Molecular Characterisation of *Toxoplasma gondii* and Development of Diagnostic Assay for Bradyzoites

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Abstract

Toxoplasma gondii (*T. gondii*) is a ubiquitous parasite that infects warm-blooded animals and humans. In humans, *T. gondii* causes encephalitis in AIDS patients, and there is no drug that can eliminate *T. gondii* infection. *T. gondii* specifically manipulates the intermediate host's behaviour favouring its transmission to the definitive feline host. Human *T. gondii* seropositivity has also been associated with mental disorders. *T. gondii* has two aromatic amino acid hydroxylases, TgAaaH (1 and 2), that convert phenylalanine to tyrosine, and tyrosine to L-DOPA, the latter being the rate-limiting step of dopamine biosynthesis. Based on this and elevated dopamine levels in brain tissue cysts and infected dopaminergic cells, it has been hypothesised that TgAaaH has a role in altering brain neuromodulation and potentially subsequently in the behavioural changes observed. As TgAaaH genes encode a signal peptide, the location of the enzyme was examined. TgAaaH was localised to outside the parasite both membrane-bound to parasites within the parasitophorous vacuole based on immunofluorescence, fractionation, and trypsin susceptibility of released parasites. Another possible role of TgAaaH in cyst wall generation was examined by testing for dopa-oxidase activity to convert L-DOPA to dopa-quinone. Dopa-oxidase activity was not detectable in infected fibroblasts, yet it remains possible that parasite produced L-DOPA is metabolised to dopaquinone by host cell enzymes such as within feline gut endothelial cells where oocysts are formed. This, and our finding that host cell dopa-decarboxylase is required for dopamine biosynthesis, suggest that the product of parasite-produced L-DOPA may be dependent upon the type of cell infected (ie. dopamine in catecholaminergic cells and dopaquinone in endothelial cells). The limitation of studying the bradyzoite stages of infection due to proliferation of tachyzoite stages was resolved by development of a new culture system with depleted tryptophan. This method was then applied to develop a novel throughput assay to identify bradyzoite inhibitors. The validity of this assay was evaluated using tachyzoite and bradyzoite specific inhibitors. This assay will help in finding an anti-toxoplasma drug for curing of infection.

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Abbreviations

2D	Two dimension
3D	Three dimension
α-7nACh	α-7 nicotinic acetylcholine
AAAH	Aromatic Amino Acid Hydroxylase
AIDs	Acquired Immune Deficiency Syndrome
AMA	Apical membrane antigens
BAG	Bradyzoites surface antigen
BSA	Bovine serum albumin
DA	Dopamine
DBA	*Dolichos biflorus* lectin
CD	Cluster of differentiation
DDC	Dopa decarboxylase
DMEM	Dulbecco's modified Eagle medium
DOPA	Dihydroxyphenylalanine
DRD	D1-like dopamine receptors
EDTA	Ethylenediaminetetraacetic acid
ELQ	Endochin-like quinolones
EM	Electron microscope
FBS	Fetal bovine serum
FITC	Fluorescein isothiocyanate
GABA	γ-aminobutyric acidic
GAD67	Glutamic acid decarboxylase 67
GFP	Green fluorescent protein
GRA	Dense granules proteins
HBSS	Hanks' balanced salt solution
HeLa	Henrietta Lacks cells
HFF	Human foreskin fibroblast cells
HPLC-ECD	High-performance liquid chromatography electrochemical

	detection
HS	Horse serum
HXGPRT	Hypoxanthine-xanthine-guanine phosphoribosyl transferase
IC50	Inhibitory concentration the cause 50% reduction of the parasite number
IDO	Indoleamine 2, 3-deoxygenize
IFNγ	Interferon γ
IL	Interleukin
MAG	Matrix antigen
MBTH	3-methyl-2-benzothiazolinone hydrazone
MICs	Micronemes
miRNAs	MicroRNAs
NMADA	N-methyl-d-aspartate receptor
OCD	Obsessive compulsive disorder
PCA	Perchloric acid
PBS	Phosphate buffered saline
PBS-T.	5% non-fat powdered milk containing 0.05% Tween-20
PBSTR	1X PBS + 0.05% Triton solution
PC12	Rat pheochromocytoma cells
PCR	Polymerase Chain Reaction
PS	Penicillin-streptomycin
PV	Parasitophorous vacuole
PBS	Phosphate buffered saline
RFP	Red fluorescence protein
RIPA	Radioimmunoprecipitation assay buffer
RON	Rhoptry neck proteins
ROP	Rhoptry proteins
RPMI	*Roswell Park Memorial Institute medium*
RT	Reverse Transcriptase-
SAG	Surface Antigen
SDS-PAGE	Dodecylsulphate- polyacrylamide gel electrophoresis
T25	T25-cm^2 vented flasks

TD-DMEM	Tryptophan depleted- Dulbecco's modified Eagle medium
TH	Tyrosine hydroxylation
TgAaaH	*Toxoplasma gondii* aromatic amino acid hydroxylase
T. gondii	*Toxoplasma gondii*
WB	Western Blot
WC	Whole cell

Chapter 1 Introduction

The Apicomplexa are a parasitic phylum, comprised of obligate intracellular eukaryotes, which can only reproduce inside the cells of their hosts. They are distinguished by a unique organelle known as an apical complex. Examples of apicomplexan parasites are *Plasmodium* spp., which cause malaria, *Toxoplasma gondii* (*T. gondii*), and *Cryptosporidium. T. gondii* an opportunistic pathogen of humans, with three common *T. gondii* strains associated with human infections: types I, II, and III (Howe et al., 1997; Aspinall et al., 2002; Ajzenberg et al., 2009). This thesis will focus on *T. gondii*, including its life cycle, latent infection and treatment.

T. gondii is a ubiquitous parasite that can infect all tested warm-blooded animals, including humans. Its human seropositivity is low for the majority of the planet, but in certain areas the seropositivity is as high as 95% (Dubey and Jones, 2008; Kamerkar and Davis, 2012; Centers for Disease Control and Prevention, 2015). *T. gondii* seroprevalence varies depending upon dietary habits and contact with felines, its definitive host (Kamerkar and Davis, 2012). The Centers for Disease Control (Atlanta, GA) (2015) stated that, in the U.S., more than 60 million people are *T. gondii* seropositive. In the United Kingdom, *T. gondii* seroprevalence is 20-40% in adults (The Advisory Committee on Microbiological Safety of Food, 2012; Ho-Yen, 2009). Epidemiological studies in Saudi Arabia from 1992 to 2013 showed that *T. gondii* seropositivity ranged between 21% and 53% (Ahmed, 1992; Yaneza and Kumari, 1994; Al-Qurashi et al., 2001; Al-Qurashi, 2004; Alqahtani and Hassan, 2012; Eisa et al. 2013).

In the Netherlands and the U.S., *T. gondii* is one of the top seven food-borne pathogens (The Centers for Disease Control, 2014 (Centers for Disease Control and Prevention, 2015; Kamerkar and Davis, 2012). The prevalence is highest in hot, humid climates and at lower altitudes. These environmental conditions may favour survival of *T. gondii* oocysts. Meerburg and Kijlstra (2009) predicted that climate change would increase the prevalence of *T. gondii* owing to the higher temperatures, drier summers, and shorter, wetter winters.

1.1 *Toxoplasma gondii* life cycle

The definitive hosts of *T. gondii* are felines, such as domestic cats. Felines become infected upon the ingestion of *T. gondii* tachyzoites, bradyzoites, or oocysts in the raw meat of intermediate hosts, such as mice or birds, (Dubey, 2002; Garcia, 2006) (Figure 1.1). *T. gondii* infections in farm animals can cause abortion, creating a major economic impact. For example, toxoplasmosis is the major cause of abortion in sheep (Louis and Kim, 2013). In the feline gut, the parasite reproduces sexually and the unsporulated oocysts shed in the faeces (Figure 1.1). Outside the definitive host, unsporulated oocysts transform into sporulated oocysts within 1–5 days (Dubey et al., 1970). Sporulated oocysts can survive in cool, moist soil for a year or longer (Frenkel, 1970). Furthermore, the definitive host get infected by ingesting the tachyzoites stage, sporulated and unsporulated oocysts.
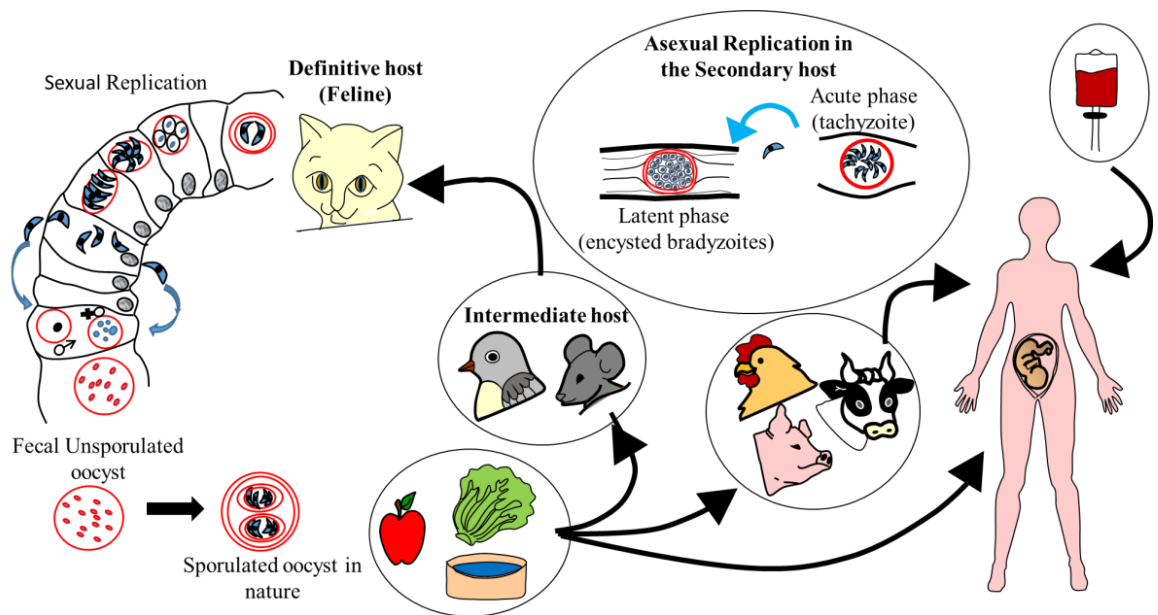
Figure 1.1. Life cycle and transmission of *T. gondii* (Adapted from Centers for Disease Control and Prevention, 2015)**.**

The secondary hosts of the parasite are all warm-blooded animals including humans (Louis and Kim, 2013) (Figure 1.1). Ingesting the sporulated oocysts from contaminated vegetables and water can infect the secondary host, as can eating raw meat that contains the cysts. In the secondary host, the parasite replicates asexually, and

then passes through the gut wall to other organs, such as the muscles and brain. The infection can also be transmitted congenitally from the mother to the foetus. Humans can also become infected by transfusion of contaminated blood that contains tachyzoites, or transplantation of organ tissue that contains a cyst (Louis and Kim, 2013).

1.2   *T. gondii* morphology and host invasion

This section will focus on the morphology and organelles of the *T. gondii* stages found in secondary hosts, and the known function of these organelles during infection.

In the secondary host both tachyzoites and bradyzoites are produced asexually (Figure 1.1). *T. gondii* can enter the host cell either by active penetration or by being phagocytised (Pulvertaft et al., 1954; Jones and Hirsch, 1972). As a member of the apicomplexan family, *T. gondii* has an apical complex in the anterior end of the parasite (Figure 1.2). The apical complex consists of a conoid structure, two polar rings, an internal microtubule, and the subpellicular microtubules that are associated with the inner membrane complex (Morrissette and Sibley, 2002). The conoid structure consists of two apical rings and a spiral of microtubules (Nichols and Chiappino, 1987) (Figure 1.2).

 There is a difference in the morphology between tachyzoites and bradyzoites but they have the same ultrastructure (Dubey et al., 1998). Tachyzoites have a crescent shape, while bradyzoites are more slender. The tachyzoite nucleus was found in the centre of the cell, and the amylopectin is either in discrete particles or absent. In bradyzoites, the nucleus was found toward the posterior end and contains several amylopectin granules. Bradyzoites rhoptries sometimes found looped back on themselves (Dubey et al., 1998).

When searching for the host cell and prior to penetration, the conoid of *T. gondii* extends, retracts, and rotates (Chiappino et al., 1984). As the parasite penetrates the host cell membrane, it forms a new subcellular compartment in the host cell, termed the parasitophorous vacuole (PV). The PV membrane mostly consists of host cell membrane (Jones and Hirsch, 1972). During invasion, the rhoptries and micronemes (MICs) are released into the nascent PV (Saffer et al., 1992; Dubremetz and Schwartzman, 1993; Sibley et al., 1995; Carruthers and Sibley, 1997). The MICs

contain microneme proteins and apical membrane antigens (AMAs) (Figure 1.2). MICs and AMAs assist the parasite in motility and in building the ring-like complex known as the moving junction, which it uses to attach to the host cell (Hehl et al., 2000; Huynh et al., 2003; Mital et al., 2005; Huynh and Carruthers, 2006; Kessler et al., 2008).

The *T. gondii* rhoptry (Figure 1.2) contains more than 30 proteins, which are divided into two groups: rhoptry proteins (ROP) and the rhoptry neck proteins (RON). As suggested by their name, RON are found at the neck of the apical tip and ROP are located in the body (Leriche and Dubremetz, 1991; Bradley et al., 2005). The RON and ROP that have been characterized so far are all kinases, phosphatases or proteases, and are generally specific to the Apicomplexa (El Hajj et al., 2006; Gilbert et al., 2007). However, many of the ROP physiological roles remain unknown (Bradley et al., 2005). As the parasite invades the host cell, the RON4 and AMA1 proteins form the moving junction interface between the parasite and host cell (Alexander et al., 2005; Lebrun et al., 2005).

Shortly after invasion, the dense granules (Figure 1.2) secrete their contents into the PV (Carruthers and Sibley, 1997). The dense granules contain nine proteins (GRA). After invasion, some of the GRAs form a stable complex with the PV membranes (Braun et al., 2008). During cell division, a single *T. gondii* apicoplast (Figure 1.2) is segregated into the nucleus, then replicates, maintaining one copy of itself per daughter cell (Striepen et al., 2000). Fichera and Roos (1997), showed that *T. gondii* plastid genome was reduced >10 folds during ciprofloxacin treatment compared to untreated parasite, and the parasite replication was inhibited. Studies showed that the division of ciprofloxacin treated parasite is inhibited immediately upon entry into the second host cell (Fichera and Roos, 1997; He et al., 2001). These studies demonstrate that the apicoplast is essential for the parasite to be infective.

Tachyzoites are the fast-replicating stage in the secondary host, during which the organism is between 2-6 μm long, and 1-5 μm wide (Dubey et al., 1998; Louis and Kim, 2013). This stage causes both acute and reactivated infection (Louis and Kim, 2013). After that, under pressure from the innate and adaptive immune responses of the host, the parasite forms slow-growing bradyzoites, which are 7 μm long and 1.5 μm wide, in soft tissues (Dubey et al., 1998; Louis and Kim, 2013). The bradyzoite cyst stage is associated with long lasting chronic toxoplasmosis.

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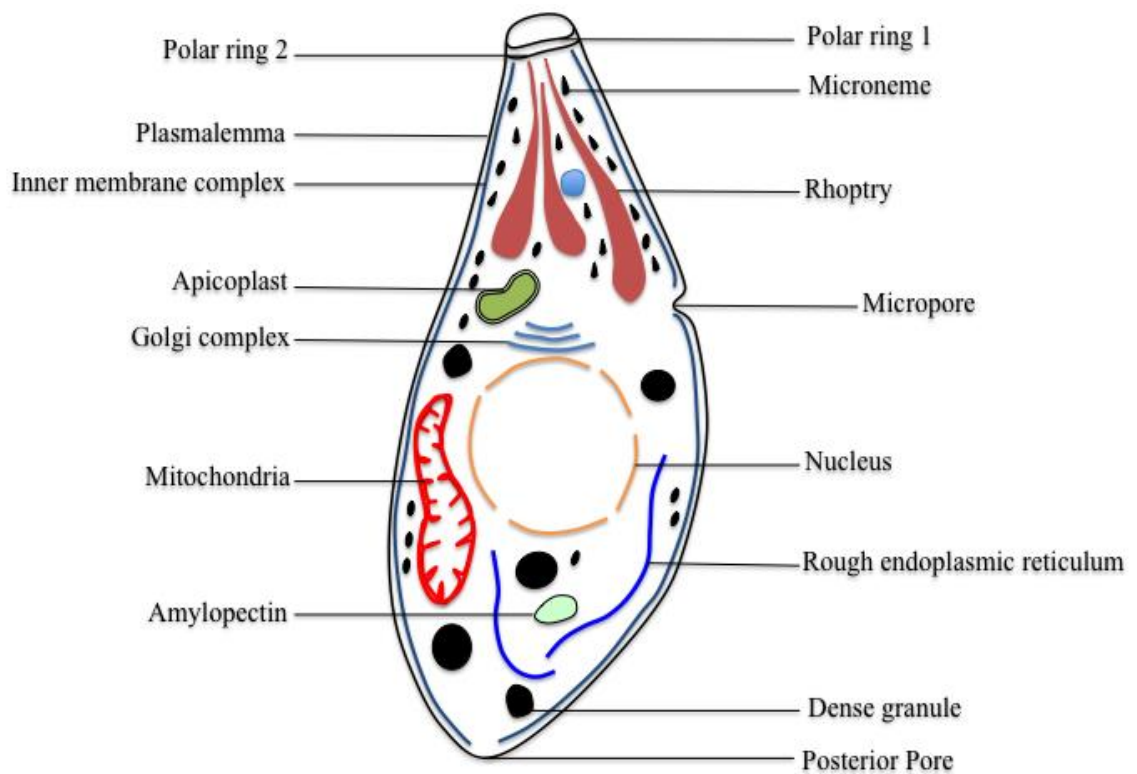
Figure 1.2. Schematic representation of the basic morphology of *T. gondii* tachyzoites and bradyzoites. The structure of *T. gondii* is composed of the following: nucleus, Golgi complex, mitochondria, apicoplast, rough endoplasmic reticulum, amylopectin, dense granules, micronemes, posterior pore, plasmalemma, inner membrane complex, and polar rings 1 and 2. This diagram was adapted from the schematic drawings of electron micrograph composites of *T. gondii* by Dubey et al. (1998).

1.3 *T. gondii* clinical disease

T. gondii causes acute and chronic toxoplasmosis in humans. Both of these stages are mostly asymptomatic in immunocompetent patients (Remington, 1974; Louis and Kim, 2013). In some patients, acute toxoplasmosis causes cold symptoms or, in rare cases, prolonged fever, fatigue, retinochoroiditis, painless cervical lymphadenopathy and seizures (Masur et al., 1978; Teutsch et al., 1979; Luft and Remington, 1984; Bowie et al., 1997; Benenson et al., 1982; Carme et al., 2009). In chronic toxoplasmosis, bradyzoite cysts are found in soft tissues such as the lungs and brain (Louis and Kim, 2013). Immunocompromised humans with latent toxoplasmosis can suffer from meningoencephalitis and mental complications (Flegr et al., 2003).

Immunocompromised patients, such as those with Hodgkin's and non-Hodgkin's lymphoma, leukaemia, solid tumours, Acquired Immune Deficiency Syndrome (AIDS), or collagen vascular disease, and post-organ transplant patients can experience acute or reactivated toxoplasmosis that is often associated with diffuse encephalopathy, retinochoroiditis, meningoencephalitis, and cerebral mass lesions (Louis and Kim, 2013). Immunocompromised patients infected with *T. gondii* may also suffer from altered mental state, motor impairment, seizures, abnormal reflexes, and other neurological symptoms such as disorientation, anxiety, depression and psychosis (Arendt et al., 1999; Louis and Kim, 2013).

Pregnant females infected with *T. gondii* can suffer from abortion or stillbirth (Louis and Kim, 2013). Congenital toxoplasmosis is severe when the mother acquires the infection in the first trimester, and the risk of transmission increases during the last trimester (Dunn et al., 1999). Infected new-borns may suffer from retinochoroiditis, cerebral calcification, and less commonly, hydrocephalus and microcephalus (Louis and Kim, 2013).

1.4 *T. gondii* host behavioural change

The manipulation hypothesis phenomenon, is phenomenon where certain parasites can alter the host behaviour for their own selective benefit to survive (Poulin, 1995; Thomas et al., 2005; Biron et al., 2005). The benefit of this manipulation is that the parasite can reach the definitive host, where it replicates sexually and ensures continuation of the parasite genes. An example of manipulation hypothesis phenomenon have seen in trematode *Dicrocoelium dendriticum*, that induces infected ants to 'freeze' at the top of a blade of grass at dusk, enhancing their consumption by livestock where it reproduce. Another example is *Euhaplorchis californiensis* that alters the behaviour of the California killifish to increase the likelihood of its consumption by birds, which is definitive host (Lafferty and Shaw, 2013). Several studies, summarized in the following section, have demonstrated that *T. gondii* can alter the intermediate host behaviour to increase the chances of transmission to cats, the definitive host, in order to complete the parasite life cycle (Figure 1.1).

1.4.1   Behavioural changes in animals

Several studies in mice and rats investigated *T. gondii*'s behavioural effects. Studies of mice infected with *T. gondii* demonstrated that learning capacity and memory were decreased compared to uninfected mice, which could be due to the loss of the recognition of new or recent stimuli (Piekarski et al., 1978; Witting, 1979). Chronically infected mice demonstrated deficient motor coordination and sensory response (Gulinello et al., 2010). Studies showed that naturally infected wild rats either became more active (Webster, 1994) or remained longer (*p*= 0.0001) in new and unprotected areas compared with uninfected rats (Webster et al., 2006). Hrdá et al. (2000) demonstrated an increased peak reaction time in *T. gondii* infected mice. Vyas et al. (2007a) observed that mice infected by *T. gondii* showed a greater reduction of learning ability than infected rats. In a review by Webster (2007), it was suggested that the higher invasion of *T. gondii* in the brains of mice than in rats during the latent infection stage could be associated with the decreased learning capacity.

Wild rats are neophobic, which means that they are innately sensitive to new stimuli, a trait that helps them avoid capture or being poisoned. It is well documented that both wild and laboratory rats have an innate aversion to cats (Blanchard et al., 2001; Dielenberg et al., 2001). Conversely, *T. gondii* infected wild rats showed indifference to new stimuli, which subsequently made them less responsive to cat sounds, scent, and sight (Webster, 1994). Further, Berdoy et al. (2000) showed *T. gondii* infected rodents were attracted to an area treated with cat scent more than an area treated with non-predator rabbit urine; the opposite of the reaction observed in uninfected rodents. Vyas et al. (2007a) also showed that *T. gondii* infected rats and mice were attracted to cat urine. All of these behavioural changes make it easier for cats to capture infected mice and rats, thus completing the parasite's life cycle by infection of the definitive host, cats, and allow subsequent reproduction of the parasite (Figure 1.1).

Meanwhile, some researchers have speculated whether these behavioural changes in rodents were specifically due to *T. gondii* infection or if they were a general reaction to illness. Webster et al. (1994) demonstrated that the loss of neophobia in infected rats had no relation to sex, weight, age, hunger or general body condition. Another study compared infections by *T. gondii* and other pathogens, including *Leptospira* spp., *Cryptosporidium parvum, Coxiella burnetti, Hymenolepis nana,* and *Syphacia muris* in

infected and uninfected rats, and the results showed that only the rats infected with *T. gondii* demonstrated higher activity levels than uninfected animals (Webster, 1994).

In (1995), Berdoy et al., showed that social status and mating success did not change in rodents that were congenitally infected with *T. gondii*. This result led to the conclusion that *T. gondii* affects the host in specific ways that benefit the parasite life cycle (Berdoy et al., 1995). Vyas et al. (2007a) observed changes in innate aversion, anxiety, and learned fear during *T. gondii* infection in rats, since the neuronal circuits involved in these three traits overlap in the brain. The same study also showed that *T. gondii* infected rats learning fear, anxiety-like behaviour, olfaction or non-aversive learning had no or very little change. Furthermore, the infected laboratory rats had higher loss of aversion to a moderate amount of cat urine (1 mL) than smaller or larger amounts (0.5 through 2.5 mL) (Vyas et al., 2007a). All these studies demonstrated that the parasite's ability to affect behavioural change is specific and not due to generalised illness (Berdoy et al., 1995; Vyas et al., 2007a; Vyas et al., 2007b).

1.4.2 Behavioural changes in humans

As previously mentioned, latent or acquired toxoplasmosis in immunocompromised patients can cause mental and neurological disorders. Human *T. gondii* seropositivity have been linked with mental disorders including schizophrenia, Parkinson's disease, obsessive compulsive disorder (OCD), and Tourette's syndrome. Celik et al. (2010) found no significant difference in *T. gondii* seropositivity between Parkinson's patients and healthy individuals. However, in the same year, Miman et al. (2010) found that *T. gondii* seropositivity was 19% higher in patients with Parkinson's compared to healthy individuals ($p = 0.006$). Meanwhile, both Carrazana et al. (1989) and Murakami et al. (2000) study showed that the symptoms of Parkinson's disease were reduced in two *T. gondii* positive AIDS patients when treated with anti-parasitic drugs. Further supporting this link, anti-parasitic treatment reduced the symptoms of *T. gondii* seropositive OCD patients (Brynska et al., 2001).

In 2010, links between *T. gondii* infection and personality disorders were evaluated in 896 psychiatric patients the primary diagnoses of schizophrenia, major depression, schizoaffective, or bipolar disorder. These patients were compared to 214 psychiatrically unaffected controls (Hinze-Selch et al., 2010) and it was found that personality disorder was significantly associated with *T. gondii* infection in people aged

more than 45 ($P=$ <0.001) and individuals who consumed raw or undercooked meat ($P=$ 0.05) (Hinze-Selch et al., 2010). Meanwhile, a study of 36 Tourette's syndrome patients found that they had higher levels of *T. gondii* antibodies compared to 30 healthy individuals ($P<0.07$) (Krause et al., 2010). Furthermore, *T. gondii* immunoglobulin G seropositivity was found to be higher in Alzheimer's patients ($p=$ 0.005) (Kusbeci et al., 2011). A large study by Pearce et al. (2012) linked bipolar disorder and *T. gondii* infection (n=41; adjusted OR: 2.4; 1.2– 4.8; $p < 0.05$), but established no link between the parasite infection and history of major depression (n=574- OR 0.8), severe major depression (n = 515- OR: 0.8), dysthymia (n= 548- OR: 1.1), or dysthymia with comorbid major depression (n =242- OR: 1.2), these results had a *p* values were > 0 .05. In (2014), Dickerson et al. found a link between *T. gondii* IgM antibodies and low cognitive functioning in bipolar disorder patients (n= 347) and individuals without a psychiatric disorder (n= 352). On the other hand, Fond et al. (2015) demonstrated that giving toxoplasmosi treatment to *T. gondii* positive bipolar patients helped reduce their depression compared to similar patients who did not receive anti-toxoplasmosis treatment.

Studies have shown that latent toxoplasmosis in immunocompetent patients causes behavioural alterations similar to those in rodents, namely, altered personality (Flegr et al., 1996; Flegr, 2007) and increased reaction times in simple computer reaction tests (Havlicek et al., 2001). In addition, patients with latent toxoplasmosis were found to have 2.65 times more at risk of being involved in a car accident compared to non-infected individuals (Flegr et al., 2002; Flegr et al., 2009). Increased reaction time in *T. gondii* patients could explain the higher risk of being involved in car accidents, although this hypothesis was not formally tested.

Several studies have established a link between suicide and *T. gondii* seropositivity. Arling et al. (2009) found that *T. gondii* seropositive patients with recurrent mood disorder who attempted suicide (n=99) had higher *T. gondii* antibody titres than those who did not attempt suicide (n=119) ($p < 0.004$). A study in Turkey also linked history of suicide attempts with *T. gondii* seropositivity ($p = 0.004$) (Yagmur et al., 2010). A strong association between suicide in postmenopausal women and *T. gondii* infection was also found ($P < 0.05$) (Ling et al., 2011). Other studies also linked *T. gondii* seropositivity with schizophrenia (Yolken et al., 2001; Torrey and Yolken, 2003;

Niebuhr et al., 2008). Yolken et al. (2001) found that first-episode schizophrenia patients (n=38) had increased levels of immunoglobulin (Ig) G, M, and A to *Toxoplasma* proteins compared to healthy control subject (n=27) ($p < 0.02$).

A meta-analysis of 42 papers from 15 countries demonstrated a link between *T. gondii* antibody measurement and schizophrenia, and indicated that *T. gondii* infection has a stronger association with schizophrenia than genetic or environmental factors (Torrey et al., 2007). Another study found that schizophrenia patients undergoing antipsychotic treatment had lower IgG antibody levels to *T. gondii* compared to non-treated patients and non-schizophrenic controls (Leweke et al., 2004).

T. gondii brain infection and schizophrenia affect similar populations of glial cells, particularly astrocytes that are lost (Cotter et al., 2001). In addition, hydrocephalus and increased ventricular size have been seen in foetal *T. gondii* infections and schizophrenia patients (Torrey and Yolken, 2003). Meanwhile, children whose mothers had elevated IgM for *T. gondii* shortly before birth later developed schizophrenia (Torrey and Yolken, 2003). All these studies support the suggestion that *T. gondii* infection is linked with human mental disorders. The question is how?

1.5   Proposed mechanisms for the behavioural changes

The studies summarised in section 1.4.1 provided evidence that *T. gondii* manipulates the intermediate host for its own benefit. But, the mystery is how *T. gondii* infection causes changes in the intermediate host behaviour. A review by McConkey et al. (2013), proposed that the parasite could be expected to have a gene or genes to aid it in conducting this behavioural change and increase the chances of predation of the intermediate host by a cat. On the other hand, Webster (2007) proposed that *T. gondii* might affect the behaviour either through the host immune response (1.5.1), the histopathological location (section 1.5.2) of the parasite in the brain*,* and/or neuromodulatory changes (section 1.5.3) during infection.

1.5.1   Immune response

This section will start with a brief summary of the immune response against *T. gondii* infection, and then will discuss the studies that support the suggestion that the immune response to *T. gondii* could alter the host behaviour.

