Elevated plus maze
ELISA	Enzyme-linked immunosorbent assay
EDTA	Ethylenediaminetetraacetic acid
EIF4G1	Eukaryotic translation initiation factor 4-γ 1
EMPReSS	European Mouse Phenotyping Resource of Standardised Screens
FBS	Foetal bovine serum
GWAS	Genome-wide association studies
GLT-1	Glutamate
Het	Heterozygotes
HVC	Homovanillic acid
HRP	Horseradish peroxidase
HFF	Human foreskin fibroblast
IgA	Immunoglobulin A
IgG	Immunoglobulin G
IgM	Immunoglobulin M
IFN-γ	Interferon-γ
IL-2	Interleukin-2
IP	Intraperitoneal
KO	Knock out
L-DOPA	L-3,4-dihydroxyphenylalanine
LGI	Leeds General Infirmary
LRRK2	Leucine-rich repeat kinase 2
MRI	Magnetic resonance imaging
MAT	Modified agglutination test
NRXN1	Neurexin-1
NRXNs	Neurexins
NLGNs	Neuroligins

OCD	Obsessive compulsive disorder
OF	Open-field
PD	Parkinson’s disease
PS	Penicillin streptomycin
PBS	Phosphate-buffered saline
PCR	Polymerase chain reaction
PPI	Prepulse inhibition
PP2A	Protein phosphatase 2A
SFDT	Sabin Feldman dye test
SNCA	Synuclein
SN	Substantia nigra
TMB	Tetramethylbenzidine
TAE	Tris acetate EDTA
TH	Tyrosine hydroxylase
VPS35	vacuolar protein sorting 35
VTA	Ventral tegmental area
VMAT2	Vesicular monoamine transporter 2
WT	Wild type

Chapter 1 General Introduction

Toxoplasma gondii is a protozoan parasite whose definitive host is the cat family Felidae; it has a wide range of intermediate hosts, including small mammals, cattle, sheep, birds, primates and humans. *T. gondii* belongs to the Apicomplexa which are a parasitic phylum of obligate intracellular eukaryotes. They are organisms solitarily reproducing inside the cells of their hosts. They have an apical complex, a unique organelle that can be characterised by. The most famous parasite *Plasmodium* spp., which cause malaria, is an example of apicomplexan parasites as well as *T. gondii*, and *Cryptosporidium. T. gondii* has three strains associated with human infections: types I, II, and III (Ajzenberg et al., 2009; Aspinall et al., 2002; Howe et al., 1997). Prevalence estimates range from around 5–90% in domestic cats (*Felis domesticus*) around the world, and 4–92% in humans (Dubey, 2009). *T. gondii* has shown dramatic differences in seropositivity in various parts of the world. For example, in the United Kingdom, the seropositivity of the parasite is estimated to be 20% (Ho-Yen, 2009), whereas it is about 25.5% in the United States (Jones et al., 2003). The incidence of *Toxoplasma* may reach a percentage of 95% (Dubey, 2009). Such variation could be due to hot weather and high humidity, which is the optimal weather for *T. gondii* oocysts (Meerburg and Kijlstra, 2009). For example, a Brazilian study conducted in 2006 showed that the seroprevalence of *T. gondii* in the rural western Amazon region was 73% in humans (Cavalcante et al., 2006). Another example seroprevalence of *T. gondii* variation is Saudi Arabia which is a country with an area of 244 000

km^2, making it nine times larger than the United Kingdom with 36 million fewer people than the population of UK; it has a *T. gondii* seropositivity range of 21–53% (Ahmed, 1992; Alqahtani and Hassan, 2012; Al-Qurashi, 2004; Al-Qurashi et al., 2001; Eisa et al., 2013; Yaneza and Kumari, 1994).

T. gondii is an apicomplexan protozoan which can infect all warm-blooded mammals worldwide. The only definitive host of this parasite is the cat family Felidae, in which *T. gondii* undergoes full gametogenesis and forms oocysts. Then, unsporulated oocysts are shed in cat faeces (Hutchison et al., 1969). These oocysts become infectious in 1 to 5 days after elimination and are converted to sporulated oocysts, which can survive for several months. Each sporulated oocyst contains two sporocysts and each sporocyst contains four sporozoites (Baron, 1996). A cat is usually infected through the ingestion of oocysts or eating infected animals. Intermediate hosts such as mice and humans are infected by the ingestion of oocysts or tissue cysts. In humans, the route of infection is through the consumption of contaminated raw meat or water and being in contact with cat faeces. In addition, congenital transmission of *T. gondii* has been reported in humans and sheep. Within the intermediate hosts, the parasite undergoes asexual reproduction (Baron, 1996). First, the tachyzoite stage occurs; within hours, tachyzoites can reach extra-intestinal tissues via the blood and lymph system. They can penetrate any type of cell and multiply there until they fill the cell, causing it to rupture and die. The tachyzoites then invade another cell and multiply. As a consequence, tissue necrosis will occur. Following this, tachyzoites are converted into the bradyzoite stage and become encysted in the brain, heart and other tissues. The host will carry those cysts for the rest of its life

(Baron, 1996). In addition, in immunocompetent hosts, tissue cysts could rupture and release bradyzoites, which reform and multiply to form tachyzoites. As a result, the host could die because of toxoplasmosis. The cause of tissue rupture is still not understood (Kamerkar and Davis, 2012).

The parasite is thought to affect the intermediate host's behaviour in order to increase its chances of transmission to the definitive host, Felidae. Potential mechanisms of the parasite include a reduction of fear of feline odour and an increase in activity in the intermediate host (Berdoy et al., 1995; Webster, 2001). Through predation, a cat ingests an infected intermediate host, allowing the protozoan to complete its life cycle (Figure 1). During the various stages of a lifecycle, a parasite goes through various cellular stages that are characterized by different morphology, behaviour, function, and biochemistry. In each of the stages, the parasite differs in shape, size, function, and location. The main function of tachyzoites is to expand the population of the parasite in the host through rapid multiplication (Hill and Dubey, 2002). Their motility and ability to multiply rapidly aid in fulfilling their role. With regard to their morphology, tachyzoites are crescent-shaped and possess a pointed front (Dubey and Jones, 2008). In addition, they are 2 by 6 micrometers with a rounded back end. They contain numerous organelles and inclusion bodies. Examples of these structural bodies and organelles include pellicle, micronemes, endoplasmic reticulum, apical rings, Golgi complex, ribosomes, microtubules, amylopectin granules, micropores, mitochondria, and dense granules (Dubey, 1998). They lack motility structures even though they have the ability to move and rotate. After multiplication, they are transported to various parts of the body through

blood streams. As the lifecycle progresses, tachyzoites convert to bradyzoites in order to form tissue cysts that are critical in the development of the parasite (Dubey, 1998).

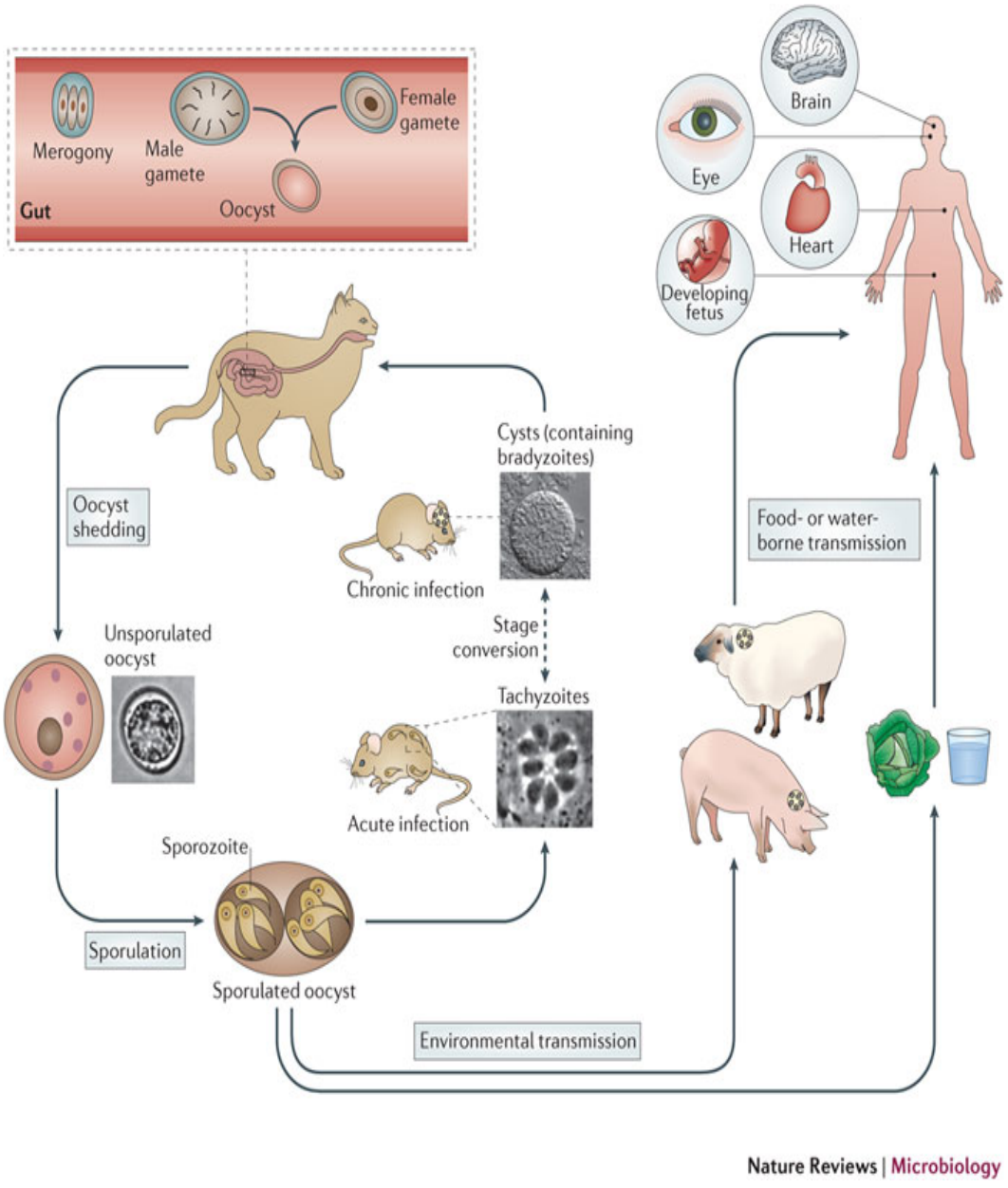

Figure 1: Life cycle of *T. gondii* (Hunter and Sibley, 2012)**.**

Unlike tachyzoites that have a central nucleus, bradyzoites have a nucleus that is located toward the cell's posterior end. They are crescent-shaped and

are larger than tachyzoites. They are about 7 by 1.5 micrometers in size (Dubey, 1998). The main function of bradyzoites is to form tissue cysts when they enter a host's cells and aid in the progression of the parasite's life cycle. They are orally infectious and therefore, play an important role in the transmission of *T. gondii* (Tenter et al., 2000).

1.1. *T. gondii* Infections

The protozoan parasite *T. gondii* commonly infects sheep, and causes early embryonic death, neonatal death and abortion. It may also cause resorption, mummification, stillbirth and foetal death. Large numbers of sheep acquire the infection after birth (Dubey and Welcome, 1988). Congenital *T. gondii* has been observed in many animal species, for instance, rodents, sheep and goats. It has also been reported in humans. It is possible to propagate the congenital *T. gondii* strain in some mouse breeds. These mice will produce about 10 generations of congenitally infected offspring (Dubey, 2008). Moreover, in humans, both acute and chronic infections are produced by *T. gondii*. Acute infection can be benign and asymptomatic (Garcia, 2007); some patients may experience influenza-like symptoms. In rare cases, seizures and painless cervical lymphadenopathy may develop (Carme et al., 2009). In most cases, chronic (or latent) toxoplasmosis will occur with no symptoms in immunocompetent humans and animals (Webster et al., 2006). The rates of *T. gondii* infection in a number of human populations considered to have a high prevalence emphasises the importance of research into the subtle effects the parasite may have during chronic infection of the human brain. *T. gondii* is established by maintaining

stability between the parasite's evasion and the host's immune response. The tachyzoite proliferation during the acute stage of infection is suppressed by interferon-γ (IFN-γ)-dependent, cell-mediated immune responses and to some degree by humoral immunity. As a consequence, the host may develop chronic infection by *T. gondii* cysts, and these are usually located in the brain. *T. gondii* can be diagnosed using biological, serological or histological methods. The type of serological tests used for humans include the dye test, enzyme-linked immunosorbent assay (Ajzenberg et al., 2009) and the modified agglutination test (MAT). Unfortunately, the clinical signs of toxoplasmosis are nonspecific, and therefore, no precise diagnosis can be made.

The treatment available for *T. gondii* in humans involves using sulphonamides with pyrimethamine (Dubey, 2009). The drug targets tachyzoites, thereby controlling the active infection. However, drugs will not eradicate chronic infection. Meanwhile, the drug doses administered usually vary depending on the patient's condition. For example, if a pregnant woman, a child or an immunosuppressed individual has the symptoms of toxoplasmosis, they will need extensive attention (Huskinson-Mark et al., 1991; McCabe and Remington, 1983)**.**

1.2. *T. gondii* host behavioural change

Certain parasites manipulate their hosts to successfully complete their lifecycles. These parasites can amend their host behaviour for the benefit of their own survival (Poulin, 1995). The trematode *Dicrocoelium dendriticum* is successful as a manipulative microorganism. It causes ants, the

intermediate host, to remain inactive at the top of a leaf; as a result, they are ingested by livestock, which are the primary hosts in which *D. dendriticum* reproduces (Lafferty and Shaw, 2013). *Euhaplorchis californiensis* is another example. This parasite can alters the behaviour of the California killifish so that birds can ingest them and it can complete its lifecycle (Lafferty and Shaw, 2013). *T. gondii* also causes behavioural changes, and its manipulation of hosts is documented (Webster, 2001).

1.2.1. Rat infection and behavioural changes

T. gondii invasion of the intermediate host brain places the parasite in a position to control and alter host behaviour. One study compared direct lifecycles, where the parasite lives and reproduces inside the host, with an indirect lifecycle, which are complex and require multiple hosts to reproduce, as in the case of *T. gondii* (Webster, 1994). It showed that *T. gondii* increased activity levels in wild laboratory (Lister hooded) hybrid brown rats, *Rattus norvegicus.* In contrast, parasites with direct lifecycles, such as *Leptospira* spp., *Cryptosporidium parvum*, *Coxiella burnetii,* and *Hymenolepis nana* did not exhibit increased activity (Webster, 1994). *T. gondii*–infected wild brown rats also display low neophobia compared to their uninfected counterparts. Predation rates increase with these behavioural changes (Erhardt et al., 2007).

The manipulation hypothesis was examined in a study with laboratory (Lister hooded) rats. The study showed that *T. gondii* infection appeared to alter a rat's innate aversion to feline odour. It also showed that the more active the

rat, the greater its attraction to the feline odour (Berdoy et al., 2000). The study showed that infected and non-infected rats' responses were normal for rat odour, water odour (neutral) and rabbit odour (non-predatory mammal). However, a difference in response was found in the infected rats' reaction when exposed to cat odour. The results suggested that the behavioural alteration in the rats represent a subtle cognitive alteration that would increase the predation risk. This study confirmed that the different response is not the result of an olfactory impairment due to a high rate of infection (Berdoy et al., 2000).

Additional studies have compared infected and uninfected Lister hooded laboratory rats' reactions to the odours of cats and other predatory mammals, such as dogs (Kannan et al., 2010) and mink (Lamberton et al., 2008). The findings demonstrated that infected rats were only attracted to the feline definitive host. This provides strong support for the theory that the behavioural changes induced by *T. gondii* are mechanisms adopted by the parasite to survive and complete its lifecycle in the definitive host.

Vyas et al. (2007) also showed that *T. gondii*–infected rats were attracted to cat urine. Additionally, elevated plus-maze (EPM) anxiety tests were used with Wistar rats that were infected with *T. gondii*. Infected rats showed a low level of anxiety compared to non-infected rats (Gonzalez et al., 2007). Anxiety levels were assessed using EPM and social interaction tests. The researchers found that the rats' anxiety levels were not affected by the rate of parasitic infection; however, greater infection resulted in general decreases in motor activity, which could hamper the detection of an

anxiolytic effect in rats. The study showed that *T. gondii* infection changes the behaviour of rats, resulting in a subtle alteration that is specific to the parasite. However, the dose of the infection was also crucial, as a higher dose could overcome this alteration, resulting in severe pathology to the host. The evidence suggested that brain cyst distribution and anti–*T. gondii* immunoglobulin G (Saeij et al., 2005) levels in serum are associated with the anxiolytic effects (Gonzalez et al., 2007). Glial and neural cells, as well as limbic areas, were mainly targeted by the parasite; this may also have been responsible for the anxiolytic effects (Afonso et al., 2012). Rodents that were congenitally infected with *T. gondii* showed no difference in behaviour in terms of social status and mating relations compared to controls. The study concluded that *T. gondii* affects the intermediate host in subtle ways that benefit the parasite lifecycle (Berdoy et al., 1995). Changes in innate aversion, anxiety and learned fear was observed during *T. gondii* infection, whereas learning fear, anxiety-like behaviour, olfaction effects and nonaversive learning showed no or extremely little change. The feline odour test was classified into three odour intensities and involved 0.5, 1, 1.5, 2 and 2.5 mL of cat urine. Infected laboratory rats exhibited a higher loss of aversion to a moderate amount of cat urine (1 ml) than smaller or larger amounts (Vyas et al., 2007).

1.2.2. Mouse infection and behavioural changes

It would be reasonable to expect that the same effects observed in rats would also be found in mice, but this is not the case. One study found that

chronically *T. gondii*-infected C57BL/6J mice at 7 week of age displayed increased exploratory locomotion, reduced fear and more hazardous behaviours than control mice (Afonso et al., 2012). These findings were consistent with the manipulation hypothesis. In contrast, a study of 8-week-old C57BL/6 mice that were chronically infected with the avirulent ME49 *T. gondii* strain showed widespread brain pathology, as well as motor coordination and sensory deficits (Gulinello et al., 2010). Brain examination showed that the brain pathology involved minimal to moderate meningoencephalitis with gliosis. Indeed, there were no changes in social interaction or cognitive behaviour (Gulinello et al., 2010).

BALB/c:C57BL/6 crossed mice at the age of 10 weeks were infected with *T. gondii*, and temporary behavioural changes and pathology was observed; the effects faded at the 12th week post inoculation (Hrda et al., 2000). Based on a double-training maze exercise, one study showed that infected mice with *T. gondii* exhibited altered learning capacity and memory, which were suppressed compared with the same characteristics in uninfected animals (Witting, 1979). *T. gondii* may also increase the predation rate by making the intermediate host more active. It is thought that cats are interested in and curious about moving and exposed objects, while they do not pay full attention to stationary objects.

Different studies have demonstrated the effects of *T. gondii* on the activity and exploratory behaviour of mice. For instance, Y-shaped mazes, running wheels and open field tests have been used (Hay et al., 1984), and the results have shown that uninfected mice are considerably less active than

infected ones. Likewise, infected mice spend a lot of time in novel areas (loss of neophobia), whereas their uninfected counterparts spend more time in familiar environments (Berdoy et al., 1995; Hay et al., 1984). In addition, chronically infected mice demonstrate deficient motor coordination and sensory responses (Gulinello et al., 2010). Moreover, *T. gondii*–infected mice demonstrate an increased peak reaction time (Hrda et al., 2000). In one study, the researchers also observed that mice infected by *T. gondii* showed a remarkable reduction in learning ability in comparison to infected rats (Vyas et al., 2007).

1.2.3. *T. gondii* in humans

Immunocompromised patients are in great risk if they develop latent or acquired toxoplasmosis. It can cause mental and neurological disorders. Human *T. gondii* seropositivity has been linked with mental disorders, including schizophrenia, Parkinson's disease (PD), obsessive compulsive disorder (OCD) and Tourette's syndrome. Behavioural alterations caused by *T. gondii* do not only occur in mice or rats. A study by Flegr's group (2007) showed that immunocompetent people with latent toxoplasmosis have shown altered personality (Flegr, 2007; Flegr et al., 1996a). They also showed that in simple computer reaction tests, infected individuals need more time to react, which would suggest reduced psychomotor performance. Furthermore, the risk of having a car accident was found to be 2.65 times greater in patients with latent toxoplasmosis than in noninfected individuals (Flegr et al., 2002; Flegr, 2007; Flegr et al., 2009). It seems that the slow

reaction time and reduced psychomotor performance in *T. gondii* patients could explain this higher risk of being involved in car accidents.

T. gondii seropositivity has also been linked with suicide. A recurrent mood disorder study included 99 patients who had attempted suicide and 119 who had not. It was shown that those who had attempted suicide had a higher titre of *T. gondii* antibodies (Arling et al., 2009). A study performed in Turkey also linked a history of suicide attempts with *T. gondii* seropositivity (Yagmur et al., 2010). Moreover, suicide in postmenopausal women and *T. gondii* infection have been linked (Ling et al., 2011).

A study of Tourette's syndrome patients found that they had higher levels of *T. gondii* antibodies compared to healthy individuals (Krause et al., 2010). Moreover, *T. gondii* IgG seropositivity was found to be higher in Alzheimer's patients (Kusbeci et al., 2011). A large study linked bipolar disorder and *T. gondii* infection (Pearce et al., 2012), while several studies have connected *T. gondii* seropositivity with schizophrenia (Niebuhr et al., 2008; Torrey and Yolken, 2003; Yolken et al., 2001). A study of 38 first-episode schizophrenia patients and 27 controls showed that *Toxoplasma* immunoglobulins IgG, IgM and IgA were higher in schizophrenia patients compared to controls (Yolken et al., 2001). A convincing link between schizophrenia and the parasite antibody was also shown in a study that analysed schizophrenic data from 17 different countries (Torrey et al., 2007). Schizophrenia is usually associated with genetic causes and/or environmental factor; Torrey et al.'s (2007) analysis showed the link between schizophrenia and *T. gondii* antibody was considerably stronger than genetic or environmental factors

(Torrey et al., 2007). Moreover, schizophrenia treatment using antipsychotic drugs has been found to decrease the IgG antibody levels against *T. gondii*. In contrast, control patients were found to have a higher antibody presence (Leweke et al., 2004). It has been shown that both *T. gondii* and schizophrenia could target the same cells, for example, glial cells and particularly astrocytes (Cotter et al., 2001). In the brain, the grey matter size is reduced in schizophrenia patients. Using magnetic resonance imaging showed that *T. gondii*–seropositive patients had a reduced grey matter size compared to *T. gondii*–seronegative patients or healthy controls (Horacek et al., 2012).

Another connection that has been found is that hydrocephalus and increased ventricular size are both found in foetal *T. gondii* infections and schizophrenia patients. This suggests that children are more likely to develop schizophrenia as a foetus or shortly after birth if mother has elevated IgM for *T. gondii* during pregnancy. This may increase the risk of developing schizophrenia later on in life and may interact with other risk factors (Torrey and Yolken, 2003).

Two different Turkish studies focused on the correlation between *T. gondii* and PD. The studies were piloted in the same district in two different cities about 60 miles apart. Miman's (2010) group showed that less than half of the cases with PD and about one-quarter of the controls were positive for *T. gondii* antibodies (total n=92). Sera samples were analysed using micro-ELISA technique to detect both IgM and IgG antibodies of *T. gondii*. PD patients were scanned with Magnetic resonance imaging (MRI) which

showed primary PD and also no family history of the disease. Factors such as age, sex, socioeconomic status were analysed statistically and resulted in no statistical significance difference. The researchers concluded that there was a statistically significant difference between the positivity rates in the PD and control groups in IgG antibodies (p = 0.006). While IgM antibodies analysis showed negative results in all samples of PD and control. No single borderline sample was detected (Miman et al., 2010a). Meanwhile, Celik's group (2010) showed that half of PD patients and 40% of controls were positive for *T. gondii* antibodies (total n=100). Sera samples were analysed using the Sabin Feldman dye test (SFDT). Factors such as cat ownership, occupation, rural or urban accommodation and cooking preference showed no statistical significance difference. PD patients were scanned with MRI, no neurological diseases and no PD family history. Thus, these researchers concluded that there was no significant difference between the two groups (p = 0.61). The study does not reported any borderline results. PD patients were scanned with Magnetic resonance imaging (MRI) which showed primary PD and no family history of the disease (Celik et al., 2010). Both studies showed no importance of predisposing factors such as cat ownership and socioeconomic status. However, sera analysis were different. Chapter 2 will discuss a similar approach in UK PD patients. The approach is similar to Miman's method with enhancement by using two different analysis kits instead of one, ELISA and direct agglutination tests (DAT). SFDT is acceptable method. Nonetheless, ELISA and DAT would be advanced and faster methods. Both studies used total of 92 or 100 sera

samples. There are limitations on samples number such as how many PD patients available and how many are willing to participate.

Antiparasitic drugs have been shown to reduce PD symptoms in individuals with acquired immunodeficiency syndrome (AIDS) (Carrazana et al., 1989; Murakami et al., 2000). In addition, antiparasitic drugs have proven effective in *T. gondii*–seropositive individuals with OCD (Brynska et al., 2001). It is vital to understand the association between exposure to *T. gondii* and related neurological disease in terms of determining a final diagnosis. It is also extremely important to investigate the level of involvement of *T. gondii* antibodies in central nervous system (CNS) diseases.

1.3 Potential mechanisms for the behavioural changes related to *T. gondii*

The host behavioural changes caused by *T. gondii* have been studied extensively, although a gap in knowledge remains concerning how the parasite manipulates its intermediate host. It has been shown that the parasite may affect the brain indirectly through the immune response (Webster and McConkey, 2010). It may also affect the brain directly through the localisation of the cysts. However, a recent study has suggested that the localisation of the cysts in the brain may not have a direct effect on the parasitic mechanisms (McConkey et al., 2013), and further investigations are needed. Moreover, it has also been found that the parasite may directly affect the brain by altering the levels of neurotransmitters, such as dopamine (Webster and McConkey, 2010). A study on mice demonstrated that acute toxoplasmosis caused the level of homovanillic acid (HVC) to rise by about

40%, whereas norepinephrine decreased by 28% (Stibbs, 1985). HVC is a metabolic stress marker, and it is associated with dopamine levels (Molero et al., 2011), whereas norepinephrine, also known as noradrenaline, is a chemical substance produced during stress that is also associated with dopamine (Glavin, 1985). In contrast, in the same study, no change in the level of dopamine was observed. Conversely, in chronic toxoplasmosis, the total brain dopamine level was found to be elevated by 14% in infected mice compared to their uninfected counterparts (Stibbs, 1985).

1.3.1 Immune response

The host responds to infection by initiating innate and adaptive immune responses. After the ingestion of *T. gondii*, the host's immune system activates macrophages to fight the parasite (Flegr, 2013). The main purpose of innate immune response is to prevent the multiplication of *T. gondii* (Tenter et al., 2000). In addition, it initiates the activation of the adaptive immune response after the ingestion of the parasite (Flegr, 2013). The adaptive immune response triggers the release of certain antibodies and effector cells whose role is to eliminate the invader (Flegr, 2013). The response causes the specialization of dendritic cells, B cells, and macrophages in order to present specific antigens for the elimination of *T. gondii*. The presentation of the antigen to T cells commences the differentiation process that leads to the development of immunological memory that protects the host from re-infection (Blanchard et al., 2015).

1.3.2 Localisation of the cysts on the brain

One of the mechanisms through which *T. gondii* manipulates host's behaviour is through localisation on certain parts of the brain. In infected hosts, cysts of the parasite are usually distributed in several brain regions (Carruthers and Suzuki, 2007). A study conducted to study the distribution of *T. gondii* cysts in the brains of CD1 mice found that cysts were localised on all brain regions including the olfactory bulb, the hippocampus, amygdala, the entorhinal, and the frontal association and visual cortices (Berenreiterova et al., 2011). Low distribution of cysts was observed in regions that include the cerebellum, myelinated axons, the pontine nuclei, and the caudate putamen. The study conducted by Berenreiterova et al. (2011) found out that 54 brain regions contained the parasite's cysts. Research has shown that during the chronic stages of *T. gondii* infection, cysts are found throughout the brain (Blanchard et al., 2015). However, the localisation of the cysts has not yet been studied in detail. Certain studies have shown that there is a high density of *T. gondii* cysts in two main brain regions namely the frontal cortex and the amygdala (Carruthers and Suzuki, 2007). Brains infected by *T. gondii* have high levels of dopamine (Lafferty, 2006). These findings have been used to explain why infected hosts exhibit changes in behaviour. According to Vyas et al. (2007), *T. gondii* manipulates host behaviour by localising in brain regions that process fear including the amygdala.

1.3.3 Effect on neuromodulator levels

Studies have proposed histopathological, immunological, and neuromodulatory hypotheses for the manipulation of host behaviour by *T. gondii*. According to the neuromodulatory hypothesis, the local immune response that is elicited to inactivate *T. gondii* alters the levels of cytokines, which influence neuromodulator levels (Lafferty, 2006). The neurological basis of anxiety has been studied in several studies to determine how *T. gondii* alters neuromodulator levels. Fearless reactions in rats have been shown to arise from the blocking of anxiogenic N-methyl-D-aspartic acid receptors in the amygdala (plays an important role in emotional behaviour). HVC and norepinephrine alter the mood, locomotor activity, and cerebral blood flow of hosts. HVC is a degradation product of dopamine that has been associated with behaviour change of infected hosts. One of the proposed mechanisms of neuromodulation involves alterations in the levels of neurotransmitter in the host (Wiser, 2010). Several studies have found out that the levels of dopamine increase in brain cells that contain cysts. *T. gondii* directly increases the quantities of dopamine in infected cells by synthesising tyrosine hydroxylase, which plays an important role in the production of dopamine. Studies have identified two tyrosine hydroxylase genes that are responsible for production of excess dopamine in the brain. For example, overregulation of TgAaaH2 gene during the differentiation of *T. gondii* to bradyzoites results in stimulation that increases the production of dopamine (Prandovszky et al., 2011). The accumulation of dopamine in

various brain regions is responsible for the behavioural changes that are observed in animals and humans infected by *T. gondii* (Lafferty, 2006).

1.4 Aims and objectives

T. gondii has been associated with many neurological disorders. However, the mechanisms involved in this complex interplay between parasite and host have not been delineated. Specifically, how the parasite alters neurotransmission in the host and the effects on host behaviour represent gaps in our knowledge.

This thesis investigated the following aspects of *T. gondii* exposure and neurological mechanisms:

1. To determine whether there is an association between *T. gondii* exposure and Parkinson's disease (PD).
2. To investigate gene x environment (GxE) interactions in transgenic mice to assess the combined effect of neurexin-1α mutation and *T. gondii*. The neurexin-1α gene has been associated with schizophrenia and other neurological disorders. The present work may lead to a better understanding of the mechanism(s) of the parasite and its effect on neurotransmitters; and
3. To assess the distribution of *T. gondii* cysts in the rat brain and its tropism toward specific regions as a plausible explanation for observed changes in behaviour with infection.

Chapter 2 Correlation between *T. gondii* and Parkinson's disease

2.1 Introduction

Parkinson's disease (PD) is a chronic neurological disorder that mostly affects people greater than the age of 60. It is a progressive neurodegenerative disease. The brain's substantia nigra (SN), which controls voluntary movement, is the most affected region with loss of dopamine. As a result, the messages controlling movement become inefficient. The aetiology of PD is still unknown, but the hallmark clinical features of this disease are rigidity, resting tremor, bradykinesia, loss of balance and loss of posture (Venda et al., 2010). It is well known that PD is a common, complex neurological disorder. The disease was first described about two centuries ago, and knowledge concerning the disease continues to emerge. PD is a neurodegenerative disease exhibiting the clinical feature of major dopaminergic neuronal death in the substantia nigra. As a consequence of dopamine loss within the basal ganglia, Parkinsonian motor symptoms become apparent. PD also causes non-motor symptoms that sometimes emerge prior to the motor dysfunction (Kalia and Lang, 2015). It is thought that the foundation of PD treatment should involve managing symptoms using drugs that target dopamine regulation, via increase in dopamine or directly stimulating dopamine receptors. However, these drugs may affect different regions of the brain and neurotransmitters other than dopamine. In the past, PD was believed to be caused by environmental

factors; however, more recent finding indicate that it might be caused by a combination of environmental factors and genetic mutations, resulting in the devastating progression of a series of symptoms.

A clinical diagnosis for the early stages of PD has not been yet been established, making diagnosis challenging during the onset of the disease. Moreover, the PD diagnosis can be verified only by postmortem pathological examination through the presence of SN pars compacta degeneration and Lewy pathology. The correlations between the development of PD pathology and Lewy body pathology are still not fully understood. These complexities of PD represent challenges for researchers and scientists.

Clinical diagnosis suggests two major subtypes of PD, namely tremor-dominant PD (no other motor symptoms) and non-tremor-dominant PD or idiopathic parkinsonism (which includes phenotypes described as akinetic–rigid syndrome and postural instability and gait disorder). Although the knowledge of PD pathogenesis is expanding, thereby helping to identify potential targets of treatments (Kalia and Lang, 2015), there is still no official classification of these PD subtypes. The tremor-dominant and non-tremor-dominant PD subtypes are associated with different disease prognoses; for example, tremor-dominant PD is often associated with a slower rate of progression and less functional disability than non-tremor-dominant PD (Jankovic et al., 1990).

Early common PD symptoms include non-motor signs, such as olfactory dysfunction, cognitive impairment, psychiatric symptoms, sleep disorders, autonomic dysfunction, pain and fatigue. It is considered that these non-

motor signs can be associated with below average personal sanitary, hygiene and health management (Duncan et al., 2014; Martinez-Martin et al., 2011). These non-motor signs usually emerge before the onset of the motor symptoms. The Premotor or prodromal phase may be prolonged for up to 12 – 14 years before parkinsonism occurs. The onset of this phase is described as involving impaired olfaction, constipation, depression, excessive daytime sleepiness and rapid eye movement sleep behaviour disorder (Postuma et al., 2012). The progression of PD is characterised by deterioration in both the motor and non-motor signs.

As the PD symptoms worsen, it becomes more complicated and difficult to treat the disease; complications related to long-term treatment and a resistance to the treatment may develop. Long-term treatment difficulties can include motor and non-motor fluctuations, dyskinesia and psychosis. If resistance to treatment accumulates, this development may be signified by prominent motor and non-motor features, including axial motor symptoms like postural instability, freezing of the gait, falls, dysphagia and speech dysfunction. Most patients who live with PD for more than a decade exhibit freezing of the gait and falls, while half of them may experience choking (Hely et al., 2005).

Late-stage non-motor signs of PD include symptomatic postural hypotension, constipation and urinary incontinence (Hely et al., 2005; Hely et al., 2008). PD patients who have had the disease for two decades will develop dementia, which has a prevalence of 83% in these individuals (Hely

et al., 2008). Moreover, treatment resistance can contribute to disability and ultimately death (Coelho and Ferreira, 2012).

T. gondii is a neurological disease that is able to infect the brains and other tissue of the body. Studies have found that *T. gondii* cysts in the mouse brain contain high levels of dopamine and tyrosine hydroxylase (TH) using immunostaining with dopamine and TH antibodies. The dopamine level was elevated three-fold in infected mammalian dopaminergic cells (Prandovszky et al., 2011). However, the source of dopamine remains unclear. The proposed route of neuromodulation for altered behaviour suggests that *T. gondii* increases dopamine via TH, which converts tyrosine to L-3,4-dihydroxyphenylalanine (L-DOPA). However, L-DOPA decarboxylase is necessary to convert L-DOPA to dopamine, and the source of L-DOPA decarboxylase is unknown. It could be that *T. gondii* encodes for L-DOPA decarboxylase or that it uses the host's L-DOPA decarboxylase. DA found in cysts and released DA from infected cells above normal physiological levels can cause cell death especially if improperly packaged in vesicles (Dias et al., 2013). PD symptoms are caused by death of dopaminergic neurones. Hence *T. gondii* could be linked to PD by making dopaminergic neurones more likely to die. Or, conversely, maybe *T. gondii* infection helps PD symptoms by producing extra DA. Interestingly, some patients with neurological diseases, such as PD, show an improvement in general health when treated with antiparasitic drugs (Brynska et al., 2001). A greater understanding of the potential link between PD and parasitic infection could help elucidate the causative agent.

2.1.1 Risk Factors for PD

The second most common neurodegenerative disorder after Alzheimer's disease is PD (Alzheimer's, 2014; Dorsey et al., 2007). It is thought that the prevalence of PD is higher in Europe, North America and South America than in other parts of the world. Moreover, it has been estimated at 66–1500 per 100 000 (von Campenhausen et al., 2005), 111–329 per 100 000 (Strickland and Bertoni, 2004) and 31–470 per 100 000 (Bauso et al., 2012), respectively. In contrast, the prevalence is estimated to be 10–43 per 100 000 in Africa (Okubadejo et al., 2006), 15–119 per 100 000 in Asian (Muangpaisan et al., 2009) and 27–43 per 100 000 in Arabic countries (Benamer et al., 2008). The incidence of PD ranges from 10–18 per 100 000 person per year (Van Den Eeden et al., 2003). The increasing life expectancy of the population and the high proportion of elderly people with PD and associated complications can pose public health concerns. It is expected that the PD will would rise by more than 50% by 2030 (Dorsey et al., 2007).

Gender, ethnicity and age are three important risk factors for PD. In terms of the gender ratio, it has been estimated that three males will be affected for every two females. Ethnicity also plays an important role in PD; for example, in the United States, the incidence of PD among those of Hispanic ethnic origin is the highest, followed by that of non-Hispanic whites, Asians and blacks, respectively (Van Den Eeden et al., 2003). Finally, age is an extremely important risk factor, and the potential risk rises as individuals

age, with 80 years of age representing the peak risk (Driver et al., 2009; Pringsheim et al., 2014).

A meta-analysis study of potential environmental factors concluded that out of 30 different potential factors, 11 environmental factors are likely to modify the risk of PD. Factors that may escalate the incidence of PD are pesticide exposure, prior head injury, rural living, β-blocker use, agricultural occupation and well-water drinking. Meanwhile, factors that may reduce the incidence of PD are tobacco smoking, coffee drinking, non-steroidal anti-inflammatory drug use, calcium channel blocker use and alcohol consumption (Noyce et al., 2012). The analysis suggested that smoking may be good for PD patients (Noyce et al., 2012). However, a recent study also showed that PD patients are able to quit smoking more easily than healthy controls. A more recent finding suggested that smoking is not beneficial to PD patients and that the reduced susceptibility to smoking addiction may be due to a declined reaction to nicotine during the early phase of PD (Ritz et al., 2014).

2.1.2 Genetics

The genetic causes of PD have been extensively studied. Research has focussed on inherited PD to identify related genes. The first discovery was the gene called α-synuclein (SNCA) (Corti et al., 2011). The SNCA gene has been linked with mutations associated with autosomal-dominant parkinsonism (Devine et al., 2011). The discovery of this gene has promoted the scientific identification of SNCA as the hallmark component of Lewy

bodies and the histological examination of neurones (Spillantini et al., 1997). This gene discovery has prompted the discovery of other genes that can be associated with monogenic forms of PD (Corti et al., 2011).

Another gene has been discovered, namely leucine-rich repeat kinase 2 (LRRK2), which is proposed to be related to mediating autosomal-dominant forms of PD. LRRK2 has been found to have different cellular functions, such as neurone outgrowth, synaptic morphogenesis, membrane trafficking, autophagy and protein synthesis; it may also play a role in the innate immune system (Paisan-Ruiz et al., 2004; Healy et al., 2008; Aasly et al., 2010). It has been shown that LRRK2 mutations are found in 4% of familial PD and 1% of sporadic PD cases worldwide (Aasly et al., 2010). The most common LRRK2 mutation results in a Gly2019Ser amino acid substitution, which increases the kinase activity of the protein (Lesage et al., 2006).

A third gene has been discovered called vacuolar protein sorting 35 (VPS35; (Zimprich et al., 2011; Vilarino-Guell et al., 2011). This gene is thought to be associated with endosomes, which are intracellular membrane-bound compartments that traffic proteins between the plasma membrane, Golgi apparatus and lysosomes (Bonifacino and Hurley, 2008). This gene is connected with inherited PD.

Another identified gene is called DNAJC13; this gene is involved in membrane trafficking through early endosomes (Postuma et al., 2012). In addition, eukaryotic translation initiation factor 4-γ 1 (EIF4G1) mutations have been discovered to be linked to PD. However, further investigations are needed (Chartier-Harlin et al., 2011). A mitochondrial protein mutation called

coiled-coil-helix-coiled-coil helix domain containing 2 (CHCHD2) has been observed in Japanese patients with familial PD, but further investigation on this topic is needed.

Autosomal-recessive forms of PD can be caused by mutations in genes called Parkin, PINK1 and DJ-1; these forms are characterised by early onset of PD at age 40 years or even less (Schrag and Schott, 2006). Parkin mutations have been found in about half of familial PD patients, while PINK1 (1–8%) and DJ-1 (1–2%) mutations are less common causes of early-onset sporadic PD (Singleton et al., 2013). A mutation in a homozygous gene or multiple heterozygous genes may result in autosomal-recessive PD. In some cases, only a single heterozygous mutation is detected (Klein et al., 2007). Further research is needed on this topic. In addition, the proteins encoded by PARKIN, PINK1 and DJ-1 are all involved in mitochondrial health (McCoy and Cookson, 2012).

2.1.3 The α-synuclein gene and dopamine

It has reported that the SNCA gene has a role in both familial and sporadic PD. The gene was documented in unconnected families diagnosed with PD. It was discovered that the gene has three mis-sense mutations (Polymeropoulos et al., 1997; Kruger et al., 1998; Zarranz et al., 2004). Autosomal-dominant PD has been associated with duplications and triplications of the wild-type SNCA locus (Singleton et al., 2003; Chartier-Harlin et al., 2004; Ibanez et al., 2004; Farrer et al., 2004). Moreover, it has been shown that patients with triplication of the mutant gene have a higher

degree of severity of non-motor symptoms, early onset of PD, additional widespread neurodegeneration and faster disease progression than patients with duplicated genes (Singleton et al., 2003; Chartier-Harlin et al., 2004; Ibanez et al., 2004; Farrer et al., 2004). The expression of α-synuclein is widely observed in the whole brain region.

PD degeneration is usually found in the CNS, specifically in SN pars compacta, where the major pathology and maximum loss of cells occurs; this results in motor signs. The dopamine homeostasis mechanism that could be affected by mutations in α-synuclein is still under investigation in PD patients. The production of dopamine in cultured cells been observed, and interaction between α-synuclein and the enzyme TH has been found to regulate the production of dopamine. TH is responsible for transforming L-DOPA in the dopamine synthesis pathway (Perez et al., 2002; Liu et al., 2008). The activity of the TH promoter has been decreased using α-synuclein overexpressers in a cell culture model (Gao et al., 2007). As a consequence, the level of TH mRNA and protein were reduced (Baptista et al., 2003; Yu et al., 2004). It has also been observed that α-synuclein binds to TH to block TH phosphorylation.

α-Synuclein has been shown to stimulate protein phosphatase 2A (PP2A), which may be responsible for preventing TH phosphorylation (Perez et al., 2002; Peng et al., 2005). Furthermore, in an α-synuclein wild-type overexpressor mouse model, reduction of TH activity has been reported (Masliah et al., 2000; Kirik et al., 2002). In contrast, in cell culture experiments, an increase in TH phosphorylation was found to lead to

escalation in TH activity when SNCA was inhibited (Liu et al., 2008). There is also evidence that SNCA could network with the catalytic enzyme L-DOPA decarboxylase. This enzyme is responsible for converting L-DOPA to dopamine (Tehranian et al., 2006). In SNCA overexpressor dopaminergic cell models, the activity of serine phosphorylation and Aromatic amino acid decarboxylase (AADC) activity was lowered; this may have been due to changing PP2A activity (Tehranian et al., 2006). Moreover, in young adult SNCA null mutant mice, a mild reduction of striatal dopamine content (18%) was observed (Abeliovich et al., 2000). In another study with the same mouse model, no change in striatal dopamine was found (Robertson et al., 2004; Schluter et al., 2003; Alerte et al., 2008). Research was reported in 24- to 26-month-old SNCA null mice and showed that striatal dopamine content was substantially decreased by about 36% when compared to the wild-type counterpart (Alerte et al., 2008; Robertson et al., 2004; Schluter et al., 2003; Al-Wandi et al., 2010). It was also observed that the expressions of TH and the dopamine transporter Martinez-Martin et al. (2011) were decreased in the striatum, but there was no reduction in TH-positive neurones in the SNc (Al-Wandi et al., 2010). In addition, studies have been conducted on synaptic vesicle function, which showed an association with α-synuclein. Nonetheless, these studies have generally investigated non-dopaminergic hippocampal neurones; thus, they did not focus on the dopaminergic midbrain neurones relevant to PD.

A human α-synuclein overexpressor in mutant mice was found to affect synaptic vesicle exocytosis in hippocampal neurones (Yavich et al., 2004). In addition, α-synuclein-transfected ventral midbrain dopaminergic neurones in

rats showed defective synaptic vesicle exocytosis in hippocampal neurones (Nemani et al., 2010).

The neurotransmitter dopamine is usually imported into vesicles containing the vesicular monoamine transporter 2 (VMAT2). If left unpackaged within the cell, it could lead to detrimental effects due to oxidation stress by oxygen or quinones in the cytoplasm. A deficiency in synaptic vesicle function and/or formation may lead to oxidative damage like that observed in PD patients. In a study using a VMAT2 inhibitor auto-oxidation of dopamine in the cytoplasm was observed (Bender et al., 2006). In addition, α-synuclein has a role in regulating VMAT2 expression (Wersinger and Sidhu, 2003). The density of VMAT2 transporters per vesicle rises while the total number of intracellular vesicles declines in human neuronal cells when the inhibitor of α-synuclein is administered (Fountaine et al., 2008).

The hypotheses on how patients may develop PD has still not been fully elucidated. *T. gondii* exposure could lead to greater risk of developing PD. However, the similarity between *T. gondii* mechanisms and features of PD, such as TH activity and the association with dopamine, could be targets of interest. To date, no comprehensive study has been carried out to evaluate the correlation between *T. gondii* and PD. The aim of this chapter was to investigate the possible correlation between *T. gondii* exposure and PD by analysing *T. gondii* IgG seropositivity in PD patients compared to healthy individuals.

2.2 Methods

A total of 105 PD blood samples were collected from the neurological clinic at Leeds General Infirmary (LGI) hospital. The samples were collected weekly when there were PD patients available. Written consent forms were signed by all of the patients. All PD and control samples were from individuals of white British ethnicity with an average age of 70. The gender ratio was 60% male and 40% female. Blood samples were taken by a clinical nursing specialist (CNS) (Dr Queenie). They were harvested into collection tubes containing EDTA anticoagulant. At 8:30 AM on the day of collection, a box filled with packed ice (-20°C) labelled ‘Dr McConkey’ was delivered to Dr Queenie’s office. Dr Queenie then collected PD samples throughout the day. At the end of the working day, at 6:00 PM, the box was collected and transferred from LGI hospital to a research laboratory in the nearby Miall building. The number of PD samples collected on each collection day depended on the number of patients attending at that time, and most importantly the number of those that agreed to participate in the study. After receiving the blood samples, each was transferred into a 15 ml Falcon tube under sterile conditions. The samples were then centrifuged at 3,000 rpm for 8 minutes. The serum (supernatant) was transferred into a Sterilin tube and stored in a freezer at -70°C. An additional 300 sera control samples and 100 plasma control samples were obtained from the blood department at LGI hospital. They were matched in terms of gender (60% males and 40% females), racial origin (white British) and age (average of 70). The blood department was asked to treat control samples the same way as PD

samples in terms of transfer-tubes and centrifugation. They were kept frozen at -70°C until the time of collection. The human controls were provided by the LGI screening service. These were random samples that were sent from NHS providers throughout West Yorkshire (same demographics as the PD group). The samples were from individuals without major illnesses and hence generally healthy.

Moreover, a total of 201 PD serum samples were obtained from Greater Manchester (GM), along with 76 control sera. The 201 PD samples came from 137 males and 64 females, while the control sera came from 18 males, 42 females and 12 individuals whose genders were not recorded. The Manchester controls were often the spouse or a care provider and hence had similar environments and the same demographics.

For the purpose of detecting *T. gondii* antibodies in all samples, direct agglutination kits (BioMérieux®) were purchased and the manufacturer's instructions were followed. Solutions were added together as shown in Table 1 below. The procedures were carried out on microtitration plates with a round-bottom. Each sample was diluted at a ratio of 1:20 (10 µl serum diluted in 190 µl phosphate-buffered saline [PBS]).

Material	Quantity
1.Diluted PD sample or control	25 µl
2.Mercaptoethanol (R3) plus PBS	25 µl
3.*Toxoplasma* antigen (R1) plus albumin buffer (R2)	50 µl

Table 1: The order of solutions added to the plate for DAT analysis of *T. gondii* IgG antibodies in both PD and controls.

It was important to keep the order of the solutions otherwise the test would not work. Test solutions were added in the order shown in Table 1, then the plates were shaken well and incubated for 15–18 hours before they were read. Each sample was tested in duplicate to ensure consistency of results. For each 22 samples tested, commercially supplied positive control and negative control samples were also tested in parallel. In other words, each 96-well microplate test run included 4 'ready to use' positive controls and 4 'ready to use' negative controls. An Abcam® anti–*T. gondii* IgG human ELISA kit was used to verify the direct agglutination kit (BioMérieux®) results, as well as to confirm the positivity or negativity of samples. The manufacturer's protocol was followed. The kit was designed to measure anti-*T. gondii* IgG antibodies quantitatively in human serum and plasma. It is a ready-to-use kit supplied with all of the reagents needed, including standards, with a 96-well plate. The 96-well plate was precoated with *T. gondii* antigen and was ready to use.

All samples – including PD and control – were prepared by dilution in PBS at a ratio of 1:1. Following this, the samples were diluted in the supplied IgG diluent at a ratio of 1:100 following the manufacturer's instructions. Each sample was tested once due to budget constraints. Diluted PD samples or controls (100 µl each) were then added to the wells and incubated for 60 minutes in the dark at 37°C. Next, samples were aspirated, and wells were washed three times with 1X washing solution. To ensure that the wells were accurately emptied, the plate was banged on a clean tissue several times. In the next step, 100 µl of *T. gondii* anti-IgG horseradish peroxidase (HRP) conjugate was added to all sample wells. Next, the plate was covered with

foil and incubated at room temperature for 30 minutes, after which the washing step was repeated three times. Following this, 100 µl of tetramethylbenzidine (TMB) substrate solution was added to all wells and the samples were incubated in the dark for 15 minutes at room temperature. At this stage, some samples turned blue, which could be seen by the naked eye. Finally, 100 µl of stop solution were added to all wells and absorbance was measured at 450 nm within 30 minutes using spectrophotometry. Any blue colour turned yellow at this stage. A second reading was measured at 620 nm for reference only.

Each ELISA plate had a well for the substrate blank and wells for standards A, B, C and D, each of which was supplied with the kit. The absorbance values for the blank and each standard were < 0.100, < 0.200, > 0.300, > 0.500 and > 1.000, respectively. All samples, including standards, were blank subtracted. A standard curve was created for each test, and the concentration of the samples was revealed (U/mL).

Microsoft Excel was used to calculate Y value from the standards absorbance. Then, concentrations were obtained by dividing the absorbance by the Y value. Concentrations of > 35 U/mL were considered to be ‘reactive’, 30–35 U/mL ‘inconclusive’ and < 35 U/mL ‘nonreactive’.

Data were analysed using IBM SPSS statistics software (version 22). Chi square and Fisher's exact test 2 sided were determined by the software. Openepi website (http://www.openepi.com/TwobyTwo/TwobyTwo.htm) was used to calculate odds ratio.

2.3 Results

2.3.1 Sample collection for Leeds Parkinson's Disease Study

Blood samples were collected from 105 patients with Parkinson's disease, by the Neurological Department, PD clinic, at LGI hospital. The patients signed a consent form and volunteered to participate in the study. The study subjects were diagnosed with PD via magnetic resonance imaging (MRI); however, they had non-complicated PD. The patients were of white British origin, and the average male age was 70.9 years, while the average female age was 72.5. Of the total of 105 participants, 60% were male and 40% were female.

A total of 100 healthy blood plasma samples as well as 300 healthy serum samples were obtained from the blood clinic at LGI hospital. They were matched in terms of ethnic background (white British), 60% male with an average age of 70.9 years, and 40% female with an average age of 72.5. The aim of the study was to detect *T. gondii* antibodies in PD patients plasma as well as control sera and plasma in order to find out if there is a correlation between PD and *T. gondii* by comparing PD seropositivity with control seropositivity.

2.3.2 Leeds Samples Analysed by Agglutination Assay

The blood samples from the patients were analysed using the BioMérieux® kit for detecting *T. gondii* antibodies. The test results were classified into three categories according to the kit manufacturer's instructions:

1. Positive result (Figure 2); will form 'agglutination of the *Toxoplasma* in a mat covering about half of the well base. The mat may show slight shrinking around the edges (irregular shape).'
2. Negative result; will form 'sedimentation of the *Toxoplasma* in a button or ring.'
3. Borderline result; will form 'agglutination of the *Toxoplasma* in a mat covering less than half of the well base' (Biomérieux, 2012).

The results for PD samples were as follows:

- 79 samples were negative (75.24%).
- 20 samples were positive (19.05%).
- 7 samples were borderline (5.71%).

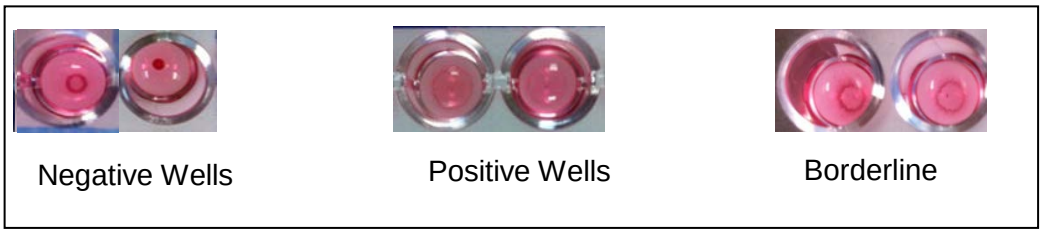

Figure 2: Representative samples of agglutination test results (wells of a 96-well plate).

The BioMérieux® kit was used to detect antibodies of the parasite in the control samples, and the results are shown below, in Table 2.

	Positive	Negative	Borderline
Number	46	337	17
Percentage	11.50	84.25	4.25

Table 2: Leeds control total sample results.

2.3.3 Leeds Serum vs. Plasma

To confirm that there were no differences between the serum and plasma controls, Fisher's exact 2-sided test was performed, yielding a *p*-value of 0.72 ($p \leq 0.05$) and hence there were no statistically significant differences between the serum and plasma control groups. Therefore both serum and plasma control samples can be used.

2.3.4 Leeds PD and Total Control Groups

The differences between the PD samples and the control samples were analysed by four crosstabulation statistical analyses- taking into account borderline samples or excluding them.

		Result			Total
		Positive	Negative	Borderline	
Group	Control	46	337	17	400
	PD	20	79	6	105
Total		66	416	23	505

Table 3: Crosstabulation 2 x 3 table of PD and control group results.

Table 3 was created using all the samples as normally found positive, negative and borderline. Pearson's chi-square test yielded a *p*-value of 0.089 ($p \leq 0.05$) was detected, indicating that there was no statistical significance between PD seropositivity and controls.

		Result		Total
		Positive	Negative	
Group	Control	63	337	400
	PD	26	79	105
Total		89	416	505

Table 4: Crosstabulation 2 x 2 table with borderline samples counted as positives.

Table 4 was created with all borderline samples entered as positives in order to determine their influence on the final results. Fisher's exact 2-sided test yielded a *p*-value of 0.049; a statistically significant result ($p \leq 0.05$). An odds ratio was used to assess the association between *T. gondii* exposure and PD outcome. The odds ratio indicated that PD patients are 1.76, 95% CI (1.048, 2.956) times more likely than healthy people to be exposed to *T. gondii*.

		Result		Total
		Positive	Negative	
Group	Controls	46	354	400
	PD	20	85	105
Total		66	439	505

Table 5: Crosstabulation 2 x 2 table with borderline samples counted as negatives.

Table 5 was generated with all borderline samples entered as negatives. This time, to investigate whether the borderline samples could play a crucial role in terms of statistical significance. Fisher's exact 2-sided test yielded a *p*-value of 0.066; an approaching statistical significant result at a significance

threshold of $p \leq 0.05$. An odds ratio of 1.81 was calculated with 95% CI (1.081, 3.221). The odd ratio was used to determine the probability that PD patients had been exposed to *T. gondii*.

		Result		Total
		Positive	Negative	
Group	Control	46	337	383
	PD	20	79	99
Total		66	419	482

Table 6: Crosstabulation 2 x 2 table with borderline samples omitted from the analysis.

Table 6 was generated with borderline samples excluded. Fisher's exact 2-sided test yielded a *p*-value of 0.05; a marginal statistically significant result ($p \leq 0.05$). An odds ratio of 1.85 was calculated with 95% CI (1.039, 3.31). It means that PD patients are 1.85 times more likely to get *T. gondii* than healthy controls. As these analyses are close to the cut-off for statistical significance, borderline samples are influential on the outcome. However, all crosstabulations agreed that there was not a highly significant association of PD with *T. gondii* seroprevalence.

2.3.5 Manchester PD and Control Samples Analysed by Agglutination Assay

Samples were obtained from Manchester in collaboration with Prof. David Mann, Professor of Neuropathology, University of Manchester. The samples were processed, and each serum sample was 50 µl in volume. A total of 277 PD sera samples and controls were obtained. Among the 201 PD samples, 68.2% came from males and 31.8% from females. In terms of the control

sera, 23.7% came from males, 60.5% came from females and 15.8% had no gender recorded.

Using the BioMérieux® kit for detecting *T. gondii* antibodies in human serum in order to determine the seropositivity of PD and compare it with healthy controls. The test results were classified into three categories according to the manufacturer's instructions, as mentioned in section 2.3.2. Manchester PD samples and control results are given in Tables 7 and 8 below.

PD	Positive	Negative	borderline	Total
Total	3	171	27	201
Percentage	1	85	14	100

Table 7: Agglutination test results for PD of Manchester.

Control	Positive	Negative	borderline	Total
Total	2	65	9	76
Percentage	3	86	11	100

Table 8: Agglutination test results for controls of Manchester.

The chi-square test was used to analyse the differences between the PD samples and the control samples. To ensure that the samples were thoroughly statistically analysed, four different analyses were conducted;

a. The data were used as normally found; positive, negative and borderline shown in Table 9.

b. All data were used with all 'borderline' samples counted as 'positive' (Tables 10).

c. All data were analysed; all 'borderline' samples were counted as 'negatives' (Tables 11).

d. Positive and negative test results were used, with borderline samples omitted entirely (Tables 12).

		Result			Total
		Positive	Negative	Borderline	
Sample	Ctrl	2	65	9	76
	PD	3	171	27	201
Total		5	236	36	277

Table 9 : Crosstabulation 2 x 3 table of Manchester PD and control group results.

Table 9 crosstabulation analysis was created with all the samples as normally found positive, negative and borderline which showed Pearson's *p*-value of 0.777 which suggest that no association between PD and *T. gondii* exposure.

When borderline samples counted as positives , Fisher's exact two-sided test yielded a *p*-value of > 0.999, a not statistically significant result at a significance of $p \leq 0.05$. (Table 10), indicating that there is no statistical significance between PD seropositivity and healthy control seropositive.

		Result		Total
		Positive	Negative	
Sample	Ctrl	11	65	76
	PD	30	171	201
Total		41	236	277

Table 10: Crosstabulation 2 x 2 table with borderline samples counted as positives (Manchester).

		Result		Total
		Positive	Negative	
Sample	Ctrl	2	74	76
	PD	3	198	201
Total		5	272	277

Table 11: Crosstabulation 2 x 2 table with borderline samples counted as negatives (Manchester).

To investigate whether borderline samples could play a crucial role in terms of statistical significance, all borderline samples were entered as negatives (Table 11). Fisher's exact two-sided test yielded a *p*-value of 0.837, which was not a statistically significant result at a significance threshold of $p \leq 0.05$. suggesting that there is no association between *T. gondii* exposure and PD.