T. gondii commonly causes human infection through the ingestion of contaminated food. In the intestine, the parasite crosses the intestinal epithelium to infect enterocytes. The infected enterocytes go through physiological and morphological disturbances, and can secrete cytotoxic molecules such as nitric oxide (Yap and Sher, 1999). After replication of the parasite, the host enterocyte ruptures and parasites are released. Then the parasite disseminates to other tissues by infecting the circulating host macrophages or CD11c+ dendritic cells (Da Gama et al., 2004; Courret et al., 2006). The initial immune response to the infection involves the release of interleukin 2 (IL-2) from macrophages, neutrophils and dendritic cells. IL 2 will result in the activation T cells, and release of interferon γ (IFNγ), and activation of CD8+ killer cells (Gazzinelli et al., 1993; Pfefferkorn and Guyre, 1984; Munoz et al., 2011). Maes et al. (1994) showed that patients with major depression have high INFγ compared to normal control subjects. However, there was no significant relationship between *T. gondii* infection and major depression (Pearce et al., 2012) (see section 1.3.2).

One of the host immune responses to suppress *T. gondii* and to keep it dormant is through an increase in cytokine levels such as interferon (Figure 1.3). During brain infection, IFNγ is produced to activate macrophages and lymphocytes (Denkers and Gazzinelli, 1998). IFNγ production lowers tryptophan levels by the activation of indoleamine 2, 3-deoxygenize (IDO), causing tryptophan starvation (Pfefferkorn et al., 1986) (Figure 1.3). Low tryptophan level might affect serotonin level, since tryptophan hydroxylation is the rate-limiting step for serotonin biosynthesis (Walther et al., 2003), Meanwhile, study by Söderpalm et al. (1989) showed that serotonin receptor antagonists (buspirone , gepirone, ipsapirone, 8-OH-DPAT, and L-5-HTP) lowered anxiety in rats during exploring a new environment in open spaces. Their result might give a clue on how IFNγ production and tryptophan starvation are linked to the loss of anxiety in rats (through serotonin depletion) during *T. gondii* infection. On other hand, Xiao et al. (2014) found an increased serotonin level in the brain during acute toxoplasmosis in mice. The increase in serotonin could be due to the cytokines IL6 and IL1 being produced in the brain by the host immune response and increasing tryptophan (Figure 1.3) (Dunn, 1988; Wang and Dunn, 1998).
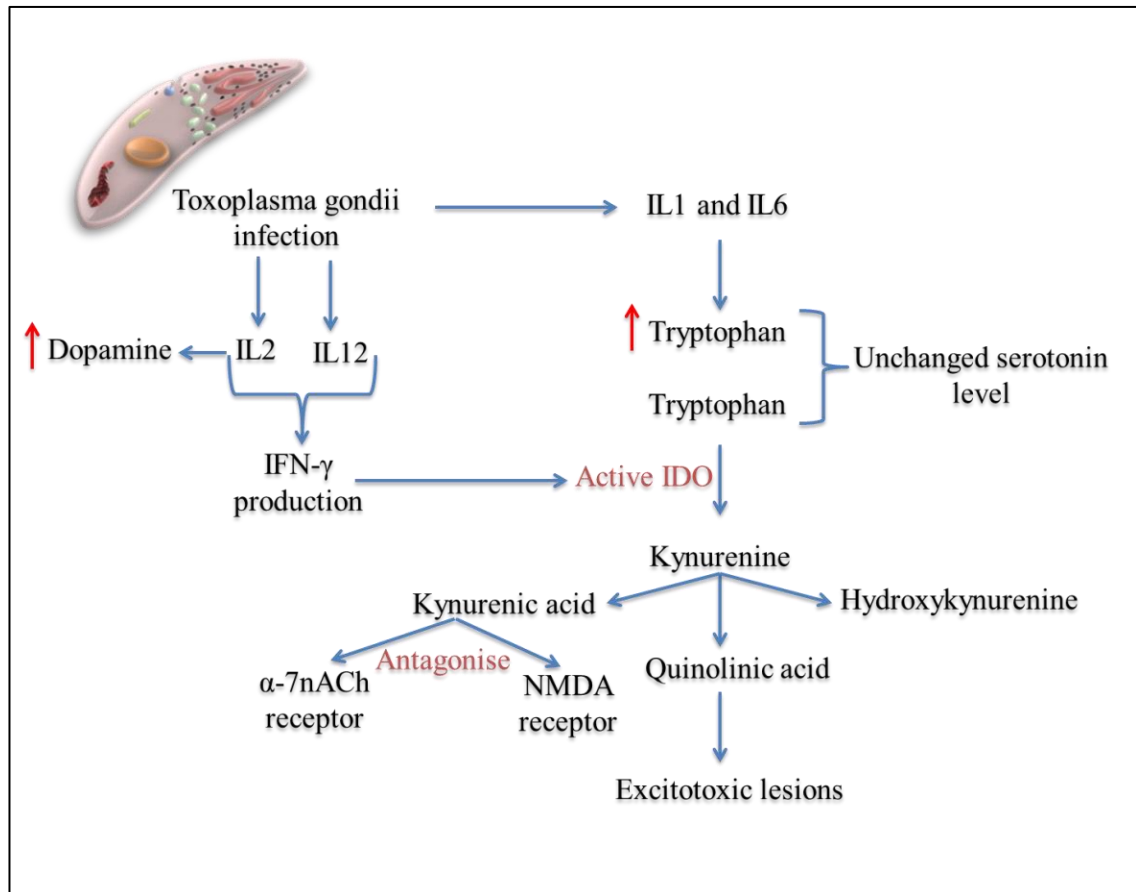
Figure 1.3. Brain *T. gondii* infection and immune response. IL2, which is secreted as a response to *T. gondii* infection, has been found to increase brain DA levels. INF-γ will then be produced, which will activate IDO, which breaks down tryptophan to kynurenine, then kynurenine can be degraded to kynurenic acid, quinolinic acid, and hydroxykynurenine. Kynurenic acid blocks both NMDA and α-7nACh. Quinolinic acid is known to cause brain lesions.

IDO catabolises tryptophan to kynurenine (Pfefferkorn et al., 1986); then, kynurenine is degraded into hydroxykynurenine and either quinolinic acid or kynurenic acid (Figure 1.3) (Schwarcz, 2004). Schwarcz et al. (1983) found that intracerebral injections of quinolinic acid in rats caused excitotoxic lesions (Figure 1.3). Meanwhile, kynurenic acid is an antagonist for excitotoxic N-methyl-d-aspartate (NMDA) receptor and α-7 nicotinic acetylcholine (α-7nACh) (Figure 1.3) (Schwarcz and Pellicciari, 2002). NMDA and α-7nACh receptors are underlie learning, memory, and other manifestations of synaptic plasticity (MacDonald et al., 2006; Albuquerque et al., 2009). Sathyasaikumar et al. (2011) demonstrated that kynurenine 3-monooxygenase, which converts L-kynurenine to hydroxykynurenine in microglial cells, has lower expression levels and enzymatic activity in schizophrenic patients.

Waguespack et al. (1994) found that IL-2 can cross the blood-brain barrier. Furthermore, insertion of IL-2 into either the frontal or the central region of mouse brains causes novelty-induced locomotion, and increased activity during maze training (Petitto et al., 1997; Zalcman, 2001), which is similar to the behaviour observed in *T. gondii*-infected rats. It has been established that IL-2 level was elevated in the cerebrospinal fluid of schizophrenia patients, and it was suggested that IL-2 might increases increased dopaminergic neurotransmission (Figure 1.3) (Licinio et al., 1993). It has been found that IL-2 increases climbing behaviour in mice, and it is linked to DA D1 and/or D2 receptor (Zalcman, 2002). Furthermore, schizophrenia patients treated with haloperidol have reduced levels of IL-2, and IL-2 levels increased when haloperidol treatment was stopped (McAllister et al., 1995).

Nevertheless, the exact mechanisms connecting toxoplasma brain infection, IL-2, and increased DA are not clear. This also applies to *T. gondii* infection, IL-2, increased DA in the brain, and behavioural changes. Do all pathogens with some link to schizophrenia affect IL-2? This would contradict the finding that the altered behaviour prompted by *T. gondii* is specific to completing its life cycle (in section 1.3.1). The studies in this section show the indirect immune response during *T. gondii* infection, but didn't fully explain how the infection causes specific behavioural alteration.

1.5.2   Histopathological location of *T. gondii* brain cyst

T. gondii cysts in the brain induce a granulomatous inflammatory response, progressive depositions of necrotic material, and subsequent vesicular occlusion, which may influence neuronal function or cause neurodegeneration (Werner et al., 1981). This section includes localisation studies that support the hypothesis that *T. gondii* can alter the intermediate host behaviour as a result of the its location within the host brain.

Hematoxylin and eosin staining of *T. gondii* cysts in chronically infected rat brains showed that the amygdala had a high density of cysts (Figure 1.4) (Vyas et al., 2007a). Hermes et al. (2008), using immunostaining of chronically infected *T. gondii* mouse brains, found increased cyst numbers in the cortex and the diencephalon, the region which contains the thalamus ($p= 0.0012$). Other studies have found that *T. gondii* infects most of the brain sections, and there are other brain areas with high cyst numbers. Gonzalez et al. (2007) found that *T. gondii* cysts predominantly ($p < 0.05$)

invaded the limbic areas. Also, this study found the largest parasites cyst and clusters in the nucleus accumbens and ventromedial hypothalamic nucleus ($p < 0.05$) (Figure 1.3)(Gonzalez et al., 2007).

Using bioluminescence imaging technology, Di Cristina et al. (2008) localised *T. gondii* that express luciferase under the control of a bradyzoite-specific promoter in mouse brains. They found that there were luminescent clusters in the cerebral cortex, colliculi, cerebellum and olfactory bulbs (Figure 1.4) (Di Cristina et al., 2008). Another study found a higher accumulation of cysts in olfactory bulb, the entorhinal, somatosensory, motor and orbital, frontal association and visual cortices, and, importantly, the hippocampus ($P < 0.001$) (Figure 1.4) (Berenreiterova et al., 2011).

The broad distribution of locations where *T. gondii* cysts may form within the host brain raises the question of whether cyst formation functions to directly affect behaviour by interfering with functions of specific areas, or if there are more general mechanisms at play. For instance, effects on fear-based behaviours and anxiety may be mediated by cysts in regions such as the amygdala and the limbic system of the hypothalamus, which plays a role in natural anxiolytic mechanisms. Blanchard and Blanchard (1972) showed that rats with lesions in the amygdala lack fear of cats ($p < 0.001$), which is similar to the behaviour observed in *T. gondii*-infected rats, as mentioned in section 1.4.1. Meanwhile, the retro hippocampus subiculum and entorhinal cortex regions of the nucleus accumbens play a role in psychomotor behaviour, and it is known that altered brain psychomotor are involved in the aetiology of schizophrenia (Chiarenza et al., 1985; Gray et al., 1991). The hippocampus is involved in anxiety, learning, and memory (Prandovszky et al., 2011). In contrast, Gulinello et al. (2010) observed behavioural change ($p < 0.005$) in chronically infected mice without major brain damage or cognitive dysfunction (see section 1.4.1). This contradicts the finding that *T. gondii*-associated behavioural changes are caused solely by general types of brain damage resulting from encystation, and supports the suggestion of the manipulation hypothesis, which says that the behavioural changes in the intermediate host are moderated by a parasite-specific effect.
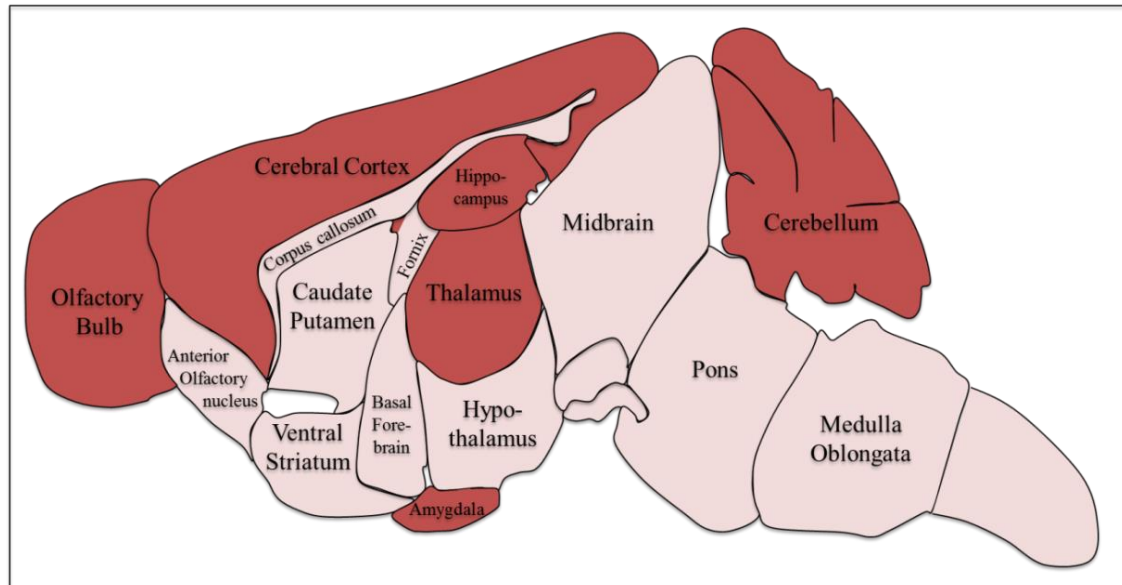
Figure 1.4. *T. gondii* **cyst localization in murine brain.** Schematic representation of a midsagittal section of a murine brain, showing *T. gondii* cyst location throughout the brain during chronic toxoplasmosis. The highest number of *T. gondii* cysts was in the olfactory bulb, cerebral cortex, hippocampus, thalamus, cerebellum and amygdala (highlighted in dark red). (Studies that provided data for this figure are Gonzalez et al., 2007; Vyas et al., 2007a; Di Cristina et al., 2008; Berenreiterova et al., 2011).

Electron microscopy (EM) of the brains of chronically infected mice, aged three, six, and twelve months, found that *T. gondii* cysts were mostly present in the neurons (~90%) (Ferguson and Hutchison, 1987a). Meanwhile, Sims et al. (1989) demonstrated that the cysts were not found in neuroglial cells in the brains of chronically infected mice using EM. Gonzalez et al. (2007) stained the cyst with hematoxylin and eosin and found it in neurons and astrocytes adjacent to neuronal nuclei. Melzer et al. (2010) studied the location of *Toxoplasma* cysts in neuron and astrocytes using immunostaining. The cyst wall was stained with fluorescein isothiocyanate (FITC) with *Dolichos biflorans*, the neurons was detected using antibody to microtubule associated protein, and the astrocytes was detected using antibody to the astrocyte specific intermediate type cytoskeletal protein. They found that *T. gondii* cyst found mostly in neurons (57 cysts; 33 positively identified as neurons) and astrocytes in *T. gondii*-infected mice brains. The same study also found astrocyte interactions with neuronal cysts. Finding *T. gondii* in neuronal cells of infected brains raises the possibility that direct neuromodulation can result in host behaviour changes.

1.5.3 Neuromodulation mechanism

One of the mechanisms proposed to explain *T. gondii*'s behavioural effect is altered DA regulation (Stibbs, 1985; Torrey and Yolken, 2003; Webster et al., 2006; Gaskell et al., 2009; Prandovszky et al., 2011). As mentioned before in section 1.4.2 several studies linked *T. gondii* infection with human neurological diseases such as schizophrenia (Torrey et al., 2007). Both schizophrenia and *T. gondii* infections are characterised by high DA levels (Stibbs, 1985; Torrey and Yolken, 2003).

Both, *T. gondii* replication and invasion were inhibited by the antipsychotic medication haloperidol and the mood stabilizer valproic acid, according to Jones-Brando et al. (2003), who suggested that this inhibition was due to prevention of calcium entry into the cells, which is needed by the tachyzoites for invasion (Jones-Brando et al., 2003). This inhibition could also be due to direct antipsychotic action on the D2 DA receptor (Seeman et al., 1997). Another study showed a reduction in altered behaviour among four groups of *T. gondii* infected rats that were treated with either haloperidol or valproic acid combined with pyrimethamine and dapsone, standard anti-*T. gondii* agents used in rodents and humans (Webster et al., 2006). All experiment rat were euthanased, and fluorescence staining of their brain showed that the number of immunohistochemically *T. gondii* positive neurons and ganglia were decreased, especially with haloperidol treatment (Webster et al., 2006).

Another study demonstrated that treating chronically *T. gondii*-infected mice with vanoxerine, a DA uptake inhibitor (GBR-12909, 1-[2-[bis (4-fluorophenyl) methoxy] ethyl]-4-(3-phenylpropyl) piperazine), prevented the behaviour alteration of reduced board hole exploration in infected males, compared to control infected mice (Skallova et al., 2006). These studies demonstrate that *T. gondii* affects the intermediate host by affecting DA levels in the brain.

In (1985), Stibbs used high-performance liquid chromatography (HPLC) to study the concentrations of DA, homovanillic acid, and norepinephrine in the brains of mice acutely and chronically infected with a virulent type I strain of *T. gondii*. In the acute stage, homovanillic acid levels increased by up to 40%, while norepinephrine was reduced by as much as 28%. No change was found in DA levels during the acute stage. Meanwhile, in chronic toxoplasmosis, the total brain DA level was elevated by 14%

relative to that in uninfected mice, although there was no change in serotonin levels (Stibbs, 1985). It is worth mentioning that an altered brain dopamine (DA) level during latent *T. gondii* infection was found by only one group (Stibbs, 1985) and this finding has not yet been reproduced. The change in DA levels during the chronic stage were not found in mice that were congenitally infected with the less virulent type III strain of *T. gondii* (Goodwin et al., 2012).

Tyrosine hydroxylase is the rate-limiting step in production of DA and catecholamines. TH transforms tyrosine into L-dihydroxyphenylalanine (DOPA) (Figure 1.5) (Flatmark and Stevens, 1999). Then, DOPA-decarboxylase (DDC) in dopaminergic neurons transforms L-DOPA into DA (Figure 1.5). Gaskell et al. (2009) found that *T. gondii* have two nearly identical genes that encode two aromatic amino acid hydroxylase enzymes, TgAaaH 1 and 2. These enzymes catalyse the hydroxylation of both phenylalanine and tyrosine (Figure 1.5). TgAaaH 1 and TgAaaH 2 has to two- to three-fold affinity for tyrosine (Gaskell et al., 2009). On the other hand, the parasite enzyme was unable to convert tryptophan to serotonin, so it does not have tryptophan hydroxylase activity.

Gaskell et al. (2009) also found that TgAaaH 1 is expressed constitutively, whereas TgAaaH 2 expression increases during bradyzoite conversion. The differences in gene expression raise the question of whether the product(s) of TgAaaH 1 and TgAaaH 2 are each needed specifically in the tachyzoite or bradyzoite stage. Knockout of TgAaaH 2 genes in *T. gondii* did not affect the parasite growth, but TgAaaH 1 and double-knockout mutants could not be isolated (Wang et al., 2015). Hence, TgAaaH may have multiple functions during infection. Wang et al. (2015) found the levels of DA was not change during PC12 infection. These cells were stressed with high pH disabling the host machinery and direct measures of enzyme activity were not performed.

The TgAaaH enzyme genes are predicted to encode a signal peptide, which could be involved with transporting or secreting the enzyme (Gaskell et al., 2009). Prandovszky et al. (2011), demonstrated through immunostaining using DA antibodies and tyrosine hydroxylase antibodies that the cysts in the brains of mice chronically infected with *T. gondii* had high levels of DA and TgAaaH. The staining was not disrupted by

competition from exogenous serotonin. Also, it was found that infected dopaminergic neuronal cells produced a hundredfold more DA than non-infected cells.

A study by Martin et al. (2015), showed that the levels of DDC (also known as aromatic-L-amino-acid decarboxylase) did not change in *T. gondii* infected neuronal cells. Also, staining infected mouse brain tissue and neuronal cells with anti-DDC antibodies demonstrated that DDC was found within the intracellular parasite cysts. All these studies demonstrate that *T. gondii* has the ability to increase DA, which might alter dopaminergic neurotransmission and subsequently result in altered host behaviour. However, the level of DA that will significantly affect the host neural processes remains unknown.

Xiao et al. (2014) studied the regulation of microRNAs in *T. gondii* infected human neuroepithelioma, and in mouse brains with acute infection. MicroRNAs (miRNAs) are noncoding RNA sequences that are involved in organizing the activity of multiple genes within biological networks, including neurodevelopment and adult neuronal processes. An upregulation in miRNA-132 was found in both human neuroepithelioma and mouse brains (Figure 1.5). MiRNA-132 is regulated by cyclic adenosine monophosphate-responsive element binding (CREB). Using target prediction and pathway enrichment analysis of the *T. gondii* infected mice transcriptome, miRNA-132 was found to be involved with downregulation (> 2 folds) of 20 genes, and some of these genes related to DA receptor signalling (Xiao et al., 2014).

Also in the Xiao et al. (2014) study, found that the brains of acutely infected mice had a decrease in the expression of D1-like dopamine receptors (DRD1 and DRD5) (Figure 1.5). Also, there was a decrease in monoamine oxidase A, which catalyses oxidative deamination of amines, and intracellular proteins, which are involved with the transduction of DA-mediated signalling (phosphorylation at threonine 34 and serine 97 by the gene DARPP-32). This study also showed that the metabolism of DA was decreased while 5-hydroxytryptamine metabolism was unchanged. High performance liquid chromatography demonstrated an increase in the concentrations of DA and its metabolites, serotonin and 5-hydroxyindoleacetic acid (Xiao et al., 2014).
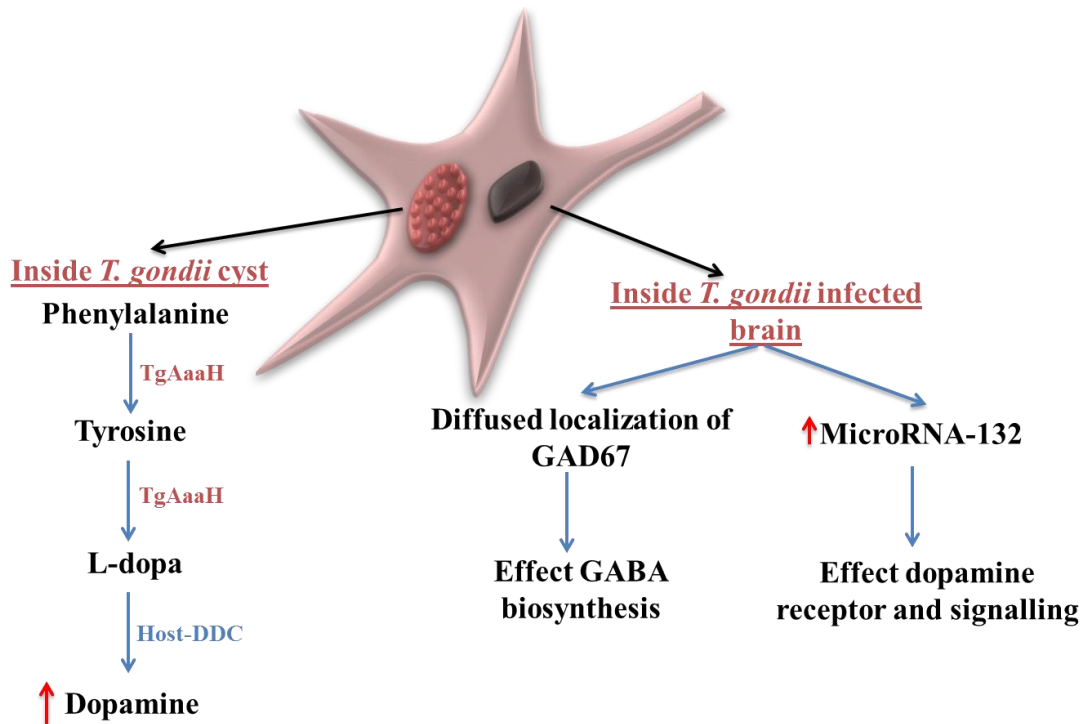
Figure 1.5. Altered neuromodulation pathway during brain infection by *T. gondii*. Increased DA was found within the brain *T. gondii* cysts. DA synthesis begins when phenylalanine is hydroxylased to tyrosine. Then, tyrosine is hydroxylased to L-DOPA. In *T. gondii*, these two steps catalysed with the same enzyme TgAaaH. *T. gondii* utilises the host dopa-decarboxylase to convert L-DOPA to DA. In *T. gondii*-infected brains, the distribution of GAD67 becomes diffuse in the neuropil instead of clustered at pre-synaptic termini. GAD67 involved in GABA biosynthesis. Infected brains have increased expression of microRNA-132, which subsequently cause a down regulation of gene involved in DA receptor signaling.

T. gondii brain infection found to indirectly effect γ-aminobutyric acidic (GABA), which is an inhibitory neurotransmitter. The effect on GABA might be part of the mechanism in which the parasite alters the brain neuromodulation process to induce the intermediate host behavioral change. Brooks et al. (2015) studied glutamic acid decarboxylase 67 (GAD67) in the brains of mice infected with type II *T. gondii*. The study showed that *T. gondii* infected brains had a more diffuse localization of GAD67 throughout the neuropil instead of GAD67 clustering at the pre-synaptic termini (Brooks et al., 2015) (Figure 1.5). GAD67 is involved in GABA biosynthesis (Figure 1.5). A study on mice with GAD67 -/- and GAD67 +/- found that they had 20 % less GAD activity and 7% GABA content ($P < 0.01$) compared to wild mice. Further, mice with GAD67 -/- died the morning of their birth, while GAD67 -/+ mice survived and

had no neurological disorders (Asada et al., 1997). Crestani et al. (1999) found that mice with genetically modified (heterozygous mutant) $GABA_A$ receptor γ^2 subunits, which had reduced $GABA_A$ mainly in the hippocampus and cerebral cortex, were less inhibited by natural aversive stimuli. This altered behaviour is similar to that observed in *T. gondii* infected mice. In mice, the hippocampus and cerebral cortex were also found to have high numbers of cysts during latent infection (see section 1.5.2) (Berenreiterova et al., 2011).These studies coincide nicely with observations of seizures in patients with toxoplasmosis (Arendt et al., 1999).

1.6   Toxoplasmosis treatment

The treatment for toxoplasmosis varies depending on the patient age, immune competence, and severity of the symptoms. For pregnant patients, it also depends on whether the infection is recently acquired or latent. In some cases, no treatment is advised for patients as they will recover with time. These cases are immunocompetent patients with acute toxoplasmosis (with symptoms of adenopathy, with or without mild fever or malaise) or for asymptomatic latent infection (Klinker et al., 1996; Louis and Kim, 2013). Similarly, ocular toxoplasmosis usually does not require treatment since it is a self-limiting disease (Holland and Lewis, 2002; de-la-Torre et al., 2011a).

If treatment for toxoplasmosis is advised, the drugs most commonly are antifolates such as sulfadiazine, sulfathiazole, and pyrimethamine (Figure 1.6) (Weiss et al., 1992; Wong and Remington, 1994; Klinker et al., 1996). Pyrimethamine and sulfadiazine are given to severely infected immunocompetent patients, for example, in case of myocarditis, encephalitis, sepsis syndrome with shock, and hepatitis (Klinker et al., 1996; Louis and Kim, 2013). This drugs combination also given in cases of active toxoplasmosis or encephalitis in immunocompromised patients with AIDS and Hodgkin's disease (Klinker et al., 1996; Louis and Kim, 2013). While ocular toxoplasmosis may not need to be treated, for active chorioretinitis and active choroidal neovascular membrane, pyrimethamine and sulfadiazine with or without corticosteroids are used to minimize the potential for damage to the retina and optic disc (Holland and Lewis, 2002; de-la-Torre et al., 2011a; Louis and Kim, 2013).
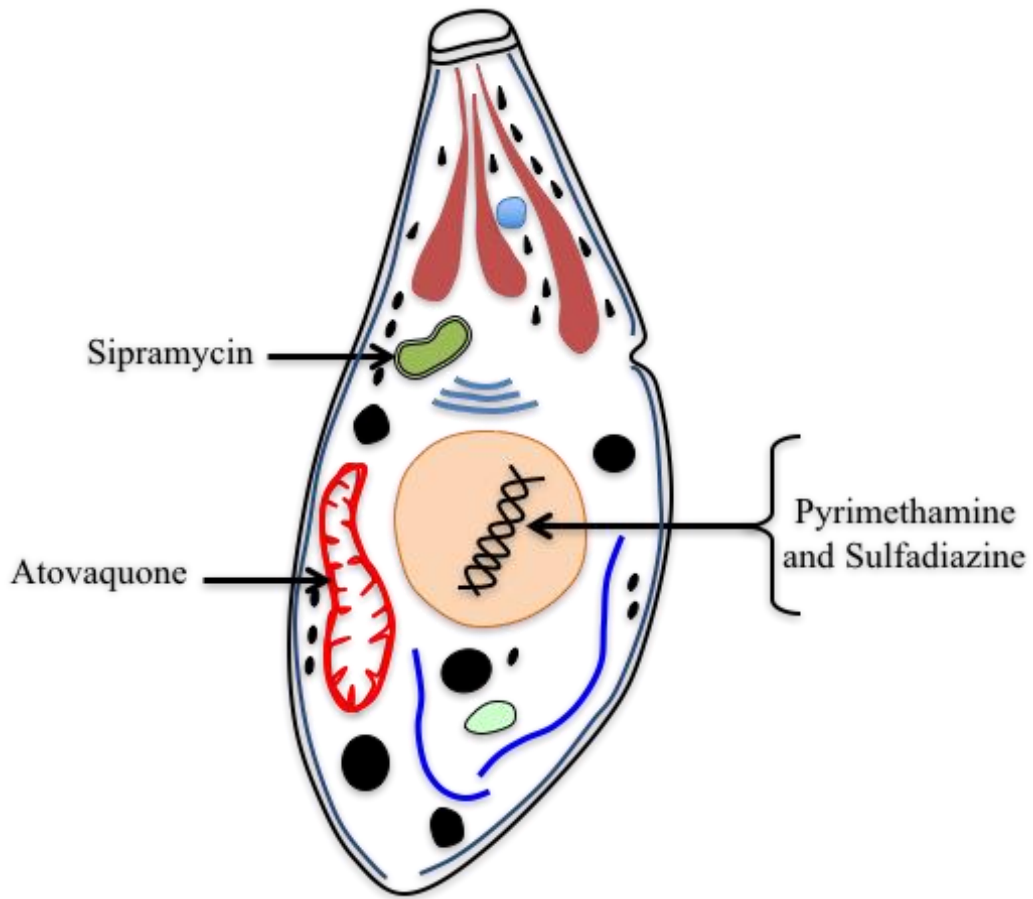
Figure 1.6. Mode of action of current treatment for toxoplasmosis. The most common treatment is pyrimethamine and sulfadiazine. Both act on folate biosynthesis and subsequently affect DNA synthesis. In cases of intolerance or resistance to pyrimethamine and sulfadiazine, atovaquone is given. Atovaquone targets the cytochrome bc1 complex in the mitochondria. Sipramycin is the choice of treatment for pregnant women and it targets the apicoplast in *T. gondii*.