		Result		Total
		Positive	Negative	
Sample	Ctrl	2	65	67
	PD	3	171	174
Total		5	236	241

Table 12: Crosstabulation 2 x 2 table with borderline samples omitted from the analysis (Manchester).

When all borderline samples excluded (Table 12). Fisher's exact two-sided test yielded a *p*-value of 0.853; this was not a statistically significant result at a significance threshold of $p \leq 0.05$, indicating that there is no association between *T. gondii* exposure and PD. Hence there is no detectable differences in seroprevalence of PD and control samples regardless of the input of the borderline samples. To ensure that these findings were not dependant on the method, I repeated testing using an ELISA assay as an independent method.

2.3.6 The Leeds PD and Control Samples Analysed by Abcam® Anti-*Toxoplasma gondii* IgG Human ELISA Assay

As BioMérieux® agglutination assay results were not consistent between Leeds PD and Manchester PD, Abcam® Anti-*Toxoplasma gondii* IgG

Human ELISA kits were used to detect *T. gondii* antibodies in PD and control samples in order to confirm the agglutination results. In this study, Leeds serum controls (300 samples) were excluded for cost reasons.

In total, 105 PD and 100 plasma control samples were tested using ELISA assay (Table 13). As a result, 36 out of 105 Leeds PD samples were found to be positive. In contrast, in the Leeds plasma control samples from healthy individuals, 37 out of 100 turned out to be positive. Two controls were inconclusive (out of 100). Pearson's chi-square test yielded a *p*-value of 0.279. This *p*-value is well below significance for any association between *T. gondii* exposure and PD disease.

Sample * Result Crosstabulation					
		Result			Total
		Reactive	Non-reactive	Inconclusive	
Sample	Ctrl	37	61	2	100
	PD	35	70	0	105
Total		72	131	2	205

Table 13: Crosstabulation 2 x 3 table of PD and control group of Leeds ELISA results.

When inconclusive samples were omitted from the statistical analysis. Fisher's exact test (two-sided) *p*-value was 0.558, hence, there were no correlations observed between the presence of PD and *T. gondii* exposure.

2.3.7 The Manchester PD Abcam® anti-*Toxoplasma gondii* IgG human ELISA Assay

Using Abcam® anti-*Toxoplasma gondii* IgG human ELISA Assay with the Manchester samples, 20 positives out of 76 controls (26.31%) were

identified, while 50 positives were found out of 201 PD patients (24.87%). Statistical analysis of the Manchester PD and control samples showed no statistical significance between the two groups, with a Fisher's exact test (2-sided) value of 0.877. There were 4 PD samples showing inconclusive results. These results do not support a correlation between patients with PD and *T. gondii* exposure.

Figure 3 shown below is a representative standard curve that was created at the time of ELISA test using the standards. Each ELISA analysis had its own standard curve which was used to calculate the serum levels.

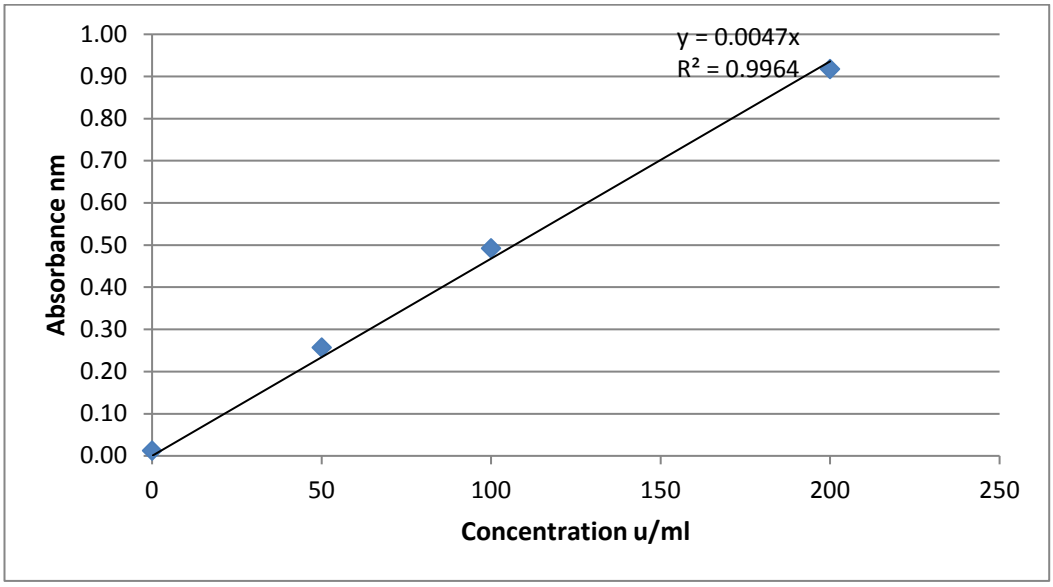

Figure 3: A representative standard curve created by Excel for ELISA analysis.

2.3.8 DAT and ELISA result reliability

Cohen's kappa (k) is a measurement used to detect the agreement between two categorical scales. In this instance the results of DAT and ELISA. To measure the agreement between the two results kappa test was used. Table

14 showed kappa crosstabulation for Leeds results. k value was 0.542 which suggest a moderate agreement between both analysis. Figure 4 below showed ELISA and DAT agreement in a graph of box-plot.

ELISA vs DAT Crosstabulation		DAT			Total
		Negative	Borderline	Positive	
ELISA	Non-reactive	130	1	0	131
	inconclusive	2	0	0	2
	Reactive	27	12	33	72
Total		159	13	33	205

Table 14: Leeds PD and control kappa crosstabulation results.

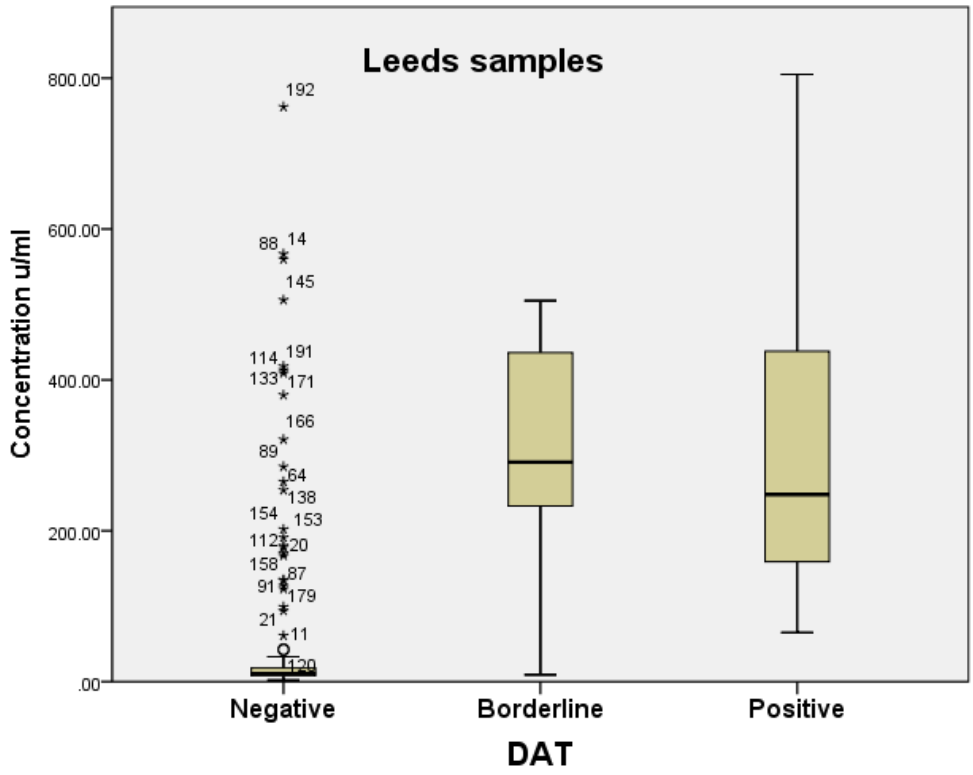

Figure 4: Box-plot of ELISA units classified by DAT category for Leeds samples.

Table 15 below showed kappa crosstabulation for Manchester results. k value was 0.183 which suggest that a slight agreement between ELSIA and DAT (Figure 5).

ELISA vs DAT Crosstabulation		DAT			Total
		Negative	Borderline	Positive	
ELISA	Non-reactive	183	10	2	195
	inconclusive	2	2	0	4
	Reactive	51	24	3	78
Total		236	36	5	277

Table 15: Manchester PD and control kappa crosstabulation results.

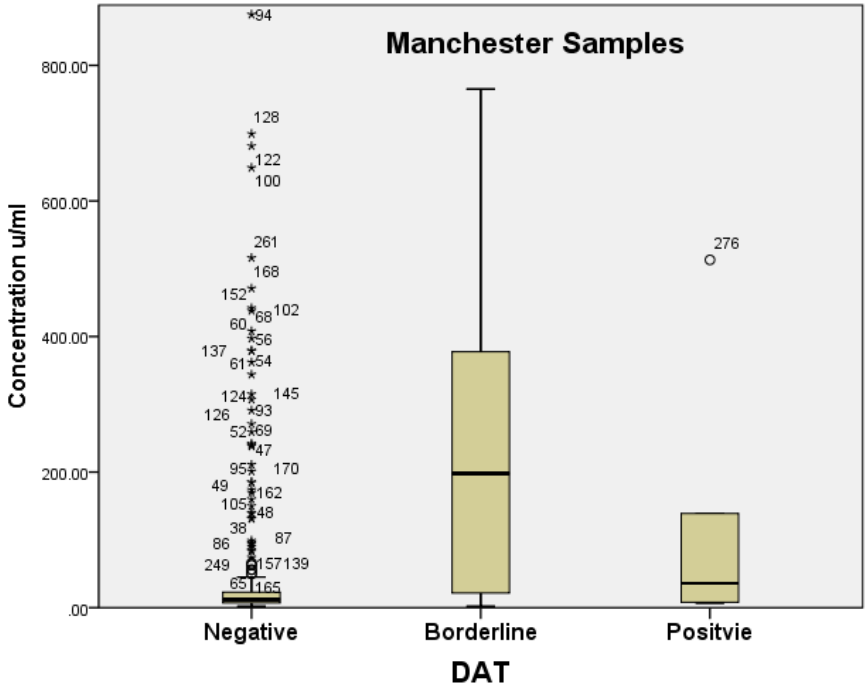

Figure 5: Box-plot of ELISA units classified by DAT category for Manchester samples.

2.4 Discussion

The aim of this study was to investigate the correlation between *T. gondii* exposure and PD. The choice of the direct agglutination test and ELISA to determine the prevalence of *T. gondii* exposure was based on previous studies. For example, the prevalence of *T. gondii* in the United states was examined using the Platelia Toxo-G IgG enzyme immunoassay (Jones et al., 2003), whereas in France, parasite prevalence was tested also using ELISA to detect IgG as well as IgM antibodies (Richomme et al., 2009). According to the manufacturer's manual, the sensitivity of the BioMérieux® direct agglutination kit used in this study is 96.22%, and its specificity is 98.80%, which is similar to the corresponding parameters in previous studies. Moreover, each sample was tested in duplicate to ensure consistency of the results, as well as to determine the sensitivity of the kit. These results were preliminary and confirmation via ELISA analysis was completed.

In this study, the difference between the positivity rates of the control sera and the control plasma was not statistically significant. Serum, unlike plasma, lacks clotting agents and fibrinogen. The results of this study suggest that there is no significant difference between plasma and serum levels of *T. gondii* antibodies. In other words, both sera and plasma are similar enough in their components that the storage and laboratory preparations did not affect the level of *T. gondii* antibodies detected in the analysis.

The study reported herein involved four different types of statistical analysis to ensure reliability. The first analysis included all results – positive, negative

and borderline. A crosstabulation 2×3 table was used. The results suggested no significant correlation between PD and *T. gondii* exposure. It is not possible to accept these results because the true nature of the borderline samples is not known; in other words, it is not known whether they are truly positive or negative. In addition, the previous studies reported did not describe borderline results in their analyses. The kit classified undefined samples as borderline, and the percentages of borderline results in this study were 5.7% and 4.25% for PD and control samples, respectively. Depending on how the borderlines are counted there is some minor significance between patients and controls but this is at best marginal.

It is important to consider population effects when determining the importance of the correlation between prior *T. gondii* exposure and PD. PD samples from Manchester were obtained to compare to our dataset, so that we had samples from a similar environment and ethnic group. Hence, the data represented northern England. The samples were analysed and the results were compared to those of the present study, although one must consider possible differences, such as rural versus urban participants, since this has been found to be a relevant factor with regard to *T. gondii* seroprevalence. Prior studies have reported that *T. gondii* seropositivity is correlated with schizophrenia and depression; thus, participants should be monitored for behavioural disorders. Hence, it is important to further analyse the participant pool in the context of parameters like length of drug treatment. In addition, the full history of patients in this study was not known, such as potentially relevant environmental factors and aspects of personal hygiene.

In this study, it was not possible to determine what was a cause and what was an effect. To overcome this in the future, one possible approach would be a longitudinal study in which blood samples were taken every five years. This could help to identify causality.

Manchester PD and control samples were provided by the Neurological Department at the University of Manchester. DAT results were not consistent based on ELISA kappa tests as well as absolute values were surprisingly low. ELISA analysis of Manchester samples showed 26.31% positives out of 76 controls were identified, while 24.87% positives were found out of 201 PD patients. Fisher's exact test yielded a *p*-value of 0.877, . There were 4 PD samples showing inconclusive results. These results indicate there was no correlation between patients with PD and *T. gondii* exposure. The Manchester samples controlled for environmental factors as both patient and control samples came from the same households although hygiene may differ for PD patients. In the DAT analysis with all borderline samples entered as negatives as well as when borderline counted as positives Fisher's exact 2-sided test revealed a *p*-value of statistically non significance. This data is not consistent with a correlation between *T. gondii* and PD. As a consequence of large number of borderlines in DAT analysis, the results of Manchester samples may be classified as unreliable.

The agglutination reaction can be influenced by different factors, such as concentrations of both antigens and antibodies, pH, temperature and surface change. The kit manual was strictly followed, and therefore I cannot explain why the Manchester samples exhibited such a large number of borderline

results and very small number of positives. It was not possible to retest all borderline samples in serial dilutions to determines the correct dilution due to budget constraints and the large number of samples that would have needed to be retested.

To ensure that the Leeds and Manchester PD samples were tested accurately and quantitatively, the Abcam® ELISA kit was used. ELISA is a method of quantitatively measuring IgG class antibodies against *T. gondii* in human serum and plasma. A plate was coated with capture antigen and the samples were added to it. A labelled HRP conjugate was then added to the samples. Nonspecific antibodies were removed by washing. Then, the TMB substrate solution bound with the sample in each well, causing it to change colour. A stop solution was added to stop the reactions, and optical density was measured using a spectrophotometer. Following this, concentrations were calculated. This is a reliable method that is widely used in the scientific field and at hospitals to investigate patient samples. The sensitivity of ELISA is 96.6%, defined as the probability that the assay will return a positive score in the presence of the specific analyte. ELISA's specificity is 98.2%, defined as the probability that the essay will return a negative score in the absence of specific analyte.

The Leeds PD results showed 36 out of 105 patients were positive for *T. gondii*. Meanwhile, in the Leeds plasma controls, 37 out of 100 healthy individuals were found to be positive. Two controls were inconclusive (out of 100). The Manchester PD samples showed 50 positives out of 201 PD patients and 20 positives out of 76 Controls. The percentages were 34.28%,

37%, 2%, 24.87% and 26.31%, respectively. Statistical analysis suggested that there were no correlations found between PD patients and *T. gondii* exposure.

Kappa test was used to find out how DAT and ELISA were agreeing in terms of positive/reactive, negative/non-reactive and borderline/inconclusive results for both sets of samples Leeds and Manchester. Kappa has shown that Leeds DAT showed a stronger agreement with ELISA results (k= 0.543) than Manchester kappa (0.183) which is a weak agreement. Borderline can be counted as positive as suggested by box-plot. Manchester samples showed a weak agreement as a result, Manchester data may not be credible.

To date, no comprehensive study has included such a large sample of PD patients and controls to determine the possible correlation between *T. gondii* exposure and PD. It was observed that the rate of seropositivity for anti–*T. gondii* IgG antibodies was slightly greater in control healthy subjects than in patients with PD, but the results showed that there may be no association between *T. gondii* infection and PD. The literature suggests that risk factors, such as genetic and environmental factors, have a possible role in the developmental and the aetiology of PD. Studies have shown that environmental factors, such as alcohol and coffee consumption; smoking; exposure to chemicals; metals; some toxins; infections; head injuries; rural living; and diet could play crucial roles in PD (Chen et al., 2004; Logroscino, 2005; Priyadarshi et al., 2001). Vlajinac (2013) observed that PD may be associated with the following diseases: mumps, scarlet fever, influenza,

herpes simplex, whooping cough, chicken pox and tuberculosis (Vlajinac et al., 2013).

Many studies have been conducted on the *T. gondii* infections that shows that it has a role in neurological diseases. For example, studies have concluded that there are possible relationships between *T. gondii* and schizophrenia, as high anti-*Toxoplasma* IgG antibody levels were found in patients compared with a control group (Alipour et al., 2011; Alvarado-Esquivel et al., 2011; Cetinkaya et al., 2007; Torrey et al., 2007). Indeed in a meta-analysis of 38 studies, a correlation overall was found of *T. gondii* antibody and Schizophrenia with an odds ratio of 2.73 (95% CI) (Torrey et al., 2012). Nevertheless, Daryani et al. (2010) were unable to find any correlation between the two diseases in a study conducted in Iran (Ahmad et al., 2010). Moreover, *Toxoplasma* infection has been associated with Alzheimer's disease (Kusbeci et al., 2011), OCD (Miman et al., 2010b), suicide attempts (Arling et al., 2009; Ling et al., 2011; Yagmur et al., 2010) and bipolar disorder (Pearce et al., 2012). Furthermore, it has been well documented that in the CNS, brain cells and neurotransmitters, such as dopamine and norepinephrine, are involved in *T. gondii* infection (Skallova et al., 2006; Yolken et al., 2009; Alipour et al., 2011; Cetinkaya et al., 2007). The parasite genome encodes two enzymes that convert L-dopa to dopamine in the brain (Prandovszky et al., 2011). During chronic infection and the bradyzoite stage of the parasite, these two enzymes are clearly expressed (Gaskell et al., 2009).

In PD patients, neuronal degeneration and low dopamine levels are common symptoms of the disease (Venda et al., 2010). It has been suggested that *T. gondii* could promote neuronal death via proinflammatory cytokines, thereby resulting in PD (Obeso et al., 2008; Olanow et al., 2009). A recent study conducted in the Iranian city of Tabriz to investigate the probable correlation between *T. gondii* infection and PD showed a high prevalence of *Toxoplasma* in PD and healthy controls. In this study, 85.3% of PD patients and 90.3% of healthy controls exhibited positivity for the anti-*Toxoplasma* IgG antibody. Nevertheless, all subjects were found to be negative for the IgM anti-*Toxoplasma* antibody (Oskouei et al., 2014). The researchers concluded that they could not observe any association between toxoplasmosis and PD. However, they found a strong association between PD and cat ownership ($p = 0.03$), as well as the use of undercooked eggs ($p = 0.004$). These factors have been shown to have a link with *T. gondii* infection. People with cats could likely ingest oocysts of the parasite when dealing with cat faeces (Oskouei et al., 2014).

In Turkey, Miman et al. (2010a) showed a statistically significant difference between anti-*Toxoplasma* IgG antibody seropositivity in the PD and control groups, while all subjects were negative for IgM antibody although the numbers (n=52, 40) were low. In another part of Turkey, a different study was unable to find a relationship between toxoplasmosis and PD using the Sabin–Feldman dye test, as the researchers found no significant differences between the control and PD groups in terms of occupation, residency, cat ownership, use of undercooked meat or *T. gondii* seropositivity (Celik et al., 2010).

In this study, the Human IgG Ready-SET-Go!® ELISA kit was purchased from the Affymetrix eBioscience company. This was almost half as expensive as the Abcam® kit. This kit has 96-well plates, but they have not been precoated with tachyzoites. In addition, the incubation periods for the different stages of the test were longer than for the Abcam® kit. Freshly grown tachyzoites were used to coat the plates, as described by Dr Rupert Quinnell, University of Leeds. Here, 100 µl of tachyzoites diluted in PBS was incubated in each well overnight in sterile conditions. Then, the samples were washed with PBS and left to dry. The kit's protocol was followed precisely. However, no results whatsoever were achieved. The optical density was always low despite the care taken to optimise the methods. The manufacturer suggested that using a different brand of plates can reduce the optical density. Thus, plates were purchased from Affymetrix eBioscience, but this did not help. The company also suggested that manual washing could cause a low signal. As a result, a washing machine for ELISA plates was used with the Affymetrix eBioscience washing buffer, but this did not help to obtain results. In addition, different dilutions of samples were used. Each step was followed and repeated comprehensively, but this did not help. The reagents were stored and used according to the manual. About three weeks and about eight plates were used, but unfortunately, nothing was achieved using this kit.

In contrast to the Human IgG Ready-SET-Go!® ELISA kit, the Abcam® kit was ready to use. All ingredients and one plate were prepared and ready to testing. It was only necessary to dilute the test samples. The incubation periods for the different stages were found to be short compared to those

needed for the other kit. The Abcam® kit was first tested with the standards and 12 PD samples in duplicate. Then, the test was repeated in exactly the same manner the next day. The kit did work promptly with high accuracy. Next, more kits were purchased within the limits of the budget were purchased, and each sample was tested separately, as described in the previous chapter.

Future work could consider the use of advanced imaging techniques such as PET and MRI could be used to evaluate brain alterations that have been linked with *T. gondii* infection although advances are needed in detection as cysts are so small. It is also possible to monitor patients and look for the development of psychiatric disorders such as PD and schizophrenia that may be linked to *T. gondii*.

Chapter 3 Investigation of the combined effects of *Toxoplasma gondii* exposure and neurexin-1α deletion on behaviour in mice

3.1 Introduction

Schizophrenia is a chronic mental illness that causes positive symptoms of hallucinations, delusions and thought disorders and negative symptoms of anhedonia, decreased motivation and cognitive deficits (Barnes et al., 1996; Elkis, 2007). The disorder is among the leading causes of disability worldwide (Lopez and Murray, 1998). Current antipsychotic medications are unable to treat positive symptoms effectively in one-third of patients, are ineffective for negative symptoms and cognitive deficits (Barnes et al., 1996; Elkis, 2007; Elvevag and Goldberg, 2000), and often have unacceptable side effects, adding considerable complexities to patient management (Deakin et al., 2010). Enlargement of the lateral ventricles is among the most frequently reported macroscopic brain structural changes in schizophrenia, although variable in extent and localization (Gaser et al., 2004). Several studies have reported a link between *T. gondii* seropositivity and schizophrenia. Indeed, it has been found that *T. gondii* poses a higher risk for schizophrenia than any other environmental factor (Torrey et al., 2007). In some cases, acute toxoplasmosis in adults has been associated with psychiatric symptoms such as delusions and hallucinations (Torrey et al., 2007). Since 1956, more than 20 studies have compared antibodies to *T. gondii* in adults with and without schizophrenia (Torrey and Yolken, 2003). An overall analysis of these studies indicates that serological evidence of *Toxoplasma* infection is

almost three times more common in persons with schizophrenia than in controls living in the same geographical region. Two additional studies report an increased level of *T. gondii* antibodies in the late-pregnancy serum of women giving birth to infants who later developed schizophrenia (Brown et al., 2000; Mortensen et al., 2005). Other studies have reported greater childhood exposure to cats among persons with schizophrenia than among controls (Torrey et al., 2000; Torrey and Yolken, 1995). In some cases, acute toxoplasmosis in adults has been associated with psychiatric symptoms such as delusions and hallucinations (Torrey et al., 2007).

Schizophrenia is a neurodevelopmental disorder in which genetic factors account for approximately 80% of the total variation in liability (McGuffin et al., 1995). Genome-wide association studies (GWAS) have shown a substantial polygenic contribution to the risk of schizophrenia, but rare genetic mutations with large contributions to the risk of schizophrenia have also been identified (McGuffin et al., 1995). For example, mutations in families with schizophrenia have been discovered in *DISC1* (Millar et al., 2000), *NPAS3* (Kamnasaran et al., 2003), *PDE4B* (Millar et al., 2005), *GRIK4* (Pickard et al., 2006) and *ABCA13* (Knight et al., 2009). It is considered that interactions between various environmental factors and genetic variants could result in schizophrenia. Understanding these interactions is thus of great importance for understanding the disease.

Oligonucleotide array based techniques for whole-genome scanning have identified chromosomal microduplications and microdeletions, known as copy number variants (CNVs), in schizophrenia patients (Merikangas et al.,

2009). One of the genes identified is *NRXN1*, encoding neurexin-1, in which deletions that eliminate exons have been found. *NRXN1* has also been linked to mental retardation and autistic spectrum disorder (ASD) (Duong et al., 2012; Harrison et al., 2011; Utine et al., 2014). *NRXN1* mutations could thus play a crucial role in the neuropathology contributing to these distinct neurodevelopmental disorders.

The CNS is the part of the body that is most affected by *T. gondii* in mammals; encysted bradyzoites can form in the CNS, as well as in other immunoprivileged tissues. *T. gondii* infection can result in miscarriages and stillbirths. Neurological symptoms, such as tremors, headshaking, seizures and incoordination, have been observed in *T. gondii*-infected animals (Wastling et al., 2000). In human beings, delusions and hallucinations have been reported in some cases of acute toxoplasmosis. In one study, 24 out of 114 cases of acquired toxoplasmosis exhibited psychiatric symptoms, such as paranoia, delusions, auditory and visual hallucinations, and thought disorder (Torrey et al., 2007). Three patients with acquired toxoplasmosis were initially diagnosed with schizophrenia (Kramer, 1966). Neurotransmitters such as dopamine and serotonin have been postulated to be involved in both schizophrenia and *T. gondii* infection (Torrey et al., 2007).

3.1.1 Neurexin 1α and Its Role in Schizophrenia

The neurexins (NRXNs) are a synaptic adhesion protein family that bind neuroligins (NLGNs) for cell adhesion and the formation of synaptic contacts

(Bang and Owczarek, 2013). One of the primary reasons they do so is to reconcile critical communications between special presynaptic and postsynaptic functions (Martinelli and Sudhof, 2011). The paralogous genes *NRXN1*, *NRXN2* and *NRXN3* encode neurexin-1, neurexin-2 and neurexin-3, respectively. Each gene is transcribed in neurones from two independent promoters to yield longer (α) and shorter (β) proteins composed of distinct extracellular domains linked to identical intracellular sequences.

Several researchers have suggested that loss of neurexin-1 is a causative factor in some cases of schizophrenia. In fact, submicroscopic chromosomal deletions that disrupt expression of neurexin-1α have been shown to increase the risk of developing schizophrenia (Wright and Washbourne, 2011). Understanding of neurexin-1 and its role in the CNS may thus aid our understanding of schizophrenia. Deletions in the *NRXN1* gene have also been found in subjects with mental retardation (Ching et al., 2010). A comprehensive review of the structure and possible function of NRXNs and NLGNs has been carried out by Owczarek et al. (2015). Vertebrate NRXNs and NLGNs are adhesion molecules, and they have a distinct synaptic function (Wright and Washbourne, 2011). They influence trans-synaptic activation of synaptic transmission, and their dysfunction impairs the properties of synapses and disrupts neural networks without completely abolishing synaptic transmission (Reichelt et al., 2012).

The discovery of a *de novo* deletion of the neurexin 1α promoter and exons 1-5 in a boy with autistic-like behaviours and mild learning disability provided the first evidence implicating *NRXN1* in neurodevelopmental disorders

(Friedman et al., 2006). Subsequently, a *de novo* deletion eliminating exons of Neurexin 1α and β in two siblings with typical autism was discovered (Szatmari et al., 2007). Kim et al. (2008) found a chromosomal excision of neurexin 1α exons 1-5 in a subject with autism and her unaffected father. Vrijenhoek et al. (2008) found neurexin 1α-specific exonic deletions in three patients with paranoid-type schizophrenia, but not in 706 unaffected control subjects. Kirov et al. (2008) found a deletion eliminating the promoter and exon 1 of neurexin 1α in two siblings with schizophrenia and their unaffected mother, but not in 372 controls. Walsh et al. (2008) found a deletion eliminating exons common to neurexin 1α and β in identical twins with childhood-onset schizophrenia, but not in 268 controls. In a larger study, Rujescu et al. (2009) found partially overlapping deletions of neurexin 1α-specific exons among 2,977 schizophrenia patients and an individual with autism as well as 33,746 control subjects, but the deletions showed a highly significant association with schizophrenia (P = 0.0027) and conferred an increased risk for disease diagnosis (odds ratio 8.97) by virtue of their greater prevalence in the patient group. The impact of *NRXN1* exonic deletions on the expression of neurexins 1α and β in the brain is unclear because human post-mortem brain tissue from deletion carriers is unavailable. However, those deletions that eliminate the promoter and initial exons of neurexin 1α, but leave neurexin 1β coding sequences intact, would be expected to produce complete absence of neurexin 1α transcripts and unaltered expression of neurexin 1β (Zahir et al., 2008). Geppert et al. (1998) reported that neurexin 1α null mutant mice are viable, fertile and indistinguishable in appearance from wild-type mice. Interestingly, female

neurexin 1α null mice display less care for their litters, regardless of pup genotype, a behavioural abnormality proposed as a model of human autism (Brodkin, 2007). Therefore, there is the need for more studies to be conducted in the context of establishing the relationship between the neuropsychiatric conditions and the gene mutations.

3.1.2 The Neurotransmitter Dopamine and the "Dopamine Hypothesis" in Schizophrenia

Dopamine (DA) is a catecholamine neurotransmitter, synthesised by tyrosine hydroxylase (TH), that creates a bridge between neurones to facilitate communication between them. It has roles in different activities, such as pleasure and emotional status. Four major dopaminergic pathways have been described. The first pathway is called the tuberoinfundibular pathway, which refers to a group of DA neurones that connect the hypothalamus to the pituitary gland (Weiner and Ganong, 1978). The second pathway is the nigrostriatal pathway in the basal ganglia motor loop, consisting of neurones originating in the substantia nigra (SN) and terminating in the dorsal striatum. Parkinson's disease is characterized, in part, by the death of dopaminergic neurones in the pars compacta of the substantia nigra (Barbeau, 1962). It is considered that the SN has a role in behavioural abnormalities (Sotak et al., 2005; Volkow et al., 2002). The third dopaminergic pathway is the mesocortical pathway, which sends out DA neurones from the ventral tegmental area (VTA) to the frontal lobes of the cerebrum, an area that has a role in cognition and emotional responses. It is

thought that this pathway is involved in the negative symptoms of schizophrenia (Olijslagers et al., 2006). The fourth pathway is the mesolimbic pathway, which originates in the VTA and transmits DA from this area to the nucleus accumbens, where the amygdala and hippocampus participate in the pathway; these regions orchestrate the reward and pleasure responses. It is thought that this pathway is involved in the positive symptoms of schizophrenia (Kienast and Heinz, 2006; Palmiter, 2008). The dopamine hypothesis of schizophrenia is a model attributing symptoms of schizophrenia (like psychoses) to a disturbed and hyperactive dopaminergic signal transduction (Kim et al., 2003). The “original dopamine hypothesis” states that hyperactive dopamine transmission results in schizophrenic symptoms. The “revised dopamine hypothesis” proposes hyperactive dopamine transmission in the mesolimbic areas and hypoactive dopamine transmission in the prefrontal cortex in schizophrenia patients (da Silva Alves et al., 2008; Walter et al., 2009).

The link between *T. gondii* and behavioural changes is hypothesized to act via the level of DA concentration. In *T. gondii*, the balance of DA is regulated by two genes (Prandovszky et al., 2011). *T. gondii* is known to increase the levels of DA in rodents (Stibbs, 1985). DA levels can be increased by cytotoxic nitric oxide, interleukin-2 (IL-2) and IL-6. These cytokines are produced at inflamed areas of the infected brain by activated leukocytes (Miller et al., 2009). The dopamine hypothesis of schizophrenia draws evidence from the observation that a large number of antipsychotics have dopamine-receptor antagonistic effects. Thus, the increased DA levels in the brains of *T. gondii*-seropositive individuals may increase their risk for

schizophrenia (Strobl et al., 2012; Webster et al., 2006). The hypothesis, however, does not posit DA overabundance as a complete explanation for schizophrenia. Rather, the overactivation of D2 receptors, specifically, is one effect of the global chemical synaptic dysregulation observed in this disorder.

It is clear that DA participates in different physiological and behavioural systems. Alterations in the dopaminergic pathway can result in neuropsychiatric disorders; however, it is extremely important to investigate how dopaminergic neurotransmission, its regulatory factors and their impairment can promote a behavioural disorder. Elucidating these topics will help in the development of effective treatment. This is why it is important to study the effects of *T. gondii* on behaviour.

3.1.3 Behavioural Effects of *T. gondii* in Mice

The persistence and behavioural consequences of *T. gondii* on the rodent host is attributed to brain encystment of parasites, which induces behavioural changes that favour felid-vectored transmission (Gatkowska et al., 2012; Webster, 2001). The response to parasite-mediated behavioural change differs between mouse strains, as shown by comparison of the behavioural responses of the BALB/c and C57BL/6 inbred strains to *T. gondii* infection (Holland and Cox, 2001; Saeij et al., 2005).

Open field (OF) test

The open-field test measures the exploratory, locomotor, and compulsive behaviour of mice (Havlicek et al., 2001). The tendency of a mouse to

explore the periphery versus the brightly lit centre of the arena is quantified as a measure of anxiety. General locomotor activity (ambulation) is also measured. It has been suggested that *T. gondii*–infected mice could be more hyperactive than controls, which would increase the predation rate and allow the parasite to be transmitted to the definitive host (Hay et al., 1983). In the open-field test, an intraperitoneal administration of *T. gondii* cysts (20 per mouse) was found to cause a significant reduction in the exploratory behaviour (p<0.001), rearing, self-grooming and locomotor activity of chronically infected male C57BL/6 mice (Gatkowska et al., 2012), although these results need to be interpreted with caution because of the sickness phenotype in infected mice.

Elevated plus maze (EPM)

The EPM is used to measure anxiety-like behaviour and is based on the approach–avoidance theory (Carobrez and Bertoglio, 2005; Hogg, 1996; Walf and Frye, 2007). It is a widely used behavioural test based on the unconditioned response, which can be seen as a natural response in wild rodents (Walf and Frye, 2007). This test may be able to detect behavioural changes caused by *T. gondii* infection (Webster, 2001). Anxiety-related behaviours in the EPM have also been found to differ between strains. O'Leary et al. (2013) found significant strain differences between C57BL/6 and BALB/c mice in the duration spent in the open arms of the EPM; overall, C57BL/6 mice spent more time in the open arms, indicating that they were less anxious than BALB/c mice. The EPM measures trait anxiety typical of a specific strain. The higher anxiety of the BALB/c strain limited the mice's free-exploratory behaviour in the EPM tests (Blanchard et al., 2003).

Increased anxiety is a symptom of both autism and schizophrenia (Dachtler et al., 2015).

Passive avoidance

Passive avoidance is a memory and learning ability test. It is used to determine whether mice have impairments in cognition by using aversive stimuli, usually an electric foot-shock. The amygdala, which controls fight or flight response, is affected by the *T. gondii* parasite. *T. gondii* infection has been shown to modulate passive avoidance. A study by Okva et al. (2013) of 40 male BALB/c mice found that those that had received intraperitoneal injections of 10 *T. gondii* cysts showed reduced passive avoidance by spending more time in the dark compartment. In contrast, C57BL/6 mice infected with 10 *T. gondii* cysts exhibited high passive-avoidance learning, as they spent more time in the light and avoided the aversive stimulus (foot-shock) in the dark compartment (Lindova et al., 2006; Nasello et al., 1998).

Object recognition memory

The object recognition test is now among the most commonly used behavioural tests for mice. A mouse is presented with two similar objects during the first session, and then one of the two objects is replaced by a new object during a second session. The amount of time taken to explore the new object provides an index of recognition memory (Leger et al., 2013). *T. gondii* alters novelty-seeking behaviours in mice, rats and humans (Webster, 2001). For example, BALB/c mice showed prolonged object exploration after peroral infection with 10 cysts (Skallova et al., 2006). Novelty-seeking behaviour and the ability to discriminate between novel and familiar objects will trigger a recognition memory concerning the familiar object (Bevins and

Besheer, 2006). Schizophrenia patients exhibit impairments in recognition memory (Pelletier et al., 2005; Danion et al., 1999). The function of recognition memory controlled by dopamine receptors represents a possible link between *T. gondii* infection and potential changes in the dopaminergic pathways (Prandovszky et al., 2011).

Marble burying

Marble burying is an anxiety-related behaviour in mice, where the repetitive digging response is a defensive burying trait that is genetically determined. Hippocampal function can influence the act of marble burying. Thus, such burying can be used to assess hippocampal activity. The serotonergic system's active compounds may lead to marble-burying inhibition by attenuating anxiety, depression or obsessive–compulsive disorder (Deacon, 2006). Marble-burying activity could represent a defensive burying mechanism (Deacon, 2006), which may be a factor in the *T. gondii* predation mechanism. It has been reported that *T. gondii* may damage the hippocampal function, since hippocampal neurones and glial cells have been invaded by the parasite (Creuzet et al., 1998). Furthermore, the innate fear of feline odour is associated with hippocampal activity (Vyas et al., 2007). In humans, it has been shown that the neuropathology of schizophrenia is influenced by hippocampal function (Harrison, 2004).

Prepulse inhibition

Deficits in attention and information processing are considered a central feature of schizophrenia, which lead to stimulus overload, cognitive fragmentation, and thought disorders (Perry and Braff, 1994). Prepulse

inhibition of the startle response (PPI) provides an operational measure of sensorimotor gating, a fundamental form of information processing that is deficient in schizophrenia patients and regulated by dopamine in rodent models (Geyer et al., 2002; Swerdlow et al., 2000; Mansbach et al., 1988). PPI is the attenuation of a startle response by a preceding non-startling sensory stimulus, usually acoustic. In the PPI test, each mouse is placed in a small chamber and exposed to a brief pulse of noise (pre-pulse) prior to a second more intense pulse. The reflexive startle response is measured in both trials (Clapcote et al., 2007).

Nest building

In rodents, nest building is considered a social or parental behaviour (Lijam et al., 1997; Peripato and Cheverud, 2002).

Three-chambered social approach test

T. gondii is considered to be a risk factor for schizophrenia patients, who tend to be less social. Sociability in mice can be assessed using the three-chambered social approach test (Clapcote et al., 2007).

Emergence Test

The emergence test identifies anxiety-like behaviour when mice have to choose between remaining within an enclosure and exploring a brightly lit open arena. *T. gondii*–infected mice tend to be more active and take more risks (Hay et al., 1983), which would increase the predation rate.

Aversion to cat odour

Mice have an innate aversion to traces of feline predators, including cat odours. However, for the parasite to complete its lifecycle, it is vital to be successfully transmitted to the feline. Rodents in the wild may face the

challenge of confronting feline odour. Testing their behaviour in such conditions could exhibit subtle behavioural changes caused by *T. gondii* (Berdoy et al., 1995; Webster et al., 2006). Fatal feline attraction is a test used to measure the natural response of rodents to cat urine odour. The assessment can be measured either by the numbers of entries to arms with odour or the time spent in zones (Lamberton et al., 2008). *T. gondii*–infected mice display no avoidance of cat odour (Kaushik et al., 2014); instead, they tend to be attracted to cat odour, a phenomenon called fatal feline attraction (Vyas et al., 2007).

In one study, infected male BALB/c mice showed no aversion to bobcat urine smeared in a section of a cage, whereas controls did show avoidance behaviour (Ingram et al., 2013). C57BL/6 mice infected with *T. gondii* have shown attraction to feline odour; they were found to prefer feline odour zones to regions with rabbit (non-predator) odour (Vyas and Sapolsky, 2010; Berdoy et al., 2000; Holland and Cox, 2001).

According to da Silva and Langoni (2009), hippocampal dysfunction due to *T. gondii* encystment suppresses the infected mice's innate aversion to cats. The innate fear of feline odour is associated with hippocampal activity, such that impairment of the hippocampal regions involved in memory contributes to the lack of aversion to cats among infected mice (Vyas et al., 2007). Research has also associated increased toxoplasmosis-related lesions in the amygdala, a brain area that controls the 'fight-or-flight response' in animals, with infected mice's attraction to feline odour (Vyas et al., 2007).

Thus, attenuated aversion to feline odours indicates amygdalar dysfunction, leading to impairment of emotions, such as fear and anxiety.

3.1.4 Neurexin-1 Behavioural Changes

Neurexin-1α homozygous knock out (KO) mice exhibit reduced PPI compared with WT mice at low prepulse levels (Etherton et al., 2009), suggesting that KO mice have deficiencies in their sensorimotor gating. In addition, KO mice exhibited longer grooming times than their WT counterparts (Etherton et al., 2009). Both genotypes were also tested for nest building, which is considered a social or parental behaviour (Lijam et al., 1997; Peripato and Cheverud, 2002). The nests built by KO mice were of significantly lower quality than those built by WT mice (Etherton et al, 2009). However, Dachtler et al. (2015) found that neurexin-1α KO heterozygous (Het) mice were able to build cotton nests as well as their WT littermates.

Grayton et al. (2013) found that neurexin-1α homozygous KO mice exhibited reduced locomotor activity in novel situations, as well as increased anxiety in the open field, light/dark box and EPM tests. The study also reported that neurexin-1α KO mice show increased social approach but decreased locomotor activity in novel environments. It has also been reported that male neurexin-1α KO mice may become more aggressive towards adolescent and adult mice (Grayton et al., 2013).

In the first phase of social approach test, which measures sociability, neurexin-1α heterozygous and WT mice both showed a preference for exploring a cylinder containing a stranger mouse (Stranger 1) rather than an

empty cylinder (Dachtler et al., 2015). In the second phase of the test, which measures preference for social novelty, mice encountered Stranger 1 (the now familiar mouse) as well as a novel mouse (Stranger 2) in the formerly empty cylinder. Neurexin-1α heterozygotes showed less exploration of Stranger 2 than did WT mice. However, there was no difference between neurexin-1α Het and WT mice in the novel object recognition test. In the passive avoidance test, female neurexin-1α heterozygotes demonstrated cognitive impairment by their faster approach to the shock chamber than WT mice. During anxiety and activity assessments, as well as in the PPI test, neurexin-1α Het and WT mice were indistinguishable (Dachtler et al., 2015).

To date, no study in the literature has been conducted on neurexin-1α Het mice exposed to *T. gondii*. Thus, the present study examined the effect of *T. gondii* on neurexin-1α Het mice, focusing on non-invasive behavioural traits and their correlation with HPLC analysis of the brain hemispheres. Scientifically, the experiment aimed to improve the understanding of the combined biological effects of an environmental factor (*T. gondii* exposure) and a genetic factor (neurexin-1α deletion) that individually increase the risk for schizophrenia. The experiment thus aimed to shed light on how the parasite contributes to the disease, and possibly identify a mechanism by which *T. gondii* alters the behaviour of the host. It was also anticipated that the experiment could help in the investigation of new treatments.

It has been shown that the *T. gondii* parasite may affect the brain indirectly through the immune response and directly through the localisation of cysts in

the brain. In another observation, it was found that the parasite could affect the brain directly by altering the levels of the neurotransmitters DA and serotonin (5-HT) (Stibbs, 1985). To successfully test such effects in mice, the following aspects needed to be considered:

- A suitable strain type, such as WT II Pru which is avirulent, was required; and
- The dose should be sufficient to induce a chronic infection in the animals but not make them ill.

The choice of mice as opposed to rats was based on the reasons described in Table 14, as well as the availability of neurexin-1α KO mice in Leeds.

Central nervous system (CNS) & immune system	Similar structure & neurochemicals to human
Straightforward to maintain	Easy to maintain
Novel stimuli	Decreased neophobia (as in humans)
Learning capacity	Severe impairment
Latent toxoplasmosis	Higher in the brain than in rats
Morbidity	Higher than rats (negative symptoms & generalised pathology)
Knock-out (KO) type	Useful & available
Parasite-induced mortality	Possible if wrong strain, high parasite load or late in infection
Short-/long-term memory (cognitive deficit)	No impact

Table 16: Advantages of Using Mice as an Experimental Model for *T. gondii* Infection (Webster et al., 2013).

3.2 Materials and Methods

3.2.1 Ethics

All procedures were approved by the University of Leeds Animal Ethical and Welfare Review Board and performed under United Kingdom Home Office Project and Personal Licences in accordance with the Animals (Scientific Procedures) Act, 1986. Replacement, reduction and refinement (the 3 Rs) were taken into account.

3.2.2 *T. gondii* Culture

Human foreskin fibroblast (HFF, Sigma-Aldrich) cells were maintained daily in T25-cm^2 (T25) vented flasks. The cells were cultured in 10 ml of fresh Dulbecco's modified Eagle's medium (DMEM, Gibco) supplemented with 10% heat-inactivated, iron-supplemented foetal bovine serum (FBS, Invitrogen) and 1% penicillin–streptomycin (PS, Sigma) antibiotic suspension at pH 7.2. At the point of confluency (75–95%), which was defined as adherent monolayer cells, the HFF cells were passaged frequently. Five ml of DMEM were discarded and 5 ml were kept in a Falcon tube under sterile conditions (a ventilated hood for *T. gondii* culturing and 75% ethanol were used as necessary). Then, the flask containing attached HFF cells was washed with 100% phosphate-buffered saline (PBS, Invitrogen), and the cells were detached by adding 5% trypsin-ethylenediaminetetraacetic acid (trypsin-EDTA, Sigma) for 90 seconds at 37°C. Following this, the free cells were collected and suspended in fresh DMEM media (10 ml). The old 5 ml

were then added to the 10 ml, and this was topped up to a total of 30 ml or 50 ml to separate the medium into 3 or 5 new flasks. The cycle was repeated to maintain cells.

The confluent HFF cells were used to culture and sustain *T. gondii* tachyzoites by serial passaging. To passage the parasite, HFF cells were released using 5% trypsin. Then, the cells were centrifuged for 10 minutes at a speed of 2500g. The supernatant was removed and the parasite pellet was collected and mixed with 100% PBS. The mixed solution was then passed through a 27-gauge needle, which resulted in the release of the intracellular parasite. The releasing step was repeated several times. The solution was then centrifuged in the same way. The parasite pellet was suspended in 5 ml of fresh DMEM medium and used to infect 75–95% confluent HFF.

3.2.3 Mouse lines

Neurexin-1α Heterozygous (Het) KO (The Jackson Laboratory, Maine, USA) male mice with a predominantly C57BL/6 genetic background were provided by Dr Steven Clapcote, University of Leeds, and were backcrossed to the C57BL/6NCrl strain (Charles River, Margate, United Kingdom). Heterozygous neurexin-1α KO mice with a 50% C57BL/6 and 50% BALB/c genetic background were bred by outcrossing C57BL/6-neurexin-1α Het males to BALB/cAnNCrl (BALB/c; Charles River) females.

3.2.4 Mouse Genotyping

After weaning at 3–4 weeks after birth, ear punching was performed to identify the pups. Each mouse was punched serially according to the University of Leeds animal unit protocol. DNA was extracted from each ear biopsy, using the steps described below.

Each ear biopsy was transferred into an Eppendorf tube (1.5 ml), to which 600 µl of TNES (1.211 g TRIS base, 23.376 g NaCl, 37.224 g EDTA, 6 g SDS with 800 ml dH_2O; pH 8 was adjusted using 10 M NaOH) and 10 µl of proteinase K (Roche, Mannheim, Germany) were added. The tube was then vortexed and incubated at 55°C for a minimum of 3 hours or overnight. After incubation, 166.7 µl of 5 M NaCl were added, and the sample was shaken vigorously for 15 seconds. At room temperature, the sample was centrifuged at 13,000 rpm for 10 mins. Following this, 725 µl of supernatant were transferred to a new tube, with care taken not to disturb the pellet. Then, 725 µl of ice cold ethanol kept at –20°C were added. The tube was inverted 10 times for mixing, after which it was spun down for 10 minutes at full speed at room temperature. The supernatant was discarded and the pellet was kept and rinsed with 700 µl of 70% ethanol by inversion, which was done about 25X. The sample was then centrifuged using the same protocol. The supernatant was discarded, and the sample was either air dried in the fume cupboard on tissue paper for about 15 minutes or inverted on clean tissue and left to dry overnight. Following this, the sample was suspended in 50 µl of TE buffer and heated in a water bath at 65°C for 10 minutes; it was then vortexed and spun down. The DNA concentration (ng/µl) was measured

using the NanoDrop 2000 apparatus. Each ear biopsy yielded up to 50 ng/µl DNA.

The following primers were designed by the Jackson Laboratory (Bar Harbor, Maine, USA):

- Primer forward WT (A): 5'-CGA GCC TCC CAA CAG CGG TGG CGG GA-3';
- Primer reverse common (B): 5'-CTG ATG GTA CAG GGC AGT AGA GGA CCA-3';
- Primer forward mutant (C): 5'-GAG CGC GCG CGG CGG AGT TGT TGA C-3'.

The primers were synthesized by Sigma-Aldrich (Poole, UK). The PCR mixture was as follows: 2 µl of DNA sample (20 ng/µl), 6.0 µl of dH_2O, 10.0 µl of Hotshot (Clent Life Science, Stourbridge, UK) and 2.0 µl of 5 µM primer mix (*Nrxn1* A+B+C). The PCR cycle for the amplification of the *Nrxn1* gene is shown in Table 17.

PCR cycle × 35	Temperature ˚C	Time
Initial denaturation	95	05:00 minutes
Denaturation	94	30 seconds
Annealing of primers	70	60 seconds
Extension	72	Repeated 35 times
Final extension	72	10:00 minutes

Table 17: Nrxn1 KO genotyping PCR thermocycling conditions

A 20X dilution of Tris/acetate/EDTA (TAE) was prepared (110 ml plus 140 ml of distilled water). One per cent agarose powder was added to 100 ml of diluted TAE, which was then boiled in a microwave oven. To visual the PCR

bands,11 µl of SYBR safe were added and mixed before the melted agarose was poured into a gel tray. Fifteen µl of each PCR reaction were mixed with 3 µl of 6X loading dye and then loaded into the second or subsequent wells of the gel. Ten µl of 100-bp DNA ladder (Promega Corporation, Madison, USA) were loaded into the first well. Electrophoresis was run at 100 V for 60–80 minutes.

3.2.5 Mouse Infections with *T. gondii*

Mice were housed five per cage with *ad libitum* access to food pellets and water; they were caged by sex except for breeding cages. Mice were checked daily, seven days a week. Their weight was measured weekly. Any mouse showing severe illness or significant weight loss (25%) was promptly culled. Mice were grouped according to treatment. Half of the mice were infected by intraperitoneal (IP) injection of *T. gondii* type II strain WT Prugniaud, an avirulent strain used in numerous mouse studies. The other half of the mice were controls, injected with 0.2 ml phosphate-buffered saline (PBS). Mice were aged 6–14 weeks at the time of injection. PCR genotyping of the mice gave inconsistent results, so a partially blind experiment was initiated while genotyping problems were resolved. The following experiments were undertaken to test the hypothesis that *T. gondii* may exacerbate schizophrenia-related behavioural symptoms in these mice which carry a deletion of neurexin-1α, a genetic risk factor for schizophrenia.

Experiment 1: C57BL/6NCrl backcross neurexin-1α mice

Experiment 1 used mice bred from neurexin-1α heterozygous males x C57BL/6NCrl females. Table 18 shows the number of mice in each round of

injections. Each round was commenced once the mice had bred and reached a reasonable number to infect.

	Infected		Control		Total	Dose
Round 1	14 (♂)	23 (♀)	0 (♂)	0 (♀)	37	500 tachyzoites
Round 2	4	0	1	0	5	50 tachyzoites
Round 3	5	3	3	2	13	50 tachyzoites
Round 4	7	8	3	4	22	50 tachyzoites
Round 5	9	10	2	11	32	50 tachyzoites
Round 6	4	4	10	1	19	50 tachyzoites

Table 18: Number of infected and control mice for all rounds.

Round 1

Tachyzoites were released from HFF and suspended in PBS. Using a haemocytometer, the concentration of tachyzoites in solution was determined. Using the same procedure, the concentration was confirmed to be 154×10^4 cells/ml by both the author and the supervisor. The parasites were diluted to 1:100 (0.1 ml into 10 ml PBS); following this, 5 ml were taken and diluted to 1:6 (5 ml into 30 ml), giving a final concentration of 2500 tachyzoites per ml. Following aseptic techniques mice were infected by IP injection of 0.2 ml (containing approximately 500 tachyzoites) (Vyas et al; 2007). PBS controls were planned for later that day but did not commence.

Round 2

Only four male mice aged 2 months were infected. Control experiments tested virulence with mouse ID number #1 injected with 50 tachyzoites, mouse #2 with 100 tachyzoites, mouse #3 with 300 tachyzoites and mouse #4 with 400 tachyzoites; mouse #5 (control) was injected with 0.2 ml PBS. This round was done to determine if there is an effect of tachyzoites number on mice symptoms and whether the virulence of parasite could be less with a lower dose.

Round 3 and 4

Bradyzoites were obtained from the brains of mice infected in round 2. Then, HFF cells were infected with these parasites and propagated as tachyzoites. Freshly released tachyzoites were kept at –70% for the next rounds of infection. It is thought that parasite strain could be less virulence due to passaging it through mice (round 2). It would be safe to use on mice C57BL/6NCrl backcross neurexin-1α mice in round 3 and 4 after round 1 was failed.

Experiment 2: BALB/cAnNCrl outcross neurexin-1α mice

Unexpected complications were found in mice bred from neurexin-1α heterozygous males x C57BL/6NCrl females. As a result, the breeding strategy was amended to cross neurexin-1α heterozygous males with BALB/cAnNCrl females. Mice bred from neurexin-1α heterozygous males mated with BALB/cAnNCrl females were injected with a dose of 0.2 ml containing 50 tachyzoites or with 0.2 ml PBS for controls (Rounds 5 and 6; Table 19).

3.2.5 Infection Test

At 65 days postinfection, mice were culled according to the UK law. Direct agglutination kits (BioMérieux®) were used to detect the *Toxoplasma* antibodies in infected and control mice, using the manufacturer's instructions. Jugular blood was collected immediately after culling mice. Blood then was centrifuged and sera were collected and kept at -20°C until DAT was performed to detect *Toxoplasma* IgG antibodies.

Genotype	Status	Sex	Number	Sex	Number
Het	Infected	Female	7	Male	6
Het	Control	Female	6	Male	6
WT	Infected	Female	7	Male	7
WT	Control	Female	6	Male	6

Table 19: BALB/c Outcross Mice in Rounds 5 and 6 Infection Test.

3.2.6 Mouse Behavioural Testing

Mice were habituated to handling for 5 minutes per day for 7 days prior to behavioural analysis. C57BL/6NCrl backcross neurexin-1α mice (experiment 1) were tested in a battery of behavioural tests in the following order, with an interval of 3 days between tests: open field > elevated plus maze > emergence test > social approach > wire suspension > vertical pole. BALB/cAnNCrl outcross neurexin-1α mice (experiment 2) were tested in a battery of behavioural tests in the following order, with an interval of 2 days between tests: open field > elevated plus maze > novel object recognition > marble burying > social approach > spatial habituation in T-maze > passive avoidance > feline odour test. Tests were initiated at 3-weeks postinfection. Seventy per cent ethanol was used to clean the arena between mice. The arena was left to dry for 3-4 minutes before commencing the next subject. Table 20 explains briefly the aim of each behavioural test.

3.2.6.1 Open Field Test

The internal OF arena had a diameter of 40 x 40 cm with a semitransparent Perspex wall. The arena floor was white plastic. To prevent the mice from seeing the surrounding room, a cylinder of white card was place around the arena 30 cm away from the walls. The ambulation of the mice was recorded

using a webcam that was placed on a tripod above the arena. The arena was divided into the following zones: outer zone, 8 cm from the outer walls; central zone of 6.4 cm^2 (16% of the total area); intermediate zone, covering the remaining area between the outer zone and the central zone (as defined by the European Mouse Phenotyping Resource of Standardised Screens [EMPReSS]). The zones were determined using the AnyMaze tracking software (Stoelting Europe, Dublin, Ireland).

Mice were always placed at the centre of the arena facing the same wall. The exploration time was 30 minutes without any interruptions or intervals. Time spent in and entries to each of the zones, as well as distance travelled, were recorded automatically using the AnyMaze tracking software. At the same time, rearing and grooming were quantified during the trial by the experimenter.

3.2.6.2 Elevated Plus Maze

EPM consisted of two open arms and two enclosed arms with dense walls. The arms were 30 cm long and 5 cm wide. The central zone where the arms are connected has an area of 5 cm^2. The experimenter placed the mouse into the central zone of the maze facing an open arm. The trial time was 5 minutes, and AnyMaze tracking software was used to track each mouse's movement in the maze. Time in and entries made to the open, closed and central zones were measured. Entry into a zone was defined as when the hind legs crossed the boundary of the zone. In addition, the number of head dips over the side of the open arms was recorded.

3.2.6.3 Emergence Test

The emergence test identifies anxiety-like behaviour when mice have to choose between remaining within an enclosure and exploring a brightly lit open arena. In the OF arena, a black plastic enclosure measuring 17 cm L x 11 cm W x 5.5 cm H was placed against the middle of one wall. The positioning of the enclosure was moved between the arena walls in different trials. The black box had a 6 x 3.5–cm opening to allow the mouse free exploration between the box and the arena. The trial time was 15 minutes, with the mouse freely moving between the black box and the arena. AnyMaze tracking software was employed to measure time spent out of the enclosure exploring the arena, latency to emerge from the enclosure and the number of entries to the arena.

3.2.6.3 Social Approach

Sociability was assessed using a three-chambered arena (60 x 40 cm) that had two openings to allow the mouse access to the left and right chamber from the central chamber (each chamber measured 40 x 20 cm; the opening measured 7 x 8 cm). The test involves using two unfamiliar mice that have been habituated in cylinders measuring 10 cm W x 10.5 cm H prior to the test. The cylinders are made of vertical metal bars separated by 9 mm, which allows air exchange and increases the possibility of contact between the test and stranger mice.

Following the protocol of Clapcote et al. (2007), a test mouse was placed into the central chamber of the three-chambered arena. The ‘habituation’ stage was carried out for 15 minutes; at the end of this time, the test mouse was moved to the central chamber and the openings to the side chambers

were blocked by guillotine doors. A cylinder was placed in both the right and the left chamber. A stranger mouse (‘stranger 1’, a young male C57BL/6NCrl) was placed in the cylinder in either the left or right chamber (balanced between treatment groups) (Figure 6). Following this, the doors were removed and ‘phase 1’ was initiated; this phase lasted 10 minutes.

Social approach was scored when the test mouse’s nose poked either the cylinder containing stranger 1 or the empty cylinder. At the end of phase 1, the test mouse was placed in the central chamber and the doors were shut. Then, a new unfamiliar mouse (‘stranger 2’) was placed in the formerly empty cylinder. At this point, phase 2 was initiated, and this stage lasted for 10 minutes. Social approach was scored when the test mouse’s nose poked either the cylinder containing stranger 1 or the cylinder containing stranger 2. The cylinders were then wiped clean with 70% ethanol. The experimenter wore nitrile gloves throughout the procedure.

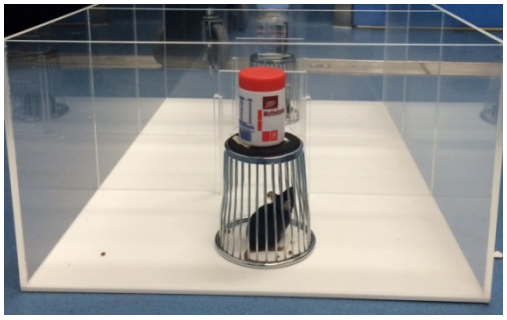

Figure 6: A stranger mouse placed in a cylinder.

3.2.6.4 Wire Suspension Test

In the wire suspension test (Figure 7), the mouse was suspended from a single wire coat hanger (2 mm diameter) by its forepaws. The time that the mouse hung on the wire was recorded, with a maximum of 60 seconds per

mouse. This test was used to assess the strength of the mice, as a measure of the general health of infected mice.