For infection during pregnancy, pyrimethamine and sulfadiazine are used to treat toxoplasmosis infection acquired after the 24 weeks of gestation or for confirmed foetal infection (Wong and Remington, 1994; Louis and Kim, 2013). If the mother acquired the infection during the first 18 weeks of gestation and no infection was found in the amniotic fluid, spiromycin is the drug of choice (Desmonts and Couvreur, 1974; Wong and Remington, 1994). Sipramycin targets the apicoplast (Figure 1.6)(Wiesner et al., 2008), and is non-toxic to the foetus because it cannot cross the placenta (Louis and Kim, 2013). If the patient acquired the infection before pregnancy, the parasite is not transmitted to the foetus and no treatment is required (Louis and Kim, 2013).

In patients with sulfonamide intolerance, the alternatives are clindamycin, clarithromycin, or trimethoprim with pyrimethamine (Derouin et al., 1992; Meneceur et al., 2008; Louis and Kim, 2013). For patients who are intolerant or resistant to pyrimethamine and sulfonamide, atovaquone is given instead (Kovacs, 1992; Romand et al., 1993; Jacobson et al., 1996; Chirgwin et al., 2002). Atovaquone treats toxoplasmosis by acting on the cytochrome bc1 complex (Figure 1.6). Unfortunately, atovaquone resistance has also been reported (Baatz et al., 2006). Meanwhile, none of these treatments can clear toxoplasma infection in patients since they are not active against the bradyzoites (cyst in tissues) stage. Therefore, a new drug that targets both the tachyzoites and bradyzoites stages would be of a great benefit to patients.

1.7 Conclusion to introduction and statement of research questions

T. gondii is a unique parasite, because it can infect all warm-blooded animals and humans. As a result of this unique trait, infection by the parasite is found almost all around the world. However, the parasite's only definitive hosts are members of the feline family. Laboratory studies described in section 1.4.1 showed that *T. gondii* alters behaviour in mice and rats, which might increase their predation by cats and, consequently, further the completion of the *T. gondii* life cycle (Piekarski et al., 1978; Witting, 1979; Webster, 1994; Webster et al., 2006; Hrdá et al., 2000; Berdoy et al., 2000; Vyas et al., 2007a; Gulinello et al., 2010). Meanwhile, it would be interesting to confirm, in nature, whether the parasite increases predation of the intermediate host by felines.

In humans, *T. gondii* seropositivity has been linked with mental disorders such as schizophrenia, OCD, Parkinson's disease, bipolar disorder and Tourette's syndrome (Brynska et al., 2001; Torrey et al., 2007; Krause et al., 2010; Miman et al., 2010; Hinze-Selch et al., 2010; Kusbeci et al., 2011). Most of these studies compared the seropositivity for the parasite between healthy individuals and patients with mental disorders. However, the design of the studies was biased, because mentally ill patients may have had a higher chance of acquiring the infection when compared to healthy individuals who were better able to care for themselves.

Toxoplasma infection in humans has no cure, and awareness of *T. gondii* infection is low (Li et al., 2014; Chandrasena et al., 2016). The low awareness could be because

both acute and chronic infections are asymptomatic in immunocompetent individuals. However, in immunocompromised patients, infants, and, sometimes, patients with ocular infections, the parasite infection is severe (Louis and Kim, 2013). Therefore, finding a cure to *T. gondii* infection is vital. Furthermore, curing the parasite infection in patients with mental disorders would help to establish whether the parasite is the cause of the mental symptoms.

It is still unclear how the parasite affects the host behaviour. The parasite causes rats to become unafraid of cats, and both mice and rats are attracted to cats (Berdoy et al., 2000; Vyas et al., 2007a). The question is why the parasite alters human behaviour. Does the parasite effect the human brains differently (manifesting as a mental disorder) because human brains are larger and more sophisticated, or because humans are not naturally afraid of cats.

Webster (2007) suggested that the parasite might change behaviour indirectly through the immune response, directly through its location in the brain during the latent infection, or by altering the brain neuromodulation pathways, but research (1.5.1 and 1.5.2) on the chronic infection has not provided a clear link between the immune response or the parasite location with the altered behaviour.

Several studies have linked chronic *T. gondii* infection and altered neuromodulation pathways. In chronic brain infection, the cysts are mostly found in neurons (Ferguson and Hutchison, 1987a; Melzer et al., 2010), and there was an increase in DA within the cyst (Prandovszky et al., 2011). The parasite's ability to increase DA was due to the activity of two TgAaaH enzymes (1 and 2) (Gaskell et al., 2009). TgAaaH can hydroxylate tyrosine to L-DOPA, which is the rate-limiting step of DA biosynthesis (Gaskell et al., 2009).

Still, whether the parasite synthesizes DA from L-DOPA through DDC enzyme activity is unclear. TgAaaH 2 was expressed more in the bradyzoite stage (Gaskell et al., 2009), and a TgAaaH knockout strain did not survive (Wang et al., 2015). These results suggest that TgAaaH could be involved in vital functions other than providing DA. Studies (1.5.3) into the latent parasite infection and its effect on the brain neuromodulation pathway have not provided a clear answer on how the parasite alters behaviour. Further, no studies have linked increased brain DA levels during latent *T. gondii* infection and altered behaviour.

This thesis focused on the following questions regarding *T. gondii* latent infection (bradyzoite cysts in the brain):

1. During *T. gondii* infection, does the parasite show DDC activity that is involved in DA synthesis?
2. Where can the TgAaaH enzyme be found during infection, and how does it correlate with the parasite increasing DA during latent infection?
3. Can the products of TgAaaH (L-DOPA and DA) be involved in synthesis of the cyst wall through formation of dopa-quinone by the dopa-oxidase enzyme?
4. Is it possible to develop a bradyzoite-specific drug-screening assay to detect drugs that are active against this stage of the parasite?

The following chapters attempt to answer these questions.

1.8 Aims

This thesis has two aims. The primary aim is to understand more about TgAaaH function and DA synthesis during *T. gondii* infection, and how they may take part in the mechanisms by which *T. gondii* affect the behaviour of the intermediate host. A secondary aim is to develop a novel culture media that induces *T. gondii* differentiation into the cyst stage, and development of a novel screening assay intended to identify potential drugs that can prevent that stage.

1.9 Objectives

The primary aim was achieved in Chapter 2 by investigating the following:

1. Evaluated DDC activity presence in *T. gondii* by *in-vitro* testing of DA production in *T. gondii*.
2. Investigated the role of TgAaaH in tachyzoite cyst wall formation by *in-vitro* testing of dopaquinone activity in *T. gondii*.
3. Documented cellular localisation of TgAaaH and DDC during *T. gondii* infection.

The second aim was addressed in Chapter 3 and 4 by the following:

1. Preparation of tryptophan depleted Dulbecco's modified Eagle medium (TD-DMEM), which is the culture media was intended to induces *T. gondii* differentiation.
2. Documented *T. gondii* ΔKU80-GFP growth and differentiation in the new culture media with depleted tryptophan using bright field and fluorescence imaging.
3. Evaluated the expression of stage specific marker of *T. gondii* growing in cultures supplemented with TD-DMEM, pH8, and DMEM.
4. Evaluated the inhibition of *T. gondii* ΔKU80-GFP cyst by ELQ271 and pyrimethamine using bradyzoites specific screening assay in TD-DMEM.
5. Evaluated the activity of ELQ271 on *T. gondii* ΔKU80-GFP cyst using bradyzoites specific screening assay supplemented with either TD-DMEM, normal DMEM, or alkaline DMEM.

Chapter 2 *Toxoplasma gondii* aromatic amino acid hydroxylase and dopamine biosynthesis

2.1 Introduction

Studies have found that *T. gondii* produces two enzymes (TgAaaH 1 and 2) that catalyse tyrosine hydroxylation (TH), which is the rate-limiting step of DA and catecholamine biosynthesis. This could alter DA and catecholamines function in neuronal pathways during brain stage of infection. Alternatively, parasite-produced L-DOPA could be used for dopaquinone. To assess the role of TgAaaH in DA biosynthesis it is important to understand the cellular location and function of these enzymes.

Gaskell et al. (2009) found that *T. gondii* possesses two nearly identical TgAaaH genes. The two genes are located on chromosome V and are separated by approximately 450 kbp. The translation of TgAaaH genes produces TgAaaH 1 and 2 enzymes, each with 565 amino acid residues. TgAaaH 1 and 2 consist of C and N terminals. The C terminal domain is responsible for the catalytic activity, while the N terminal plays a role in substrate specificity. Most described amino acid hydroxylases are soluble, cytosolic, and lack a signal peptide. On the other hand, TgAaaH genes produce insoluble enzymes. Also, the enzyme coding genes encode a signal peptide in the N-terminus. It was suggested the signal peptide could be involved in transporting TgAaaH 1 and 2 to the parasite's outer membrane and/or secretion of the enzymes outside the parasite (Gaskell et al., 2009).

To better understand TgAaaH function, Gaskell et al. (2009) studied the activity of recombinant TgAaaH 1 and 2 expressed as N-terminal His-tagged fusion proteins under control of the T7 promoter. It was found that TgAaaH was involved in the production of L-DOPA, which is a DA precursor. L-DOPA is produced by hydroxylation of phenylalanine to tyrosine and tyrosine to L-DOPA. Also, recombinant TgAaaH enzymes have a two to three-fold preference for tyrosine, compared to recombinant rat

tyrosine and phenylalanine hydroxylase. Furthermore, TgAaaH was unable to utilise tryptophan (Gaskell et al., 2009).

To determine the *in vivo* expression of TgAaaH 1 and 2 during tachyzoite and bradyzoite stages, Gaskell et al. (2009) used RNA Reverse Transcriptase-PCR (RT-PCR) with stage specific stage primers surface antigen 1 (SAG1: tachyzoite marker), bradyzoites surface antigen 1 (BAG1), and surface antigen 4 (SAG4) (BAG1 and SAG4 are bradyzoite marker). It was demonstrated that TgAaaH 1 expression level didn't change between the tachyzoite and the bradyzoite stage, while TgAaaH 2 was expressed more in the bradyzoite stage. It was also found that TgAaaH requires tetrahydrobiopterin as a cofactor. Webster and McConkey (2010) suggested that the first enzyme provides tyrosine as a nutrient for the parasite's protein synthesis in the proliferative tachyzoite stage and the second enzymes provides L-DOPA for use during chronic stage. Preliminary work to localise *T. gondii* TgAaaH by Gaskell et al. (2009), using immunostaining of infected fibroblasts with both tachyzoites and bradyzoites showed that TgAaaH enzymes were localised in the parasite membrane and PV, but these results needed further confirmation.

Prandovszky et al. (2011) determined DA levels for infected (*T. gondii* bradyzoites stage) and non-infected PC12 cells using HPLC electrochemical detection (ECD). It was found that *T. gondii* infected PC12 cells had higher levels of DA, compared to non-infected cells. Furthermore, Prandovszky et al. (2011) localised TgAaaH and DA in the paraformaldehyde fixed brain section of 6-8 week infected mice by immunofluorescence with DA and customised TgAaaH antibodies. TgAaaH antibody was customised (Genscript, Piscataway) to assess the parasite enzyme in animals. This antibody is designed to bind to a unique sequence (CIRSSPDPLDLRKMT) in the TgAaaH terminal domain, a sequence that is not found in mammalian tyrosine hydroxylase and has no significant similarity to any protein in the predicted mammalian proteome or other proteins of the *T. gondii* proteome. TgAaaH antibody specificity to *T. gondii* was confirmed with uninfected controls. Immune-stain brain section of chronically infected mice with TgAaaH antibody showed that TgAaaH was found within the tissue cyst of infected neuronal cells (Prandovszky et al., 2011). DA was localised within *T. gondii* tissue cysts and cyst periphery in the infected mouse brains. Meanwhile, TgAaaH location within the cyst was unclear as staining could not

resolve between protein localisation on the surface of parasites and protein found in the PV not attached to parasite membranes (Prandovszky et al., 2011).

Wang et al. (2015) examined TgAaaH 1 and 2 roles by constructing two strains, one without the TgAaaH 1 gene and one without the TgAaaH 2 gene. The TgAaaH 1 knocked out strain could not survive which indicated that TgAaaH 1 might have an essential role. Strain growth, differentiation, host infection and horizontal transmission to wild type strains of TgAaaH2 knock outs showed that TgAaaH 2 is not essential for any of these functions. Meanwhile, Wang et al. (2015) failed to directly detect TgAaaH protein using WB and immunofluorescent staining. To detect TgAaaH with WB and immune-fluorescent staining, Wang et al. (2015) constructed a Ty-tagged TgaaH 2 protein overexpressor strain using a BAG1 promoter. A Ty epitope band was detectable in the Ty-tagged TgAaaH2 protein overexpressor strain with WB techniques. Also, the immunofluorescent stain of these strains showed that TgAaaH 2 is detectable in the cyst matrix.

The mentioned earlier in this chapter introduction that Prandovszky et al. (2011) found that *T. gondii* brain cyst was associated with DA, and DA level was increased in infected dopaminergic cells. The increase of DA could be due to the tyrosine hydroxylase activity that converts tyrosine to L-DOPA, but the parasite needs DDC to convert L-DOPA to DA. The question is whether *T. gondii* has DDC enzyme activity. Bio-informatics search (metaTiger data base: Whitaker et al., 2009) indicates that the *T. gondii* genome doesn't contain the DDC gene sequence. The ability of *T. gondii* to produce DA will be assessed by adding tyrosine to the parasite cell extract and running the samples into the HPLC-ECD to detect DA. This experiment is used to explore the possibility of *T. gondii* having DDC activity. The difference in TgAaaH 1 and 2 gene expression (Gaskell et al., 2009) and non-survival on TgAaaH 1 knockout strain (Wang et al., 2015) raised a question: can it be that L-DOPA and DA have another role during the tachyzoite stages, such as wall biosynthesis?
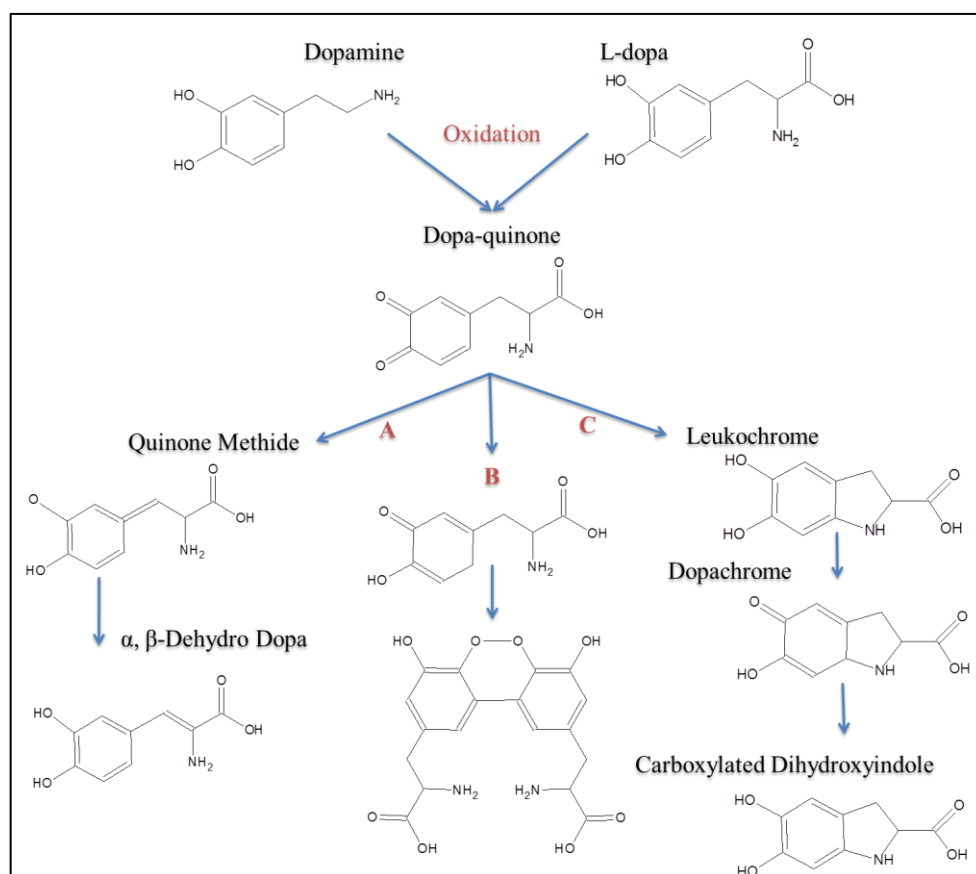
Figure 2.1. Cross-linked polymers from dopaquinone. L-DOPA and DA can be oxidised to dopa-quinone by enzymes such as tyrosinase or peroxidase. A: dopa methide is formed by DOPA quinone tautomerisation, then α, β-dehydro dopa, is formed after the release of α proton. Finally, cross-linking will occur by a pathway alike to that happening in insect cuticle sclerotisation. B: Generation of aryloxy free radical generation, phenol coupling and oxidation will form cross-linked polymer. C: Leukochrome is formed by internal cyclisation, and then rearrangement of cyclized DOPA will form dopachrome and carboxylated dihydroxyindole. The end of pathways A, B, and C will result in the generation of a crosslinked polymer (Adapted from Lee et al., 2002).

It is known that L-DOPA is involved in forming intermolecular covalent bonds and hardening of DOPA containing proteins. L-DOPA and DA can be oxidised to the reactive chemical form dopaquinone (Figure 2.1). Dopaquinone is known to be involved in several chemical reaction pathways that result in cross-linking (Figure 2.1). L-DOPA can be transformed to dopaquinone by several enzymes such as tyrosinase, catechol oxidase, laccases, peroxidase and chemical oxidants, such as hydrogen peroxidase and periodate (Waite, 1990; Rzepecki et al., 1991; Lee et al., 2002). Aryloxy radical generation and diphenol coupling of dopaquinone will result in the

formation of catechol polymer (Lee et al., 2002). The release of α proton from tautomerized dopaquinone (quinone methide) is capable of a cross-linking similar to that occurring in insect cuticle (Lee et al., 2002). Meanwhile, intramolecular cyclization between the catechol side chain and the amine group in DA will form leukochrome which can be polymerised in the same pathway to polymerise melanin formation (Lee et al., 2002). All these informations show how L-DOPA and DA and the formation of dopaquinone involved in the formation of crossed linked polymer. The question is does this process occur in *T. gondii*, and does L-DOPA and DA takes part in that process.

In this chapter, several questions have been addressed regarding TgAaaH location and function. First, the probability that L-DOPA, which is the end product of TgAaaH activity, and DA are converted to dopaquinone was investigated. This will give an insight if L-DOPA and DA production have a role in the polymer cross-linking in the process of membrane formation. To address the this question, dopa-oxidase activity was assessed in free *T. gondii* tachyzoites and bradyzoites. Secondly, this chapter assessed DA synthesis in liberated *T. gondii* using HPLC-ECD. Thirdly, the probable intracellular, extracellular or membrane-bound location of *T. gondii* TgAaaH was investigated. Knowing TgAaaH location during infection will help in understanding its role in DA formation. To address these possibilities, samples of non-dopaminergic cells were infected with *T. gondii*. The location of TgAaaH *in vitro* was determined by the separation and analysis of cell components (particularly membrane), accompanied by WB, enzymatic assay analysis and immune-fluorescence staining. Fourth and finally, DDC location was assessed during *T. gondii* dopaminergic cell infection using WB protein analysis probed with anti-DDC primary antibody in WB. Overall, these experiments allowed elucidation of mechanisms of DA formation during *T. gondii* infection and provided information about the function of TgAaaH.

2.2 Materials and Methods

2.2.1 Cultivation of cells and *T. gondii* strains

Human foreskin fibroblast cells (HFF, Sigma Aldridge) and Henrietta Lacks cells (HeLa, a kind gift from Mark Stead, Leeds University) were cultured and maintained by serial passaging in T25-cm2 vented flasks (T25) as a confluent adherent monolayer. HFF and HeLa cells were passaged every 3-4 days. For the passaging of 75–95% confluent HFF cells, the cells were washed in 100% phosphate buffered saline (PBS: Invitrogen), detached by adding 5% trypsin-ethylenediaminetetraacetic acid (trypsin-EDTA, Sigma) for 90 seconds. The free cells were suspended in fresh Dulbecco's modified Eagle's medium (DMEM, Gibco), supplemented with 10% heat-inactivated iron-supplemented fetal bovine serum (FBS, Invitrogen) and 1% penicillin-streptomycin (PS, Sigma) antibiotic solution at pH 7.2. The passaged cells in the fresh media were transferred to three or four new T25 flasks. The newly passaged cells were incubated at 37°C in 5% CO_2. Passages 1–42 of HFF cells were used according to the manufacturer's recommendation.

Rat pheochromocytoma cells (PC12, from the European Collection of Cell Cultures, ECACC) were cultured and maintained by serial passaging in T25 flasks as free rounded cell clusters, and passaged every three to four days, as recommended by the manufacturer. Cell passaging was started by freeing the cells from the cluster by pipetting. Then, cells were collected by centrifugation at 800xg for 10 minutes. The supernatant was discarded and the cell pellet was suspended in fresh Roswell Park Memorial Institute medium (RPMI, Gibco), supplemented with 10% horse serum (HS, Invitrogen), 5% FBS and 1% PS antibiotic solution at pH 7.2. The cell suspension was then transferred to three new flasks and incubated at 37°C in 5% CO_2. Cell cultures with passage numbers between 1 and 20 according to the manufacturer's recommendation.

T. gondii Prugniard (kind gift from David Roos) and RH ΔKU80-GFP strain (kind gift of David Bzik, Dartmouth) were used in this chapter's studies. RH ΔKU80-GFP is a strain that lack hypoxanthine-xanthine-guanine phosphoribosyl transferase (HXGPRT) and has green fluorescent protein (GFP) fused to the BAG1 promoter. This strain stably expresses bradyzoite specific-GFP (Fox et al., 2011).

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The parasites were cultured and maintained by serial passaging in confluent monolayer HFF cells. The parasite was passaged by freeing the HFF cells with 5% trypsin and then the cells were centrifuged at 2500xg for 10 minutes. The supernatant was discarded and the cells were suspended in 100% PBS. Then, the intracellular parasite was released by passing the cells through a 27-gauge needle several times, and the suspension was centrifuged again. Finally, the parasite pellet was suspended in 5 mL of fresh DMEM medium and used to infect 75–95% confluent HFF.

2.2.2   *In vitro T. gondii* bradyzoites inductions

The *T. gondii* tachzoite stage was alkaline shocked to induce bradyzoite conversion in two experiments: dopa-oxidase and TgAaaH immune-staining. Bradyzoites induction was done as previously described by Prandovszky et al. (2011), where they shocked the parasite in alkaline (pH 8) RPMI without serum. Briefly, tachyzoites were liberated by passing through a 27 gauge needle several times and cells collected by centrifugation at 2500xg for 10 minutes. Then, the pellets were suspended in RPMI supplemented with 1% PS antibiotic solution at pH 8, and incubated for 16–18 hours at 37 °C. After the incubation is done, the cell pellet was collected by centrifugation at 2500xg for 10 minutes, then suspended in RPMI (pH 7.4) containing 10% HS, 5% FBS and 1% PS.

2.2.3   De Novo Synthesis of Dopamine in *T. gondii*

It is known that *T. gondii* is able to transform L-DOPA to DA in cells that have the DDC enzyme. To better understand the role of TgAaaH and DA formation during *T. gondii* infection, *T. gondii*'s ability to form L-DOPA or DA in HFF cells, which don't have the DDC enzyme, was determined. This was determined using TH activity as previously described by Naoi et al. (1988) with modifications. Briefly, 1 μL of 20 mM tyrosine was added to the collected parasites (50 μL), then both L-DOPA and DA was detected using HPLC-ECD.

Cell Harvesting: Confluent HFF cells and PC12 cells infected with 2-3 x10^5 *T. gondii* Prugniard were incubated for three to four days at 37°C in 5% CO2. Infected HFF cells were collected by scraping and centrifugation at 2500xg for 10 minutes. PC12 pellets were collected by centrifugation at 1000xg for 10 minutes. Each cell pellet was suspended in 50 μL 10 mM potassium phosphate buffer and sonicated using an MSE sonicator at 2/3 of full power for 10 seconds. The following chemicals were made

freshly and used on the date of the experiment: 10 mg/mL catalase (Calbiochem) in potassium phosphate buffer pH 7, 200 mM L-tyrosine (Sigma, Aldrich) in sodium acetate – acetic acid buffer, and 100 % glacial acetic acid diluted 1:100 in dH_2O.

Dopamine assay: The assay was started by adding 26 µL of sodium acetate-acetic acid (200 mM) to 50 µL of the sonicated free parasites (pH 6.0). Thereafter, 1 µL glacial acetic acid-catalase solution (Sigma; 1 mg/mL) was added. Then, 1 µL of 20 mM L-tyrosine solution and 20 µL of 10 mM tetrahydrobiopterin (Schicks Laboratories, Jona, Switzerland) in 1 M beta-mercaptoethanol (Sigma Aldrich) were added and incubated for 10 minutes at 37°C. The reaction was stopped by adding 100 µL of 0.1 M perchloric acid (PCA, Sigma Aldrich). Finally, the samples were run through HPLC-ECD with a flow rate of 0.8 mL/minutes to detect the DA peak.

HPLC analysis was performed as described previously by Prandovszky et al. (2011) with C18 Acclaim 120 column (5 mm x 4.66150 mm). The mobile phase buffer consisted of degassed 57 mM anhydrous citric acid (Fisher Scientific, Loughborough), 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 samples run included controls and standards. The standards were DA (Sigma) and L-DOPA (Sigma) with the following concentrations 0.015, 0.007, 0.0039 or 0.0019 µM.

2.2.4 Dopa-oxidase activity (MBTH assay) in *T. gondii*

The probability that *T. gondii* have dopa-oxidase activity was tested using dopa-oxidase activity assay (Winder and Harris, 1991). Dopa-oxidase enzyme activity will enable the transformation L-DOPA and DA to dopaquinone, which have been shown to be involved in the formation of crossed linked polymer and might be involved in the parasite cyst wall synthesis(Figure 2.1).

Cell harvesting: HFF and PC12 cells infected with 2-3 x10^5 *T. gondii* Prugniard. Also, HFF cells were infected with 2-3 x105 shocked *T. gondii* Prugniard. HeLa and PC12 cells were cultured to be used as negative control. The cells were harvested after three to four days of infection. Harvesting attached cells, such as HeLa cells, differs from free PC12 cells. HFF and HeLa cells were harvested by washing with PBS, and then

scraping. The cells were then collected by centrifugation at 1000xg for 10 minutes. The supernatant was discarded and the pellet was suspended in NaCl/Pi (137 mM NaCl, 3 mM KCI, 8.1 mM Na2HPO4, and 1.5 mM KH2PO4+ 0.2% trypsin-EDTA, pH 7.3). Meanwhile, free PC12 cells were harvested by centrifugation at 1000xg for 10 minutes and the supernatant was discarded. After supernatant was discarded, the pellet was suspended in NaCl/Pi. After collection and resuspension, all cell types (HeLa, and PC12/ infected and non-infected) were washed twice with ice cold NaCl/Pi. After washing, the cell pellet was frozen at -70°C. After freezing, the pellets were rapidly thawed, and then 5X the pellet cell volumes of cold dopa-oxidase buffer (50 mM sodium dihydrogen phosphate, pH 6.9) was added. The cells were sonicated on ice for 30 seconds (3x10 second bursts at 2/3 full strength, followed by resting on ice for 30 seconds). Then, the cell pellet was collected by centrifugation at 9000xg for 30 minutes at 4°C.

Bovine serum albumin (BSA) and Bradford reagent (Sigma) in 96-well plate (Costar 3603 by Corning Incorporated, New York) was used in the Bradford assay to estimate the protein concentration (Bradford, 1976). Briefly, the absorbance of BSA standard (1-5 μg/ml) and the samples was determined using SPECTRA MAX, Molecular devices (at 595 nm). The regression line curve of the BSA standards concentrations and absorbance at 595 nm was drawn. The samples concentration was estimated using BSA standards curve regression line and equation.

Dopa-oxidase assay: The assay was done in a 96-well plate (Costar) and each well contained a reaction mix of the following: 50 mM sodium phosphate pH 6.9, 2% (by vol.) N,N'–dimethylformamide (Sigma), 1 mM L-DOPA and 6 mM 3-methyl-2-benzothiazolinone hydrazone (MBTH, Sigma), 10 μl water, and 20 μl sample. Then the plate was incubated at 37°C for 10 minutes. The absorbance at 505 nm was measured using SPECTRA MAX, Molecular devices. Dopa oxidase activity was measured for at least three biological replicates for each sample type and then run as two technical replicas. PC12 and HeLa cells were used as negative control. With each experiment, a reaction mix with no substrate (control negative) and one with mushroom tyrosinase (Sigma) (control positive) was performed. The absorbance of the samples was standardized against their protein concentrations.

2.2.5 Localising TgAaaH using Western blotting

For better understanding of TgAaaH function, the intracellular, extracellular, and membrane location of TgAaaH was assessed. An experiment was set up to detect TgAaaH protein in trypsin treated and untreated cells using Western blots (WB) probed with general anti-Aromatic Amino Acid Hydroxylase (Anti-AAAH) primary antibody. Also, TgAaaH was detected in *T. gondii* cellular fractions (soluble and non-soluble).