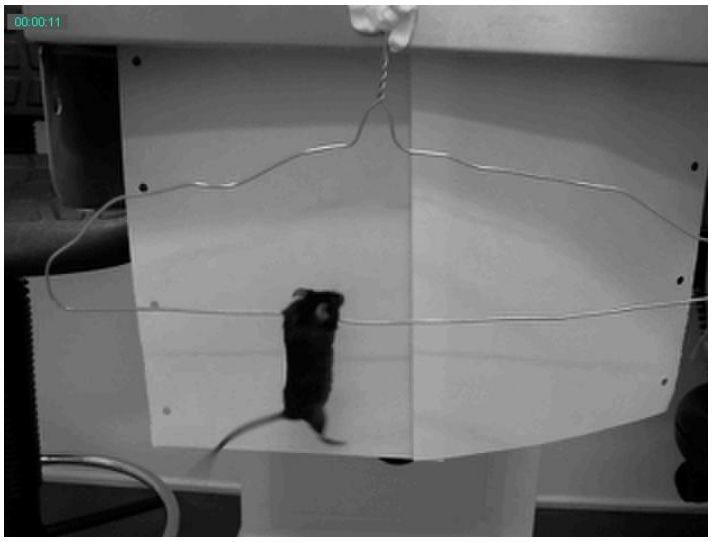

Figure 7: wire test assessment

3.2.6.5 Vertical Pole Test

In the vertical pole test (Figure 8), a mouse was placed facing up on a cloth-tape-covered pole (1.9 cm in diameter, 43 cm in length). The end of the pole was lifted to a vertical position, and the time a mouse stayed on the pole was recorded. Performance in the test was scored as follows: fell before the pole reached a 45° angle = 0; fell before the pole reached a 90° angle = 1; fell after 0–10 seconds = 2; fell after 11–20 seconds = 3; fell after 21–30 seconds = 4; fell after 31–40 seconds = 5; fell after 41–50 seconds = 6; fell after 51–60 seconds = 7; stayed on 60 seconds and climbed halfway down the pole = 8; climbed to the lower half of the pole = 9; climbed down and off in 51–60 seconds = 10; climbed down and off in 41–50 seconds = 11; climbed down and off in 31–40 seconds = 12; climbed down and off in 21–30

seconds = 13; climbed down and off in 11–20 seconds = 14; and climbed down and off in 1–20 seconds = 15.

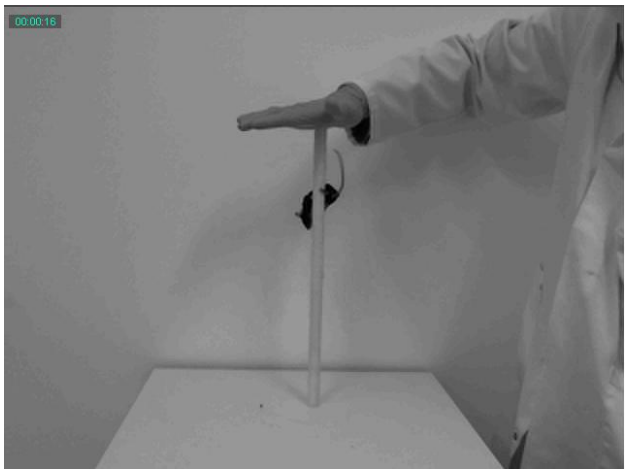

Figure 8: Vertical pole test.

3.2.6.6 Novel Object Recognition

For this test, the OF arena was used. The mouse was placed in the centre of the arena for a 5-minute session (habituation phase). The activity was recorded using the AnyMaze tracking software. Following this, the subject was placed in a holding cage for 2 minutes. Two objects were placed in a designated area about 5 cm from the arena corners. Next, the mouse was returned to the arena and was freely left to explore the objects for 5 minutes; which was called the trial phase. The time the mouse spent exploring the objects was recorded. The mouse was then returned to the home cage. After 3 hours, the arena was prepared with two objects in opposite corners; one was one of the original objects (familiar), and the other was novel. The objects were swapped between the right and left corners in between the tested mice. The mouse was then allowed to explore, and the time spent exploring each object was recorded during the 5-minute session.

3.2.6.7 Marble Burying

In a large cage (rat cage), 12 marbles were placed in a consistent pattern on wood-chip bedding that was lightly tamped down to make a flat, even surface. The mouse was placed in the cage and left for 30 minutes. The number of marbles buried up to two-thirds of their depth was counted after 30 minutes.

3.2.6.8 Spatial Habituation in the T-Maze

The T-maze was constructed from opaque Perspex. The runway forming the stem of the T was separated by a guillotine door from the arms forming the bar at the top of the T. The mouse was placed in the runway and left to explore for 10 minutes with the AnyMaze tracking software recording. Following this, the door was opened and the time spent in novel area (the arms) was tracked for 5 minutes.

3.2.6.9 Passive Avoidance

MedPC software connected to a Med Associate Shuttle Box (Med Associates, Fairfax, USA) was used to carry out the passive-avoidance test. The box consists of two soundproof chambers (10 × 10 × 20 cm) separated by a guillotine door controlled remotely. There was also a fan that delivers background noise. One chamber (the dark side) was covered with a black cloth; the other chamber (the light side) was illuminated. The conditioning trial started by placing the mouse on the light side while the door was open. After 10 seconds, the door to the dark side was opened, and the latency to passing to the dark side was measured. When the mouse fully entered the dark side, the door closed, and after 10 seconds, a 3-second 0.45 mA foot shock was delivered. After 30 seconds, the mouse was removed. Trial 2

commenced after 24 hours; here, the latency to cross to the dark side was measured, but no shock was given in this phase. If the mouse did not cross to the dark side, the trial ended after 5 minutes, and the mouse was returned to the home cage.

3.2.6.10 Feline Odour Test

The feline odour test was conducted in the T-maze apparatus (see above), following the protocol of McGirr et al. (2016). In each trial, one arm at the top of the T was contained a petri dish of food pellets, and the opposite arm contained 3 drops of bobcat urine (Maine, USA). The arms were used evenly between treatment groups. The mouse was then placed in the main runway (stem of the T). The entries to each arm were measured over 10 minutes.

Behavioral test	Outcome summary
Open Field	Ambulation, anxiety and wall hanging.
Elevated plus maze	Anxiety
Social approach	Sociability and recognition
Wire suspension test	Health and fitness
Vertical pole	Health and fitness
Novel Object Recognition	Novelty seeking and memory
Marble burying	Anxiety
Spatial Habituation in the T-Maze	Space habituation and memory
Passive avoidance	Learning and long memory

Table 20: Summary of behavioural test performance.

3.2.7 Statistical Analysis

Minitab software (version 17) was used for all analysis. The distribution was tested to meet the assumptions of normality. If a distribution is normal, a parametric test (Analysis of Variance) would be used; while a non-parametric test (Kruskal Wallis) would be used if the data were revealed to be non-normally distributed and the data after transformation was still skewed. Minitab was used to test the following main effects and interactions: genotype (neurexin-1α heterozygous versus WT), sex (male versus female), and infection (*T. gondii* versus PBS control). Non-significant interactions were removed from the model. Bonferroni corrections were used in behavioural tests that were 10 or more phenotypes in order to eliminate any false positives results.

3.2.8 Sacrifice of Mice

After finishing the behavioural testing, mice were sacrificed by cervical dislocation. Immediately after culling, the brains were rapidly removed from the skull. Using a scalpel, brains were sectioned into two hemispheres by

making a longitudinal incision. The left half was immediately stored in RNA-later solution (Sigma-Aldrich, Poole, UK) in a 1.5 ml polypropylene tube at room temperature for 24 hours; it was then stored at –20°C. The right half was sectioned into three regions, namely cerebellum, middle cortex and frontal cortex. Each region was covered separately with foil marked with the region name and mouse number and then immediately snap frozen in liquid nitrogen. Then brains were transferred to a freezer at –70°C until biochemical analysis was performed.

3.2.9 Neurochemical Analysis

The second hemisphere was coronally divided into three regions: frontal cortex, mid-brain and cerebellum. Each brain region was briefly weighed and then immediately kept at 4°C all the time during the process. It was then homogenised in 10X the volume of 0.1 M perchloric acid, as previously described by Stibbs (1985), and sonicated for 10 seconds. Following this, the region was centrifuged at full speed in a cold room for 15 minutes. The supernatant was filtered out, then the brain region transferred into HPLC vial and run. The mobile phase buffer consisted of degassed 57 mM anhydrous citric acid (Fisher Scientific, Loughborough) and 43 mM sodium acetate buffer (Dionex, Sunnyvale) containing 0.1 mM EDTA (Sigma Aldrich), 1 mM sodium octane sulphonate monohydrate and 10% methanol. The pH was adjusted to 4. Each sample was analysed using HPLC-ED performed with a C18 Acclaim 120 column (5 mm x 4.66150 mm) at a flow rate of 0.8 ml/minute. Each region was run three times depending on the volume of the

sample. Standards (Sigma-Aldrich) for each neurotransmitter were included in each run. HPLC used to detect neurotransmitters such as dopamine, noradrenaline and serotonin.

3.3 Results

3.3.1 Mouse genotyping

In order to genotype the mice, a PCR-based assay was used. The PCR yielded a wild type band of 440 bp and a mutant band of 390 bp in neurexin-1α heterozygous mice (Figure 9). At the beginning of PCR assay, there were complications and there was not consistency in results. Then, a couple of samples were repeated due to faint bands. Therefore, random samples were re-tested to double check the results. However, all mice were finally genotyped successfully.

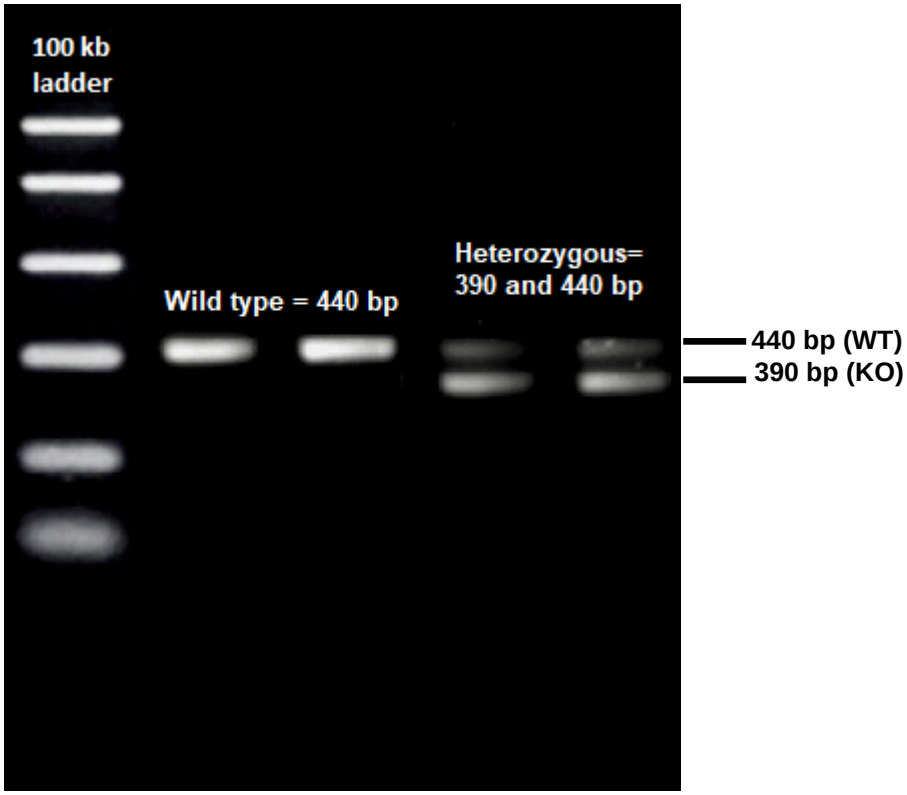

Figure 9: Neurexin-1α genotyping gel image. A 100-bp ladder was used. The 2% agarose gel shows bands of 390 bp (KO) and 440 bp (WT).

3.3.2 Infection of C57BL/6NCrl backcross neurexin-1α mice

Experiment 1

Round 1

Thirty-seven mice (23 females and 14 males), bred from neurexin-1α heterozygous males x C57BL/6NCrl females, were infected with *T. gondii* type II strain wild type Prugniaud, an avirulent strain. However, inexplicably, at one-week post infection, 5 mice died, and 2 mice were culled due to deterioration in health. At 10 days post infection, some mice presented with dull eyes, hunched backs and were sluggish, and some of them were unable to move, eat or drink. We classified these mice as requiring culling because of their deterioration in health. Overall at 10 days post infection, 10 mice were healthy (33.3%), 10 mice were ill (33.3%), seven mice were dead (23.4%), and three mice had been culled (10%). At this point, the study was terminated for mouse welfare reasons. The university veterinarian performed a post-mortem examination in the author and co-supervisor's presence and found no evidence of incorrect procedures regarding injections.

Round 2

Five male mice aged 2 months, bred from neurexin-1α heterozygous males x C57BL/6NCrl females, were infected to determine whether there was a difference in parasite virulence between doses. All infected mice showed influenza-like symptoms such as sluggish movements, dull eyes and mild hunched backs after 11-13 days. As the experiment commenced, one mouse injected with fewer tachyzoites showed paralysis in a hind leg. All mice were culled 10 weeks post infection. Their brains were extracted and bradyzoites were released in HFF which proliferated to tachyzoites to infect

the next group of mice. It was considered that the virulence of the parasite strain may have increased due to continuous laboratory passage of tachyzoite (i.e. proliferative) stages and hence cultivation of parasites from brain encysted parasites was performed. The control mice were healthy and thriving.

Rounds 3 and 4

Behavioural tests (experiment 1)

Twenty-three mice bred from neurexin-1α heterozygous males x C57BL/6NCrl females were infected using a dose of 50 tachyzoites in 0.2 ml, while 12 control mice were injected with 0.2 ml PBS. The open field, elevated plus maze, emergence and social approach tests were conducted on this group. However, the number of mice decreased over time due to the parasitic illness, as described in Tables 21 and 22. This suggests that the C57BL/6 genetic background of these mice made them particularly susceptible to *T. gondii*. As a result, assessments of the general health and fitness of the remaining mice were conducted using the wire suspension and vertical pole tests.

Round 3 health deterioration post-infection.		**Survival**
Day 9 - 11	First signs of infection for example tiredness.	8
Day 14	A female found dead.	7
Day 20	Two males showed abnormal movement but were able to eat, and drink.	7
Day 26	Male was culled due to deterioration in health.	6
Day 27	Female was culled due to deterioration in health.	5
Day 120	Experiment terminated.	0

Table 21: Round three conducted in total of 8 infected mice.

Round 4 health deterioration post-infection.		Survival
Day 11	First signs of infection for example tiredness.	15
Day 24	4 males showed abnormal movement.	15
Day 25	One male was culled.	14
Day 26	Two males and two females were culled.	10
Day 29-30	One male and one female were culled	8
Day 38 – 41	Two males and two females were culled.	4
Day 57	Experiment terminated.	0

Table 22: Round four conducted on a total of 15 infected mice.

Wire Suspension and Vertical Pole

In the vertical pole test, the Mann-Whitney U test revealed no statistically significant difference between infected mice and control mice (P=0.055) (Figure 8). In the more physically demanding wire suspension test, the Mann-Whitney U test revealed a statistically significant difference in hanging time between infected and control mice (P=0.015). Infected mice (n=8) were unable to remaining hanging for 60 seconds, unlike controls (n=15) (Figure 5). Three of the eight infected mice were only able to hold on to the wire for one second. Given their poor performance in the wire suspension test, the *T. gondii*-infected mice were deemed unfit for further behavioural testing, so were culled by cervical dislocation. Their brains were extracted and stored in RNA-later solution for further analysis.

3.3.3 Infection of BALB/cAnNCrl outcross neurexin-1α mice

Experiment 2

Rounds 5 and 6

A total of 51 mice were bred from neurexin-1α heterozygous males mated with BALB/cAnNCrl females. These BALB/c outcross neurexin-1α mice

included male and female neurexin-1α heterozygous and wild-type mice. The aim of this study was to assess the behavioural effects of *T. gondii* infection in neurexin-1α heterozygous mice with a genetic background less susceptible to the parasite. To confirm infections with *T. gondii,* DAT was performed in duplicates. Most infected mice showed positive anti-*Toxoplasma* antibodies and most control showed negative results, apart from three mice (two infected and one control) which showed borderline results. Sera obtained from mice was in very low quantity and it was not possible to re-test the borderlines. Figure 10 shows mouse body weight (g) in the period of 1-5 weeks postinfection. The lack of significant effects of *T. gondii* infection on mouse body weight suggests that infected mice were healthy after clearing the acute infection.

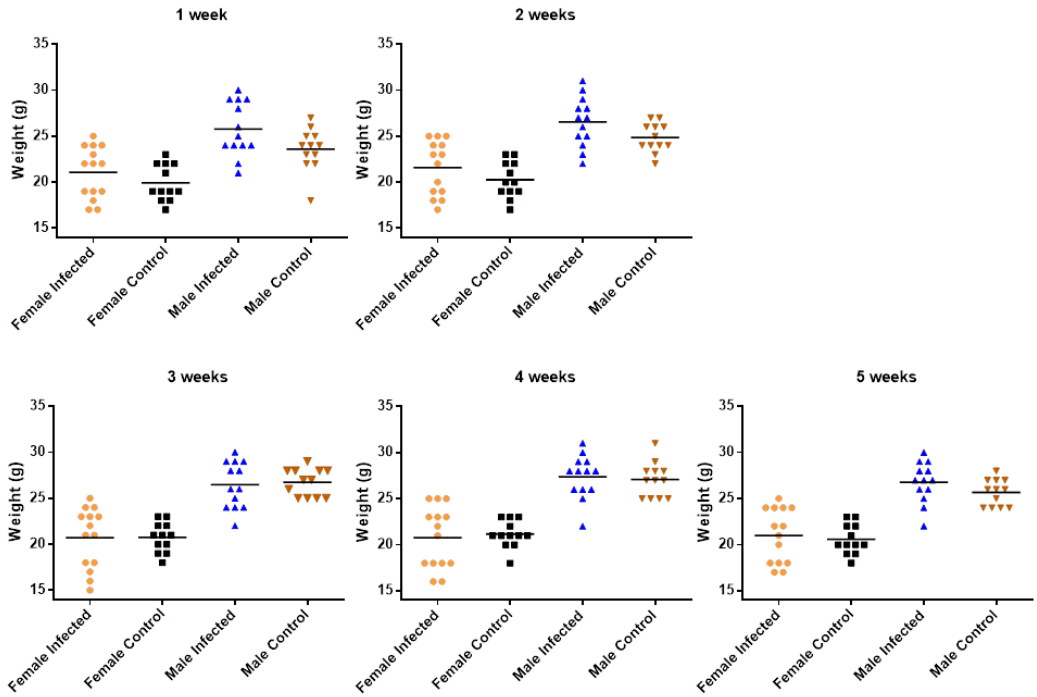

Figure 10: Body weight (g) of BALB/cAnNCrl outcross neurexin-1α mice (rounds 5 & 6) at intervals of 1 to 5 weeks postinfection.

Behavioural tests (experiment 2)

Open field

The open field is a simple test to observe locomotion of mice. Table 23 shows the *P*-values obtained. It is believed that the state of being active could enhance the transmission of the parasite from intermediate host to definitive host where the parasite completes its life cycle (Webster, 2007).

At a Bonferroni corrected significance level of $P < 0.003$, there was a significant main effect of genotype on grooming time in all zones ($P = 0.001$) and grooming time in the outer zone ($P = 0.003$), as heterozygous mice groomed more than their WT counterparts (Figure 11). Time in all zones, freezing time, total rearing, rearing in all zones, total distance travelled and distance travelled in all zones were not significantly different between genotype, sex and infection.

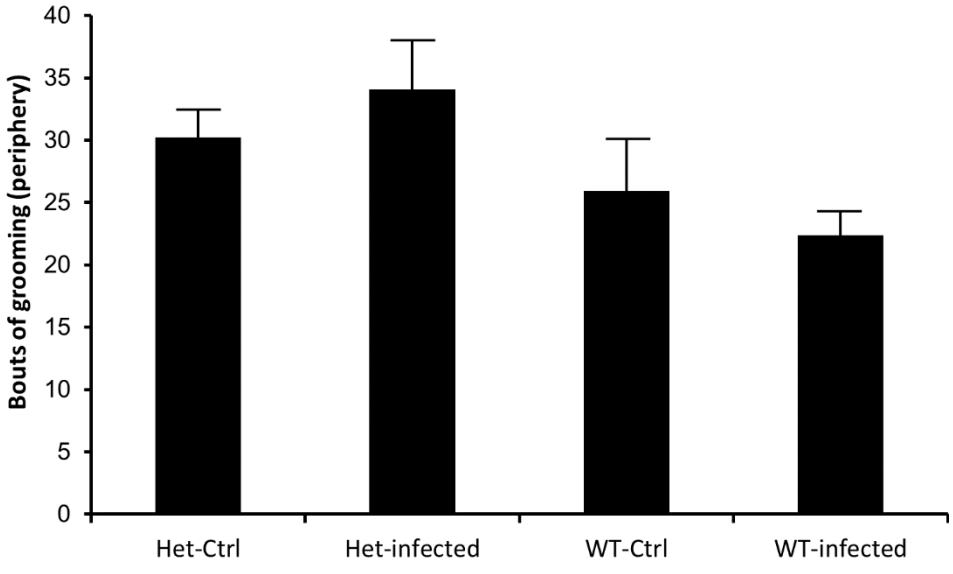

Figure 11: Shows grooming time (Y-axis) in the outer zone (X-axis). Genotype effect was observed (Mean with standard error (SE))(n= Het-ctrl=12, Het-infected= 13, WT-ctrl= 12 and WT-infected= 14).

Parameter	Effect	P-value	Parameter	Effect	P-value
Freezing time	Genotype	0.180	**Time in central zone**	Genotype	0.044
	Sex	0.163		Sex	0.082
	Infection	0.905		Infection	0.587
Grooming time in all zones	Genotype	0.001	**Grooming in central zone**	Genotype	0.071
	Sex	0.364		Sex	0.257
	Infection	0.470		Infection	0.928
Time in outer zone	Genotype	0.102	**Rearing in central zone**	Genotype	0.041
	Sex	0.161		Sex	0.731
	Infection	0.242		Infection	0.513
Grooming time in outer zone	Genotype	0.003	**Time in intermediate zone**	Genotype	0.211
	Sex	0.338		Sex	0.142
	Infection	0.604		Infection	0.196
Rearing in outer zone	Genotype	0.135	**Rearing in intermediate zone**	Genotype	0.074
	Sex	0.717		Sex	0.108
	Infection	0.770		Infection	0.100
Grooming in intermediate zone	Genotype	0.135	**Total rearing time**	Genotype	0.491
	Sex	0.898		Sex	0.405
	Infection	0.200		Infection	0.964
Total distance travelled	Genotype	0.791	**Distance travelled in central zone**	Genotype	0.269
	Sex	0.965		Sex	0.030
	Infection	0.690		Infection	0.078
Distance travelled in outer zone	Genotype	0.457	**Distance travelled in intermediate zone**	Genotype	0.591
	Sex	0.534		Sex	0.225
	Infection	0.311		Infection	0.326

Table 23: Tests for effects of genotype, sex and infection on various parameters in the open field test.

A Bonferroni correction of p-value of 0.003 (0.05/16) was calculated.

Elevated plus maze

The EPM was used to assess anxiety in the mice. Table 24 shows parameters tracked in the EPM. Anxiety was assessed as the mouse entered and explored the open arm. Frequent head dips were also counted as an anxiolytic effect. A heterozygous infected female was excluded from this test as a statistical outlier. The EPM revealed no difference between infected and control mice (Figure 12), although there was a significant effect of genotype on distance travelled (P = 0.011) as heterozygous distance travelled (m) more than WT mice (Figure 13).

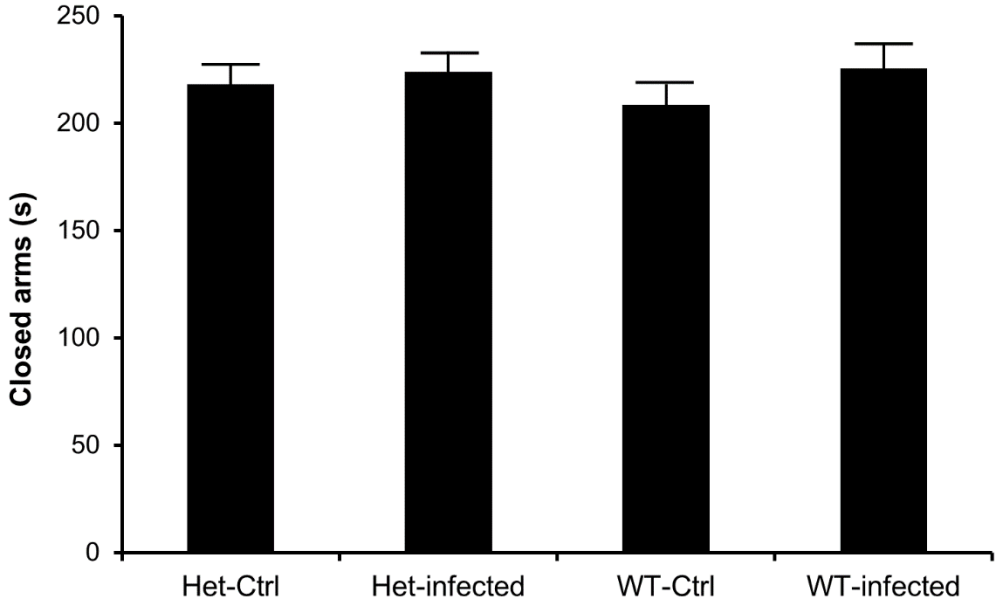

Figure 12: Time spent in the closed arms of the elevated plus maze (n=51). (Mean with SE).

Parameter	Effect	*P*-value	Parameter	Effect	*P*-value
Head dips	Genotype	0.279	**Open arms (s)**	Genotype	0.727
	Sex	0.154		Sex	0.366
	Infection	0.149		Infection	0.770
Head dip time	Genotype	0.930	**Closed arm entries**	Genotype	0.426
	Sex	0.285		Sex	0.816
	Infection	0.573		Infection	0.084
Central zone entries	Genotype	0.447	**Closed arms (s)**	Genotype	0.740
	Sex	0.824		Sex	0.326
	Infection	0.145		Infection	0.267
Open arm entries	Genotype	0.860	**Distance travelled (m)**	Genotype	0.011
	Sex	0.563		Sex	0.117
	Infection	0.515		Infection	0.225

Table 24: Parameters in the elevated plus maze that were tested in terms of genotype, sex and infection.

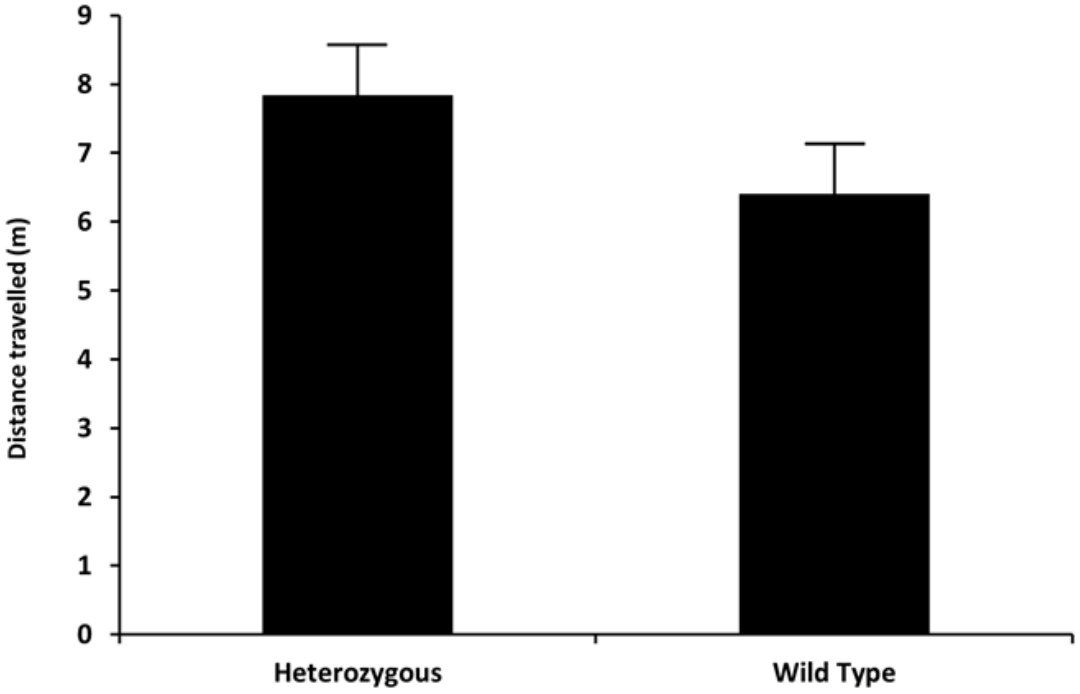

Figure 13: Distance travelled in the elevated plus maze. Data pooled across sex and infection (n=25 vs 26). (Mean with SE).

Novel object recognition

Novel object recognition is a spontaneous memory test. It is derived from the curiosity to explore a novel object (Table 25; Figure 14). The novel object recognition test showed sex effects (Figure 15) when infected and control mice were pooled together in terms of time inactive, novel object and time active. Females were more curious than males toward the novel object (*P* = 0.010). No effect was found on familiar object time.

Parameter	**Effect**	***P*-value**	**Parameter**	**Effect**	***P*-value**
Total object time	Genotype	0.829	**Time active**	Genotype	0.839
	Sex	0.088		Sex	0.083
	Infection	0.120		Infection	0.134
Novel object time	Genotype	0.777	**Total approaches**	Genotype	0.578
	Sex	0.010		Sex	0.093
	Infection	0.225		Infection	0.909
Familiar object time	Genotype	0.532	**Familiar % time**	Genotype	0.562
	Sex	0.710		Sex	0.781
	Infection	0.149		Infection	0.423
Novel % time	Genotype	0.562	**Distance travelled**	Genotype	0.701
	Sex	0.781		Sex	0.794
	Infection	0.423		Infection	0.497

Table 25: Novel object recognition parameters that have been tested in order to find a significant difference in the main effects.

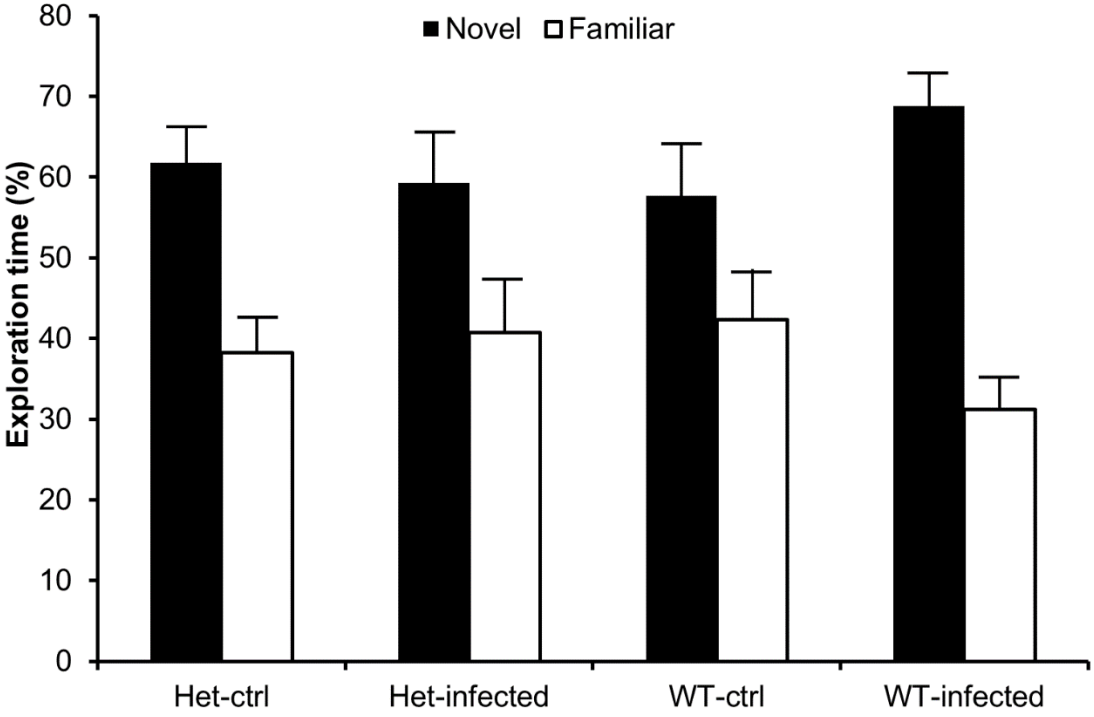

Figure 14: Exploration time (% of total time) in the novel object recognition test. No statistically significant differences between experimental groups were detected (n = 51). (Mean with SE).

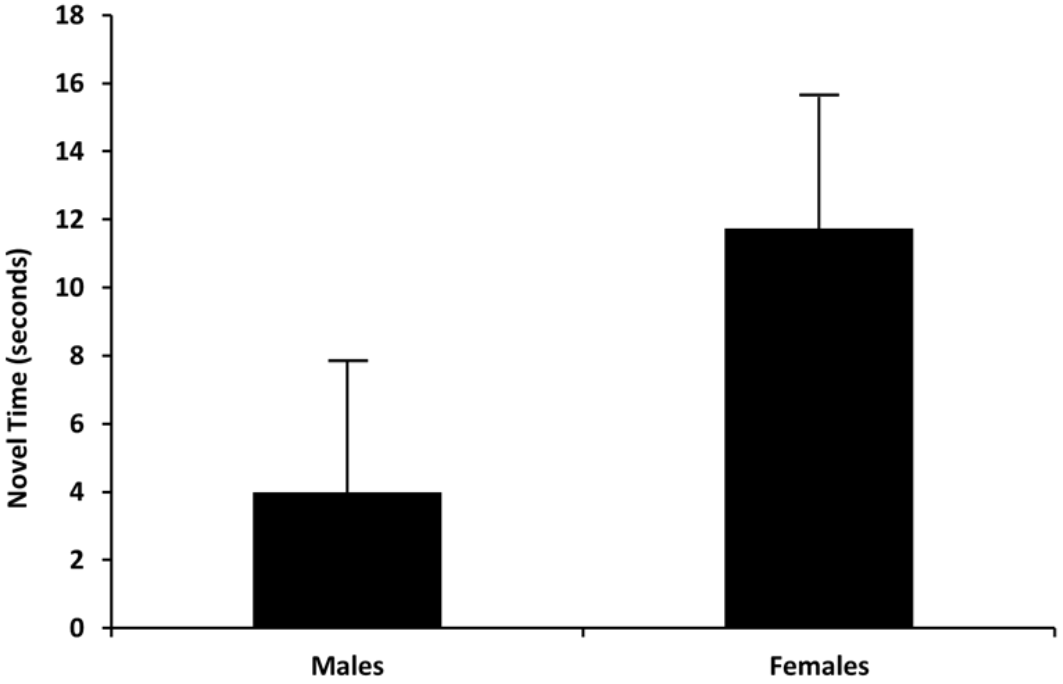

Figure 15: Time exploring the novel object (male versus female) (n = 25 vs 26). (Mean with SE).

Marble burying

Digging and burrowing are typical behaviours of mice. They dig to find food or to build a refuge to hide from predators. In the laboratory, mice dig enthusiastically in deep bedding such as wood chips. This behaviour could be subtly changed when they are infected by the parasite *T. gondii*. There were significant main effects of sex (P = 0.024) and infection (P = 0.038) on the number of marbles buried (Figure 16). Less marbles were buried by infected animals, which may indicate a sickness phenotype. In term of sex effect, male mice buried more marbles than female mice regardless of their infection condition. Kruskal Wallis test (non-parametric) was used in this analysis.

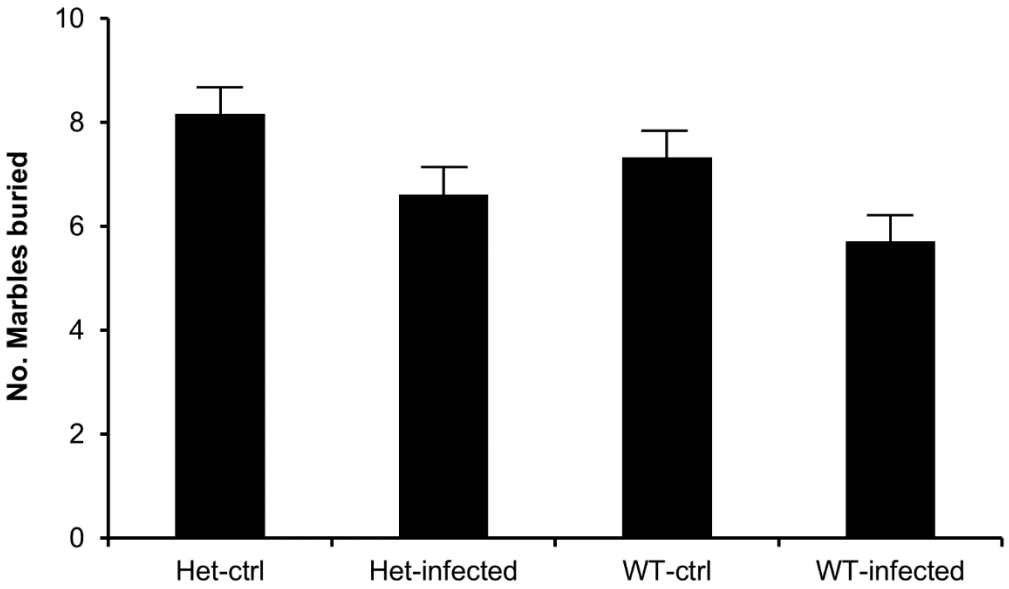

Figure 16: Number of marbles buried by mice (control versus infected) (Median with SE). (n= Het-ctrl=12, Het-infected= 13, WT-ctrl= 12 and WT-infected= 14).

Social approach

Social approach was conducted by using a three chambered arena. This test likely involves both cognitive and motivational components. In Phase 1 (Figure 17) and phase 2 (Figure 18) of the social approach test there were no significance differences in all parameters tested (Table 26). Bonferroni correction of p-value of 0.003 (0.05/18) was calculated to eliminate any false positive results. Nose poking of empty cage, stranger 1 and stranger 2, as well as distance travelled, time active and discrimination times, were not significantly different between genotype, sex and infection. However, infected mice showed a non-significant trend toward novel mice interaction compared to control mice in both phases. A heterozygous infected female, a WT infected female and a WT control female were excluded from this test as they were statistical outliers (phase 2).

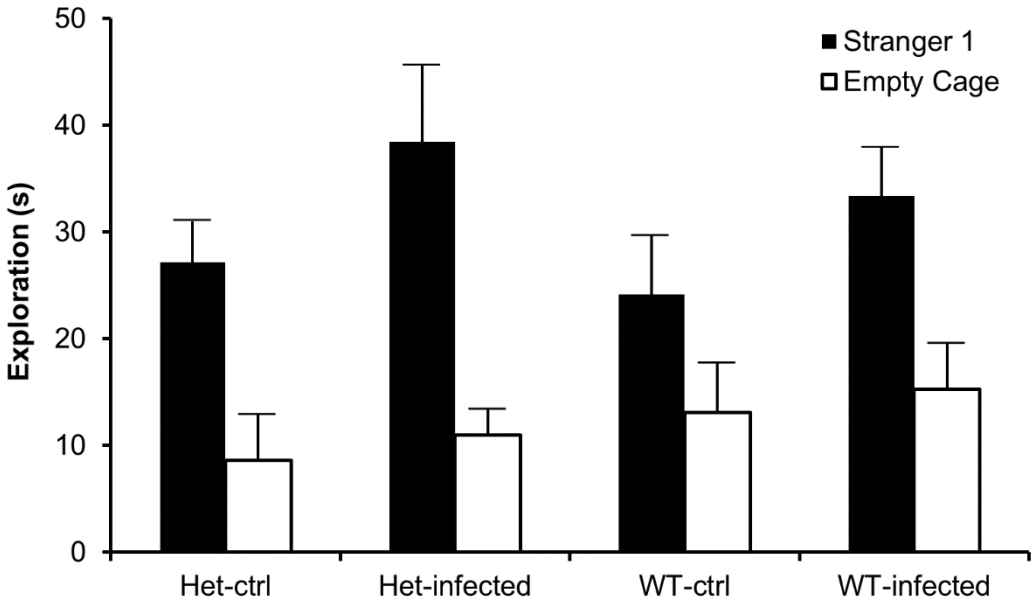

Figure 17: Phase 1 of the social approach test (stranger 1 versus empty cylinder) (n= 51). (Mean with SE).

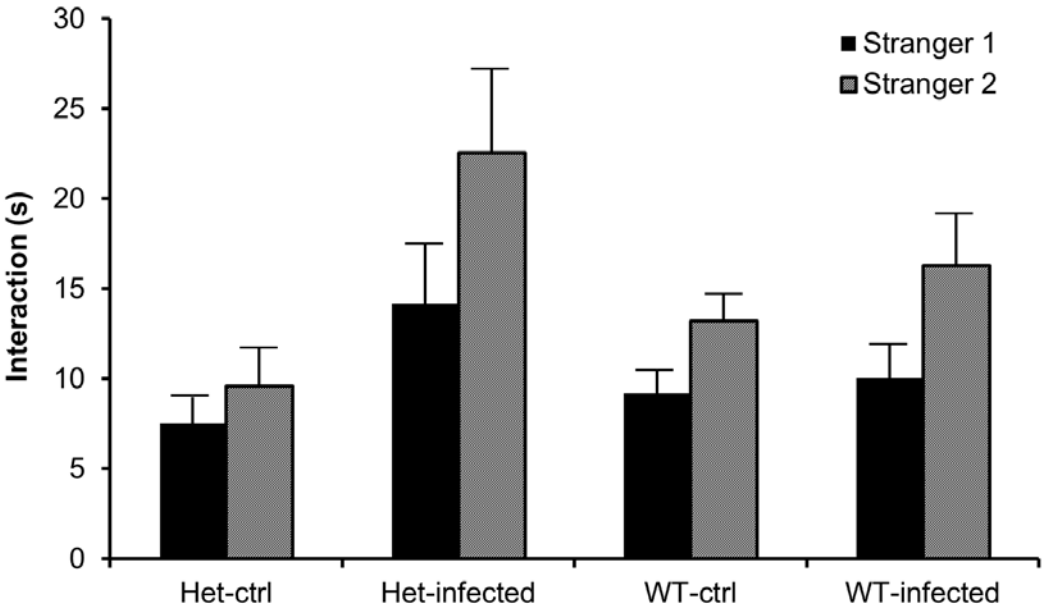

Figure 18: Phase 2 of the social approach test (stranger 1 versus stranger 2) (n= 48). (Mean with SE).

Parameter (Phase 1)	Effect	*P*-value	Parameter (Phase 2)	Effect	*P*-value
Stranger 1 nose poke time (s)	Genotype	0.457	**Stranger 1 nose poke time (s)**	Genotype	0.502
	Sex	0.948		Sex	0.279
	Infection	0.066		Infection	0.094
Empty cage time (s)	Genotype	0.284	**Stranger 2 nose poke time (s)**	Genotype	0.248
	Sex	0.940		Sex	0.657
	Infection	0.578		Infection	0.013
Stranger 1 nose pokes	Genotype	0.355	**Stranger 1 nose pokes**	Genotype	0.901
	Sex	0.088		Sex	0.293
	Infection	0.421		Infection	0.623
Empty cage nose pokes	Genotype	0.475	**Stranger 2 nose pokes**	Genotype	0.915
	Sex	0.989		Sex	0.493
	Infection	0.250		Infection	0.049
Time active (s)	Genotype	0.970	**Time active (s)**	Genotype	0.519
	Sex	0.930		Sex	0.784
	Infection	0.092		Infection	0.011
Distance travelled	Genotype	0.759	**Distance travelled**	Genotype	0.475
	Sex	0.119		Sex	0.031
	Infection	0.356		Infection	0.790
Total nose pokes	Genotype	0.771	**Total nose pokes**	Genotype	0.893
	Sex	0.201		Sex	0.937
	Infection	0.203		Infection	0.116
Discrimination time	Genotype	0.124	**Discrimination Time %**	Genotype	0.500
	Sex	0.654		Sex	0.115
	Infection	0.722		Infection	0.359
Discrimination nose pokes	Genotype	0.103	**Discrimination nose pokes**	Genotype	0.829
	Sex	0.253		Sex	0.162
	Infection	0.692		Infection	0.258

Table 26: Parameters tested in social approach behavioural test for analysis of genotype, sex and infection effects.

A Bonferroni correction of the p-value of 0.003 (0.05/18) was calculated.

Spatial Habituation in the T-maze

Exposure to a spatial location leads to habituation of exploration such that, in a novelty preference test, rodents subsequently prefer exploring a novel location to the familiar location. In the T-maze (Table 27), a significant difference in novel arm time was observed between infected (n = 27, median = 213.6) and control (n = 24, median = 183.3) mice (*P* = 0.022). As such, there was also a significant difference in familiar arm time (*P* = 0.022) between infected (median = 116.75) and control (median = 86.40) mice. Infected mice spent more time in the novel arms than in the familiar arms (*P* = 0.022) (Figure 19), thus demonstrating spatial habituation. Males spent more time in the familiar arm (*P* = 0.037) and travelled further in the T-maze (*P* = 0.004) than females (Figure 20). Kruskal Wallis test (non-parametric) was used in this analysis.

Parameter	**Effect**	***P*-value**	**Parameter**	**Effect**	***P*-value**
Familiar arms (s)	Genotype	0.407	**Freezing time (s)**	Genotype	0.105
	Sex	0.037		Sex	0.046
	Infection	0.022		Infection	0.940
Novel arm (s)	Genotype	0.412	**Novel arm entries**	Genotype	0.872
	Sex	0.037		Sex	0.312
	Infection	0.022		Infection	0.472
Familiar arms (m)	Genotype	0.486	**Familiar arm entries**	Genotype	0.806
	Sex	0.004		Sex	0.253
	Infection	0.187		Infection	0.416
Novel arms (m)	Genotype	0.066	**Total distance travelled (m)**	Genotype	0.792
	Sex	0.266		Sex	0.086
	Infection	0.313		Infection	0.651

Table 27: Spatial habituation parameters for sex, genotype and infection effects.

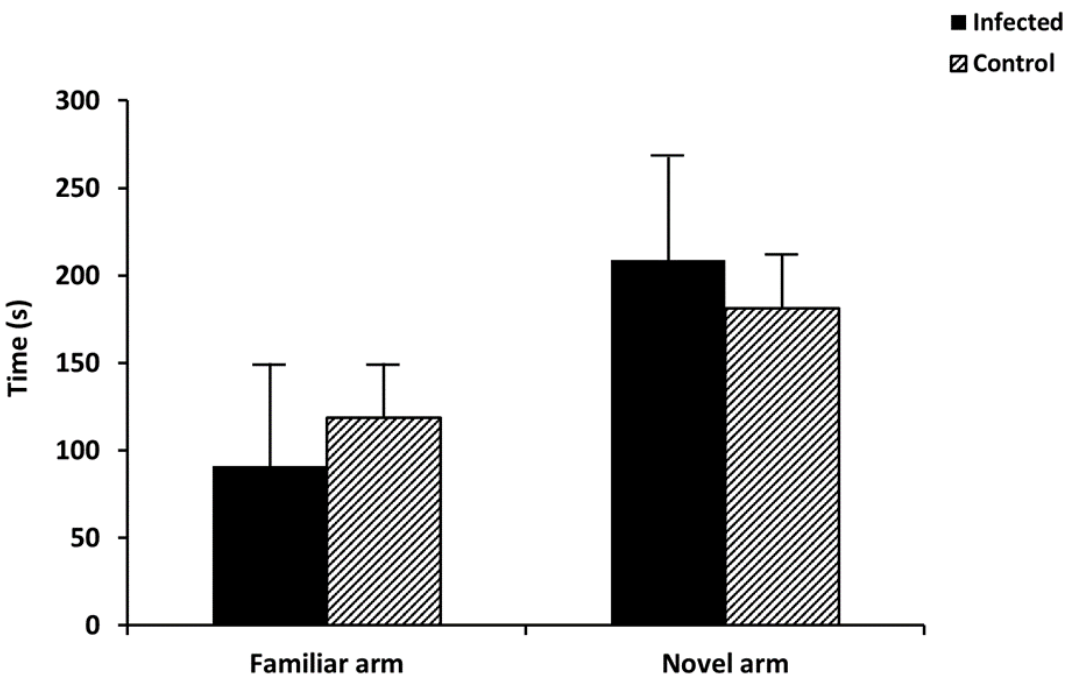

Figure 19: Infected mice showed more interest in the novel arms than the familiar arm irrespective of sex and genotype (n infected=27 vs ctrl= 24). (Median with SE)

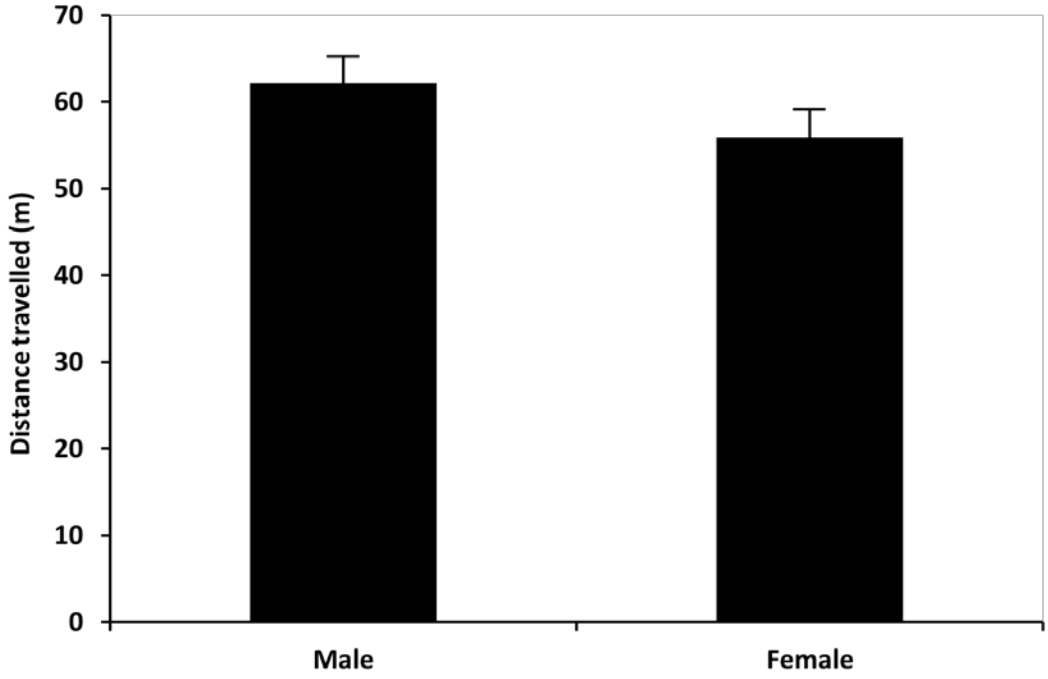

Figure 20: Distance travelled in familiar arm between male mice and female mice (n= 25 vs 26). (Median with SE)

Passive avoidance

Passive avoidance is a fear-aggravated test used to evaluate learning and memory in mouse models of CNS disorders. In this test, subjects learn to avoid an aversive stimulus, a previously administered foot-shock. Latency to cross to the dark side is shown in (Figure 21). All experimental groups remembered the foot-shock and hesitated for a long time before either crossing to the dark side or the maximum time was reached (300 seconds) (Table 28). Kruskal Wallis test (non-parametric) was used in this analysis.

Training (foot-shock)	**Effect**	**P-value**	**Test (no foot-shock)**	**Effect**	**P-value**
Latency to cross	Genotype	0.361	**Latency to cross**	Genotype	0.118
	Sex	0.152		Sex	0.214
	Infection	0.152		Infection	0.610

Table 28: Passive avoidance parameters. Both training and test showed no statistically significant differences between experimental groups.

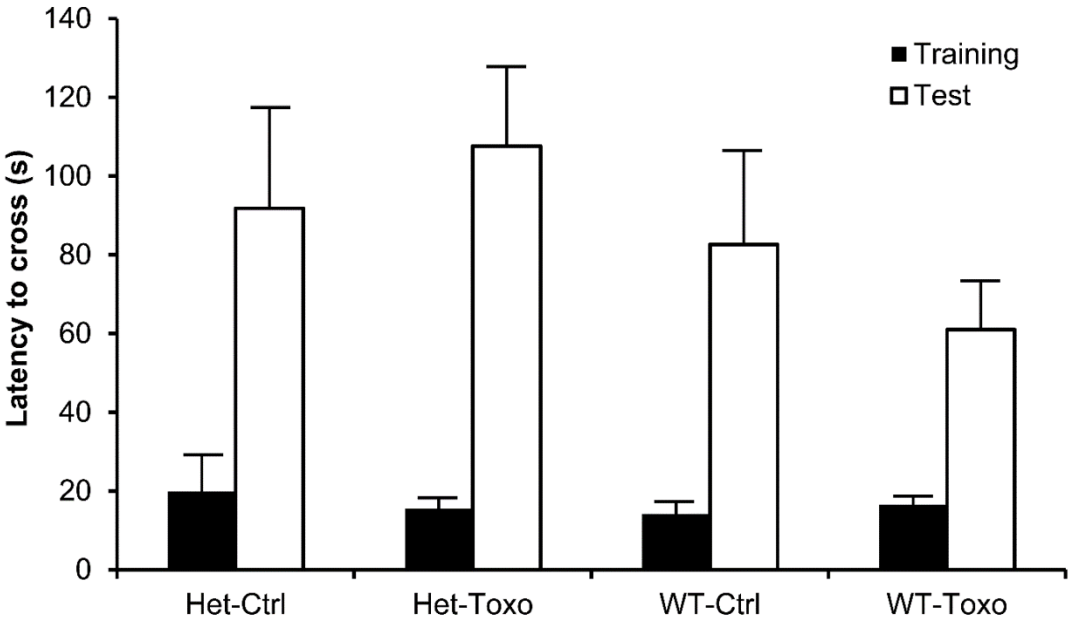

Figure 21: Passive avoidance test. Latency to cross to dark side (n= 51). (Median with SE).

Feline odour test

Mice naturally fear and avoid felines, even after many generations of domestication. Mice smell cat odours and act accordingly, but they are naturally attracted to food smells. In this test, mice were subjected to two odour choices in the T-maze arena. None of the experimental groups showed a preference towards the cat urine or to the food pellets (Table 29) (Figure 22). A Bonferroni correction of the p-value of 0.003 (0.05/14) was calculated. No obvious effect of sex, genotype or infection was detected. Entries to each zone, time in each zone and distance travelled all showed no effects of genotype, sex and infection.

Parameter	**Effect**	***P*-value**	**Parameter**	**Effect**	***P*-value**
Cat urine arm entries	Genotype	0.127	**Central zone entries**	Genotype	0.045
	Sex	0.526		Sex	0.091
	Infection	0.373		Infection	0.470
Cat urine arm (s)	Genotype	0.921	**Central zone (s)**	Genotype	0.181
	Sex	0.180		Sex	0.005
	Infection	0.414		Infection	0.149
Cat urine arm entries (%)	Genotype	0.738	**Empty arm entries**	Genotype	0.004
	Sex	0.504		Sex	0.838
	Infection	0.976		Infection	0.051
Cat urine arm time (%)	Genotype	0.980	**Empty arm (s)**	Genotype	0.260
	Sex	0.145		Sex	0.012
	Infection	0.495		Infection	0.798
Food pellet arm entries	Genotype	0.263	**Food pellet arm time (%)**	Genotype	0.833
	Sex	0.123		Sex	0.946
	Infection	0.952		Infection	0.389
Food pellet arm (s)	Genotype	0.320	**Empty arm time (%)**	Genotype	0.308
	Sex	0.533		Sex	0.027
	Infection	0.483		Infection	0.981
Food pellet arm entries (%)	Genotype	0.388	**Distance travelled (m)**	Genotype	0.435
	Sex	0.550		Sex	0.344
	Infection	0.173		Infection	0.215

Table 29: Feline odour test parameters for main effects of sex, genotype and infection.

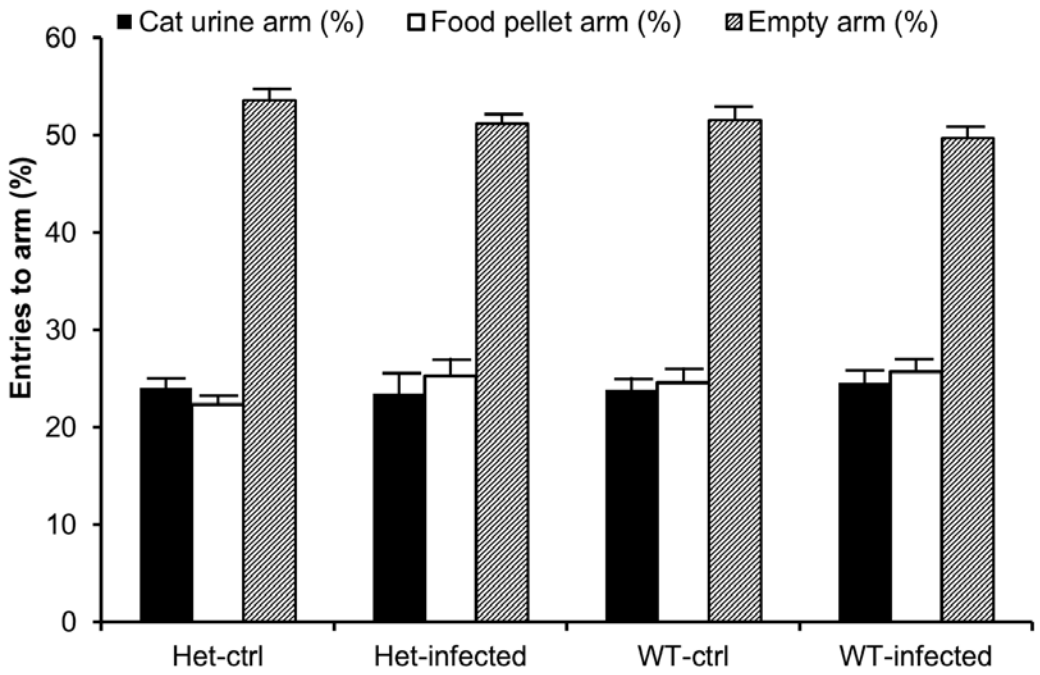

Figure 22: Feline odour test. Percentage of all arm entries to each arm (n = 51).

Summary of behavioural tests

A summary of statistical significance results of all behavioural tests is shown in Table 30. Note that there were no significant genotype*infection interaction effects after Bonferroni correction.

Behavioural test	Genotype	Sex	Infection
Open Field	Grooming time in all zones and outer zone (heterozygous)	No effect	No effect
Elevated plus maze	Distance travelled (heterozygous)	No effect	No effect
Novel object recognition	No effect	No effect	No effect
Marble burying	No effect	♂ higher than ♀	Fewer marbles (infected)
Social approach	No effect	No effect	No effect
T maze habituation	No effect	No effect	No effect
Passive avoidance	No effect	No effect	No effect
Feline test	No effect	No effect	No effect

Table 30: Summary of behavioural test results on all main effects.

3.3.4 Neurochemical analysis results

The neurotransmitters dopamine, noradrenaline and serotonin were measured using HPLC-ECD. Standard curves were created for all neurotransmitters in order to calculate the sample concentration.

Dopamine in cerebellum was detected in 8 males and 8 females. In mid-brain DA was detected in 7 males as well as in 10 females (Table 31)..

DA in frontal cortex was detected in 31 mice in total (16 male and 15 female) (Figure 23).

Noradrenaline in cerebellum was in 5 males and 9 females (Figure 24).

Noradrenaline in mid-brain was detected in 2 males only and 10 females. While in 11 females noradrenaline in frontal-cortex was detected (Table 32).

Serotonin in cerebellum was detected in 8 males and 7 females. Serotonin in mid-brain was detected in 7 males and 10 females (Table 33).

Serotonin in frontal-cortex was detected in 16 males and 15 females (Figure 25).