Cell harvesting: For the cellular localisation of *T. gondii* TgAaaH using WB, four T25 HFF flasks were infected with 2-3 x10^5 *T. gondii* Prugniard and incubated for 3-4 days. The parasites were collected by scraping and and pelleted by centrifugation at 2500xg for 10 minutes. The cells were washed with 10 ml PBS and the parasites released by passing the pellet through a 27-gauge needle. Then the cells were collected by centrifugation at 2500xg for 10 minutes. The collected pellet was used to localise TgAaaH in *T. gondii* using trypsin treatment or cell fraction localisation.

Trypsin treatment: The four different *T. gondii* pellets were treated with 0.5 ml trypsin-EDTA for 5, 10, or 15 minutes at 37°C. Then DMEM medium containing FBS was added to neutralise trypsin, and cell pellets were collected by centrifugation for 10 minutes at 2500xg. The cells were washed with PBS and pellets collected by centrifugation at 14000xg for 5 minutes. The cell pellets were suspended in radioimmunoprecipitation assay buffer (RIPA; Caymen Chemicals, Ann Arbor, MI) with protease inhibitors (Complete Mini EDTA-free cocktail; Roche Life Sciences, Burgess Hill, UK).

TgAaaH in cell fractions: The parasite cell pellets were suspended in a RIPA buffer. Then the samples were separated to soluble and non-soluble fractions by centrifugation at 14000xg for 5 minutes after sonication. Finally, the protein concentration of all samples was estimated with the Bradford assay as explained in 2.2.4.

WB localisation of TgAaaH: Five to twenty-five micrograms of protein was separated in 12% sodium dodecylsulphate- polyacrylamide gel electrophoresis (SDS-PAGE) following standard protocols. The proteins were transferred to the nitrocellulose membrane and blocked with 5% non-fat powdered milk containing 0.05% Tween-20 (PBS-T. vol/vol) for one hour. Then the blot was incubated with either 1:250 mouse anti- AAH (Millipore, UK) or 1:5000 mouse anti-actin antibodies (Milliopore, UK) as

primary antibody at 4°C overnight. The blot was washed with PBS-T, then incubated with 1:5000 anti-mouse (Biorad, UK) conjugated horseradish peroxidase antibody at room temperature for one hour. Blots were then washed as above and developed using Supersignal West Pico Chemiluminescent detection kit (Fisher Scientific). Bands were visualised with an X-Omat film system. The WB analysis was done for at least 3 biological replicates. The band pixel intensity was obtained using ImageJ and then plotted on GraphPad Prism (column graph-one way ANOVAtest).

2.2.6 Localisation of TgAaaH using HPLC-ECD

The localisation of TgAaaH produced by *T. gondii* Prugniard was determined by comparing TH activity between intracellular and extracellular parasites, and soluble and non-soluble parasite components.

Cell harvesting: For localising TgAaaH using TH activity assay and HPLC, 2-3 x10^5 *T. gondii* Prugniard strains were left to grow in a monolayer of HFF for three to four days. HFF cells were harvested by scraping, and both HFF and PC12 (control positive) cells were centrifuged at 2500xg for 10 minutes. Cells were washed with 100% PBS. Intracellular parasites were released for some samples using 27-gauge needles. Next, the samples were centrifuged at 2500xg for 10 minutes, and the supernatant was discarded. Then 50 µL of 10 mM potassium phosphate buffer (pH 7.4) was added to the cell pellets and each sample was sonicated for 30 seconds. After sonication, several samples were separated to soluble and non-soluble fractions by centrifugation at 14000xg for 5 minutes. The protein level was measured by the Bradford assay, as explained in 2.2.4.

TH activity assay and HPLC-ECD: TH activity was determined for each sample as previously described in Section 2.2.3. Twenty-six µL of 200 mM sodium acetate-acetic acid buffer (pH 6.0) was added to each sample. Each sample was analysed in the HPLC-ED with a flow rate of 0.8 mL/minutes as explained in 2.2.3. All samples were run with standards and controls. L-DOPA standard was run with samples at 0.25, 0.125, 0.0062, 0.031, 0.015, 0.007, 0.0039, and 0.0019 µg /ml. The retention times of each L-DOPA standard were determined. Then the total area under the HPLC trace for 8 different L-DOPA concentrations was measured to create the reference curve for subsequent quantitative analysis of L-DOPA amounts.

2.2.7 Immuno-fluorescence localisation of TgAaaH

In vitro localisation of TgAaaH using immuno-staining was done for HFF cells infected with *T. gondii* Prugniard and ΔKU80-GFP growing on coverslips, using TgAaaH specific customised antibodies (Genscript, Piscataway). This antibody specifically designed to bind *T. gondii* TgAaaH enzyme as described in this Chapter introduction.

Cell infection: Confluent HFF cells growing on sterile 12 mm glass coverslips (SLS coverslip) were infected with 1 x10^5 shocked *T. gondii* Prugniard or ΔKU80-GFP and left to grow in pH8 DMEM for three to four days. Then the infected cells were fixed with 4% paraformaldehyde.

Immunostaining: Immunofluorescence staining was performed as previously described (Prandovszky et al., 2011) with some modifications. Briefly, the slides were washed with 1X PBS + 0.05% Triton solution (PBSTR), and then blocked with 10% cold fish gelatin in PBSTR. The slides were incubated with anti-TgAaaH primary antibody (1:1000 in 5% PBSTR) at 4°C overnight. The slides were washed with PBST and the secondary antibody Alexa Fluor®555/red fluorescence protein (RFP) goat anti-rabbit (2:1000 in 10% cold fish gelatine PBST: Life technologies) was added to slides for 1 hour at room temperature. The slides were washed, then 12 µg/ml Hoechst 33258 (Invitrogen) was added to each slide, followed by incubation for 20 minutes at room temperature. Then the slides were washed and mounted (Vector shield mounting) at 4°C for 4 hours. For each staining, a slide was negatively stained by not adding the primary antibody. All slides were kept at 4°C for 4 hours in the dark before imaging using a Zeiss LSM 880 laser scanning inverted confocal microscope with 40X and 63X oil immersion objective . The three dimension (3D) reconstructions of several Z-stack images were generated with the same equipment using the LSM imaging software. The settings for brightness and contrast were unified for all images.

2.2.8 Western blot localisation of DDC in *T. gondii*

Western blot localisation of DDC was performed to verify if PC12 produced DDC was transferred into *T. gondii* cyst by the parasite.

Cell harvesting: To localise DDC, PC12 cells were infected with 1:1 *T. gondii* Prugniard. Dopaminergic PC12 whole cells (WC) were used as positive control and HFF WC were used as a negative control. Intracellular parasites were then collected by

centrifugation at 2500xg for 10 minutes. The parasite was released using 27-gauge needles and parasite pellets collected by centrifugation at 2500xg for 10 minutes. The cell pellet was suspended in RIPA buffer and sonicated for 10 seconds. Protein concentration was estimated using Bradford reagent, as explained in 2.2.4.

WB localisation of DDC: WB was done as detailed in section 2.2.4; briefly, ten to twenty μg protein was separated by SDS-PAGE and transferred to nitrocellulose. Rabbit anti-dopa-decarboxylase 1:5000 (Abcam, UK) was used as a primary antibody. Goat anti-rabbit HRP conjugate (Sigma) 1:5000 was the secondary antibody. The film was developed from the blot using Supersignal West Pico Chemiluminescent detection kit. Bands were visualised with an X-Omat film system.

2.3 Results

2.3.1 Dopa-oxidase activity in *T. gondii*

Dopa-oxidase activity assay was measured in *T. gondii* cell extract to test the possibility that L-DOPA (end products of TgAaaH enzyme activity) and DA have a role in *T. gondii* cyst wall cross linking by forming dopaquinone as a result of dopa-oxidase enzyme activity. Dopa-oxidase activity in the parasites was determined by measuring the formation of dopaquinone. When dopaquinone is formed, 3-methyl-2-benzothiazolinonehydrazone hydrochloride (MTBH) will react with it and form a dark pink pigment that can be measured at 505 nm. The final absorbance measurement is an average of three biological replicates (Table 2.1). No dopa-oxidase activity was detected in both infected PC12 and HFF cells with *T. gondii* tachyzoites. HFF cells infected with the *T. gondii* induced to bradyzoites also showed no enzymatic activity. The reaction mix with mushroom tyrosinase (control positive) showed dopa-oxidase activity (absorbance at 505 nm >0.2). Dopa-oxidase activity was not detected when the substrate was absent from the reaction mixture or in PC12 and HeLa cells (control −ve) which is consistent with the absorbance data reading obtained from the original reference paper (Winder and Harris, 1991).

2.3.2 De-novo synthesis of dopamine in *T. gondii*

To determine whether *T. gondii* can produce DA in non-dopaminergic cells. DA synthesis in *T. gondii* was assessed by HPLC (Figure 2.2) after adding tyrosine and tetrahydrobiopterin to the free sonicated cell extract. HPLC chromatogram of *T. gondii* pellet had L-DOPA but no DA peak. On the other hand, the positive control cells (PC12) had both L-DOPA and DA peaks. HFF negative control cells had no DA or L-DOPA peak.

Cells/ controls	Absorbance at 505nm
Mushroom tyrosinase	1.3 ± 0.40
RM (no DOPA)	0.11 ± 0.017
RM (no mushroom tyrosinase)	0.095 ± 0.013
HeLa	0.098 ± 0.0015
PC12	0.098 ± 0.0067
PC12 TGT	0.096 ± 0.0042
HFF TGT	0.096 ± 0.0082
HFF TGCL	0.095 ± 0.0037

Table 2.1. *T. gondii* dopa-oxidase assay. The table shows mean spectrophotometric absorbance of at least three biological replicates (fixed sample volume). Mushroom tyrosinase is positive control , PC12 and HeLa cells are negative controls. TGT: *T. gondii* Tachyzoites, TGCL: *T. gondii* cyst-like stages. RM stands for reaction mixture. From the absorbance reading, none of the infected cells exhibited dopa-oxidase activity.
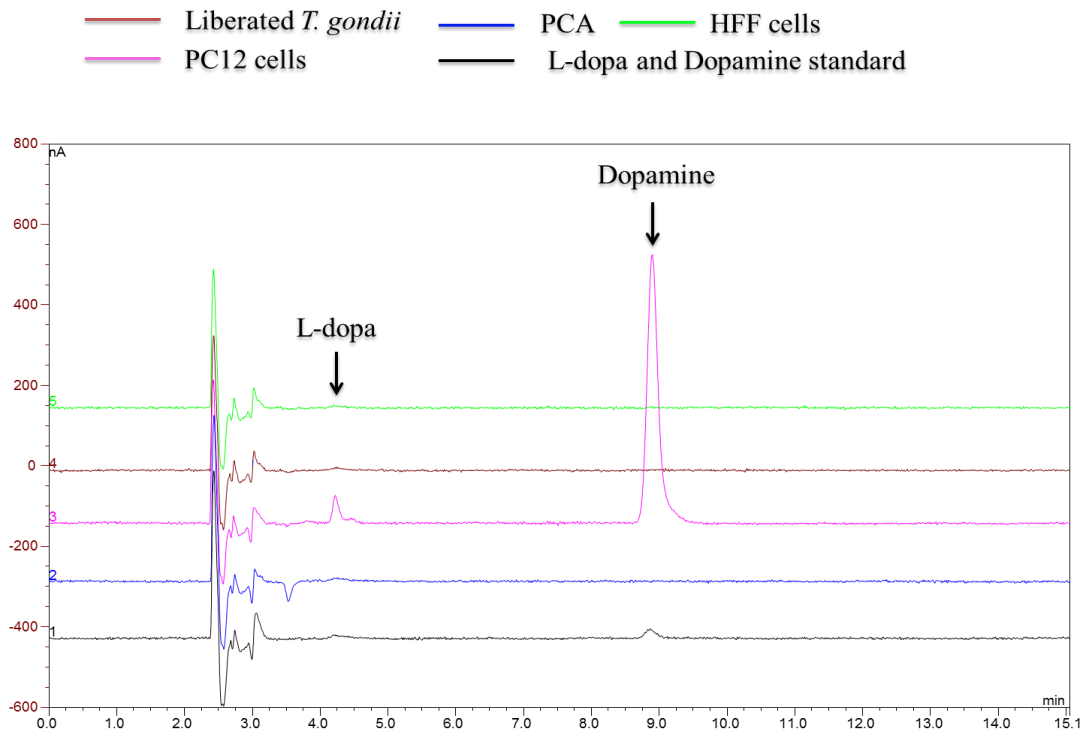
Figure 2.2. Overlay of HPLC-ED chromatograms derived from HFF cells infected with *T. gondii* Prugniard. The base line values from PC12 cells, PCA and L-DOPA were shifted ~150 nA apart for better comparisons, although all baselines were around zero. HPLC-ED chromatogram shows the following: blank (PCA) (peak time 2-3.5 minutes), L-DOPA (peak time 4-4.5 minutes), DA standard (peak time 8.5-9.5 minutes), and HFF cells (negative control). PC12 used as a positive control since it is a dopaminergic cell line, and contains all the machinery for DA synthesis, packaging, and release. HPLC ED chromatogram shows that DA is found in PC12 samples while it is not found in *T. gondii* samples. X-axis in the retention time in minutes and Y-axis is peak height μA.

2.3.3 Western blot localisation of TgAaaH

Knowing the location of TgAaaH during *T. gondii* infection will help in understanding its function in DA synthesis during the parasite infection. To localise TgAaaH, the levels of TgAaaH produced by *T. gondii* were observed before and after trypsin treatment by WB probed with anti-AAA antibody to localise TgAaaH (Figure 2.3 and Figure 2.4). Additionally, the levels of these enzymes were observed at different times (5, 10, and 15 minutes) following the exposure to trypsin. Trypsin will break down extracellular TgAaaH, which then lower its detection by WB. Accordingly, the results of this experiment will indicate whether TgAaaH is secreted out side the parasite. The WB results showed that the TgAaaH band was thinner or altogether absent after adding trypsin. There was also a proportional relationship between the low levels of extracellular TgAaaH detected by WB and an increased cells exposure time to trypsin. Band pixel intensity was analysed using ImageJ, then data analysed in GraphPad Prism using one-way ANOVA and Mann-Whitney non-parametric tests of at least three biological replicates (Figure 2.4).

Also, TgAaaH was detected in the *T. gondii* cellular component, both soluble and non-soluble fractions, on WB probed with AAAH antibody (Figure 2.5). The WB showed that TgAaaH can be detected in both fractions. Meanwhile, analysing WB band pixel intensity of four biological replicates suggested that there is no change in TgAaaH levels between soluble and non-soluble fraction (*T. gondii* parasites, pellets or supernatant). There was a slight change in L-DOPA concentration between intracellular and free parasites but it wasn't significant (*p*= 0.15).

2.3.4 HPLC-ECD Localisation of TgAaaH:

Further confirmation of TgAaaH location, The activity of TH produced by intracellular and extracellular *T. gondii* (parasites, pellets or supernatant) was monitored by observing the levels of tyrosine transformed to L-DOPA by HPLC for at least three biological replicates. L-DOPA was detected in *T. gondii* and its cellular component, but no major change was detected in the estimated L-DOPA concentration between WC, soluble and non-soluble fraction (Table 2.2). There was a slight significant change in L-DOPA concentration between intracellular and free parasites.
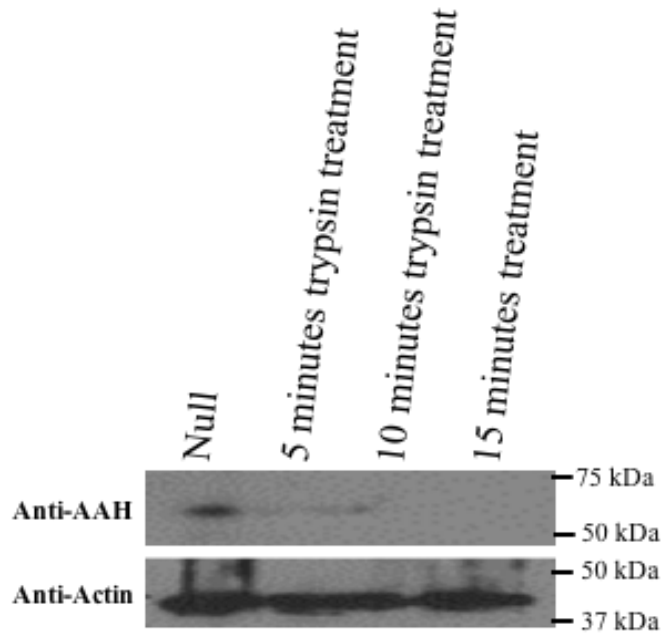
Figure 2.3. Detection of *T. gondii* TgAaaH after trypsin treatment. Blot of extracts was probed with anti-AAAH antibody to detect TgAaaH (~64.1 kDa) produced by free *T. gondii* before and after trypsin treatment. The top is a representative blot that shows that the TgAaaH band is absent when the liberated *T. gondii* is treated with trypsin. Null sample is untreated free *T. gondii* (no trypsin: positive control). Microscopic examination of the sample treated with trypsin for 15 minutes showed that the parasites were still intact. To further confirm that the parasites were still intact, WB detection of actin (housekeeping control) using anti-actin antibody (~36 kDa) showed that actin was not affected by trypsin treatment.
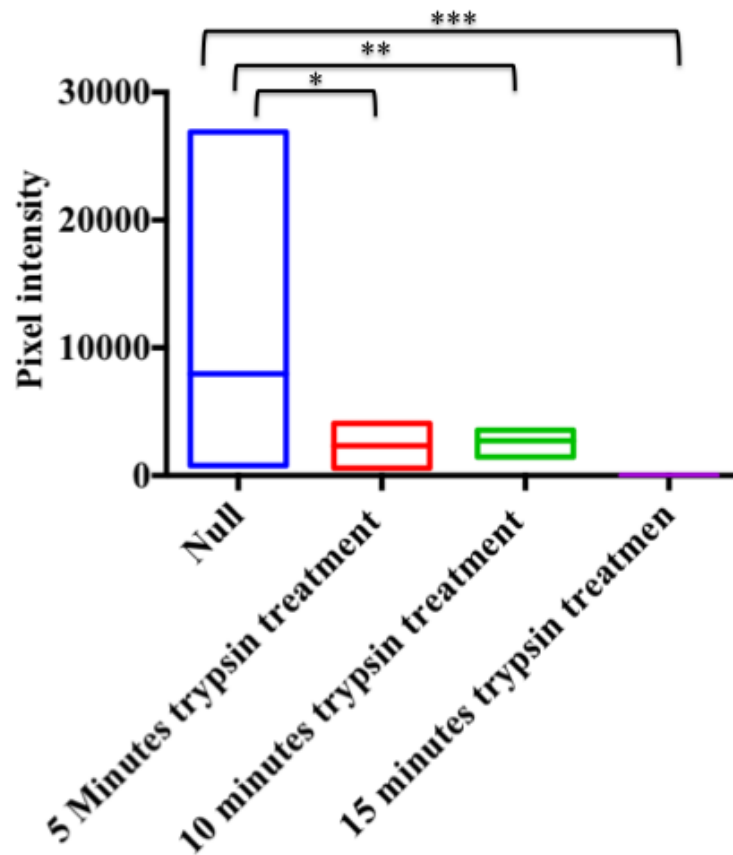
Figure 2.4. Extracellular localisation of TgAaaH. WB localisation of the protein was performed with liberated parasites that were trypsin-treated for 5, 10, or 15 minutes (biological replicates= 4) (Figure 2.3). The pixel intensity of the WB bands was measured using ImageJ software. The column graph shows the measurement obtained from ImageJ and plotted with GraphPad Prism column graphs (one-way ANOVAand Mann-Whitney non-parametric test). The graph shows that, with increases in trypsin treatment, the level of TgAaaH decreased (*P= 0.09, **P= 0.78, **P= 0.007).
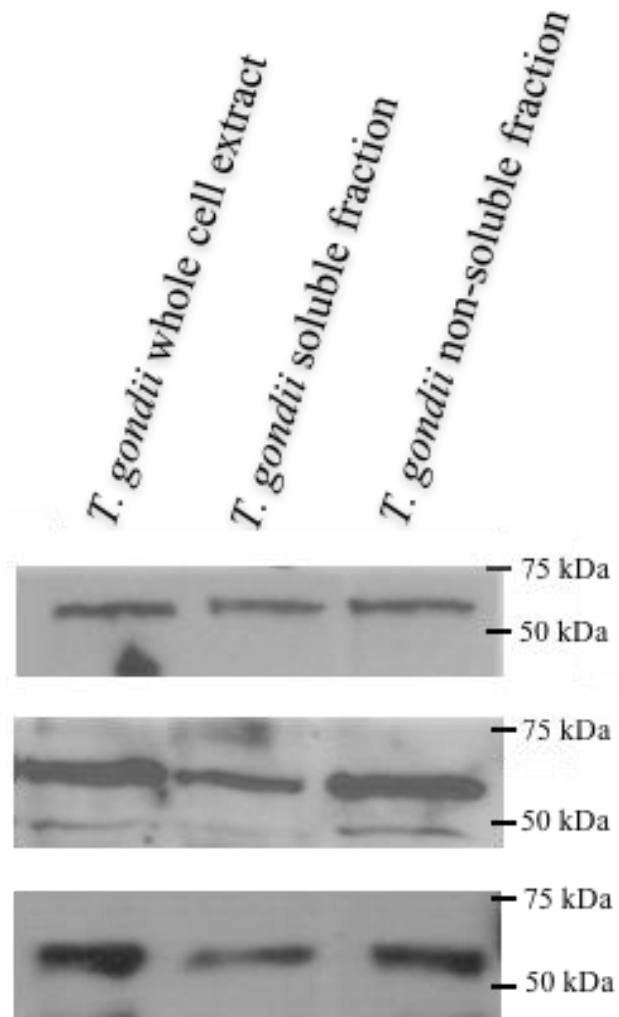
Figure 2.5. WB analysis of *T. gondii* TgAaaH in soluble and non-soluble fraction. Blots of *T. gondii* Prugniard whole cell, soluble and non-soluble fractions probed with anti-AAAH antibody to detect TgAaaH. The anti-AAAH antibody detects TgAaaH protein (~64.1 kDa) in both soluble and non-soluble fractions.
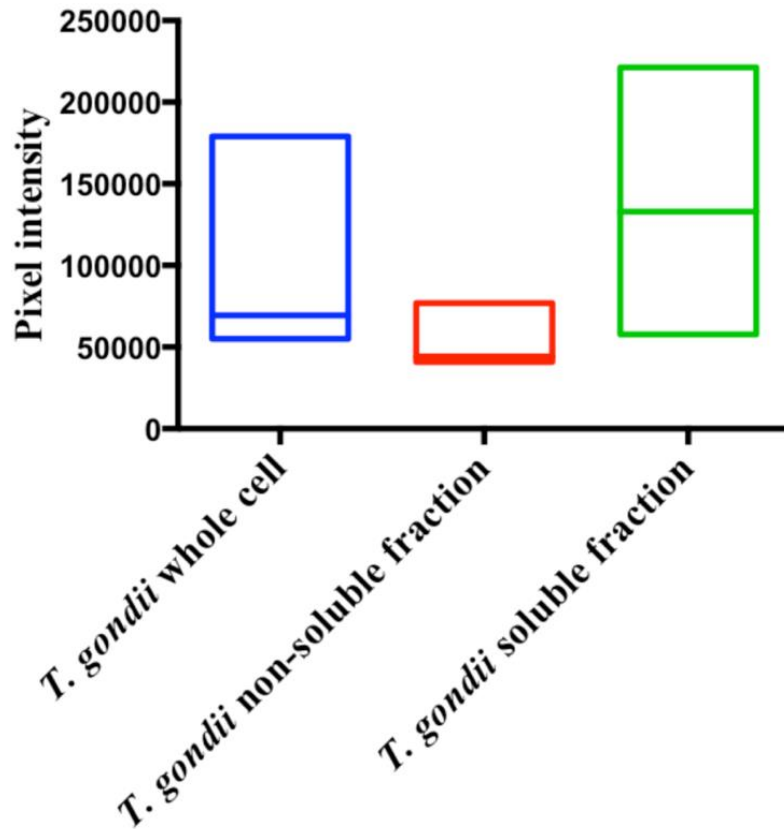
Figure 2.6. Cellular localisation of TgAaaH. WB localisation of the TgAaaH was performed on liberated parasites and different cell components of infected cells (soluble and non-soluble fractions; (biological replicates=3) (Figure 2.5). The WB band pixel intensity was measured using ImageJ software. The column graph shows the measurement obtained from ImageJ and plotted using GraphPad Prism column graphs (one way Anova). The plot analysis shows that TgAaH is detected in all cell components, and no difference was found between soluble and non-soluble fraction ($P = 0.15$).

Cells	Estimated L-DOPA concentration in µg/ml
PC12 Cells	2.25±0.84
HFF cells	0
Intracellular *T. gondii* WC	0.12±0.043
Intracellular *T. gondii* soluble fraction	0.12±0.040
Intracellular *T. gondii* insoluble fraction	0.11±0.039
Free *T. gondii* WC	0.31±0.086
Free *T. gondii* soluble fraction	0.26±0.096
Free *T. gondii* insoluble fraction	0.21±0.090

Table 2.2. Localisation of TgAaaH activity in parasite fractions. The table shows the mean ± standard deviation L-DOPA concentration (µg/ml) of at least three biological replicates. L-DOPA is produced as a result of tyrosine hydroxylation by *T. gondii* TgAaaH. L-DOPA was found in free and intracellular *T. gondii* WC extract, pellets, and supernatant. PC12 cells were used as a positive control and HFF cells were used as a negative control. Each result (sample) was obtained from one flask. WC stands for whole cell.

2.3.5   Localising TgAaaH using immunostaining

TgAaaH specific antibody was used to detect TgAaaH in HFF cells infected with shocked *T. gondii* Prugniard and ΔKU80-GFP to induce cyst formation. *T. gondii* ΔKU80-GFP expresses bradyzoite specific GFP. The two dimension (2D) images of TgAaaH stain and GFP have the same patterns (Figure 2.7). Meanwhile, 3D images showed the different pattern of TgAaaH stain and GFP (Figure 2.10). Staining was not apparent in control slides that were treated with only Alexa Flour 555/RFP (Figure 2.8). From Figure 2.7, Figure 2.9, and Figure 2.10 it is apparent that TgAaaH (stained in red) was found within the PV and inside the parasites. Figure 2.10 shows 2- and 3-D images of TgAaaH staining in ΔKU80-GFP strain which expresses bradyzoites specific GFP. The 3-D images clearly show that TgAaaH is clearly found within the PV.
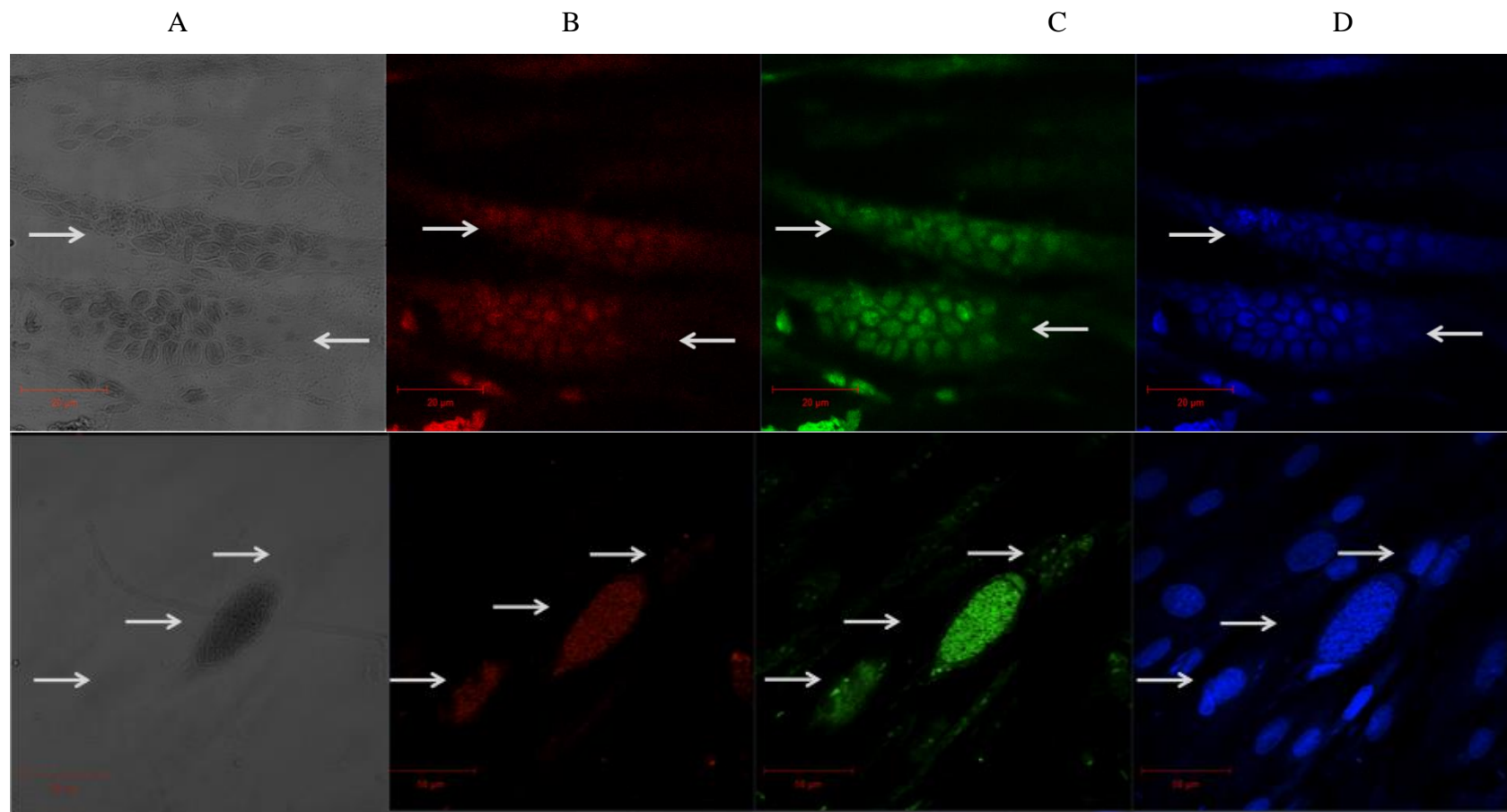
Figure 2.7. Immuno-staining Localisation of TgAaaH in *T. gondii* ΔKU80-GFP . Paraformaldehyde fixed and immunostained HFF cells infected with cyst-like *T. gondii* ΔKU80-GFP cultured in DMEM (pH 8). A: bright field image. B: *T. gondii* TgAaaH was detected using rabbit anti-TgAaaH as a primary antibody (1:1000), then Alexa Fluor®555/RFP goat anti-rabbit (2:1000) (Red: wavelength range 555-565) . C: *T. gondii* ΔKU80-GFP strain that expresses GFP under control of BAG1 promoter (Green: wave length range 433-541). D: DNA staining with Hoechst (blue: wavelength 346-441). Arrows show the location of the cyst-like *T. gondii*.