Dopamine concentration (nM)					
Cerebellum infected	**Cerebellum control**	**Mid - brain infected**	**Mid-brain control**	**Frontal cortex infected**	**Frontal cortex control**
0.006	0.077	0.025	0.090	0.010	0.005
0.052	0.069	0.071	0.021	0.017	0.003
0.069	0.003	0.043	0.109	0.040	0.003
0.001	0.001	0.001	0.057	0.063	0.152
0.001	0.003	0.001	0.069	0.069	0.947
N/D	0.001	0.005	0.036	0.087	0.066
N/D	0.001	0.001	0.001	0.052	0.815
N/D	0.002	0.002	0.001	0.001	0.958
N/D	0.002	N/D	0.002	0.008	0.000
N/D	0.001	N/D	0.002	0.002	0.002
N/D	0.001	N/D	0.001	0.042	0.382
N/D	N/D	N/D	0.001	0.001	0.002
N/D	N/D	N/D	N/D	0.001	0.002
N/D	N/D	N/D	N/D	0.002	0.002
N/D	N/D	N/D	N/D	0.003	0.003
N/D	N/D	N/D	N/D	N/D	0.001

Table 31: DA concentrations of DA in cerebellum, mid-brain, and frontal cortex. Females (black), Male (red), and N/D means concentration were not detectable.

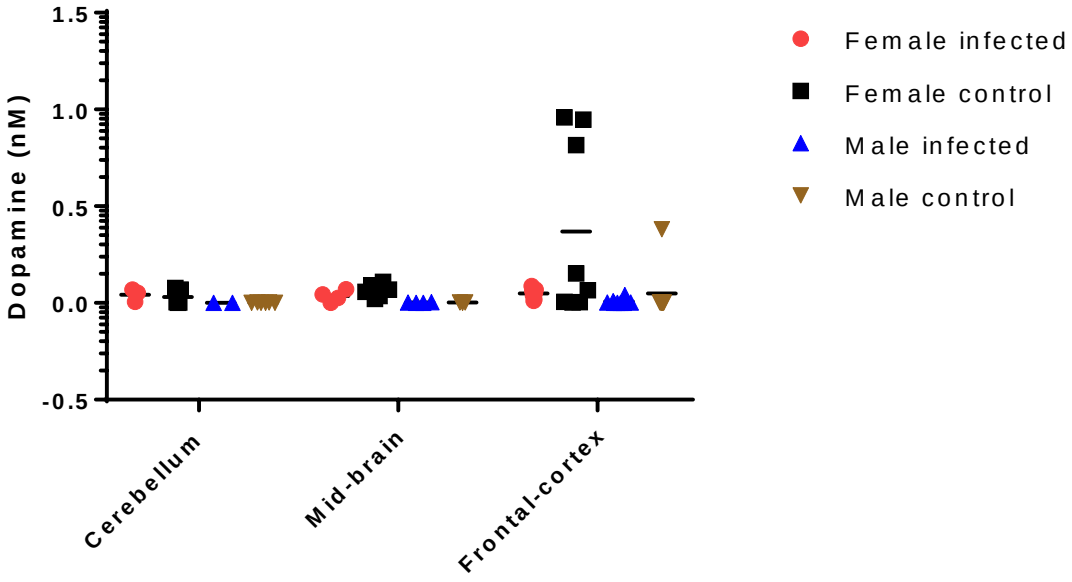

Figure 23: Mean concentration (nM) of dopamine in cerebellum, mid-brain and frontal cortex.

Noradrenaline concentration (nM)					
Cerebellum infected	**Cerebellum control**	**Mid-brain infected**	**Mid-brain control**	**Frontal cortex infected**	**Frontal cortex control**
0.006	0.010	0.617	0.086	0.686	0.050
0.033	0.002	0.089	0.005	0.003	0.009
0.021	0.141	1.115	0.004	0.004	0.168
N/D	0.004	0.002	0.018	0.004	0.003
N/D	0.487	N/D	0.731	0.005	0.209
N/D	0.003	N/D	0.789	0.045	0.926
N/D	0.325	N/D	0.257	N/D	0.063
N/D	0.003	N/D	0.002	N/D	0.055
N/D	0.022	N/D	N/D	N/D	0.005
N/D	0.004	N/D	N/D	N/D	0.002
N/D	N/D	N/D	N/D	N/D	0.002

Table 32: Noradrenaline concentrations in cerebellum, mid-brain, and frontal cortex. Females (black), Male (red), and N/D means concentration were not detectable.

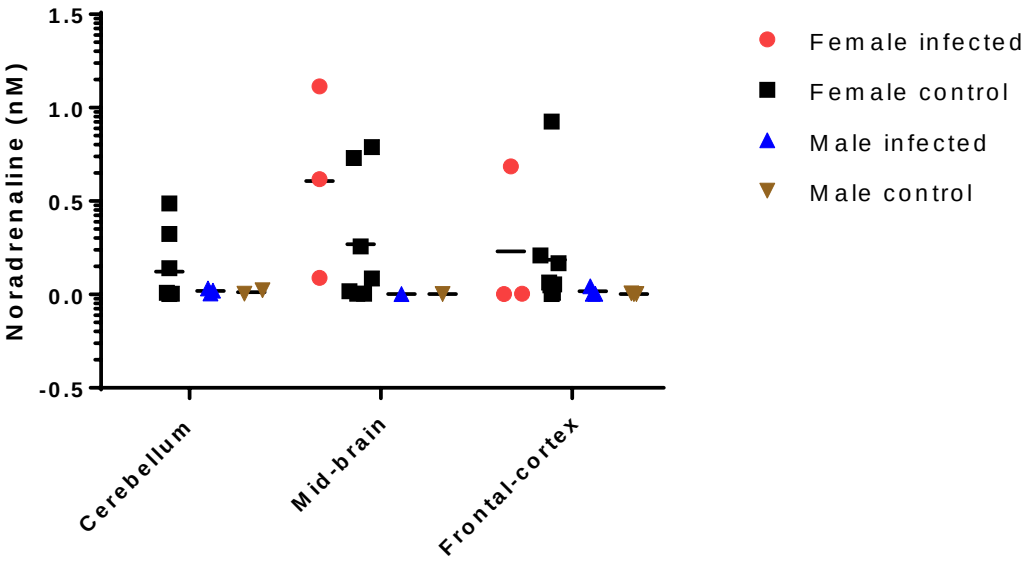

Figure 24: Mean concentration (nM) of noradrenaline in cerebellum, mid-brain and frontal cortex**.**

Serotonin concentration (nM)					
Cerebellum infected	**Cerebellum control**	**Mid-brain infected**	**Mid-brain control**	**Frontal cortex infected**	**Frontal cortex control**
0.059	0.076	0.126	0.194	0.051	10.736
0.080	0.051	0.084	0.067	0.084	40.418
0.084	0.257	0.080	0.109	0.093	15.999
0.143	0.109	0.055	0.059	0.067	0.101
N.D	15.999	0.084	2.358	0.114	73.257
N.D	0.059	0.072	0.059	0.059	24.419
N.D	N.D	0.051	0.059	0.059	0.118
N.D	N.D	N.D	N.D	0.081	0.063
N.D	N.D	N.D	N.D	0.059	0.135
N.D	N.D	N.D	N.D	0.076	0.059
N.D	N.D	N.D	N.D	0.051	0.118
N.D	N.D	N.D	N.D	10.104	0.042
N.D	N.D	N.D	N.D	0.059	0.089
N.D	N.D	N.D	N.D	0.227	0.051
N.D	N.D	N.D	N.D	8.420	0.093

Table 33: Serotonin concentrations in cerebellum, mid-brain, and frontal cortex. Females (black), Male (red), and values of (0) concentration were not detectable.

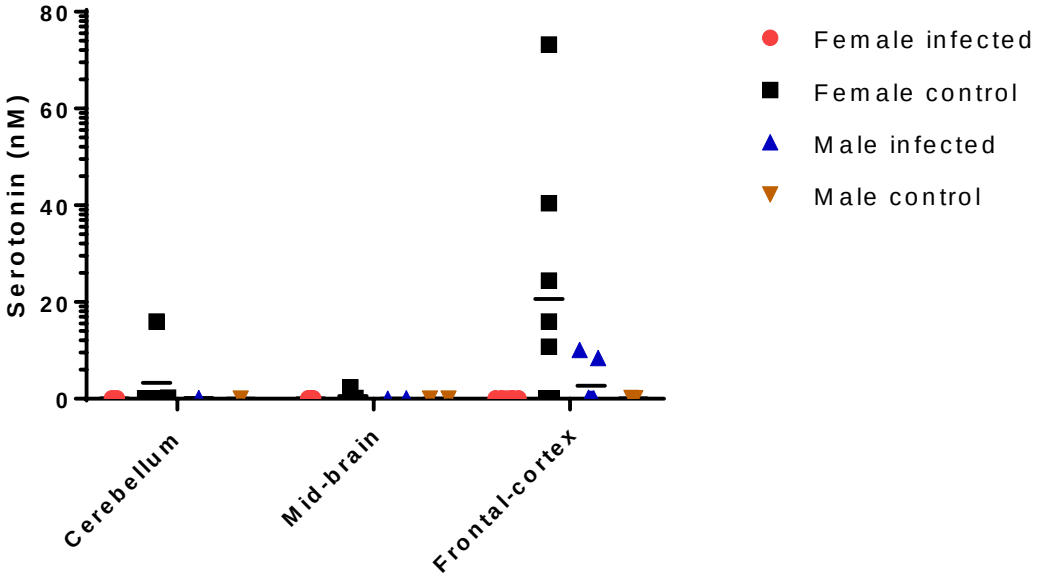

Figure 25: Mean concentration (nM) of serotonin in cerebellum, mid-brain and frontal cortex.

3.4 Discussion

Mouse studies have been used to try to understand the behavioural changes induced by infection with the parasite *T. gondii* and how it increases the likelihood of successful transmission to the definitive host in the cat family *Felidae* (Afonso et al., 2012; Gulinello et al., 2010; Hrda et al., 2000; Witting, 1979). Also, associations between *T. gondii* exposure and different neuropsychiatric diseases have been studied (Kusbeci et al., 2011; Torrey and Yolken, 2003; Yolken et al., 2009). Schizophrenia is one of the neuropsychiatric diseases that have been linked with *T. gondii*. This experiment was designed to look closer at the effect of the parasite on a mouse model of a genetic risk factor for schizophrenia: heterozygous deletion of neurexin-1α.

C57BL/6NCrl females were mated with neurexin-1α KO heterozygotes to generate heterozygous and WT mice for testing. Mouse genotyping was done using the PCR-based assay. At the beginning, there was a persistent problem with the genotyping when using standard Promega GoTaq® Green PCR mix. Upon the recommendation of Dr James Dachtler (University of Durham), an alternative PCR mix called Hotshot Diamond was tried. This mix is designed so that the Taq polymerase is inactive until it has been incubated at 95°C for 5 minutes, which ensures greater specificity. While this PCR method improved the genotyping results by more than 70%, genotyping was still problematic for some samples. This led to having to repeat the process on some samples. The decision was then made to do some of these samples in serial dilution: 50, 40, 30 ,20 and 10 ng/µl. The

remaining samples were successfully genotyped at 20 ng/µl with a 99% success rate.

The *T. gondii* dose, parasite strain and route of infection employed were chosen based on previous studies. For example, Xiao et al. (2012) used BALB/c mice infected intraperitoneally with 400 tachyzoites of Prugniaud strain. Kannan et al. (2010) and Vyas et al. (2007) used the same mouse strain, *Toxoplasma* strain, dose and route of infection. There were almost no reports of mice dying as a result of this infection. Only Gulinello et al. (2010) reported that 10 out of 20 of the infected mice died within three weeks of the initial infection. Unfortunately, in this experiment, mice of the C57BL/6N background suffered severely following *T. gondii* injection. The dose was 500 tachyzoites per mouse intraperitoneally of Prugniaud strain (Vyas et al., 2007). In round one, mice showed deterioration in just five days. Some of them were sluggish with dull eyes and had hunched backs. In day 8 post-infection, out of a total of 37 mice, there were: 13 healthy (35%), 13 ill (35%), eight dead (22%), and three culled (8%). On Day 10, the study was terminated and post-mortem examinations by the veterinarian showed no evidence of incorrect procedures regarding injections. It is suspected that the parasite strain used had increased virulence due to continual passaging in the laboratory. It was thus necessary to pass the parasite through mice, harvest brain stages, and re-administer to uninfected mice to recover a lower virulence stock. In Round 2, five mice were used to determine whether the number of tachyzoites injected was critical. The mouse injected with the lowest dose exhibited paralysis in one hind leg. A dose of 50 tachyzoites was then administrated in the next experiment. Rounds three and four were

with female mice with a C57BL/6NCrl background. The tachyzoites were released from the previously infected mice, which could reduce the virulence of the strain. In general, the health of the mice improved compared to the first round. However, it was not possible to conduct behavioural experiments as mouse health was declining every day. As a result, neurological assessments were done. The Mann-Whitney U test showed a significant difference between wire suspension time and mouse health (infected vs control) (P =0.015). Infected mice showed weakness and some of mice were not able to hang for 1 second. Overall, the health of the mice was compromised and their fitness was too low for behavioural testing. The experiment was therefore terminated, and the genetic background of the mice was reconsidered. A visit to a lab in the USA revealed that when C57BL/6 background mice were infected with *T. gondii,* they were treated with anti-parasitic drugs in order to eliminate the tachyzoites. The drugs did not eliminate bradyzoites. Nonetheless, this fact was not reported in literature and would have prevented the losses that had happened.

The decreased susceptibility of *T. gondii*-infected mice with an F_1 hybrid (50% C57BL/6N, 50% BALB/c) genetic background suggests that they were carrying dominant ‘protective’ alleles from the relatively resistant BALB/c inbred strain and recessive ‘risk’ alleles from the more susceptible C57BL/6N inbred strain. The protective alleles from BALB/c could, in principle, be mapped in the N_2 progeny of F_1 hybrid (50% C57BL/6N, 50% BALB/c) mice backcrossed to the C57BL/6N strain. Protective alleles can suppress disease in otherwise susceptible individuals (Nadeau, 2003), and the protein products that they encode could form the basis for novel

therapeutic interventions. For example, loci regulating survival time after infection with African trypanosomes that cause sleeping sickness have been identified in mice (Goodhead et al., 2010).

The open field test in *T. gondii* infected mice revealed hyperactivity in females and hypoactivity in males (Xiao et al., 2012). Eells et al. (2015) and Gatkowska et al. (2012) have both reported that infected mice, regardless of sex, are more active than control counterparts. In the present study, throughout the whole 30 minutes of the open field test, infected and control groups showed similar levels of activity. Grooming is a frequent behaviour in mice which may reflect anxiety, and strain and genotype could influence the behaviour (Kalueff et al., 2007). Previous research has revealed that *T. gondii* infected mice groomed less than controls (Gatkowska et al., 2012). Neurexin-1α heterozygous mice in this experiment exhibited more grooming (P =0.001), and were more active in the outer-zone (P =0.003) of the open field arena compared with WT mice. It has been reported that neurexin-1α KO mice groomed twice as much as than WT mice (Rabaneda et al., 2014). The outer zone is the closest to the walls, and thus the heterozygous mice were exhibiting thigomotaxis (wall-hugging).

T. gondii infected mice entered the open arm more than the controls in the elevated plus maze, an indication that they were less anxious (Correa et al., 2014). This could show that *T. gondii* is altering mouse behaviour, which, in turn, could lead to an increase in the predation rate. In contrast, another study showed that both infected and controls showed similar open arm entries, but infected mice covered a relatively larger distance than controls

(Afonso et al., 2012). In this study, there were no differences between the infected and control mice in entries to open/closed arms. The distance travelled by heterozygous mice was larger than that of WT mice (*P* = 0.012), a possible genotype effect which was not reported previously.

In the novel object test, Eells et al. (2015) showed no differences in interaction with the novel object between infected and control mice. In the present study, sex effects were detected. Females spent a longer time exploring the novel object than males (*P* = 0.003) regardless of their infection. There are factors such as oestrus cycle and hormonal changes in females that could influence these differences between male and female mice. It seems normal to find sex differences in any behavioural interactions.

Marble burying tests have shown an effect in *V1aR* KO mice. This gene is associated with autism and schizophrenia. They showed less marble burying than WT mice (Egashira et al., 2007). Rats infected with the TgTH overexpressor *T. gondii* strain showed no difference from control rats, but males buried (mean 25 out of 30) more marbles than females (mean 14.1) (Kaushik, 2014). There are no previous studies using the marble burying test with *T. gondii* infected or neurexin-1α mutant mice. The present study showed that infected mice buried fewer marbles than the controls (*P* = 0.038). This might show that infected mice were less anxious than the controls, but could also be the result of a subtle motor function deficit. Also, males buried more marbles than females (*P* = 0.024).

Sociability has been linked with *T. gondii*. A study in humans showed that *T. gondii* seropositivity was associated with personality profile and behaviour

(Flegr, 2013). Also, infection has been linked with social interaction in rats, where infected rats showed a longer duration of social interaction than controls (Gonzalez et al., 2007). In a recent study (Dachtler et al., 2015), heterozygous neurexin-1α mice showed a preference for exploring a novel mouse over an empty cylinder in phase 1. In phase 2, they showed less interaction with the second novel mouse, stranger 2 (Dachtler et al., 2015). In phase 1 and phase 2 in the present study, no effect of sex, genotype or infection was detected using three chamber social interaction test. However, infected mice showed a non-significant trend toward stranger 1 (Figure 17) and stranger 2 (Figure 18) which could indicate that *T. gondii* may increase sociability in these mice. This could be a possible mechanism to increase predation rate.

The T-maze test was used to test spatial habituation, such that mice with a normal memory should investigate novel areas more than familiar areas. In this study, infected mice showed a preference for the novel arm ($P = 0.022$), indicating intact spatial habituation.

The long-term fear memory of *T. gondii*-infected mice was also not obviously impaired. In the passive avoidance test, mice were cautious did not cross immediately to the dark side. They remembered the foot-shock and avoided it. It was reported that congenitally infected mice showed impaired memory (Wang et al., 2011), possibly due to the load of parasite administrated to mice. In the present study, however, infected mice showed intact passive avoidance. In another study, it was shown that widespread pathology of the could affect the memory of mice (Witting, 1979).

The feline odour test was also performed in this study. Previous studies have shown partiality of *T. gondii*-infected mice to bobcat urine (Xiao et al., 2012; Kannan et al., 2010; Haroon et al., 2012). Most studies have conducted this test in an open field arena, but the present study conducted it in a T-maze, following the protocol of McGirr et al. (2016). In the present study, infected mice did not show a preference towards either bobcat urine or food pellets. The T-maze configuration could have affected each mouse's behaviour because the empty arm (the stem of the T) was furthest away from the cat urine. Thus the mice may have decided to stay in the empty arm rather than exploring the other arms at the top of the T. This study found that all experimental groups entered each arm at the top of the T roughly equally.

In regards to neurotransmitters, measure levels were very low and variable between mice. Because of this, statistical analysis of these data was not carried out. This might be because some of the mice's brains were analysed in the absence of important ingredients for the HPLC-ECD run. As a result, no signals were detected. During HPLC training, a small hidden bottle that have to be filled with HPLC water was left behind by mistake. The experiment was run for few samples until the mistake was spotted.

Some of the neurotransmitters had fewer number of subjects (mice) than the others which could also have weakened the results. In other words, neurotransmitters had not been detected in all mice brains (n=51). The reason could be due to handling of the brains, neurotransmitters are delicate molecules which can be affected by various reasons such as imbalance of temperature and brain processing method. Although care when handling

mice brain was taken into account. Brain samples were kept in 4°C all the time. Centrifugation was done in a cold room (4°C).

In the present study, infected mice showed less anxiety-like behaviour compared with control mice in the open field and marble burying tests. It is known that *T. gondii* alters the mouse intermediate host in a manner that can increase the predation rate, enhancing parasite transmission. However, the open field is a less reliable test of anxiety than the EPM anxiety test, in which infected mice did not show less anxiety. Infected mice were more sociable than controls in the social approach test, with more exploration of novel strangers, which could be deemed risk-taking. In humans, there are reports that *T. gondii* infected individuals show risk-taking actions and are involved in more traffic accidents (Flegr et al., 2002). Infected mice also showed normal short and long memory in the present study, in the passive avoidance and T-maze tests.

In terms of a genotype effect, the results showed that neurexin-1α heterozygous mice spend more time engaged in self-grooming ($P = 0.002$) in the open field, and travelled more ($P = 0.012$) in the EPM. Increased self-grooming is considered to be an autistic-like behaviour in mice (Rabaneda et al., 2014). A heterozygous genotype trend was also observed in sociability toward novel mice. Despite the fact that heterozygous mice might be less sociable than WT.

The present study found no evidence of an additive behavioural effect of neurexin-1α deletion and *Toxoplasma*-infection in mice. Epidemiological studies of schizophrenia have identified *T. gondii* as a significant

environmental risk factor and neurexin-1α deletion as a significant genetic risk factor. It was therefore hypothesised that *T. gondii* infection may induce or exacerbate schizophrenia-related symptoms in subjects positive for neurexin-1α deletion. However, the present study found limited evidence of a compound effect of neurexin-1α deletion and *Toxoplasma* infection on behaviour in mice, and no conclusive evidence supports the hypothesis above. In this study, *T. gondii*-infected mice showed less anxiety-related behaviour compared with control mice in marble burying tests. This suggests that, in some tests of anxiety-related behaviour, *T. gondii* could affect risk-taking decisions of infected mice, possibly in order to increase the predation rate. It may be involved in immune response and inflammatory components in order to manipulates the host behaviour. These findings provide further support as to the sophistication and specificity of the parasite manipulation induced by *T. gondii* and the involvement of the parasite in schizophrenia.

Future work plan may include brain samples that was already stored in Sterilin tubes containing RNA-later solution for 24 at 4 °C then they were transferred to - 20 °C for indefinite storage. RNA-later stabilizes and protects cellular nucleic acids in intact form without the need to immediately snap freeze samples in liquid nitrogen for later processing. RNA-later preserves the material for future analysis using western blotting, microarray tests, epigenomic analyses such as ChIP, and q-PCR.

Moreover, Different developmental stages of the *T. gondii* life-cycle specifically tachyzoite and bradyzoite may be combined yielding the effect on host behaviour. Type I and Type III strains could be analysed in mice.

Also the PD samples could be strain typed although this is difficult. It is also possible to use genetically modified *T. gondii* strains for example, TgTH overexpressor and find out the effect of this strain on mutant mice.

Behavioural assays are important in order to detect subtle behavioural changes caused by *T. gondii.* Balance beam test can be used to assess motor coordination of infected mice. Previous studies showed that *T. gondii* infected mice showed motor coordination deficit. This test would be useful in future work because it could detect deficit in motor caused by CNS deficit or due to genetic risk factors such as schizophrenia.

Another important behavioural test is Morris water maze which is a prolonged spatial learning and reference memory. It is sensitive to hippocampal synaptic plasticity. *T. gondii* thought to affect hippocampal activity. A brain region that is involved in anxiety control. It is also relevant to schizophrenia as patients suffer from loss of memory. This test provides a relatively non-invasive means of testing the link between *T. gondii* infection and schizophrenia.

The five choice serial reaction time test normally uses visual stimuli, but could be adapted to include other stimuli such as odours. This test provides the possibility to test the effect of *T. gondii* infection and to measure accuracy of discrimination, impulsivity, perseverative responses and response latencies. The test is sensitive to discrete brain lesions and neurotransmitter depletions. It is a relevant behavioural assay that could determine association of schizophrenia and *T. gondii*. It was originally

adapted from a human task and has been successfully extended to mice and primates.

Chapter 4 The distribution of *Toxoplasma gondii* cysts in rat brain and the potential of its tropism toward specific brain regions

4.1 Introduction

Rat brain regions and their possible contribution to subtle behavioural changes during *T. gondii* infection have been described in many studies (Berdoy et al., 2000; Erhardt et al., 2007; Gulinello et al., 2010; Vyas et al., 2007). This is because of the observed different functions of the brain in different regions. The cerebellum is responsible for motor coordination and movement. The hippocampus controls long-term memory and is involved in learning, while the frontal cortex manages different functions, such as short-term memory, recognition and movement (Bear et al., 2007). The thalamus is the part of brain that controls sensation. It plays a role in every sensation except smell, which is regulated by the olfactory bulb. The hypothalamus is another important region; it maintains body temperature and controls the sex drive. The rhombencephalon, also known as the hindbrain, is involved in a wide variety of sensory and motor functions in coordination with the cerebellum. The nucleus accumbens is where the mesolimbic pathways of dopamine and serotonin occur. This region is responsible for the reward circuit. Previous studies found that the amygdala is responsible for the analysis of the sensory inputs from the environment and its elements (Bear et al., 2007). Experiments on rats show that there is a direct correlation between the operation of the amygdala and the production of fear-related responses such as fight-or-flight and freezing behaviour patterns. It has

been shown that *T. gondii* strains have a different effect on mice. For example, ME49-infected mice exhibited impaired memory while Prugniaud strains did not (Kannan et al., 2010). The connection between sickness and parasite genotype in animals is not fully understood (Dubey, 2009). In Europe and the United States, it is believed that people are affected by Type II strains (Su et al., 2012). It has recently been reported that atypical *T. gondii* genotypes were associated with immunocompetent patients (Su et al., 2012). Changes in the host behaviour, which parasites may be responsible for according to the assumptions of the behavioural manipulation theory (Lim et al., 2013), can be seen when observing infected rats. This includes behaviour involving the recognition of a predator threat, such as perception of cat odour.

Several studies point to the possibility that the phenomenon of behavioural changes caused by *T. gondii* is being influenced by the location of the parasites (Swierzy et al., 2014). Particularly, the effects on certain brain areas may provide a mechanism to explain how *T. gondii* alters the behavioural patterns of the host; to the point where the host disregards its essential habits, such as the instinct of survival. The parasite could target specific brain regions and infection could disable or lyse neurones that would alter behaviour. Indeed, one publication found that infection reduced density and length of dendritic extensions in the somatosensory complex and hippocampus with lower levels of two synaptic proteins, PSD95 and synaptophysin (Parlog et al., 2015).

The link between the location of *T. gondii* in particular brain areas and the effects that the parasite has on the rats' processing of fear have been investigated. Upon infection *T. gondii* was observed to have some measurable tropism towards the amygdala, although encysted parasites were prominent in other regions as well. With cyst development, the olfactory abilities of the host were reduced significantly, and the behavioural patterns of the host changed gradually. This was correlated with a higher density of infection in the amygdala (Vyas et al., 2007). This suggested a tropism for the amygdala although the difference was 0.10 ± 0.01 cyst per cubic millimetre (p>0.15).

One study indicated a decline in the nucleus accumbens density specifically a sub-region called the core in compared to control rats (p=0.01) and proposed this as the primary tool for *T. gondii* to alter the behavioural patterns of the host (Tan et al., 2015). For instance, Tan et al. (2015) stated that there is a direct correlation between the effects of *T. gondii* on rat nucleus accumbens and the subsequent changes in their behaviour. Tan et al. (2015) attributed the observed effect to a reduction in the rats' ability to identify the smell of the cat.

The research suggests that it is not the absence of fear but the delay in the reaction that can be defined as the primary effect of *T. gondii*'s influence. How the delay is caused by the parasite remains unclear. A retraction of neuronal dendritic extension has also been observed in *T. gondii* -infected rats, that may impair brain functions, and were observed primarily in the sensory system with a reduced production of corticosterones (Mitra et al.,

2013). Corticosterone performs functions related to energy production and regulation, as well as the provision of the responses necessary to regulate the stress levels in the rat.

It has been shown that *T. gondii* affects not only the fear-related instincts in rats but also their cognitive functions, which may thereby reduce their ability to remember certain behavioural patterns and use the acquired knowledge to their advantage. Consequently, rats may become incapacitated to perform the essential cognitive functions that allow them to analyse the environment efficiently and detect the elements that pose an immediate threat to their wellbeing (Daniels et al., 2015). Although several papers showed *T. gondii* in the forebrain areas of rats (Parlog et al., 2015), Berenreiterová et al. (2011) concluded that the parasite has tropism towards the olfactory bulb, frontal cortex, hippocampus, and amygdala in mice (Berenreiterova et al., 2011); areas with dopaminergic regulation (Arias-Carrion et al., 2010). Little is known about whether *T. gondii* specifically infects brain regions in order to manipulate the behaviour of the intermediate host. Further studies are needed to validate these findings. Most of these studies are restricted by the number of infected animals studied or the widespread infection found in mouse species.

This chapter presents the largest analysis yet of the location of *T. gondii* cysts in the brains of rats through cytological analysis. This was performed to clarify parasite tropism in the brain in a host that is resistant to serious consequences of infection (unlike mice that are susceptible to *T. gondii*) and therefore similar to human infections with *T. gondii*. Several strains of

parasite were used to investigate differences in tropism and infection density.

4.2 Materials and Methods

In the laboratory of J.P. Dubey at the USA, 150 female Sprague-Dawley rats (NTac:SD) were used. The control group consisted of 10 out of those 150 rats, and the remaining 140 rats were infected orally with an equal number of oocysts using 11 strains of *T. gondii* (Types I, II, III and atypical genotypes) (Table 34). The rats were sacrificed 8-9 weeks post-infection, according to animal welfare law, and their brains were dissected. The brains of 109 of the 150 rats were mid-sagittal sectioned. One-half of the brain, and an approximately 2–3 mm longitudinal slice of the other half of the brain, was fixed and embedded into paraffin slides for histology. Then, the samples were stained with an anti-bradyzoite (anti-BAG1) antibody and counter stained with haematoxylin. They were then sent to us at the University of Leeds for processing via the Virtual Scanned Slides application available at http://slides.virtualpathology.leeds.ac.uk (Figure 26). These scans were read by myself. The brains were divided into cerebellum, rhombencephalon, mesencephalon, hippocampus, thalamus, hypothalamus, subpallium, frontal cortex, and olfactory bulb regions, as well as a region consisting of the colliculus inferior (auditory) and colliculus superior (visual) combined. The regions were determined and classified using a rat brain atlas (Paxinos and Watson, 2006). Individual cysts were then located and counted on the virtual scanned slides. Each cyst was annotated for each slide (Figure 27) and the total numbers of cysts were collated. Twenty brain images were chosen systematically to be measured, out of a total of 109. The slides chosen were 1, 7, 11, 16, 26, 30, 35, 40, 45, 51, 57, 62, 67, 71, 77, 82, 87, 95, 106, and 109. The order was initially intended to be every fifth slide, but the next slide

was chosen instead where it was difficult to identify the individual regions of the designated slide. Where present, these difficulties resulted from the processing of the actual brains onto slides (Dubey et al., 2016).

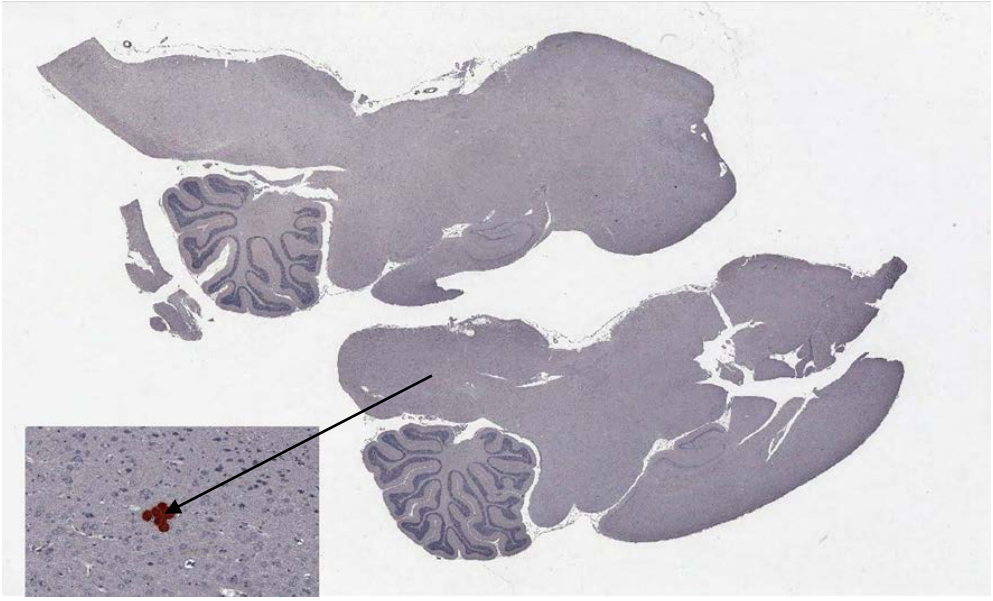

Figure 26: A sample of a virtual slide and a cyst shape (40x magnification).

***T. gondii* strain**	**Genotype**	**Origin Country**	**Host**	**Infected rat number**
GT1	I	USA	Goat	15
TgCtCO2	atypical	Colombia	Cat	15
ME-49	II	USA	Sheep	10
TgNmBr1	II	Brazil	Rabbit	10
VEG	III	USA	Human	10
TgGoatUS4	III	USA	Goat	15
TgBbUS1	atypical	USA	Black bear	15
TgPigUS15	atypical	USA	Pig	10
TgRabbitBr1	atypical	Brazil	Rabbit	20
TgCtPRC3	atypical	China	Cat	10
CT1	I	USA	Cattle	10

Table 34: Strain types summary (Dubey et al., 2016)

Automated scores were sought but, due to the sensitivity of the cyst stain, it was not possible to accurately count the cysts using automated devices.

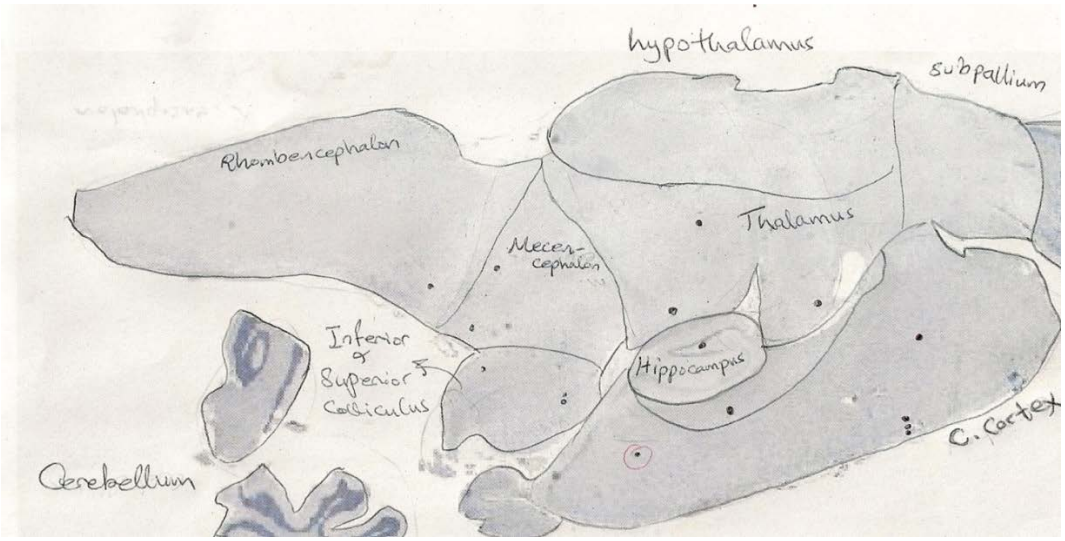

Figure 27: A sample of a rat brain section, with cysts annotated (black dots).

Table 35 shows the size measurement percentages, calculated via ImageJ to determine the pixel counts of all areas individually (cyst density) and via Microsoft Excel to calculate percentages. The following equation was used:

(Pixel size of the specific region ÷ Total pixel size of all brain regions) × 100.

Instead of reporting the number of cysts, the cyst density was calculated in order to remove bias as a result of the size of the brain region. IBM's SPSS programme was used to determine the statistical analysis. The Kruskal–Wallis (KW) test (p-value ≤ 0.05) was used to compare group medians. Kruskal-Wallis analysis was used after revealing that the samples were not normally distributed. Transformation of the data was done, however, the distribution was still skewed.

Region	Percentage
Cerebellum	15
Cerebral Cortex	23
Colliculus	4
Hippocampus	3
Hypothalamus	8
Mesencephalon	7
Olfactory Bulb	7
Rhombencephalon	15
Subpallium	9
Thalamus	9
Total	100

Table 35: Brain area pixels as percentages. Using average of systemic assigned 20 brains**.**

Mann-Whitney multiple pairwise tests were performed. Using the Bonferroni correction in order to reduce the chances of obtaining false-positive results due to multiple testing. Two pairwise comparisons were done, first between regions vs cyst density at a Bonferroni correction *p*-value of 0.001, and then between strains and cysts number at a Bonferroni correction *p*-value of 0.009 (Dubey et al., 2016).

4.3 Results

The encysted parasites, from herein termed “cysts” were counted in total of 109 brains. Two brains were control and cyst-free, all 107 infected rats had brain cysts except 6 brains that had no cysts. There was a statistically significant difference between the brain regions and the cyst density overall (KW test; p = < 0.0001). Most of the infected rats showed tissue cysts in all brain regions (Figure 28 and 29). However, the cyst density in the brain regions fluctuated between the 107 infected brains. The colliculus was the most highly infected region and the hypothalamus was the least infected region. Mann-Whitney pairwise comparisons revealed that 12 regional comparisons were significant with a p-value <0.001 (Table 36). In terms of higher density, the cerebral cortex, thalamus and cerebellum had higher tissue cyst densities than the hypothalamus or the subpallium. In contrast, the cyst densities in the hypothalamus were lower than the cyst densities in the cerebral cortex, thalamus, cerebellum, and rhombencephalon. Particularly, rats infected with the strains TgRabbitBr1 and TgCTPr-C3 showed a significant difference between brain regions (p<0.003, KW test). Both strains showed high cyst density in the colliculus and the olfactory bulb.

There was a statistical significance difference between the *T. gondii* strains and the number of cysts per brain (KW p< 0.0001) (Figure 30).

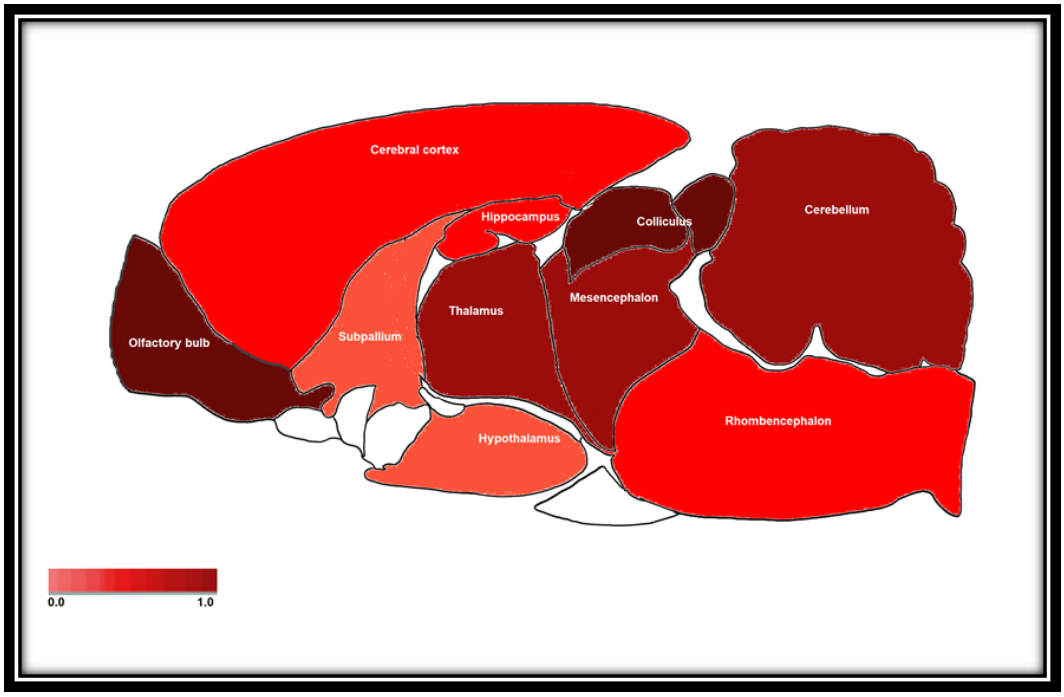

Figure 28: Brain atlas heat map showing the higher density of cysts in various brain regions. The darker the colour the higher the cyst density**.** Colour represents cysts median. Figure was recreated using atlas by Paxinos and Watson, 2006.

The brains infected with the TgCTPr-C3 strain had a higher number of cysts than the brains infected with the other strains. This strain was derived from cat and is an atypical strain. In contrast, the brains infected with the TgPigUS15 strain had few cysts ($p<0.001$). This strain was derived from an infected pig and is also an atypical strain. The number of cysts found in the strains that are most often used in the laboratory, namely ME-49 and VEG, were not significantly different than the other strains.

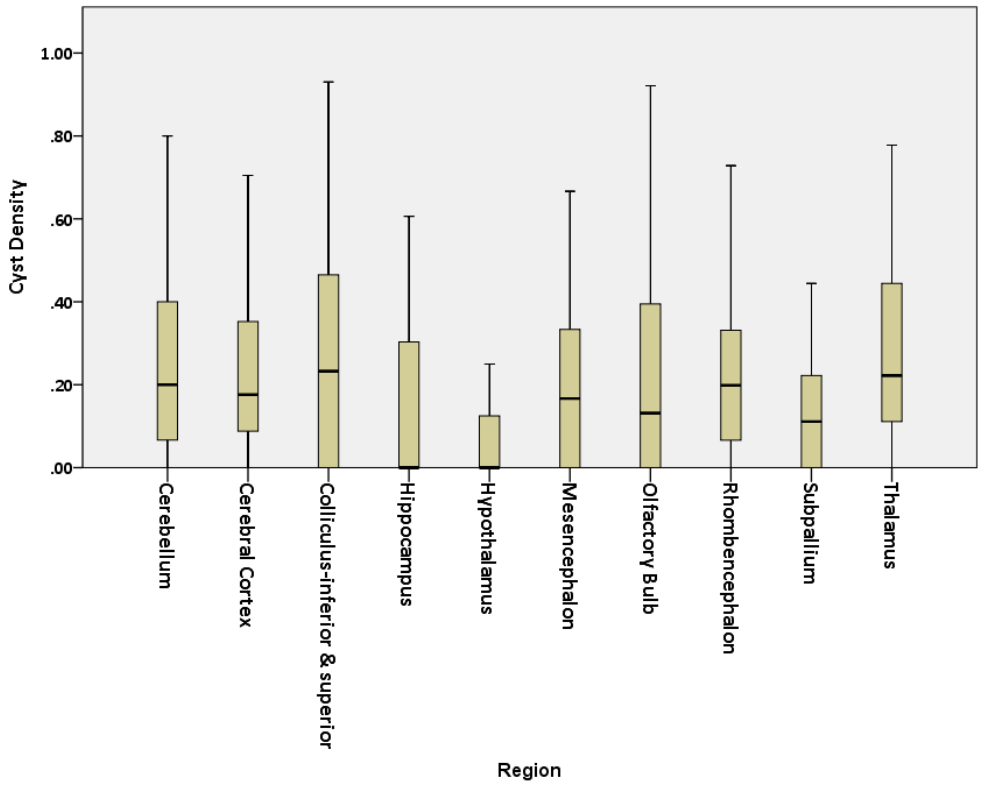

Figure 29: Boxplot of cyst distribution comparing the densities of cysts (median) with minimum, maximum and first and third quartiles (shaded) in each brain region.

	Regions	1	2	3	4	5	6	7	8	9	10
1	Cerebellum										
2	Rhombencephalon	0.030									
3	Colliculus	0.020	0.210								
4	Mesencephalon	0.010	0.130	0.550							
5	Cerebral cortex	0.280	0.780	0.140	0.010						
6	Hippocampus	0.000	0.010	0.490	0.370	0.000					
7	Hypothalamus	0.000	0.000	0.030	0.010	0.000	0.150				
8	Subpallidum	0.000	0.000	0.090	0.210	0.000	0.870	0.640			
9	Olfactory	0.010	0.110	0.470	0.650	0.080	0.230	0.000	0.020		
10	Thalamus	0.970	0.200	0.070	0.010	0.500	0.000	0.000	0.000	0.170	

Table 36: Cyst density vs. region using the Mann-Whitney test. *P*-values shown (cut-off for significance due to multiple testing is as per the Bonferroni correction of *p*-value of 0.0011 (0.05/45)).

	Strains	1	2	3	4	5	6	7	8	9	10	11
1	CT-1											
2	Me-49	0.001										
3	TgCat Co1	0.001	0.919									
4	GT-1	0.005	0.493	0.522								
5	TgCTPr-C3	0.000	0.000	0.000	0.000							
6	TgPigUS15	0.000	0.000	0.000	0.000	0.000						
7	TgRabbitBr1	0.293	0.007	0.005	0.038	0.000	0.000					
8	TgGoatUS4	0.000	0.562	0.465	0.206	0.002	0.000	0.002				
9	TgNmBr1	0.863	0.004	0.003	0.013	0.000	0.000	0.341	0.001			
10	TgBbUS1	0.193	0.11	0.097	0.273	0.000	0.000	0.549	0.038	0.19		
11	VEG	0.003	0.777	0.748	0.419	0.001	0.000	0.012	0.632	0.006	0.123	

Table 37: Cyst count vs. strains using the Mann-Whitney test. *P*-values shown (cut-off for significance due to multiple testing is as per the Bonferroni correction of *p*-value of 0.001 (0.05/55)).

Even with Bonferroni multiple testing correction establishing a cut-off of *p*-value <0.001 for significance (Table 37) statistically significant differences between cyst number and *T. gondii* strain type are still maintained. First, there is a statistical difference between strains CT-1 and TgCTPr-C3, TgPigUS15, and TgGoatUS4. Second, there is a statistically significant difference between strains TgCatCo1 and TgCTPr-C3 and TgPigUS15. Third, there is a difference in cyst number between strains GT-1 and TgCTPr-C3, and TgPigUS15.

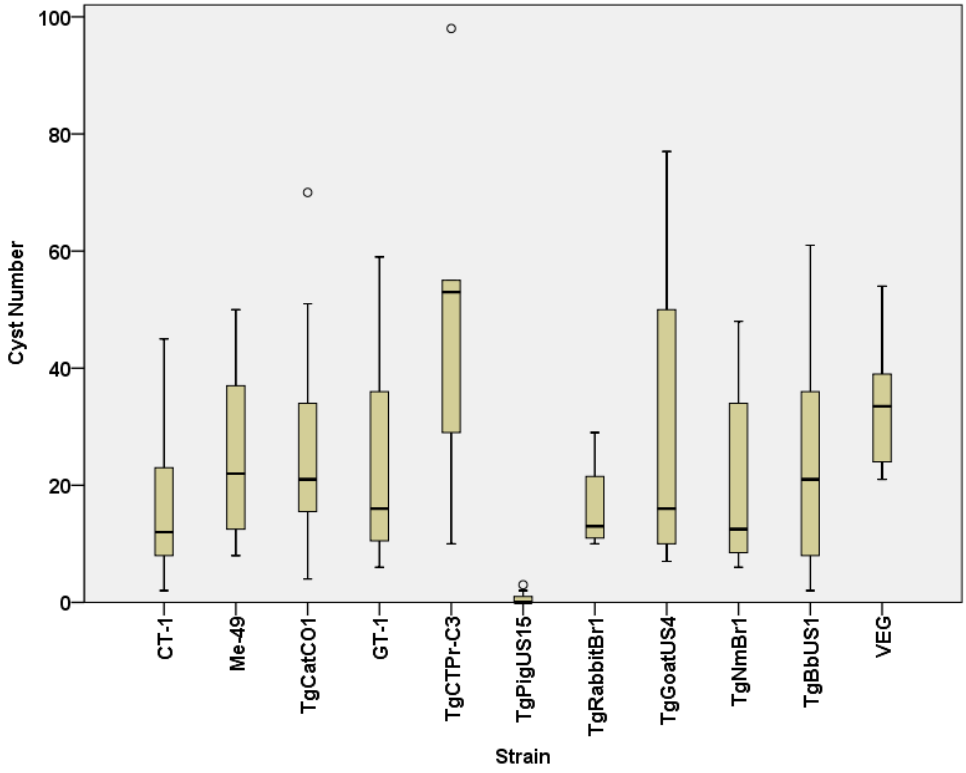

Figure 30: Boxplot of cyst distribution comparing the numbers of cysts (median) with minimum, maximum and first and third quartiles (shaded) in each strain. 107 infected rats with eleven strains with n=9-10 rats per strain.

4.4 Discussion

Whether cyst location has a consequential effect on host behaviour has remained unclear and hence the importance of this study. This study found some minor tropism in a few brain regions. One hypothesis has been that cyst location affects animal behaviour, such as parasites targeted to the amygdala could affect processing of the fear (Misslin, 2003). A study of fatal feline attraction suggested that this phenomenon is indeed arbitrated by the amygdala (House et al., 2011). One study that focused on the amygdala found that cysts accumulated in this region, however, most of the brain was invaded similarly by the parasite and tropism was marginally significant (Vyas et al., 2007). In the current study, 109 infected female rat brains were scored manually. The virtual scanned slides were a convenient method that was easy to access, and different magnifications were available to help identify the cysts and maintain bearing in the slices. The count was repeated three times for each slide to minimise user errors. Two slides (with two slices per slide) were absent of cysts. ImageJ was used to measure the pixel size of each region in an effort to fairly compare tropism for differently sized regions. Although SPSS was used in an attempt to normalise the data distribution, it remained skewed to the left. As a consequence, Kruskal-Wallis analysis was used as an alternative to one-way ANOVA. The analysis determined that the sample medians were different and found that the p value was < 0.0001 so there was a statistical difference between cyst distribution and brain regions overall. There are several key findings from this study. First, there were a statistically significant higher number of cysts (in cyst density) between the cerebellum and the hippocampus,

hypothalamus, and subpallium. Secondly, the rhombencephalon had a higher cyst density than the hypothalamus. Thirdly, the cerebral cortex was higher in cyst density with higher cysts number than the hypothalamus and subpallium. Fourthly, the thalamus (high cyst density) was greater than the hippocampus and hypothalamus. This study's findings match those of previous studies in terms of the higher number of cysts in the cerebral cortex, but did not observe the higher number in the hypothalamus and thalamus (Berenreiterova et al., 2011). This may be because of the limited number of animals that they studied (n=5) and their study used infected mice. However, new findings of higher numbers of cysts in the cerebellum and rhombencephalon could aid our understanding of the subtle and specific behavioural changes caused by *T. gondii*. Dopamine neurones are found within the rhombencephalon but not the cerebellum (Bjorklund and Dunnett, 2007). The cerebellum does have metabotropic glutamate receptors and utilises glutamate that could be involved in behaviour changes as GLT-1 was found altered with infection (David et al., 2016). Alternatively, the higher numbers may be areas that are more accessible for infection. The study also found that there was no significant difference in the number of cysts in any of the brain regions for ME-49 and VEG, which are the strains that are most commonly used in laboratory studies. Seeing that the amygdala connects to the thalamus, creating the amygdalofugal pathway, it remains plausible that there is a link between the location of *T. gondii* and the further progress of the disease, particularly, the progressing control of the fear-related functions in rats. Figure 28 showed the average number of cysts in rat brain regions. Cerebral cortex was the highest number of average cysts at 6 cysts. While

cerebellum and rhombencephalon showed an average of 4 cysts. Thalamus showed 3 cysts, olfactory bulb showed 2 while the remainder of the regions showed an average of one cyst only. In general the parasite was distributed in almost all regions.

It therefore seems that *T. gondii* has limited tropism towards the cerebellum, cerebral cortex, colliculus and thalamus although it is still unclear whether the route of infection, the dose, and parasite strain could be factors that affect the parasite distribution in the brain of the intermediate host; in this instance the rat. And definitive experiments, rather than correlations, are necessary to determine whether this tropism has any link to the behaviour changes observed. Indeed, if the behaviour changes are due to a host response such as the immune system then the parasite location may not be of great importance.

TgCtPRC3 which is an atypical strain contained the highest average number of cysts in the cerebral cortex amongst the strains. The highest average number in cerebellum and cerebral cortex was 8 produced by strain ME49, although this strain was not statistically different toward any specific region. While the lowest average cyst density in the cerebral cortex was observed with strain TgPigUS15. ME49 in a study by Afonso (2012) showed no specific preference toward a region. However, the cerebellum and cerebral cortex were largely encysted in their study. TgCTPr-C3 strain was higher in cyst number than any other strain. In contrast TgPigUS15 strain was the lowest of cyst number in comparison to all strains. Little, if anything, is known about most strains and their preferences.It has been shown that the

TgCTPr-C3 strain was lethal to mice and they died 5 days after given a large dose of the strain. It is an atypical strain which could explain the high number of cysts. TgPigUS15 has also been examined in mice and it was lethal regardless of the dose administered or the mouse breed (Dubey et al., 2012). This contrasts the pathology in rats highlighting differences between susceptibility in rats and mice where rats maintain a long-standing (i.e. lifelong) infection without pathology. The rats number, route of infection and stage of parasite could impact tropism. These findings are in line with Berenreiterova study (Berenreiterova et al., 2011).

It has been shown that *T. gondii* infection induces hypomethylation of the arginine vasopressin promoter in the amygdala (Hari Dass et al., 2012). Also the regulation of corticosterone (Lopez et al.,1999), serotonin (Herman et al.,2005), norepinephrine (Sara, 2015) and DA (Prandovszky et al., 2011), within the amygdala and cortex regions have an effect on behaviours such as emotions and fear. The immune response and inflammatory response are also potential causes of behavioural changes. In conclusion, this chapter has shown a limited tropism of *T. gondii* toward brain regions such as the colliculus. No tropism was found in the amygdala as in earlier studies with mice. It may indicates that *T. gondii* may have a role in provoking immune response and hence manipulate the intermediate host behaviour.

Chapter 5 General Discussion

The aims of this thesis were to investigate and shed light on the association between the zoonotic disease *T. gondii* and neurological disorders specifically PD and schizophrenia using two different approaches. Both diseases were associated in prior studies and have a potential link with *T. gondii* in the role of dopamine.

Possible interactions between *T. gondii* infection and PD considered were that either *T. gondii* infection speeds up the death of dopaminergic neurones with production of extra, unpackaged DA, which may enhance PD symptoms and dopaminergic neurone loss or, alternatively, infection might permit replacement of some of the loss of dopamine and dopaminergic neurones by production of extra dopamine, as has been observed (Prandovszky et al., 2011). PD is a complex neurological disease and genetics, gender, ethnicity and age are four important risk factors for PD. (Kalia and Lang, 2015).

Abnormal neurotransmitter levels are found in patients with other neurological diseases, such as schizophrenia, particularly dopamine, glutamate and gamma-aminobutyric acid (GABA); importantly, direct and indirect evidence has shown that *T. gondii* affects dopamine and other neurotransmitters in rodents and humans (Novotná et al., 2005; Prandovszky et al., 2011; Skallova et al., 2006). Miman et al. (2010) compared this finding with previous studies conducted on schizophrenia (Alvarado-Esquivel et al., 2006; El-Sahn et al., 2004; Zhu et al., 2007) which showed high seropositivity for *T. gondii.* The authors suggested that the

degeneration of dopamine production as well as non-dopaminergic system failure in PD and schizophrenia patients could be due to the production of dopamine by *T. gondii*. Hence, PD patients may develop levodopa-resistant symptoms that lead to motor complications. This suggestion by Miman et al. (2010) could be true; however, it is unclear whether *T. gondii* was mechanistically involved in PD as only seropositivity was detected and studies in this thesis do not support a link between *T. gondii* and PD. *T. gondii* is ubiquitous parasite with importance in human and animal health. Seropositivity of *T. gondii* in both healthy individuals and people affected by neurological disorders are of high importance and post a hazard to public health. *T. gondii* as a “neglected disease” requires more attention in terms of solving the parasite mechanism and developing an effective treatment for the chronic stage. My thesis design and techniques provided a meaningful tool for assessing *T. gondii*’s association with the neurological diseases PD and schizophrenia. The PD study found that *T. gondii* was found in both sets of samples (Leeds and Manchester) PD and healthy individuals. There was not a significant difference in seroprevalence. Generally practicing good hygienic measures appears to be the best option to minimize transmission of *T. gondii* to humans as no vaccine is available at the moment.

T. gondii clearly modifies the behaviour of its rodent intermediate hosts and the behavioural manipulation hypothesis is a central mechanism describing *T. gondii* transmission. In humans, schizophrenia has been linked with *T. gondii*. *T. gondii* may exacerbate schizophrenia symptoms. My study focused on this matter by examining mice with a mutation contributing to the risk of schizophrenia and its interaction with infection, and examining the

distribution of *T. gondii* cysts in the intermediate rat host since tropism toward certain brain regions could be related to pathology. It is important to note that no brain region was excluded with cysts found in all brain regions. Hence the parasite has access to all brain regions. Hence, location does not readily explain the specific behavioural changes observed in infected animals nor associations with schizophrenia.

In respect to *T. gondii* in humans, rats are a good model in terms of ocular toxoplasmosis. Lesions were found in eyes of 25% of infected rats in our publication (Dubey et al., 2016). It was similar to incidences that involved *T. gondii* contaminated water (Silveira et al., 2015) (Burnett et al., 1998). The most common site of clinical disease is the retina in human toxoplasmosis, however, other tissue may be infected without obvious signs. In some rat eyes, tissue cysts were seen in retinal tissue without inflammation; another similarity with humans. The rat model might be useful to further study the pathogenesis of human ocular toxoplasmosis (Dubey et al., 2016).

Previous studies found that the olfactory bulb and amygdala were highly infected in rodents. However, my data did not show tropism toward the amygdala (Vyas et al., 2007) or hippocampus (Berenreiterova et al., 2011). It is important to note that Vyas et al. (2007) used different mice and rat breeds, while Berenreiterova et al. (2011) conducted their study in mice. My study also used 11 different strains of *T. gondii*, while Vyas et al. (2007) used the Prugniaud strain only, a type II strain similar to ME-49 used in my study.

The route of infection and rat breed could influence the distribution of the cysts although this was not found in my samples and larger sample pools would be required for definitively addressing this question. Hence, further research associating pathology and neurological affects with parasite strain are needed especially as type II strains are found in humans and novel atypical strains have been identified (Su et al., 2012).

Studies conducted on *T. gondii* to investigate behavioural changes have focused either on the effect of brain cyst localisation or on the effect level of infection on neuronal cell biology such as neurotransmitter synthesis and gene expression. It is challenging to associate these with the role of *T. gondii* infection in the behaviour changes and the pathogenesis of psychiatric disorders. Direct and indirect mechanisms of infection such as immune responses, e.g. including the long-lasting glial cell activation and the recruitment of peripheral immune cells (Cotter et al., 2001), could be contributing to the role of *T. gondii* in neurological disorders.

Neuropathological studies have found that glial cells, specifically astrocytes, are affected in both toxoplasmosis and schizophrenia, reinforcing their involvement in the emergence of *T. gondii*-related disorders.

T. gondii could reach the brain within seven days post-infection and crosses the blood-brain barrier through a “Trojan horse mechanism”. More recently, it has been hypothesised that it crosses the blood-brain barrier in infected endothelial cells (Konradt et al., 2016). Once the CNS is invaded by the parasite, it is able to penetrate and replicate in all nucleated cells. The immune system then initiates the activation of microglia and astrocytes,

which results in pro-inflammatory cytokines. In animal models anti-inflammatory components were found to be infected, for example, 30% of microglial cells and 10% of neurons and astrocytes (Konradt et al., 2016). Activation of microglia and astrocytes correlates with the local production of cytokines, which contributes to immune cell enrolment. The effective control of parasite replication is thought to be mediated by cytokine signals (Courret et al., 2006).

The findings in this thesis support the concept that *T. gondii* infection impacts the brain and therefore can moderate behaviour. I found that Infected mice buried fewer marbles than the controls which suggests decreased anxiety although alternative anxiety testing did not find a difference with infection. Hence, treatment and screening for *T. gondii* infection could have therapeutic and diagnostic impact in schizophrenic patients.

Understanding the impact of *T. gondii* infection has direct application to screening for the parasite and preventing it from infecting humans and their infection sources, such as livestock and domestic animals, including cats. Educating healthcare practitioners about preventing the infection may help change practices during the tissue cyst and oocyst stages (Rybakowski et al., 2005). Screening policies are widely different between countries at the moment, and many guidelines for screenings procedures have been widely disseminated. These procedures could be affected by many factors, including sampling method, sample size and serological techniques used (Winstanley, 2011). If research can make progress in understanding the

effect of latent *T. gondii* infection, it can influence policies regarding resources devoted to improving screening methods and standardising screening policies between countries for this highly ubiquitous parasite.

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