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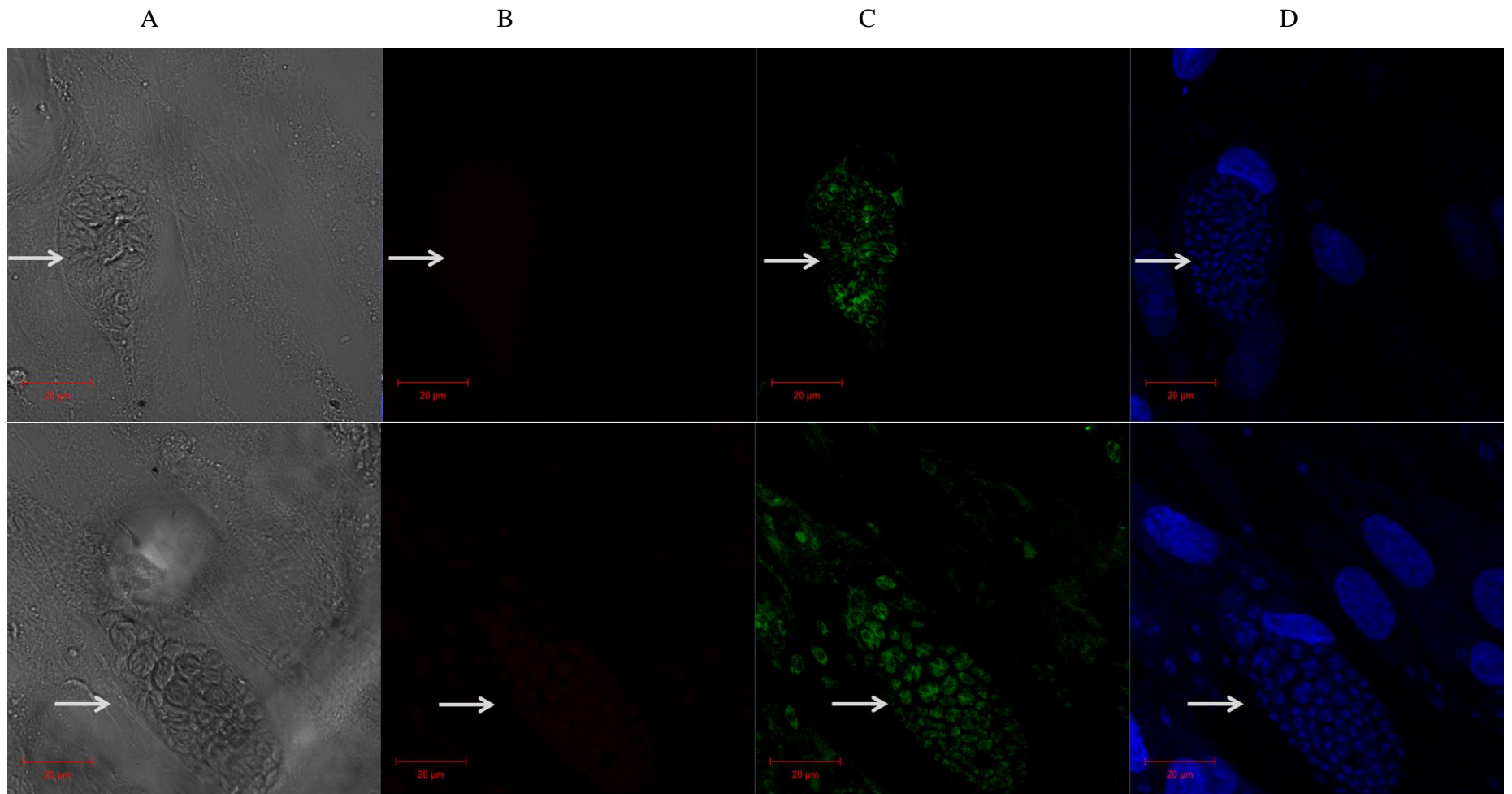
Figure 2.8. TgAaaH immunostaining negative control. Paraformaldehyde-fixed and immunostained *T. gondii* ΔKU80-GFP in HFF cells cultured in DMEM (pH 8). No primary antibody control with secondary antibody Alexa Fluor®555/RFP goat anti-rabbit (2:1000). A: images of bright field. B: red filter (wavelength 555-565). C: GFP bradyzoite marker with green filter (wavelength range 433-541). D: Blue filter showing DNA staining with Hoechst (wavelength 346-441). Arrows show the location of the cyst-like *T. gondii*.
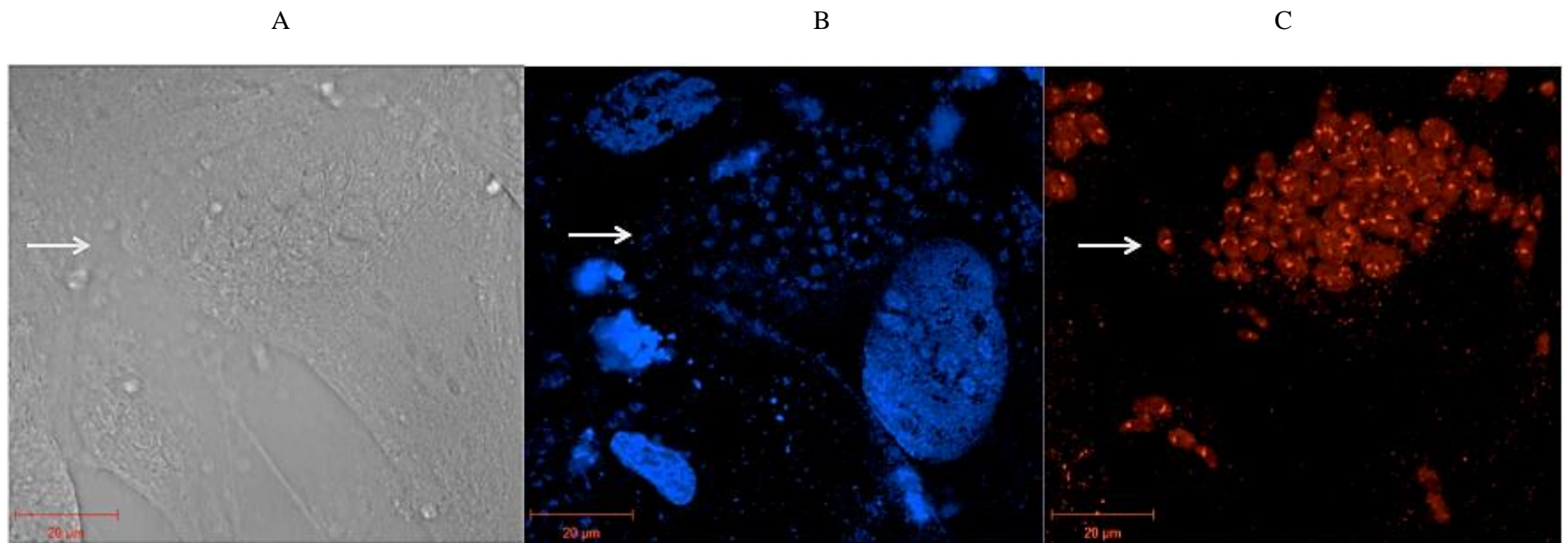
Figure 2.9. Localisation of TgAaaH in *T. gondii* Prugniard. Paraformaldehyde fixed and immunostained HFF cells infected with *T. gondii* Prugniard cultured in DMEM (pH 8). A: bright field image. B: Blue filter showing Hoechst staining of the DNA (wavelength 346-441) C: Red filter (wavelength 555-565) showing *T. gondii* TgAaaH that was detected using anti TgAaaH as a primary antibody (1:1000), then Alexa Fluor®555/RFP goat anti-rabbit (2:1000). Arrows show the location of the cyst-like *T. gondii*.
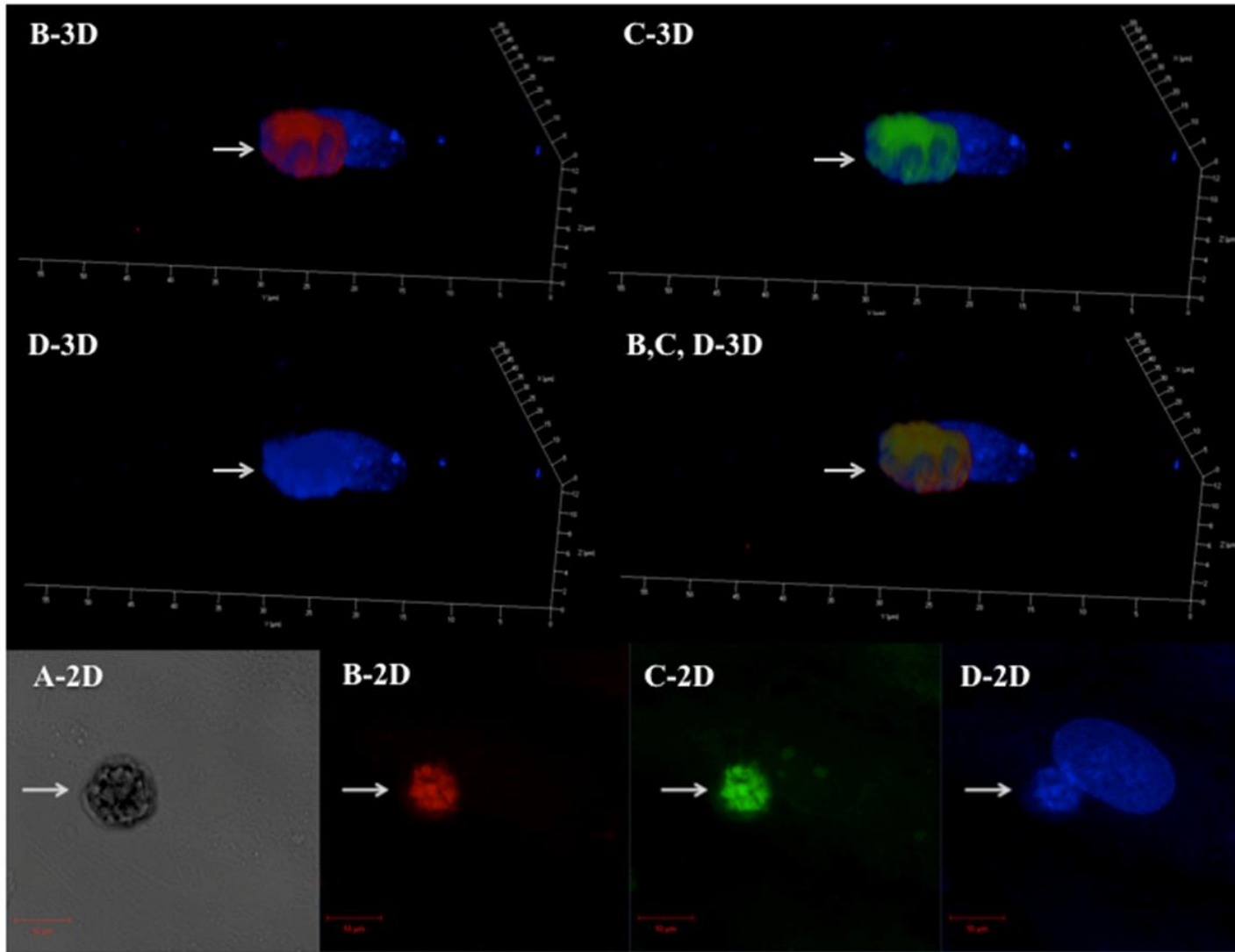
Figure 2.10. 2-D and 3-D images of TgAaaH Immuno-stain. Immuno-stained HFF cells infected with cyst-like *T. gondii* ΔKU80-GFP cultured in pH8 DMEM. The figure shows the 2 and 3-D images of TgAaaH immune-staining taken by Zeiss LSM 8800 A: bright field image. B: Detected *T. gondii* TgAaaH using anti TgAaaH used as a primary antibody (1:1000), then Alexa Fluor®555/RFP goat anti-rabbit (2:1000) (Red filter: wavelength 555-565). C: *T. gondii* ΔKU80 cyst-like GFP (Green filter; wavelength range 433-541). D: DNA staining with Hoechst (Blue filter: wavelength 346-441). Arrows show the location of the cyst-like *T. gondii*. 3-D images were reconstructed using LSM software from Z-stack serial images taken by Zeiss LSM 8800. The Y and X-axis in 3-D images have 5 μm scales. The Z axis in the 3-D images has a 2 μm scale. Arrows show the location of the cyst-like *T. gondii*.

2.3.6 Dopa-decarboxylase detection in *T. gondii*

DDC was detected from liberated *T. gondii*, which was growing in parasites from PC12 cells, by WB probed with anti-DDC antibody. The WB showed that DDC can be detected in the free parasite and, as expected, DDC can be detected in PC12 and HFF cells passed through a 27 gauge needle. DDC was detected in the dopaminergic PC12 WC that had DDC as a control positive, while it was not detected in HFF cells WC extract (Figure 2.11).
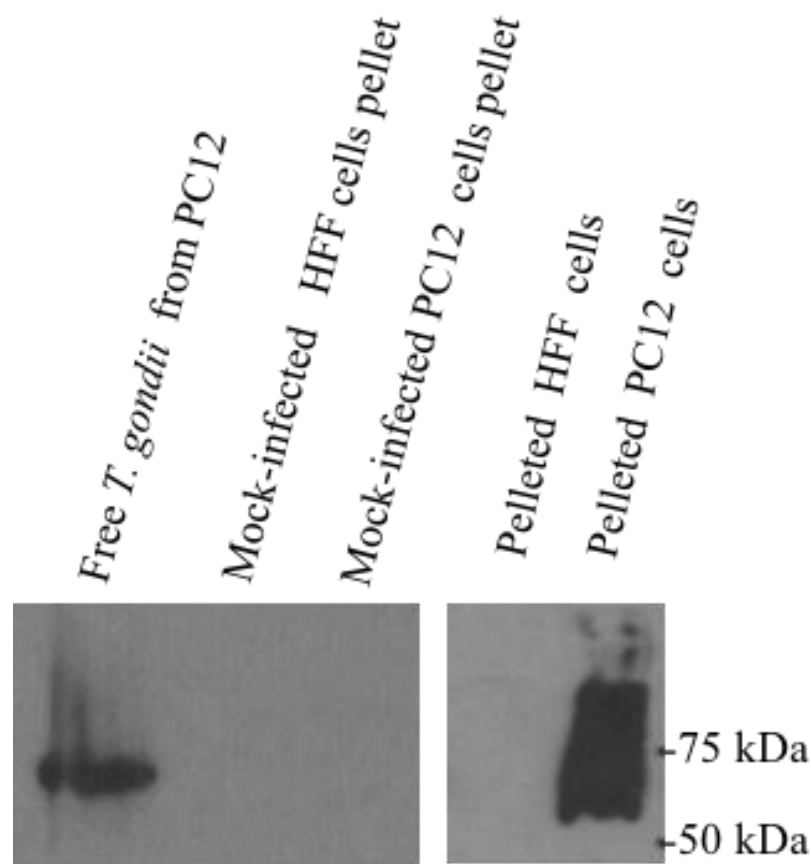
Figure 2.11. WB localisation of DDC during *T. gondii* infection. Blot of *T. gondii* Prugniard probed with rabbit anti-DDC antibody. From the blot DDC (~53 kDa) is detected in *T. gondii* and the positive control PC12 cells. DDC was not detected in the negative control HFF cells, mock infected PC12 and HFF (have been lysed by passing through a 27 gauge needle).

2.4 Discussion

In this chapter, the involvement of L-DOPA or DA in dopaquinone synthesis was evaluated in *T. gondii* by testing the dopa-oxidase activity in the parasite. Meanwhile, Dopa-oxidase assay measures the formation of the end product of L-DOPA or DA oxidation (dopaquinone). The results have shown that both liberated tachyzoites and bradyzoites from dopaminergic and non-dopaminergic cells do not have any dopa-oxidase activity (Table 2.1). These results agree with the bioinformatics information (metaTiger data base:Whitaker et al., 2009) that *T. gondii* does not have a gene that is homologous to dopa-oxidase. These data refute the probability that TgAaaH enzyme could be involved in providing L-DOPA to be converted to dopa-quinone during tachyzoites or the bradyzoites stage, and hence dopaquinone will not be utilised in cell wall synthesis. Previously, di-tyrosine and L-DOPA was found in oocyst stage wall in *Eimeria maxima* parasite (Belli et al., 2003a; Belli et al., 2003b). So, in the oocyst stage of *T. gondii*, TgAaaH might still have a function in providing di-tyrosine and L-DOPA as a part of the parasite wall biosynthesis.

T. gondii ability to form DA in non-dopaminergic cells was tested. Bioinformatics information (metaTIGER data base) indicates that the *T. gondii* genome does not encode a gene homologous to DDC. *T. gondii* infected human fibroblast cells (non-dopaminergic cells) were found to synthesise L-DOPA but not DA, whereas neither L-DOPA nor DA were detected in uninfected controls (Figure 2.2). Hence, the parasites can only increase DA in cells containing endogenous DDC, such as brain dopaminergic neurones. Similar outcomes were seen for both tachyzoites and bradyzoites, supporting the bioinformatics database information.

Bioinformatic searches have predicted that the *T. gondii* TgAaaH gene encodes signal peptides (Gaskell et al., 2009). Understanding the subcellular distribution of TgAaaH during *T. gondii* infection may contribute to our understanding of its function, such as the increase in DA production. Localisation of TgAaaH was done by growing the wild-type parasite (Prugniard) in HFF cells, then collecting the free parasites. Analysis of trypsin treatments of the free parasites allowed evaluation of extracellular vs. intracellular presence of the enzyme. Trypsin will destroy any enzyme outside the

parasite, and hence initial presence of extracellular enzyme and subsequent loss due to trypsin digestion can be examined using WB probed with anti–AAAH. As expected, extracellular TgAaaH produced by *T. gondii* was reduced or eliminated by trypsin treatment (Figure 2.3). Semi-quantitative analysis of the WB results by measuring the pixel intensity of the bands and plotting the intensities in column graphs (Figure 2.4: one way ANOVA test) showed that there is a significant decrease in TgAaaH after 15 minutes of trypsin treatment ($P= 0.0079$). Subcellular localisation of TgAaaH was done by fractionating the free parasites into their cellular components using WB probed with anti–AAAH. Detecting the presence of TgAaaH in these fractions will allow determining whether the enzyme is membrane bound or extracellular. WB showed the TgAaaH was found in both the free parasites soluble and non-soluble fractions (Figure 2.4 and Figure 2.5). These result indicate that TgAaaH was found to be membrane bound and was execrated outside the parasite.

Moreover, TH activity for the lysed and fractionised free and intracellular parasites allowed further evaluation of subcellular localization of TgAaaH enzyme. The results in Table 2.2 showed that the TH activity can be detected in the intracellular and free parasites and their cellular components. There was a slight difference in the estimated L-DOPA concentration between intracellular and free *T. gondii* parasites and their cellular components. This suggests that there is an increase in TH activity in free parasites more than the intracellular parasites (T- test: $P=0.008$). These finding confirms the initial localisation of TgAaaH to be membrane bound and outside the parasite.

TgAaaH was localised in *T. gondii* Prugniard and ΔKU80-GFP strains using immuno-fluorescence staining. To facilitate TgAaaH localisation during the bradyzoite stage, *T. gondii* ΔKU80-GFP strain was used in the immunostaining because it expresses bradyzoite specific GFP (Fox et al., 2011). The TgAaaH specific immuno-fluorescence staining (Figure 2.7 and Figure 2.9) further supported the finding that TgAaaH was found within the PV and the parasite. Meanwhile, better localisation was obtained when 3D imaging was used to verify that the individual signals seen through the GFP filter or red filters did not overlap.

The WB, tyrosine hydroxylase activity, and immunofluorescence results demonstrated that TgAaaH produced by *T. gondii* was likely to be found inside the parasite, membrane-bound and within the PV. The latter findings confirm initial observations by Gaskell et al. (2009) and Prandovszky et al. (2011) that the enzyme was found within the PV. Meanwhile, Wang et al. (2015) could not directly detect TgAaaH using WB, tyrosine hydroxylase activity evaluation, or immunofluorescence staining. They have suggested that this could be due to the low expression level of the enzyme. However, in this chapter, the localisation of TgAaaH using these techniques was achieved successfully.

Recently, DCC immune-fluorescence localisation showed DCC within the cyst in *T. gondii* infected PC12 cells (Martin et al., 2015). The current chapter showed DDC detection in the liberated *T. gondii* parasites from dopaminergic PC12 cells by WB probed with anti-DDC primary antibody. The WB results demonstrated that DCC is found within the tissue cyst. DDC can be detected in PC12 (positive control), but not in HFF whole cells (as a negative control). Coppens et al. (2006) demonstrated that *T. gondii* actively import the host endosomal system to the PV for nutrients. Similarly, an Apicomplexan family member, Plasmodium falciparum, was found to import D-aminolevulinate dehydratase from the cytoplasm of erythrocytes into parasites (Bonday et al., 1997).

2.5   Conclusions

This chapter has shown that the increase of DA due to *T. gondii* TgAaaH will be restricted to monoaminergic cells with DDC. Meanwhile, the conversion of L-DOPA and DA to dopaquinone was unlikely. The chapter research has also shown the cellular location of TgAaaH to be within the PV, parasite, and membrane bound. Furthermore, DDC was found within the parasite during dopaminergic cell infection with *T. gondii*.

Further research is needed to understand the molecular mechanism by which the parasite TgAaaH and the host DDC interact together during infection to make DA. Furthermore, to understand how DA is involved in the mechanism that induces the behavioural changes in the intermediate host. Answering these questions will help in understanding the connection between *T. gondii* infection and human mental disorders,

especially considering that there is no current cure that clears the parasite infection totally. The next chapter will explore the use of a new bradyzoite differentiation methods using TD-DMEM (chapter 3) that will be used in the developing of a novel bradyzoite specific drug screening assay (chapter 4).

Chapter 3 Effect of tryptophan-depleted DMEM on the differentiation of *Toxoplasma gondii*

3.1 Introduction

T. gondii human infection has two stages – acute tachyzoite stage and latent brayzoite cyst stage. Several methods, such as alkaline media and heat shock, have been used in studies to induce the parasite to form the bradyzoite stage *in vitro* (Soete et al., 1994; Guimarães et al., 2008; Guimarães et al., 2009). However, outcomes from these methods include both tachyzoite and bradyzoite growth, which has been shown to affect the data produced (Singh et al., 2002; Fouts and Boothroyd, 2007). Studies have demonstrated that tryptophan starvation will inhibit parasite growth- detail how this was performed is shortcoming (Pfefferkorn et al., 1986). This chapter focuses on the effect of tryptophan depletion on *T. gondii* conversion to bradyzoite, and use of tryptophan depletion as an bradyzoite induction method. This was done using tryptophan depleted- Dulbecco's modified Eagle's medium (TD-DMEM). Following a brief introduction about tryptophan starvation and the techniques used for differentiating *T. gondii*, results of *T. gondii* differentiation in TD-DMEM using bright field imaging, fluorescence imaging, and qPCR analysis will be presented.

Tryptophan is an amino acid found to be involved with the attachment process of TgMIC2 (Kappe et al., 1999). According to bioinformatics website metaTiger (Whitaker et al., 2009), *T. gondii* does not have the enzymes required for the synthesise of tryptophan. Pfefferkorn et al. (1986) studied the effect of IFN-γ treatment on HFF cells infected with *T. gondii* tachyzoites, and found that *T. gondii* tachyzoite growth was inhibited due tryptophan starvation. Tryptophan starvation occur when IFN-γ activates host IDO, which breaks down tryptophan *N*-formylkynurenine and kynurenine. This effect was reversed when tryptophan was supplemented in the media (Pfefferkorn and Guyre, 1984; Pfefferkorn et al., 1986). These results show that *Toxoplasma* is tryptophan auxotrophic, where acquisition of tryptophan from the host cytosol or media is essential for the growth of the parasite (Pfefferkorn and Guyre,

1984; Pfefferkorn et al., 1986). Pfefferkorn and colleagues (1984, 1986) demonstrated how IFN-γ treatment for two days reduces the host cell levels of tryptophan, and subsequently tachyzoite growth was inhibited. Here we interested to see the direct effect tryptophan depletion has on the bradyzoites differentiation.

Fox et al. (2004), studied the effect of depletion of the non-essential amino acid arginine on the growth of mutant *T. gondii* strain that cannot synthesise arginine *in vitro*. Tachyzoite growth rate in arginine free media was assessed by counting the parasite number per PVs after 36 hours post infection. Surprisingly, arginine starvation slowed parasite growth rate. Furthermore, both SAG1 and biotinylated *Dolichos biflorus* lectin (DBA) staining of the parasites that have been growing in arginine free media for two and seven days showed that 99% of the vacuole where SAG1 negative and DBA positive (Fox et al., 2004). These results demonstrated that arginine starvation induce tachyzoite to bradyzoite switching, and give rise to a new question: Will *in vitro* tryptophan starvation force *T. gondii* to differentiate?

Current methods reported to induce parasite differentiation *in vitro* are heat (43°C), pH stress (pH 6.6–6.8 or 8.0–8.2), chemical treatment (sodium arsenite), and differentiated myotube cultures (skeletal muscle cells) (Soete et al., 1994; Guimarães et al., 2008; Guimarães et al., 2009). Singh et al. (2002); studied gene expression in tachyzoite to bradyzoite differentiation of mutants strains compared to wild type strain. In their study only 10% of the mutant strains were able to differentiate and 85 to 90% of wild type strain were able to convert to bradyzoites using alkaline induction methods. In the meantime, Fouts and Boothroyd (2007) evaluated host gene expression of *T. gondii* infected and uninfected host cells in alkaline media, and they found that it was not easy to analyse the gene transcription levels of cells infected with bradyzoites and tachyzoites. The data analysis was not easy since the differentiation of bradyzoites in HFF cells decreases with increasing the parasite infection multiplicity in high pH media (pH 8.2). To avoid heterogonous stage growth only 20% of cells were allowed to be infected with bradyzoites, which resulted in low bradyzoite stage gene expression. Additionally, the high pH was found to affect host gene transcription (Fouts and Boothroyd, 2007). Complications during data analysis are a limitation of current methods used to induce *T. gondii* differentiation (such as alkaline pH, high temperature, and arsenic), as they result in heterogonous growth of both bradyzoites and tachyzoites

in vitro (such as in Fouts and Boothroyd, 2007). Therefore, in this study, the development and use of TD-DMEM as an alternative to induce *T. gondii* differentiation was investigated.

Successful induction of bradyzoite formation using TD-DMEM without tachyzoite growth will improve our ability to study the bradyzoite stage and develop bradyzoite screening assays. In this chapter, we explored the *in vitro* effect of tryptophan starvation on *T. gondii*. The viability of *T. gondii* in TD-DMEM was observed using light microscopy. Then, conversion of the parasite from tachyzoite to bradyzoite in TD-DMEM was investigated by fluorescent imaging of *T. gondii* ΔKU80-GFP strain with bradyzoite-specific GFP. Furthermore, the expression of stage-specific markers SAG1, SAG4, BAG1, and MAG1 mRNA was assessed and compared between parasites growing in TD-DMEM, normal DMEM, and DMEM with an alkaline pH.

3.2 Materials and Methods

3.2.1 Cultivation of cells and *T. gondii* strains

The HFF cells used in this study were maintained and passaged as previously explained in the Methods section of Chapter 2. *T. gondii* ΔKU80-GFP strain was a kind gift from David Bzik, Dartmouth. The parasites were cultured and maintained as explained in Chapter 2 Method section. Tachyzoites were induced to convert to the cyst stage using high pH (pH 8), as explained previously in the Chapter 2 Methods section.

3.2.2 Preparing TD-DMEM

To make TD-DMEM, 50 mL FBS was dialysed in 10× Hanks' balanced salt solution (HBSS; Life Technologies) for one, three hours, and then left overnight. All the materials used in the dialysing step were autoclaved. For the dialysis, Bio Design Dialysis Tubing (BioDesign Inc) was used. Before dialysis, the Bio Design Dialysis Tubing was soaked in distilled water for four hours, with a change of water after one hour, to remove traces of glycerol. Then the tube was soaked in 1 mM EDTA for a total of four hours with a change of solution at one hour to remove heavy metals. Finally, the tube was placed in a beaker with distilled water and autoclaved before use.

TD-DMEM (500 mL) consists of 50 mL of 10× HBSS, 5 mL of 100× MEM vitamin (Life Technologies), 5 mL 100× MEM non-essential amino acid solution (10 mM, Life Technologies), 10 mL 50× amino acid mix (Life Technologies), 282 mg solid L-glutamine, and 1.75 g glucose (pH 7.2), and 400 ml dH2O. The solution was adjusted to pH 7.2 using 10% NaHCO3, brought to 500 mL with dH2O. This mixture was filtered, and then added to it the dialysed FBS (50 mL) and 1% SP. The prepared TD-DMEM was stored at 4ºC. The final concentration of tryptophan is TD-DMEM is 0.1mg/500ml.

3.2.3 *T. gondii* differentiation in TD-DMEM with bright field microscopy

To see whether the parasite can grow and form bradyzoite cysts in the TD-DMEM, parasite growth in HFF was observed using bright field confocal microscopy. Four HFF cells flasks were infected with 2.5×10^5 shocked *T. gondii* ΔKU80-GFP. Two of the infected HFF flasks were supplemented with alkaline DMEM, and the other two HFF flasks were supplemented with TD-DMEM (pH 7.2). The four flasks were incubated at

37°C in 5% CO_2 for nine days. On day nine, light microscopic images were obtained from each flask.

3.2.4 *T. gondii* differentiation in TD-DMEM with fluorescent imaging

Parasite growth in TD-DMEM and the formation of cysts in HFF cells was further assessed by observing the induction of *T. gondii* ΔKU80-GFP cysts, which express bradyzoites specific GFP, using confocal fluorescent microscopy. Confluent HFF cells growing on sterile 12-mm glass coverslips were infected with 1×10^3 shocked *T. gondii* ΔKU80-GFP and allowed to grow in TD-DMEM or alkaline DMEM (pH 8) for nine days. Then, the infected cells were fixed with 4% paraformaldehyde for 10 minutes at room temperature and then incubated with Hoechst for 20 minutes at room temperature. Finally, the slides were washed and mounted (Vector shield mounting) at 4°C for 4 hours. Images of the slides were taken using a Zeiss LSM 880 laser scanning inverted confocal microscope with 40× and 60× oil immersion objectives.

3.2.5 Analysis of bradyzoite-specific genes for *T. gondii* growing in TD-DMEM

Finally, to evaluate the induction and formation of bradyzoites cyst in TD-DMEM was done using semi-quantitative RT- PCR. The bradyzoite- and tachyzoite-specific gene expression for *T. gondii* Prugniard growing in TD-DMEM was detected and compared with the gene expression of parasite growing in normal and alkaline DMEM.

Cell culture and harvesting: Confluent HFF cells flasks were infected with $1–2 \times 10^5$ shocked parasites, then the infected cells were grown in TD-DMEM, DMEM, or alkaline DMEM for nine days. The parasite pellet was collected on days three, six, and nine. The RNA was extracted from the parasite samples and converted to cDNA. Finally, bradyzoite and tachyzoites-specific genes in these samples were detected by quantitative polymerase chain reaction (qPCR). This experiment was repeated at least twice for each culture medium.

RNA extraction: Infected cells were scraped, and the cell pellets were collected by centrifugation at $25000 \times g$ for 10 minutes. Most of the medium was discarded, and the cells were suspended in the media left. The RNA was extracted from each sample using Direct-zol™ RNA MiniPrep kit (Zymo Research).The RNA was extracted according to the manufacturer's instruction. Briefly, the cells were lysed using 1:1 TRI reagent and

homogenised by pipetting and vortexing. The sample was centrifuged at $10000 \times g$ for 1 minute and the supernatant transferred to a new tube. Then, 1:1 volume ethanol (95-100%) was added to the mixture and mixed thoroughly. The mixture was transferred to a spin column, which was placed into a 2-mL collection tubes and centrifuged at $10000 \times g$ for 30 seconds. The flow-through was discarded and the column was washed with 400 µL Direct-zol™ RNA PreWash and centrifuged at $10000 \times g$ for 30 seconds. The flow-through was discarded and the column washed twice with 700 µL of wash buffer. After each wash, the column was centrifuged at $10000 \times g$ for 2 minutes. Finally, the column was carefully transferred into an RNase-free tube and the RNA was eluted by adding 50 µL of DNase/RNase-free water directly to the column matrix and centrifuging the sample at $10000 \times g$ for 30 seconds. The RNA concentration was estimated using NanoDrop 2000 (Thermo Scientific).

Synthesis of cDNA: To construct a single strand of cDNA from mRNA, a Maxima First-Strand cDNA synthesis kit (Thermo Scientific) was used and RT-qPCR was performed according to the manufacturer instructions. Briefly, cDNA was synthesised by mixing 100 ng of mRNA, 4 µL of 5× reaction mixture, 2 µL of maxima enzyme mix, and RNAse-free water to make a total reaction volume of 20 µL. The sample was run in a 2720 thermal cycler PCR machine (Applied Biosystems) for 10 min at 25°C followed by 15 min at 50°C. The reaction was terminated by heating at 80°C for 5 minutes, and then cDNA concentration was estimated using NanoDrop 2000.

Detection of stage-specific genes by quantitative PCR: To compare the stage-specific gene expression in parasites growing in TD-DMEM, DMEM, and alkaline DMEM (pH 8), qPCR was used with primers designed specifically to amplify these genes. In a white 96-well qPCR plate (Bio-rad), a qPCR reaction mixture was made as follows: 12.5 µL SYBR® Green PCR Master Mix (Thermo Fisher Scientific), 0.5 µL forward primer, 0.5 µL reverse primer, and 10.5 µL DNase/RNase-Free Water. The initial concentration of all the primers was 100 nM except for BAG1, which was made at 50 nM. The following primers were used: *T. gondii* glyceraldehyde 3-phosphate dehydrogenase (GAPDH; forward: 5′- CAAGGGTGGCAAAGATTGGC -3′ and reverse: 5′-GCC TGAATCTTTGCCACCCTTG-3′), SAG1(P30; forward: 5′-CGACAG CCGCGGTCATTCTC-3′ and reverse: 5′-GCAACCAGTCAGCGTCGTCC-3′) (Xiong et al., 1993), SAG4 (P18; forward: 5′-GCTGGACCTACGATTTCAAG AAGGC-3′

and reverse: 5′-GCTGCGAGCTCGACGGGCTCATC-3′) (Ödberg-Ferragut et al., 1996), BAG1 (forward: 5′-GAGAAACGGGCGAGTAGCACCTGAGGAGA-3′ and reverse 5′-TGG GTCTACGTCGATGGCATGACAAC-3′), and Matrix antigen 1 (MAG1; forward 5′-CCC GAT TAC AGC CGC AAA TG-3′ and reverse 5′-ATG ACG TCG CCA AGT CTG TT-3′). The qPCR plate was incubated in a G1000 Thermal cycler (Bio-Rad) at 50°C for 2 minutes; 95°C for 10 minutes; followed by 40 cycles of 95°C for 15 seconds, 55–70°C for 30 seconds (depends on the primer); and 72°C for 30 seconds. It was finally followed by a melting curve. The annealing temperature for each primer was as follows: 66°C for GAPDH, 67.5°C for MAG1, and 69°C for both SAG1 and SAG4. All qPCR runs were done in parallel with the housekeeping gene GAPDH.

3.3 Results

3.3.1 *T. gondii* differentiation in TD-DMEM with bright field microscopy

Initial assessment of *T. gondii* growth and differentiation in TD-DMEM in HFF cells was done by bright field microscopy (Figure 3.1). HFF cells infected for nine days with shocked *T. gondii,* growing in TD-DMEM or alkaline DMEM, were observed by confocal bright field light microscope. The images show that the shocked parasites growing in TD-DMEM form intracellular tissue cysts. There were none of the classic tachyzoite rosette forms observed in the TD-DMEM cultures, while this form was observed in alkaline media. On the other hand, shocked *T. gondii* cultured in alkaline DMEM developed into both tachyzoite and bradyzoite stages (Figure 3.1) with lysis of the HFF cell monolayer due to tachyzoite replication (data not shown). Parasite growth and differentiation in HFF cells was monitored for at least two biological replicates in each media.

3.3.2 *T. gondii* differentiation in TD-DMEM with bradyzoite-specific GFP fluorescent imaging

Further assessment of *T. gondii* growth and differentiation in TD-DMEM was carried out using fluorescence microscopy of a *T. gondii* strain expressing GFP under the control of a bradyzoite-specific (BAG1) gene promoter (Fox et al., 2011) (Figure 3.2 and Figure 3.3). HFF monolayers cultured on coverslips were infected with *T. gondii* in TD-DMEM and incubated for nine days, after which the slides were fixed and visualised. A key observation in the ability of the TF-DMEM medium is the maintenance after this incubation period of a complete HFF cell monolayer and the lack of visible free tachyzoites from lysed cells. Hoechst staining of nuclei exhibited blue fluorescence of HFF cell nuclei and pinpoint staining of parasite nuclei. All of the parasite nuclei visible are associated with GFP-fluorescing bradyzoites (Figure 1.2 and Figure 3.3). Hence, *T. gondii* growing in TD-DMEM exclusively formed bradyzoites in intracellular cysts expressing GFP and no tachyzoites were detected. After the nine days of infection, parallel cultures in alkaline DMEM had the complete monolayer of HFF cells lysed and free tachyzoites floating in the media.
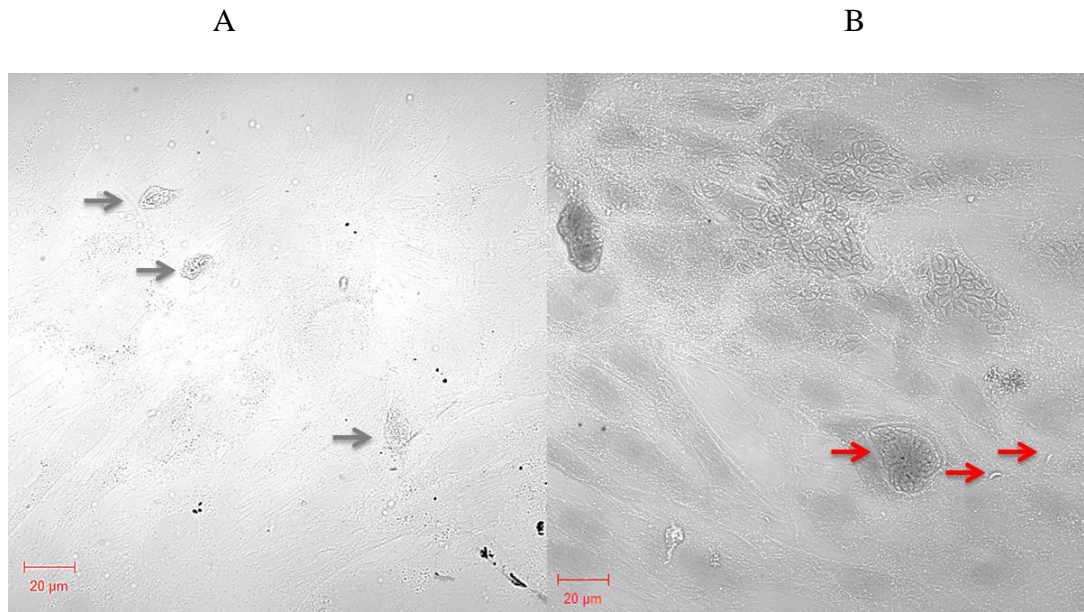
Figure 3.1. *T. gondii* growth and differentiation in TD-DMEM under a bright field microscope. A: Shocked *T. gondii* cultured in TD-DMEM for nine days, grey arrows show cysts. B: Shocked *T. gondii* growing in alkaline DMEM for nine days, red arrows show classic tachyzoite rosette and free tachyzoites. The images show that parasites cultured in alkaline DMEM started to convert to the tachyzoite form and free tachyzoites were detected. Meanwhile, the parasite forms intracellular cysts in TD-DMEM.
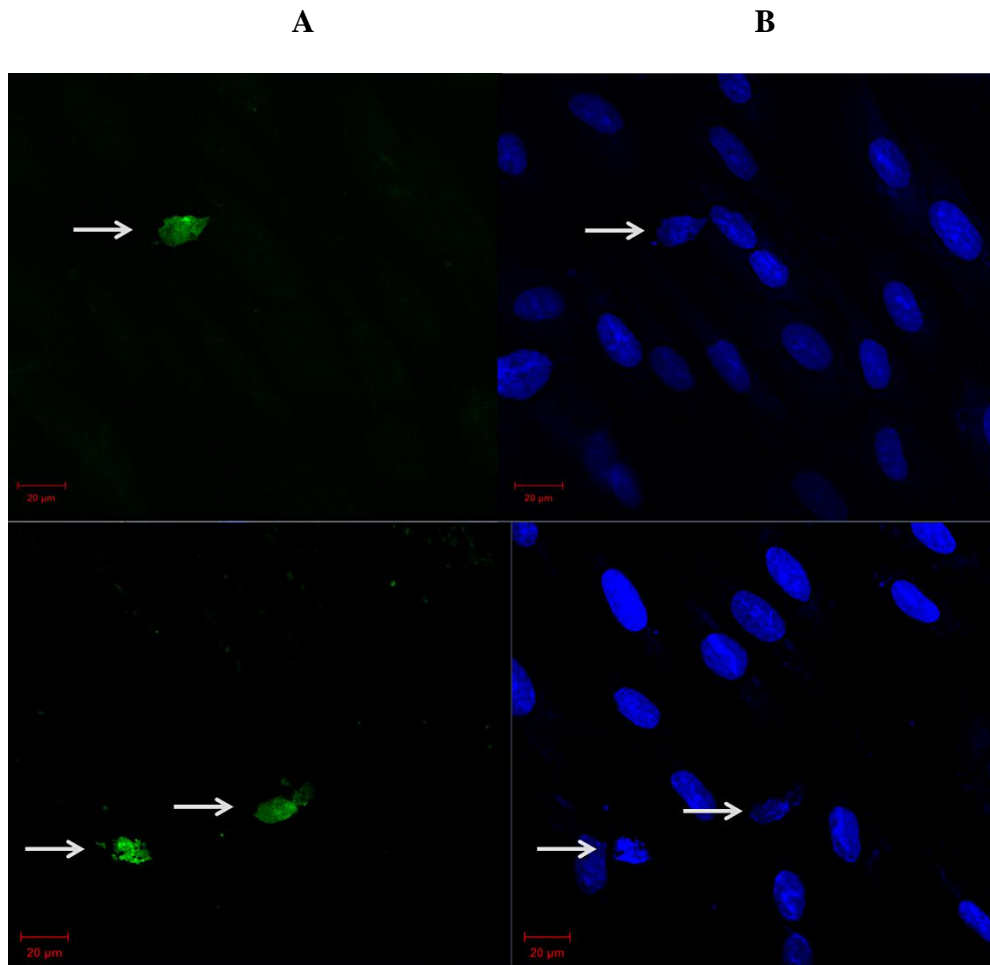
Figure 3.2. *T. gondii* **differentiation in TD-DMEM under a fluorescent confocal microscope (40X).** The images show HFF cell monolayers infected with shocked *T. gondii* expressing GFP under a BAG1 promoter cultured in TD-DMEM for nine days. Images of the differentiated parasite were obtained using an LSM 880 laser scanning inverted confocal microscope 40X oil objective. A: shows the bradyzoites cysts fluorescing in green (Green filter: wave length range 433-541). B: DNA stain with Hoechst identifies HFF and parasite nuclei (blue filter: wavelength 346-441). Note that no tachyzoites (small blue dots that do not fluoresce green) are visible. Arrows indicate differentiated *T. gondii*.
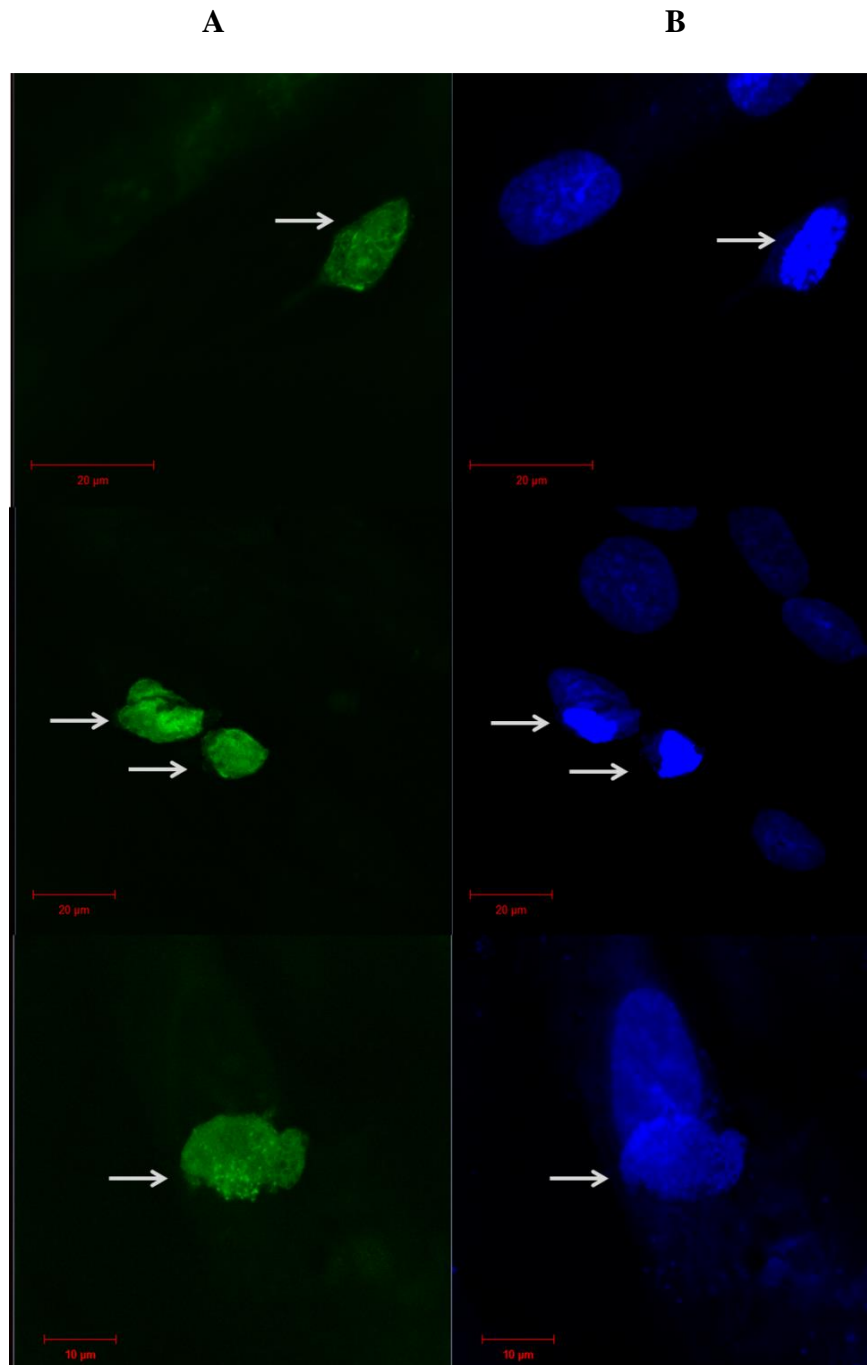
Figure 3.3. *T. gondii* **differentiation in TD-DMEM under a fluorescent confocal microscope (60X).** Images taken with LSM 880 laser scanning inverted confocal microscope (60X oil objective) of HFF cell monolayers infected with shocked *T. gondii* and cultured in TD-DMEM for nine days, Parasites are expressing bradyzoites specific GFP. A: shows the bradyzoites GFP (Green filter: wave length range 433-541). B: HFF and parasite nuclei DNA stained with Hoechst (blue filter: wavelength 346-441). Arrows show the differentiated *T. gondii*.

3.3.3 Bradyzoite-specific gene analysis of *T. gondii* growing in TD-DMEM

To evaluate whether the parasites in TD-DMEM differentiated into bradyzoites, the expression of bradyzoite (BAG1) and tachyzoite (SAG1) specific genes were assessed using qPCR. Expression of SAG1 in *T. gondii* decreased from day three to nine of cultivation in TD-DMEM. Whereas there was an increase in BAG1 expression from days three to nine (Figure 3.4, Figure 3.5, and Table 3.1). Other bradyzoite and tissue cyst markers, SAG4 and MAG1 respectively, were inconsistent in control parasite cultures in DMEM or alkaline DMEM, and hence were not included in the analysis. *T. gondii* growing in TD-DMEM formed intracellular cysts visible microscopically on day nine (Figure 3.1). Parasite cultures in pH8 media induce bradyzoite conversion in a proportion of parasites but other parasites remain vegetative tachyzoites and replicate and lyse cells. This was observed by loss of the HFF cell monolayer after three and four days of cultivation (data not shown). This is reflected by a trend in the increase in expression of SAG1 from days three to nine for *T. gondii* growing in alkaline DMEM although this trend was not statistically significant (t- test: $p=0.37$) (Figure 3.4 and Table 3.1). BAG1 was induced at day three of cultivation in pH8 medium as found in published data (Jerome et al., 1998) but day six and nine time points have inconsistent results between repeats most likely due to the large degree of host cell lysis in these cultures with active tachyzoite replication.
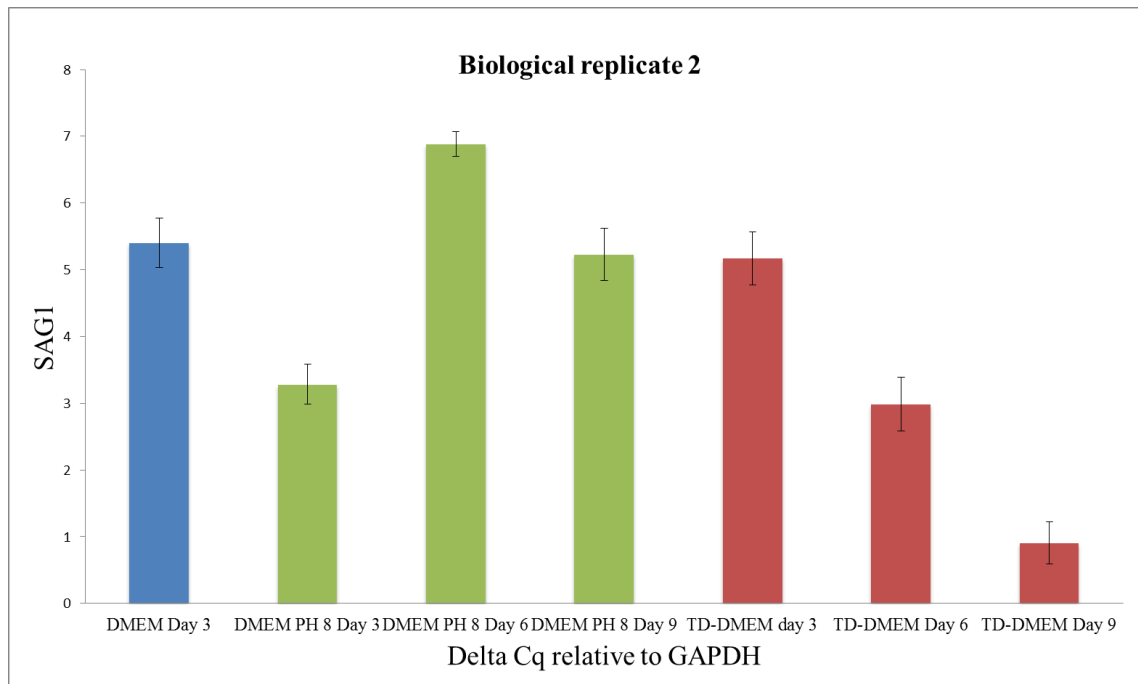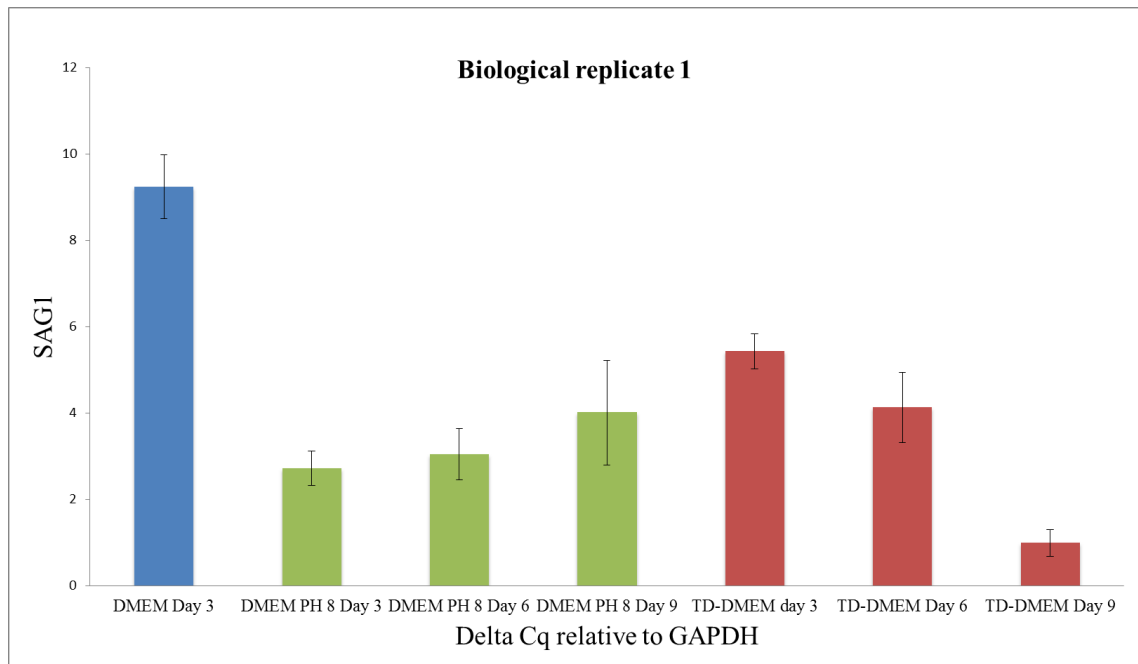
Figure 3.4. *T. gondii* **tachyzoitee-specific gene expression in TD-DMEM (SAG1) by RT-qPCR.** The graphs present expression of the tachyzoite marker SAG1 (n=3) in *T. gondii* growing in normal DMEM (blue columns), alkaline DMEM (green columns), or TD-DMEM (red columns) at days three, six, and nine. The expression of SAG1 was monitored using qRT-PCR. The graphs show low SAG1 expression in both biological replicates at day nine. In alkaline media, SAG1 expression increased between day three and day nine.
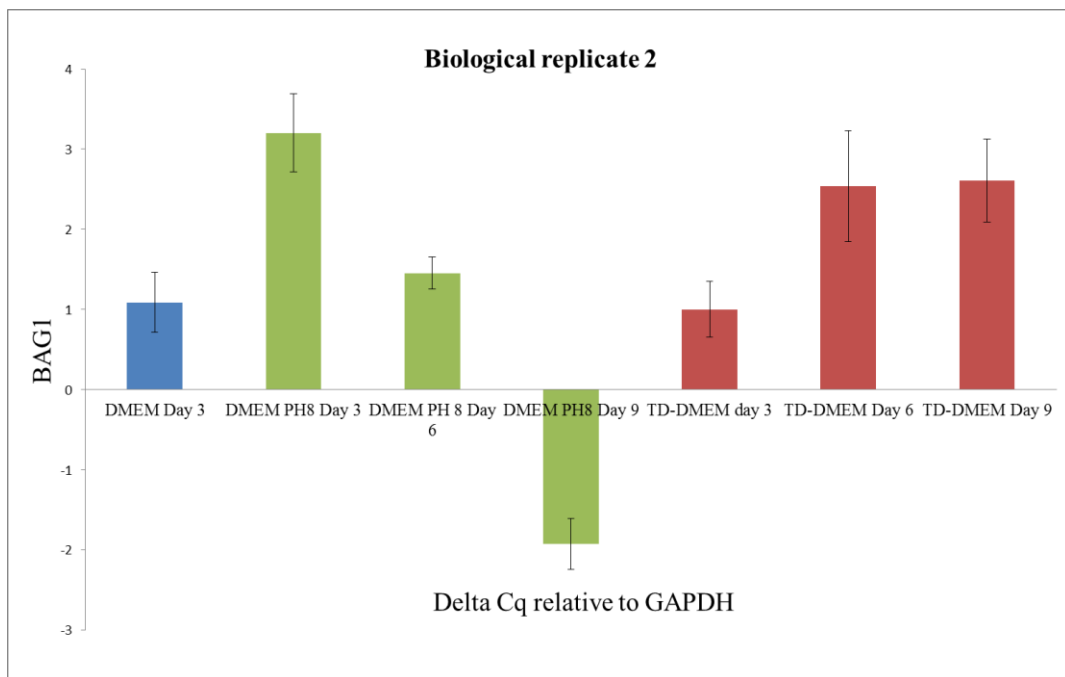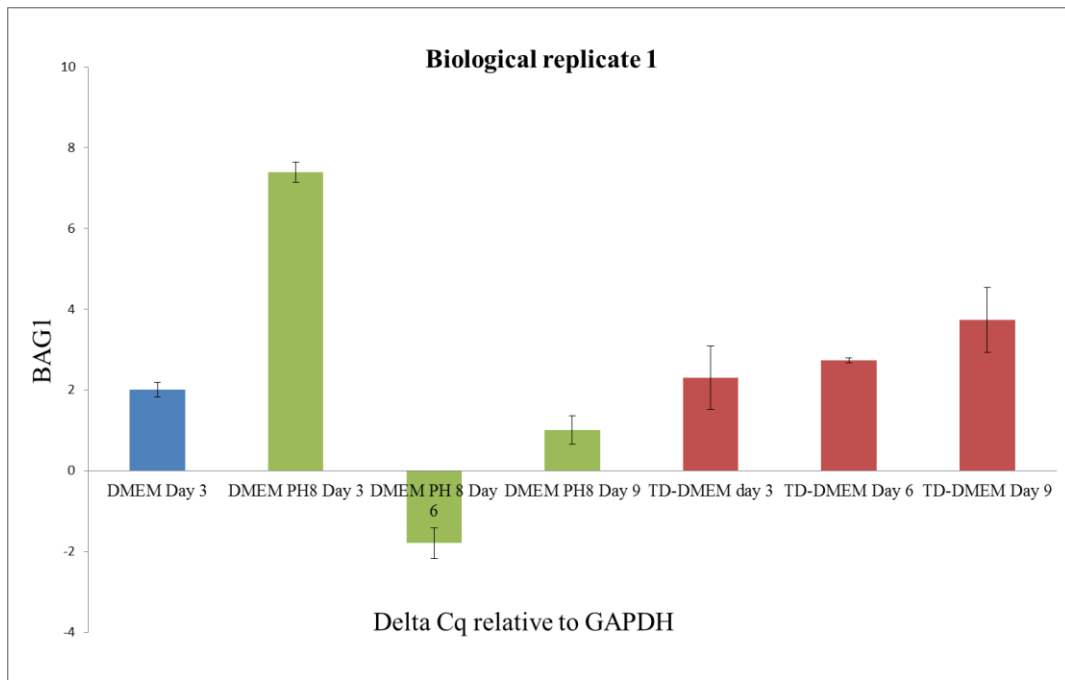
Figure 3.5. *T. gondii* **bradyzoite-specific gene expression in TD-DMEM by RT-qPCR.** The graphs show BAG1 gene expression from the differentiated *T. gondii* growing in normal DMEM (blue columns), alkaline DMEM (green columns), or TD-DMEM (red columns) at days three, six, and nine. The expression of BAG1 was determined using RT qPCR (n=3). The graphs show that BAG1 expression increases in both biological replicates at day nine at TD-DMEM, while BAG1 expression decreased in alkaline.
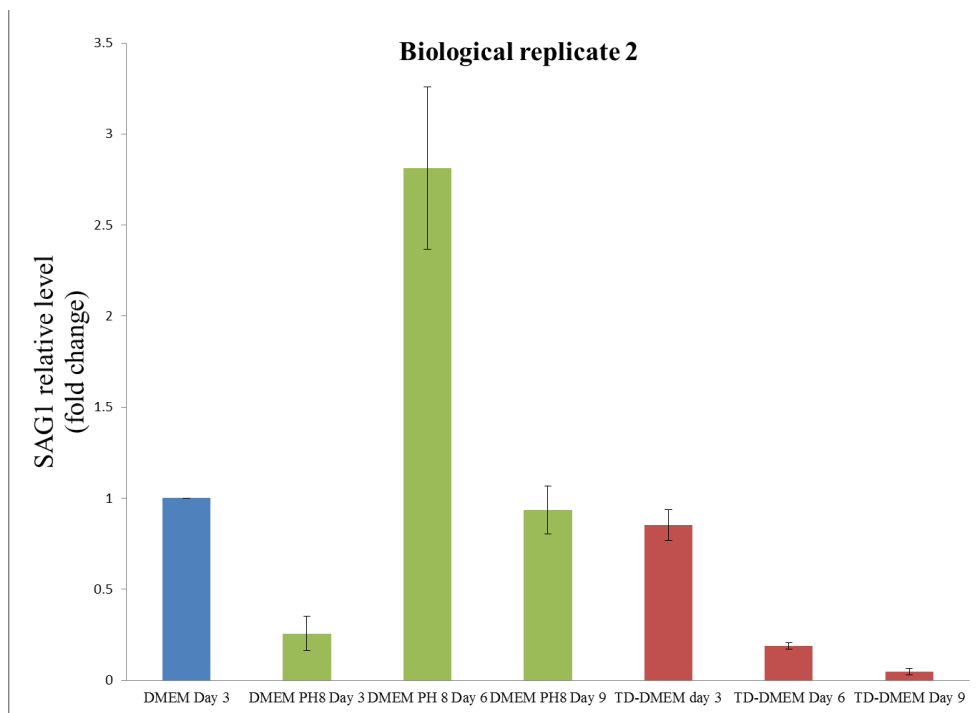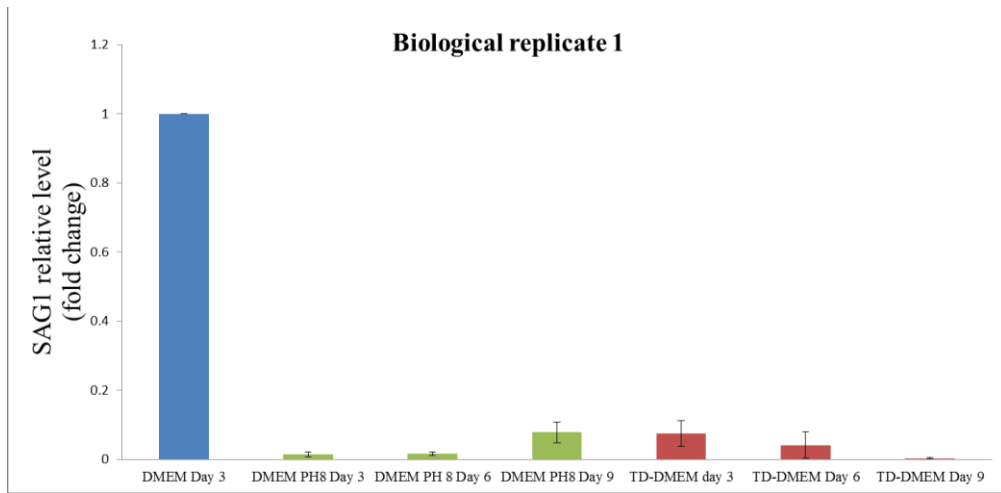
Figure 3.6. SAG1 fold change. The graphs show the average fold change of SAG1 (n=3) mRNA in the shocked parasite growing in normal DMEM (blue columns), alkaline DMEM (green columns), and TD-DMEM (red columns). The columns represent fold change value in alkaline DMEM and TD-DMEM relative to that of DMEM, which was converted to 1. The graph shows that SAG1 decreased in the parasite growing in TD-DMEM media compared normal media at day three and alkaline media at day nine. Meanwhile, SAG1 at day nine in alkaline media levels was decreased in biological replicate 1 and slightly decreased in biological replicate 2 and compared to normal media at day three.
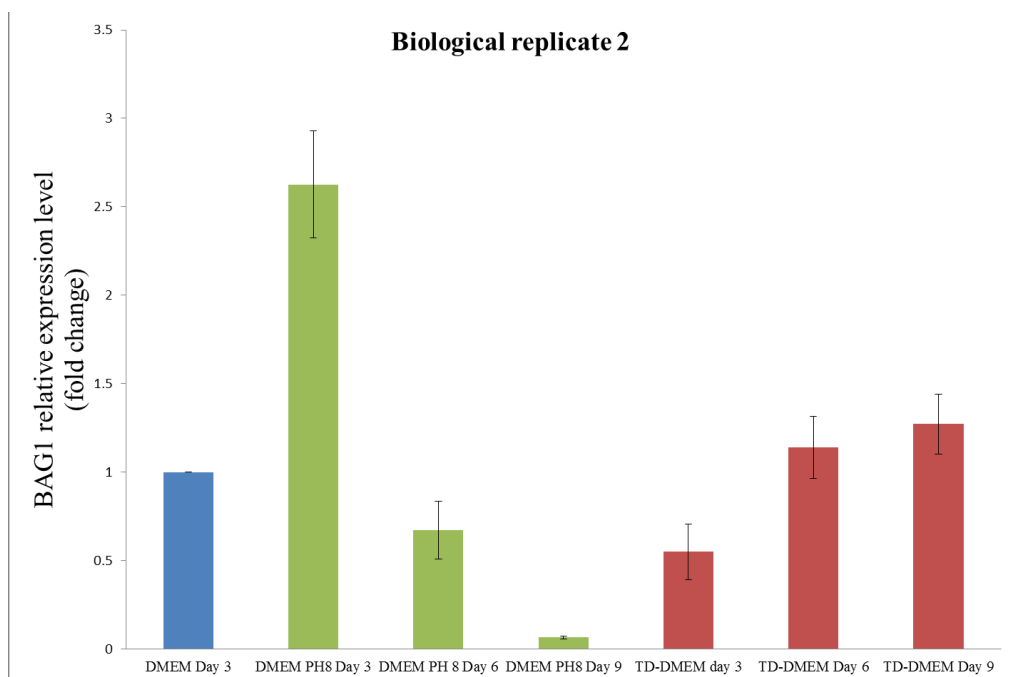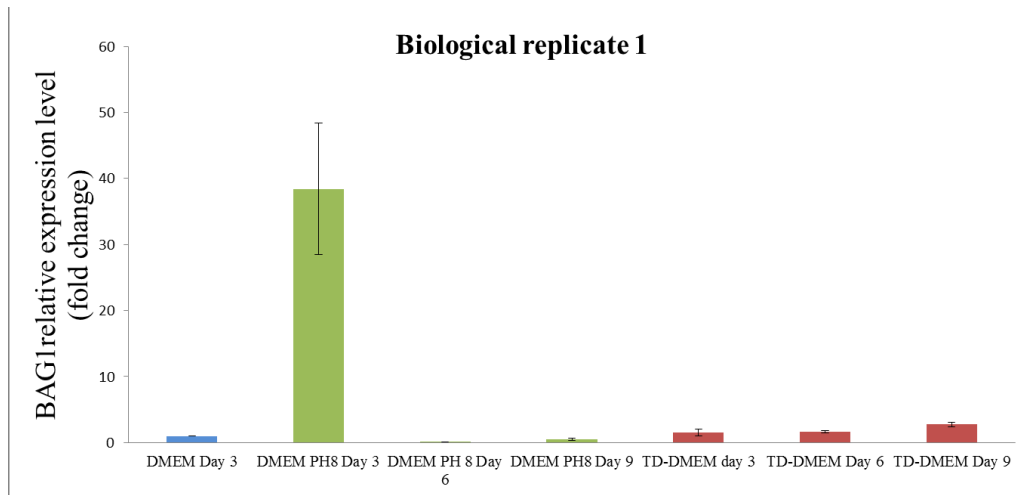
Figure 3.7. BAG1 fold change. The graphs show the average fold change of BAG1 (n=3) mRNA in the shocked parasite growing in normal DMEM (blue columns), alkaline DMEM (green columns), and TD-DMEM (red columns). The fold change value of DMEM and TD-DMEM represent the fold change relative to that of DMEM, which was converted to 1. The graph shows that BAG1 increased in the parasite growing in TD-DMEM media compared to normal media at day three. Meanwhile, BAG1 was decreased in alkaline media at day nine compared to normal media at day three.

	Normal DMEM	DMEM with pH8			TD-DMEM		
	day 3	day 3	day 6	day 9	day 3	day 6	day 9
SAG1 BR1	9.2±1.0	2.7±0.40	3.04±0.59	4.0±1.2	4.6±1.2	2.8±0.81	0.99±0.30
SAG1 BR2	5.4±0.37	1.8±0.58	6.9±0.19	5.2±0.39	5.2±0.40	2.9±0.40	0.90±0.31
BAG1 BR1	2.02±0.18	7.3±0.25	-1.8±0.38	1.01±0.35	2.3±0.78	2.7±0.057	3.73±0.80
BAG1 BR2	1.09±0.37	3.3±0.30	1.4±0.20	-1.9±0.31	1.0±0.35	2.5±0.69	2.6±0.51

Table 3.1. Tachyzoite and bradyzoite specific *T. gondii* gene expression in different media. The table shows the expression of tachyzoite (SAG1) and bradyzoite (BAG1) specific genes of *T. gondii* cultured in HFF cells supplemented with normal DMEM, alkaline DMEM, and TD-DMEM at days three, six, and nine of infection. These data represent the mean ΔCq of three technical repeats of each gene for two biological samples. ΔCq was calculated by subtracting the Cq of the target gene from the Cq of the house keeping gene. BR1 means biological replicate 1, BR2 means biological replicate 2.

3.4 Discussion

T. gondii lacks enzymes needed for the synthesise tryptophan (according to metaTiger data base:Whitaker et al., 2009), and supplementation with tryptophan is essential for the growth of this parasite (Pfefferkorn and Guyre, 1984; Pfefferkorn et al., 1986). Tryptophan breakdown by IDO has been shown to slow the tachyzoite growth (Pfefferkorn and Guyre, 1984; Pfefferkorn et al., 1986). Meanwhile, arginine starvation induced bradyzoite conversation (Fox et al., 2004). In this chapter, a TD-DMEM induction method was developed to avoid the heterogeneous growth of bradyzoites and tachyzoites, which has been observed when using current methods such as treatment with alkaline media, heat, or arsenite (Soete et al., 1994) and differentiated myotube culture (Guimarães et al., 2008; Guimarães et al., 2009).

To investigate the effect of tryptophan depletion on *T. gondii* differentiation, HFF flasks were infected with liberated, alkaline-shocked *T. gondii* and assessed by light microscopy after nine days of cultivation (Figure 3.1). Light microscopy observations showed that the parasites formed intracellular cysts, with no detectable free tachyzoites in TD-DMEM (with 0.1 mg tryptophan in 500ml DMEM). In contrast, in high pH media containing tryptophan (16 mg tryptophan in 500 ml DMEM), the parasite formed tachyzoite rosettes and caused lysis of the host cells (data not shown), releasing free tachyzoites (Figure 3.1). When HFF cells were infected with a *T. gondii* strain expressing bradyzoite promoter-specific GFP, bradyzoite cysts fluoresced (Figure 3.2 and Figure 3.3) and only the bradyzoite form of the parasite was seen when cultured in TD-DMEM. In parallel cultures containing tryptophan under standard alkaline conditions, severe host cell lysis was observed and many extracellular tachyzoite forms were observable by microscopy. Sizes of the cysts seen by confocal fluorescent microscopy in TD-DMEM ranged from 10-25 μM, which is similar to brain cyst size at day 21 post infection in mice (Ferguson and Hutchison, 1987b). The effect of tryptophan depletion on the parasite and bradyzoite cyst formation is similar to the effect of arginine starvation studied before by Fox et al. (2004), where arginine starvation converts SAG1 positive tachyzoites to SAG1 negative bradyzoites.

To further assess the effect of tryptophan depletion on parasite differentiation, quantitative RT-PCR was performed with the stage-specific primers SAG1 (tachyzoite marker)(Kasper et al., 1984) SAG4, and BAG1 (bradyzoite marker)(Tomavo et al., 1991; Bohne et al., 1995), and MAG1 (bradyzoite and tachyzoite marker)(Parmley, 2002). The results for SAG4 and MAG1 were inconsistent in control parasite cultures in DMEM and alkaline DMEM and hence were not included in the analysis. The inconsistence in MAG1 expression in the control cultures could be due the fact that it is a bradyzoite and tachezoite marker (Parmley, 2002). Meanwhile, inconsistent SAG4 results could be as a results of the heterogonous growth in those cultures. In TD-DMEM, the parasite showed 20 to 70 % increase in expression of bradyzoite-specific gene BAG1 as infection progressed, and 96 to 100 % decline in the tachyzoite-specific gene SAG1, strongly indicating a shift in the predominant form of the parasite to the bradyzoite form. Meanwhile, the shocked parasite growing in alkaline or normal DMEM continued to express both bradyzoite (60 to 90 % decrease) and tachyzoite (10 to 92 % decrease) specific genes, but both decreased with time. This decrease might be an indirect result of the active multiplication of tachyzoites, as Soete et al. (1994) observed HFF cells were totally destroyed by day nine, due to tachyzoite multiplication, and our microscopic observations also showed low numbers of intact cells at day nine. Meanwhile, studies have found that bradyzoite is converting to tachyzoites when the parasites were induced in alkaline media (Singh et al., 2002; Fouts and Boothroyd, 2007). Our data show that BAG1 was induced by day three of cultivation in TD-DMEM medium, in keeping with published data (Jerome et al., 1998). From day six to day nine there a slight increase in BAG1 expression level, this could be due to the early up-regulation of BAG1 mRNA during the differentiation (Bohne et al., 1995). Even though confocal fluorescent microscopic imaging of ΔKU80-GFP strain infected cells demonstrated that only bradyzoite cysts are formed in TD-DMEM media, RT-qPCR detected SAG1 expression in parasites growing in TD-DMEM at day nine. This may be derived from the initial infection of shocked tachyzoites that is used to induce bradyzoite formation.

This chapter RT-qPCR results shows that SAG1 can be detected in alkaline media from day three, and HFF cells started lysing by day nine. Soete et al. (1994) found

that bradyzoite cysts induced in the same way didn't express SAG1 or the bradyzoite specific marker p21 after four days. Furthermore, they also found that the host HFF cells had been completely lysed by day nine due to active tachyzoites replication. Interestingly, noted that bradyzoites induced by alkaline media had lower amount of micronemes and amylopectins compared to brain cysts in mice observed in Ferguson and Hutchison study in (1987b). It will be interesting to study the effect of TD-DMEM induction method on the parasite ultrastructure.

Current methods known to induce bradyzoite cyst formation, such as treatment with alkaline PH, sodium arsenite, or heat, and differentiated myotube culture result in heterogeneous growth (Soete et al., 1994). Myotube induction methods are further limited by the use of one specific cell line. The TD-DMEM induction method introduced here induces only bradyzoite growth according to bright field microscopy and confocal fluorescent imaging. Furthermore, RT-qPCR demonstrated that SAG1 expression was decreased in parasites grown in TD-DMEM.

3.5   Conclusions

These findings demonstrate that this TD-DMEM induction method consistently stimulates *T. gondii* differentiation. Therefore, this induction method is suitable for production of bradyzoites to be used in studies aiming to understand the mechanism of differentiation of this parasite and for comparing the tachyzoite and bradyzoite transcriptomes over long periods. Furthermore, it is an easy and cheap method that could be used for *in vitro* studies of *T. gondii* chronic infection. Parasite differentiation using TD-DMEM may be a helpful method for the development of a drug screening assay for bradyzoite stages, which is explored in Chapter 4 of this thesis.

Chapter 4 Novel tryptophan-depleted DMEM bradyzoite screening assay

4.1 Introduction

Seropositivity for *Toxoplasma gondii* ranges from 25% to 90% globally (Montoya and Liesenfeld, 2004; Dubey and Jones, 2008; Alqahtani and Hassan, 2012; Centers for Disease Control and Prevention, 2015) and seroprevalence in HIV/AIDS patients ranges from 20% to 70 % (Blaser and Cohn, 1986; Grant et al., 1990; Aydin et al., 2011; Daryani et al., 2011; Domingos et al., 2013) yet no treatments exist to eradicate the chronic stages of infection. Indeed, the majority of AIDS associated toxoplasmosis cases are due to cyst reactivation (Porter and Sande, 1992; Renold et al., 1992). The tissue cysts associated with chronic infection can last the lifetime of the host. There are several current treatments for toxoplasmosis including sulfathiazole, pyrimethamine, atovaquone, and spiramycin yet these cannot eliminate the tissue cyst stage (ie. encysted bradyzoites) (Fernandez-Martin et al., 1991; Katlama et al., 1996; Jacobson et al., 2001; Chirgwin et al., 2002). Furthermore, cases of drug resistance and intolerance have been reported to these drugs (Haverkos, 1987; Leport et al., 1988; Aspinall et al., 2002; Baatz et al., 2006). A new drug that can radically cure toxoplasmosis is required. A bradyzoite-specific drug screening assay will aid in the discovery of new drug that can clear latent bradyzoite tissue cysts. In the next paragraphs, a brief review of the current treatments for toxoplasmosis and drug screening assays will be given. Then, possibilities of using the TD-DMEM cultures described in Chapter 3, which induces *T. gondii* differentiation, in the development and evaluation of an *in vitro* bradyzoites specific screening assay-using will be explored.

The standard treatment for toxoplasmosis as mentioned briefly in the introduction (section 1.6) is a combination of sulfonamide and pyrimethamine with folic acid (Wong and Remington, 1994; Louis and Kim, 2013). This combination is used in the case of severe acute infection in competent patients, active infection in immunocompromised patients, severe ocular toxoplasmosis, congenital infection,

and acute infection of pregnant women in or after the 24th week of gestation with confirmed foetal infection (Desmonts and Couvreur, 1974; Weiss et al., 1992; Wong and Remington, 1994; Klinker et al., 1996; Holland and Lewis, 2002; de-la-Torre et al., 2011b). Acutely infected pregnant women during 1–18 weeks of gestation (with no infection in the amniotic fluid) are treated with spiramycin, since the drug cannot cross the placenta and has no adverse effects on the (Desmonts and Couvreur, 1974; Wong and Remington, 1994). Also, an *in vivo* mouse study and *in vitro* study found that a combination of pyrimethamine and dapsone has anti-toxoplasma activity (Derouin et al., 1991) against tachyzoite stages. Meanwhile, a combination of dapsone, pentamidine or trimethoprim-sulfamethoxazole was found to prevent toxoplasma encephalitis in AIDS patients due to suppression of tachyzoite proliferation (Torres et al., 1993; Bozzette et al., 1995). Pyrimethamine alone was found to be effective as a prophylaxis for *T. gondii* seropositive patients undergoing heart organ transplantation to prevent propagation of replicating tachyzoite stages (Wreghitt et al., 1992).

Toxoplasma encephalitis in AIDS patients can be treated with sulfonamide and pyrimethamine (Louis and Kim, 2013). However, some patients have developed adverse reactions, and infection relapse was reported in cases of toxoplasma encephalitis in AIDS patients treated with this drug combination (Haverkos, 1987; Leport et al., 1988; Fernandez-Martin et al., 1991; Katlama et al., 1996; Jacobson et al., 1996; Chirgwin et al., 2002). In such cases, they were given other drug combinations, such as pyrimethamine with either clindamycin (Katlama et al., 1996), clarithromycin (Fernandez-Martin et al., 1991), atovaquone (Chirgwin et al., 2002), or azithromycin (Jacobson et al., 1996). Pyrimethamine with either clindamycin or azithromycin for toxoplasma encephalitis in AIDS patients was found to be less effective than sulfonamide and pyrimethamine (Katlama et al., 1996; Jacobson et al., 2001). Meanwhile, atovaquone is used as a treatment for toxoplasma encephalitis in AIDS patients in cases of pyrimethamine or sulfonamide intolerance (Kovacs, 1992; Romand et al., 1993; Jacobson et al., 1996; Chirgwin et al., 2002). Meanwhile, death cases in immunocompeten patients with organ transplant (such as hearth and steam) taking anti-toxoplasma treatment have been reported (Wreghitt et al., 1992; Martino et al., 2000). Furthermore, atovaquone resistance has been reported in cases of long-

term treatment of ocular toxoplasmosis in immunocompetent patients (Baatz et al., 2006).

Laboratory studies have shown some *T. gondii* strains were resistant to most available drugs such as sulfonamide (Pfefferkorn et al., 1992a; Pfefferkorn et al., 1992b), spiramycin (Pfefferkorn and Borotz, 1994), clindamycin (Camps et al., 2002), and atovaquone (Pfefferkorn et al., 1993). Moreover, Aspinall et al. (2002), isolated a sulphonamide resistant *T. gondii* strain from clinically infected patients. It is important to note that all drugs currently used for the treatment of toxoplasmosis target the tachyzoite stage and are used only to clear the symptoms of the disease. There is no cure for toxoplasmosis, a since all the current treatments are unsuccessful at clearing the bradyzoite containing tissue cysts. All these studies shows that current drugs used to treat toxoplasmosis associated with adverse reaction, relapse, resistance and or death.

Recently, a new chemical (ELQ271: Doggett et al., 2012) that has activity against the cyst stages *in vivo* has been developed, but has never been tested against the cyst stage *in vitro*. In (1951), Gingrich et al., found that endochin drugs have anti-toxoplasmic activity and treatment with these drugs delayed death in acutely infected birds and mice. In (2008), Winter et al. found that endochin-like quinolones (ELQ) are active against *Plasmodium falciparum* (IC50= 1.2 nM) and targets cytochrome bc1 complex. Further testing on these drugs showed that they had poor performance in mammalian systems because they were unstable in the presence of murine, rat, and human microsomes (Winter et al., 2011).

Doggett et al. (2012) studied the activity of two ELQ analogues (ELQ316 and ELQ271, Figure 4.1) against *in vivo* toxoplasma infection in mice (chronic and acute) and *in vitro* HFF cells infection with the acute stage. Acutely infected mice orally treated with ELQ316 and ELQ271 had ED50 values of 0.14 mg/kg and 0.08 mg/kg, respectively. The *in vitro* activity of ELQ316 and ELQ271 against *T. gondii* infection had an IC50 value of 0.007 nM and 0.1 nM, respectively. No sign of overt toxicity was shown when the acutely infected mice that were given a high dose of either ELQ cehmicals (50 mg/kg). Meanwhile, treating 5-week-infected mice with ELQ chemicals for 2 weeks showed a 76% decrease in cyst number in the brain for ELQ316 and 88-84 % for ELQ271 (P < 0.0001). Furthermore, ELQ271 was found to

target *T. gondii* cytochrome bc1 complex; this was established by *in vitro* testing of ELQ271, atovaquone, and endochin activity in the presence of purified *T. gondii* mitochondria (Doggett et al., 2012). These results demonstrate that ELQ271 and ELQ316 is active against both tachyzoites and bradyzoites stages. Targeting cytochrome bc1 complex in *T. gondii*, which is found in the inner membrane of the mitochondria, will affect pyrimidine biosynthesis and oxidative phosphorylation (Vercesi et al., 1998).
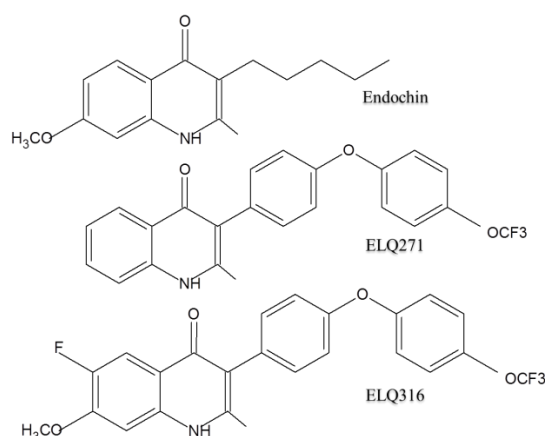
Figure 4.1. Endochin, ELQ271, and ELQ316 chemical structures (adapted from Dogget et al., 2012).

The process of discovering drugs active against *T. gondii* consists of two main parts. The first one is the chemicals screening assay to recognise chemicals that are active against the tachyzoite stage *in vitro*. The second part is testing the chemicals compounds activity against either the tachyzoite and braydyzoite stages using animal models (Araujo et al., 1988; Derouin et al., 1991; Derouin et al., 1992). Current screening assays are designed in microtiter plate with different tachyzoite viability measuring techniques. Some of these techniques that have been used are as follows: *T. gondii* labeling with radioactive uracil (Pfefferkorn and Pfefferkorn, 1977), the use of *T. gondii*-specific antibodies in an enzyme linked immunosorbent assay (Merli et al., 1985; Derouin and Chastang, 1988), and transgenic expression of the bacterial β-galactosidase reporter gene (McFadden et al., 1997). These assays have disadvantages such as the use of a radioactive compound with the uracil *T. gondii* labeling assay, or the use of or the need for external factors to visualize the signal.

Also, most of these assays allow only a single time point measurement. Meanwhile, another screening assay was developed based on parasite fluorescent labeling and fluorescence activated cell sorting (FACS) to monitor parasite growth but requires extensive time and equipment running costs (Gay-Andrieu et al., 1999). In (2003), Gubbles et al., developed a microtiter plate-based growth assays where a highly fluorescent transgenic *T. gondii* strain (YFP) is used to monitor the parasite growth. The advantage of this method is the continuous monitoring of parasite growth in the presence of the tested chemicals, no need for external factors to observe the parasite growth, and can rapidly examine multiple chemicals (Gubbels et al., 2003). Still, all of these screening assays are targeted at discovering chemicals that inhibit the growth of the tachyzoite stage. There is no assay to screen chemicals activity against the bradyzoite stage *in vitro*. The only way to test the chemical activity against the bradyzoite stage is animal models.

There is a need for a novel chemical that can cure for toxoplasmosis, since all the current treatments are unsuccessful at clearing the bradyzoite containing tissue cysts. Current *in vitro* toxoplasma chemical screening assay are tachyzoite based, and only detect the chemical activity against that stage. Animal models are the only means to assess the chemical activity against the bradyzoite stages which are time consuming, expensive and laborious. So in this chapter, a new *in vitro* bradyzoite screening assay using TD-DMEM induction of *T. gondii* differentiation was developed and assessed. This purpose of this assay is to find a drug that is able to clear the bradyzoite stage.

4.2   Materials and Methods

4.2.1   Cultivation of cells and *T. gondii* strains

The HFF cells and parasites were maintained and passaged as previously explained (Chapter 2). The *T. gondii* ΔKU80-GFP (kind gift of David Bzik, Dartmouth) strains were maintained, passaged, and shocked, which will induce bradyzoite formation, as explained previously (Chapter 3). TD-DMEM used in the screening assay was prepared as described previously (Chapter 3).

4.2.2   Cell cultures, chemicals dilutions, and screening assay design

Cell cultures: HFF cells were cultured in black bottom 96-well plates (Costar) in DMEM supplemented with 10% FBS and 1% PS at 37°C in 5% CO2 until they became 100% confluent for three to four days.

Chemicals Dilutions: To test the activity of ELQ271 and Pyrimethamine drug against *T. gondii*, both were dissolved in DMSO to make a stoke of 50 mM and stored in -20 °C freezer. Prepared a two-fold serial dilutions of each chemical form 0.97 to 250 μM in TD-DMEM.

Screening assay design: This screening assay was designed to be in 96-well clear bottom with black sides plates with confluent HFF cells infected with *T. gondii* ΔKU80-GFP strains, which expressed bradyzoite specific GFP.

4.2.3   Differentiated *T. gondii* inhibition assays

Confluent HFF cells in black side 96-well plates were infected with $5X10^3$ shocked *T. gondii* ΔKU80-GFP that were liberated from cells by 27-guage needle and quantitated by hemocytometer slides and shocked as in chapter 2. These cultures were incubated for three days at 37°C in 5% CO2. Then the nine two-fold dilutions of the chemicals were added. Plates were incubated at 37°C in 5% CO2 and read daily in a Molecular Devices fluorescence plate reader, for a total of six days of evaluations. To preserve sterility, the plates were read with lids in place, excitation is done from the bottom (492 nm) and fluorescence is read from the top (520 nm). The fluorescence intensity measurement was plotted using GraphPad Prism software (Sigmoidal dose-response variable slope) to calculate the IC50 value and the percentage of growth inhibition. The ELQ271 *T. gondii* inhibition screening assay

was performed for at least three biological replicates, and for each replicate the fluorescence reading was measured three times at each time point.

4.2.4   ELQ271 activity in different media

To further evaluation the newly developed TD-DMEM- bradyzoite screening assay, ELQ271 activity against *T. gondii* ΔKU80-GFP growth in infected HFF cells in TD-DMEM, alkaline DMEM, or normal DMEM was determined. This was done using the same methods in section 4.2.3 with some modification. Briefly: added eight two-fold chemical dilutions were added to the cultures. Then, each well was infected with $5X10^3$ shocked *T. gondii* ΔKU80-GFP. Plates were incubated at 37°C in 5% CO2 and read daily in a Molecular Devices fluorescence plate reader. Calculation of the IC50 was determined using GraphPad Prism. The screening assay was done in triplicate. Furthermore, each replicate was done at least twice.

4.3 Results

4.3.1 *T. gondii* differentiation inhibition by ELQ271

To see whether the TD-DMEM bradyzoite screening assay can detect chemical active against bradyzoite stages, ELQ271 (active against tissue cysts) and pyrimethamine (tachyzoite specific toxoplasmosis drug) activity was tested using a TD-DMEM bradyzoites screening assay (Figure 4.2). The novel TD-DMEM bradyzoites screening assay detected ELQ271 activity against the bradyzoites stage. The IC50 values were ranged from 59 to 60 μM (Table 4.1), which shows that there were no significant changes in the IC50 in the different incubation periods. The optimal concentration-response curves were obtained at day five and six. As expected, there was insignificant inhibition of bradyzoites differentiation by pyrimethamine, and its IC50 values could not be determined.
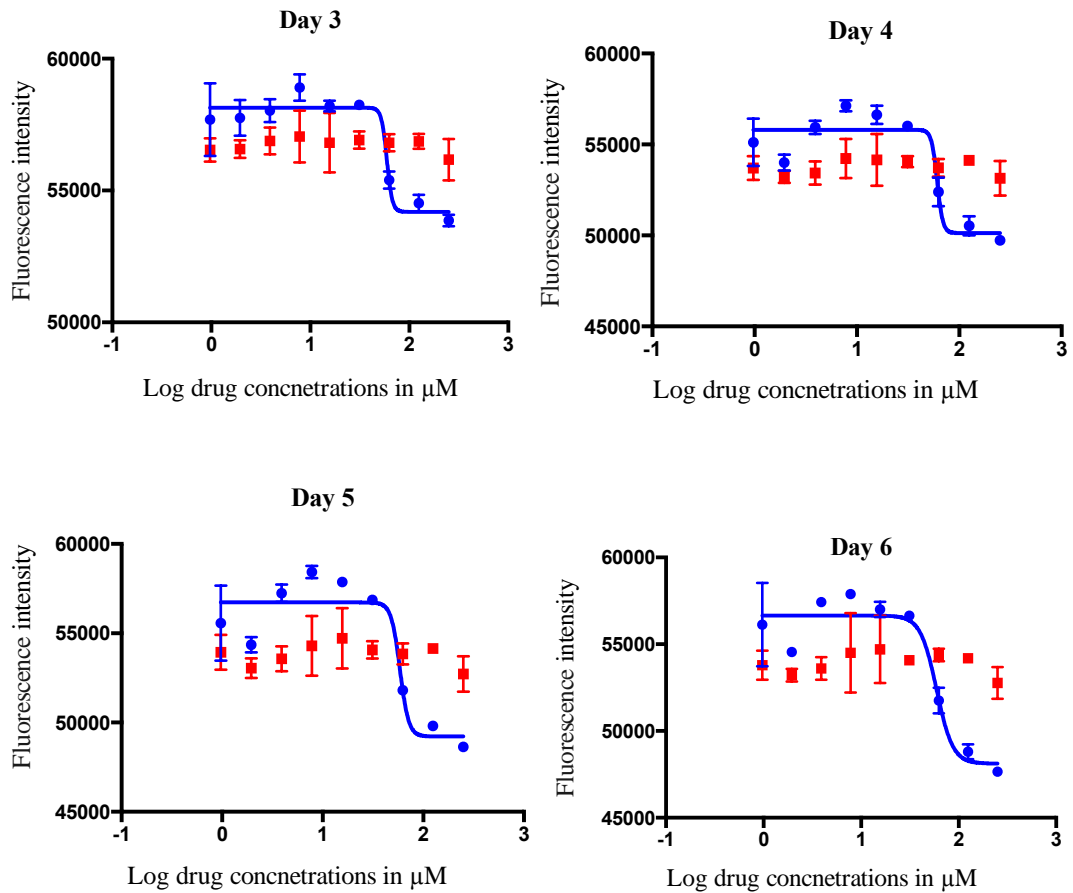
Figure 4.2. *T. gondii* **bradyzoite growth inhibition assay with ELQ271.** Parasite cultures were treated with ELQ271 (2- 250 μm). the number of bradyzoites based on a bradyzoite-specific GFP marker was measured at days three, four, five, and six with at least three biological replicates. The graphs show non-linear regression for fluorescence at the drug concentrations tested (with standard error bars). Graphs were plotted using GraphPad prism. blue denotes ELQ271; whereas red denotes pyrimethamine (tachyzoite-specific).

	Chemicals IC50 (μM)	
Day	ELQ271	Pyrimethamine*
3	59 ±6	>250
4	61±4	>250
5	59±0.2	>250
6	59±0.03	>250

Table 4.1: IC50 of ELQ271 and pyrimethamine inhibition of differentiated *T. gondii*. The table shows ELQ271's calculated IC50 by non-linear regression analysis using GraphPad Prism. *The highest concentration tested was 250 μM.

4.3.2 ELQ271 activity in different media

To further assess the use of TD-DMEM media in the screening assay, ELQ271 activity in TD-DMEM was compared to alkaline DMEM and normal DMEM. DMEM at pH 8.2 has been used in innumerable studies to induce bradyzoite differentiation although it is only promotes conversion of part of the culture. Data in Table 4.2 shows the results of this assay performed in triplicate, with each triplicate repeated twice. In assays with added ELQ271 activity in TD-DMEM, the inhibitor IC50 was between 43 and 49 µM from days three to six (Table 4.2). There was a slight difference between the IC50 of ELQ271 obtained. The IC50 value for ELQ271 in DMEM varied from 83 to 109 µM, and the IC50 in DMEM pH8 was 58–98 µM (Table 4.2), hence it was inconsistent with the other media. this is likely due to the high number of tachyzoites that will be present in these cultures and the high amount of cell lysis. These data show that assays with added ELQ271 in TD-DMEM had lower IC50 values compared to the IC50 values with experiments in DMEM and DMEM-pH8. Furthermore, a sigmoidal concentration response curve of EL271 was obtained in TD-DMEM, whereas with alkaline or normal DMEM data has a weak fit to the four-parameter logistic equation to calculate a sigmoidal curve. Indeed, based on the poor curves, the data obtained with DMEM and DMEM-pH8 is unreliable.
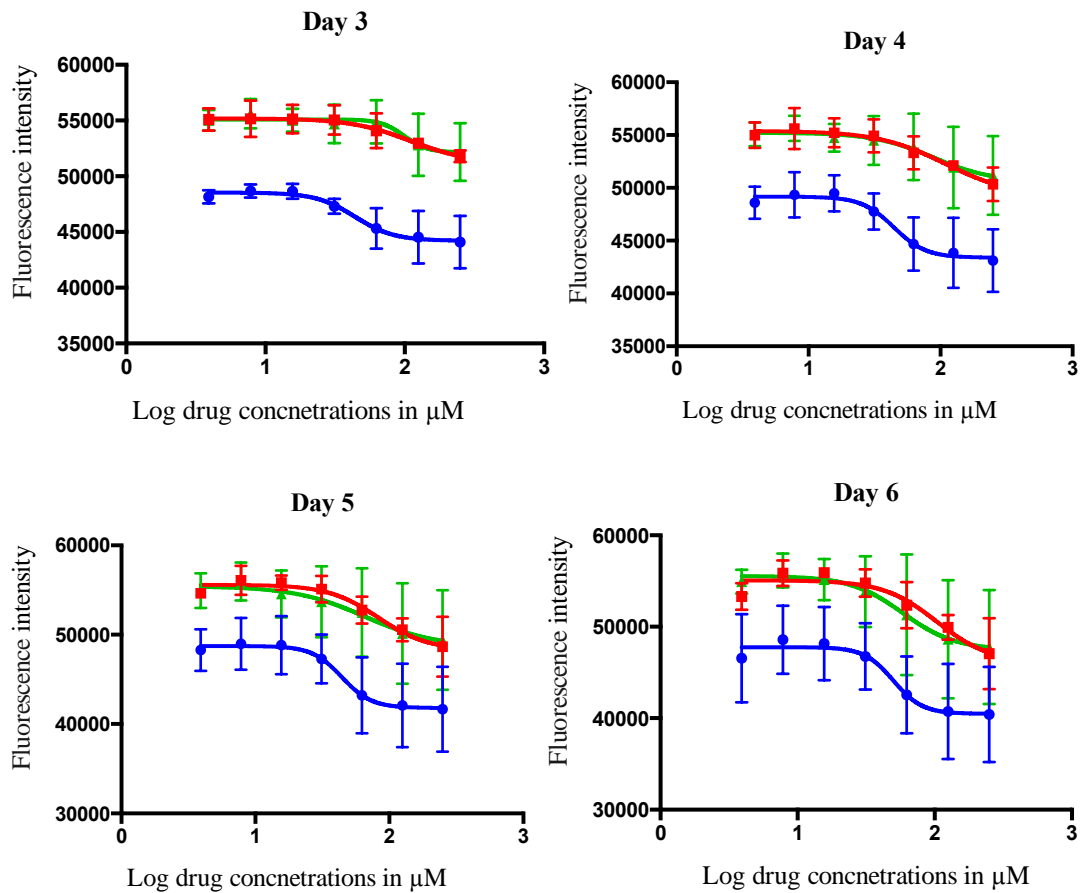
Figure 4.3. ELQ271 activity in different media. Cultures of the parasite and the chemical (2- 250 μM) in TD-DMEM (blue), DMEM (red), or DMEM pH8 (green) were tested in parallel. measurement of fluorescence indicating bradyzoite number was measured at days three, four, five, and six with at least three biological replicates. The fluorescence at different chemical concentrations was plotted using GraphPad.

	ELQ271 IC50 (μM)		
Day	TD-DMEM	DMEM	DMEM-pH8
3	44±0.1	109±0.3	99±0.2
4	44±0.1	109±0.4	89±0.5
5	44±0.2	83±0.5	66±0.5
6	50±0.2	103±0.3	58±0.3

Table 4.2. ELQ271 *T. gondii* killing IC50 values in different media: The table shows the calculated IC50 from the plotted graph of the chemical concentrations and the fluorescence parasites intensity in GraphPad Prism.

4.3.3 ELQ271 *T. gondii* percentage inhibition

The timing of addition of the inhibitor during the parasite differentiation was assessed. The percentage of growth inhibition of the differentiated *T. gondii* treated with ELQ271 was measured when the inhibitor was added at Day 1 and when the inhibitor was added at Day 3 (Figure 4.4). The second experiment permitted the parasite to differentiate in TD-DMEM for 72 hours in bradyzoite development before the inhibitor was added. The IC50 values calculated when the parasite was added three days post infection was 67 ± 0.08 μM at day five and 68 ± 0.06 μM at day six (Table 4.3). Meanwhile, the IC50 obtained from *T. gondii* percentage growth by ELQ271 when the chemical was added at the time of *T. gondii* infection was 65 ± 0.07 μM for day five and 57 ± 0.06 μM for day six. Hence there is insignificant difference based on the standard deviations from whether the parasites differentiate for three days prior to treatment and been treated at the same day. This is encouraging news for treatment of tissue cysts as described in the discussion.
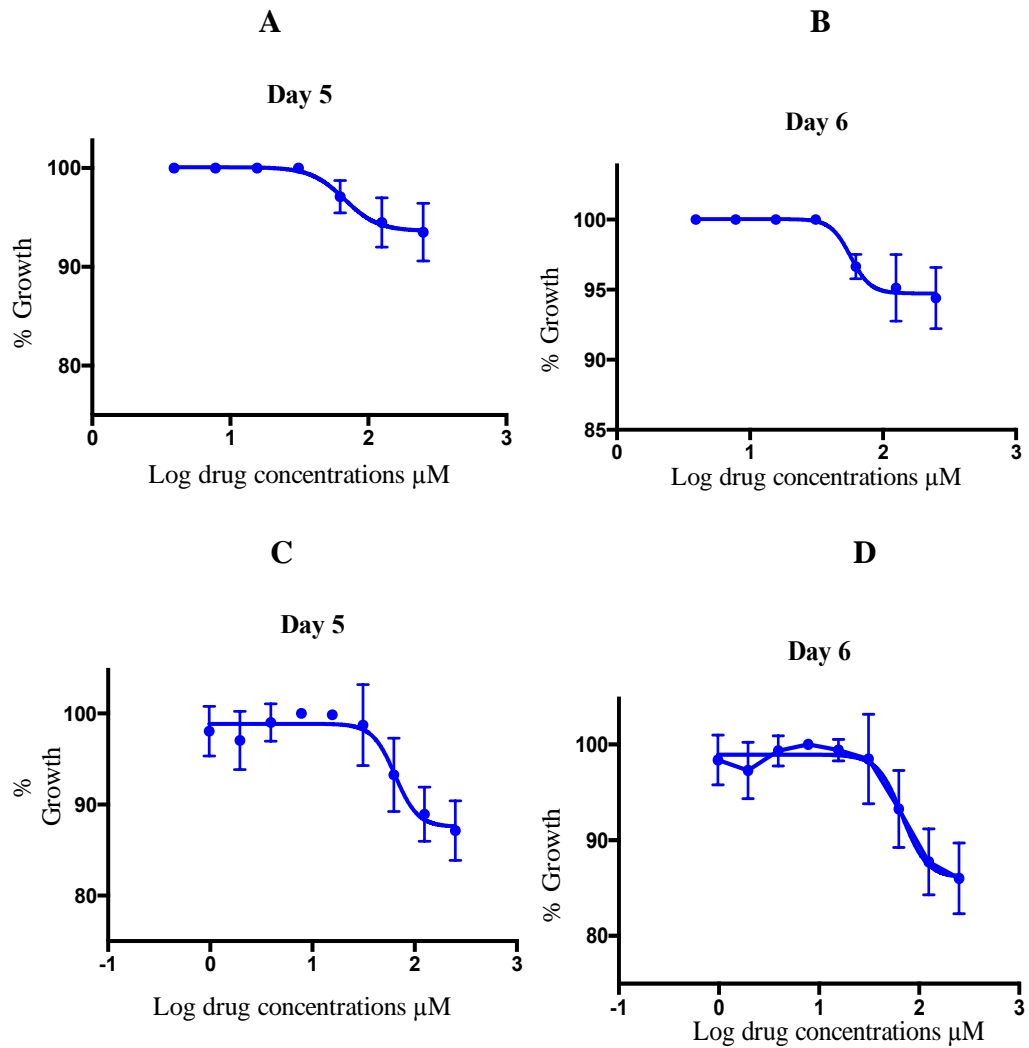
Figure 4.4. The efficacy of ELQ271 against differentiated *T. gondii* after growth. The graphs show the percentage of growth of *T. gondii* in the presence of ELQ271. The percent of untreated growth was determined and plotted at days five and six. The graphs on the top (A and B) examine the effect of ELQ271 on differentiation from time point zero, whereas graphs C and D examine the chemical effect on bradyzoite number with treatment following three days of differentiation.

	IC50 from *T. gondii* % growth (µM)	
Day	**Chemical added at time zero**	**Chemical added after 72 hours**
5	67± 0.08	64±0.07
6	68±0.06	57±0.06

Table 4.3. IC50 values from the *T. gondii* comparing times of addition of chemical ELQ271.

4.4   Discussion

T. gondii infection cannot be radically cured by the currently available drugs, as these drugs can only clear the tachyzoite stage or cannot permeate the blood brain barrier. Because of this limitation, immunocompromised patients are susceptible to infection relapse or death (Wreghitt et al., 1992; Fernandez-Martin et al., 1991; Katlama et al., 1996; Martino et al., 2000; Jacobson et al., 2001; Chirgwin et al., 2002). A drug is needed that targets both the tachyzoite and bradyzoite stages. To screen drugs against the bradyzoite stage, a straightforward drug screening assay was developed and evaluated by employing parasites stably expressing GFP in the bradyzoite stage. The screening assay was designed to be in 96-well black sides clear bottom plates with a growing monolayer of HFF cells infected with a *T. gondii* strain that expresses GFP as a bradyzoite. The chemical dilutions were added to infected cultures and the GFP intensity was measured daily until day six.

To evaluate the screening assay, two chemicals were tested against the bradyzoite stage using the assay. ELQ271 is as an active chemical against the brain cyst stage that is still in development (Doggett et al., 2012), and pyrimethamine is the standard treatment that has no activity against the chronic stage. The screening assay demonstrated that ELQ271 has activity against the bradyzoite stages from day three to day six, while pyrimethamine had no effect on the parasite. Meanwhile, the calculated IC50 values for ELQ271 ranged from 59 to 61 µM (standard error range ± 0.2 to 6). These data show that ELQ271 IC50 was not significantly changed with increase of infection period. Doggett et al. (2012), found that ELQ271 had an IC50 of 0.1 nM against tachyzoites stages, and that it lower cyst number in the brain by 84-88 % but doesn't clear the infection. The IC50 of ELQ271against bradyzoite stages observed in our TD-DMEM screening assay correlate with the 84-88% removal of brain tissue cyst observed by Doggett et al. (2012).

To further assess the TD-DMEM screening assay, ELQ271 activity against the parasite was determined in TD-DMEM, DMEM at pH8, and normal DMEM. The fluorescence readings from this experiment found higher activity of inhibitor in TD-DMEM (IC50 was < 50 µM), but importantly an uninterpretable concentration response curve in normal or alkaline DMEM due to lack of a sigmoidal curve.

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Importantly, in alkaline and normal DMEM, parasites will convert to tachyzoites (Soete et al., 1994; Singh et al., 2002; Fouts and Boothroyd, 2007) leading to cell lysis, which could explain the observed fluorescence loss in those media. Furthermore, the high pH in alkaline DMEM could affect ELQ271 activity against *T. gondii*, consistent with previous observations where different pH can affect *Mycobacterium tuberculosis* drug activity. Meanwhile, TD-DMEM media in the screening assay maintains the parasite in the bradyzoite stage and thus only detects ELQ271 activity against the bradyzoite stage.

To see whether the chemical would effect the parasite early entry and invasion or/ and latent stages, ELQ271 IC50 was determined when adding the chemical at the same time of infection and three-day post infection. The IC50 of ELQ271 in TD-DMEM was similar when adding the chemical at the same time of infection and three-day post infection (Table 4.3). Drug time based screening assay on HIV virus have also shown that IC50 varied when the drug was added at different times (Daelemans et al., 2011). This suggests that inhibitor may work well against tissue cyst stages. These stages are slowly replicating (Watts et al., 2015).

TD-DMEM bradyzoite screening assay is a medium to high throughput drug screening assay. This method allows multiple drugs screening at multiple concentrations. There is no need for additional chemicals in TD-DMEM bradyzoite screening such as with radioactive uracil screening assay (Pfefferkorn and Pfefferkorn, 1977). Meanwhile, the equipment used to measure the parasite bradyzoite specific GFP is cheap and easy to use. This is can not be said about the use of FACS in screening assay that detect parasite labelled with fluorescent (Gay-Andrieu et al., 1999). Currently, a animal models are used to screening drugs against the bradyzoite stage which take time and laborious. TD-DMEM bradyzoite screening assay will lower the necessity for animal testing for all the drugs that might work against that stage. Only drugs active against bradyzoite stage *in vitro* will be tested with animal. Furthermore, the newly developed screening assay has detected ELQ271 activity against the parasites developing into braydzoite and parasites that have been differentiated to bradyzoite for three days.

4.5 Conclusions

The TD-DMEM and GFP-based *T. gondii* screening assay combines the advantages of using a stage specific reporter and TD-DMEM to induce the parasite to differentiate. The drug activity in the screening assay will be specific against the bradyzoite stage, since TD-DMEM will keep the parasite in the bradyzoite stage without affecting the drug activity, and only the bradyzoite-specific GFP is measured. Meanwhile, the advantage of this method is that the parasite growth can be monitored at multiple time points without interfering with parasite development. This advantage might aid understanding the drug function by detecting any delayed death effect such as was seen by Gubbels et al in (2003) where a continuous fluoresce tachyzoite screening assay were used detect clindamycin delayed death effect. On the other hand, TD-DMEM based screening assay is fast, easy, and less time consuming, and therefore it is recommended for screening drugs that might be active against the cyst stage.

Chapter 5 General Discussion and Future Work

This thesis approached several important aspects of the parasite bradyzoite stages, particularly those involving the production of L-DOPA by the parasite. Further, as there are no drugs for elimination of the chronic stages of infection, a new culture method was developed for identifying inhibitors of bradyzoite stages. These advances in our understanding and new technologies will permit development of treatment to cure toxoplasmosis.

5.1 *Toxoplasma gondii* aromatic amino acid hydroxylase and dopamine biosynthesis

As L-DOPA and DA can be transformed to dopaquinone by oxidation, and dopaquinone has been shown to play a role in the formation of polymer cross-linking in other biological systems (Rzepecki et al., 1991; Waite, 1990; Lee et al., 2002). It is possible that similar cross-linking occurs in tissue cysts or potentially in oocyst formation. L-DOPA is involved in forming intermolecular covalent bonds and hardening of dopa-containing proteins in other organisms. The ability of *T. gondii* to oxidise L-DOPA and DA in order to utilise dopaquinone in cyst wall cross-linking was addressed in this thesis. Colorimetric dopa-oxidase activity assays of *T. gondii* tachyzoites and bradyzoites demonstrated that *T. gondii* does not have dopa-oxidase activity (Table 2.1). This finding concurs with data obtained from metaTiger bioinformatics search engine (Chapter 2) that the *T. gondii* genome does not contain a gene that is homologous to dopa oxidase (according to metaTiger search; Whitaker et al., 2009). These facts indicate that it is unlikely that *T. gondii* converts L-DOPA to dopaquinone. Recent RNA sequencing analysis found a high concentration of TgAaaH expressed in oocysts (See ToxoDB Fritz et al., 2012 data). A possible function of TgAaaH in *T. gondii* is to provide L-DOPA that might be used in oocyst wall cross-linking. *Eimeria maxima* oocyst wall, which was collected from chicken faeces, contains di-tyrosine and DOPA based on HPLC and fluorescence (Belli et al., 2003a; Belli et al., 2003b). Furthermore, peroxidase enzyme activity was found in the wall

forming bodies of the macrogamete, and it was suggested that it involved in the process of oocyst wall formation by catalysing dityrosin cross linking (Belli et al., 2003a; Belli et al., 2003b). The epithelial cells of the gastrointestinal tracts contains dopa-oxidase activity (tyrosinase) as a component of the melanin pathway (Morris et al., 2002). Accordingly, it will be interesting to see whether *T. gondii* oocysts growing in cat gut might contain L-DOPA in the oocyst wall by using HPLC with UV and fluorescence detection.

To better understand the function of TgAaaH, its location during *T. gondii* infection was investigated (see Chapter 2). TgAaaH genes encode a protein with a predicted signal peptide at the N-terminus, which was originally proposed to be involved in transporting TgAaaH 1 and 2 either to the parasite's outer membrane or for secretion from the parasite into the PV (Gaskell et al., 2009). Localisation of TgAaaH was done using immunofluorescence with a specific anti-TgAaaH antibody (Figure 2.7, Figure 2.9 and Figure 2.10), WB with a general AAAH antibody (Figure 2.3, Figure 2.4, Figure 2.5, and Figure 2.4), and TH activity assays (Table 2.2). Immunofluorescent localisation of TgAaaH found the enzyme within the PV, supporting what has been observed in brain tissue cysts by immunostaining Prandovszky et al. (2011). Detection of TgAaaH after extracellular trypsin treatment and cell component fractionation showed that TgAaaH is membrane-bound and/or transported outside the parasite. The finding is supported by detection of TH activity in the parasite fractioned cellular components (Table 2.2). Further study is needed to investigate whether TgAaaH is transmembrane or is secreted within the cyst but outside the parasite. This can be done by WB detection of TgAaaH and the known *T. gondii* transmembrane proteins, such as MIC transmembrane protein, after trypsin treatment.

Meanwhile, the present study did not show the direct involvement of a signal peptide in the translocation process. N-terminal signal peptides are known to be involved in directing proteins to the endoplasmic reticulum, where they are cleaved to permit the mature protein polypeptide to fold and be exported. Experiments that can be performed to find the direct link between TgAaaH localisation and signal peptides, including signal peptide deletion construct, fluorescent tagging, and pulse chase labelling. TgAaaH could be localised using WB and fluorescence techniques after knocking out the signal peptide gene. To test the influence of the signal peptide presence or absence,

pulse chase analysis of wild type TgAaaH could reveal evidence for signal peptide cleavage during pulse. The combination of the two experiment should yield a clear answer.

Wang et al. (2015) suggested that the expression of TgAaaH was very low in tachyzoites and bradyzoite since the TgAaaH protein they could not detect the protein by WB or immunofluorescence. However, in our hands TgAaaH can be detected using these techniques (Figure 2.3, Figure 2.5, Figure 2.7, and Figure 2.9). These finding indicate that TgAaaH is synthesised within the parasite and transported outside the parasite but within the PV. TgAaaH may have multiple roles: those involved in dopamine synthesis that is proposed to be involved in latent stage behavioural changes in rodents (Webster et al., 2006), or DOPA for oocyst wall biosynthesis (Belli et al., 2003a; Belli et al., 2003b). Future work is needed to specify parasite-produced L-DOPA and DA during latent stage infection and its location, which might be free and going through auto-oxidation and the formation of quinones and semi-quinones (Hald and Lotharius, 2005).

Although parasite-encoded TgAaaH activity was present for synthesis of the elevated DA levels observed in earlier studies (Skallova et al., 2006; Prandovszky et al., 2011), DDC is needed to convert L-DOPA to DA. During the period of this thesis research, DDC was found within cysts during dopaminergic cell infection using immune staining (Martin et al., 2015). This DDC appeared to be the host DDC. Still the question arises whether *T. gondii* encodes an enzyme with L-DDC activity or simply uses the host's L-DDC. Sensitive bioinformatics searches (metaTiger: Whitaker et al., 2009 :Chapter 2) did not identify an orthologue but there is the possibility of an enzyme with L-DDC activity that does not have significant homology. The potential of a *T. gondii* enzyme with L-DDC enzyme activity was tested *in vitro* by testing for the endproduct DA using HPLC of liberated parasites that had been grown in non-dopaminergic cells. HPLC results showed that L-DOPA is synthesised by liberated parasites but DA was not detectable (Figure 2.2) concurs with the bioinformatics data and demonstrates that parasites do not encode L-DDC activity. Meanwhile, WB localisation of DDC in free *T. gondii* collected from dopaminergic cells with anti-DDC antibody showed that DDC was found within the PV (Figure 2.11). The absence of DA in liberated parasites that have been grown in non-dopaminergic cells suggests that the parasite is only able to synthesise DA in dopaminergic cells. Meanwhile, DDC presence in the PV in

dopaminergic cells supports Martin et al. (2015) findings that the parasite can recruit host DDC into the parasitic vacuole where the parasite encoded TH is found. The two findings suggest that the parasite's ability to increase DA does not occur randomly in any cells, it only occurs in dopaminergic cells containing the DDC enzyme, such as dopaminergic brain neurones. Martin et al. (2015) suggested that DDC recruited within the PV to prevent L-DOPA breakdown in the cytosol and aid in forming DA. It would be interesting see whether *T. gondii* TgAaaH and the host DDC form a multi protein complex to form DA similar to PC12 DA and DDC (Cartier et al., 2010). Moreover, studies are required to see whether DA biosynthesis is the mediator of the behaviour changes, and if immune and/or endocrine response to the infection was involved in that change. Though a recent study showed that mice infected with attenuated *T. gondii*, which lack the protein (ROP5) that activate the immune response, are still attracted to cat urine (Ingram et al., 2013).

5.2 Effect of tryptophan-depleted DMEM on the differentiation of *Toxoplasma gondii*

In this thesis's Chapter 3, a newly-developed TD-DMEM media was used to induce *T. gondii* differentiation to aid in studying the bradyzoite stages. TD-DMEM induction methods were developed to overcome the limitations of the current methods used to induce *T. gondii* differentiation (such as alkaline pH), which result in heterogeneous growth of both bradyzoites and tachyzoites and eventually lyse all the host cells (such as obsereved in Soete et al., 1994; Fouts and Boothroyd, 2007). The new induction method was evaluated using light microscopy (Figure 3.1) and fluorescence imaging of a *T. gondii* strain that expresses GFP only in bradyzoites (Figure 3.2 and Figure 3.3). The light microscopic and fluorescent images show that only bradyzoite cysts grow in TD-DMEM (Figure 3.1, Figure 3.2, and Figure 3.3). These results were reinforced by examining markers of bradyzoites (BAG1) and tachyzoites (SAG1) for *T. gondii* growing in TD-DMEM (Figure 3.4, Figure 3.5, Figure 3.6, and Figure 3.7). These results showed that TD-DMEM induced the switch from fast growing *T. gondii* to slow growing bradyzoites. This effect of TD-DMEM on the parasite is similar to the effect seen in arginine starvation where 99% of the parasites switch to the bradyzoite form after seven days from infection (Fox et al., 2011). Limitation of depleting arginine is

that this amino acid is needed for nitric oxide host response, which involved in controlling the parasite growth (Khan et al., 1997; Scharton-Kersten et al., 1997). TD-DMEM is an easy and inexpensive method that could be used for *in vitro* studies of the *T. gondii* chronic stage where the parasite can be incubated for a long period without the concern of growth heterogeneity seen in on other induction techniques such as alkaline media, chemical, and heat shock (Soete et al., 1994; Guimarães et al., 2008; Guimarães et al., 2009). Also, this method can be used with any cell line, unlike the differentiated myotube induction method (Guimarães et al., 2008; Guimarães et al., 2009). The TD-DMEM differentiation method can be used in stage comparison studies that include both tachyzoites and bradyzoites. Also, the TD-DMEM media closely mimics the environment created by immune response in the brain and maintains the parasite in a differentiated state. Meanwhile, it would be interesting to determine if the depletion of other amino acids such as tyrosine and phenylalanine would affect the parasite invasion, replication, cyst wall formation, or egression. Discovering the answer to this question would also help in understanding the essential role of TgAaaH, since TgAaaH utilises both phenylalanine and tyrosine and a recent study by Wang et al. (2015) found that the parasites with TgAaaH 1 knockouts were unable to survive.

5.3 Novel tryptophan-depleted DMEM bradyzoite screening assay

In this thesis TD-DMEM-based parasite differentiation was used for developing a bradyzoite specific screening assay (Chapter 4). The current toxoplasmosis treatments cannot clear latent infection, and or have side effects (Haverkos, 1987; Leport et al., 1988). Cases of relapse have been observed in AIDS patients treated with all of the current anti-toxoplasma drug combinations (Kovacs, 1992; Romand et al., 1993; Jacobson et al., 1996; Chirgwin et al., 2002). A new drug that targets the cyst stage is needed to cure toxoplasma infection. A novel screening assay to detect drugs that are active against the bradyzoite stages has been developed. Current methods for identifying bradyzoite stage lead compounds is by testing in mice. The assay was based on the use of a *T. gondii* strain that expresses GFP in the bradyzoite stage, and TD-DMEM media to induce parasite differentiation. ELQ271, which caused an 84-88 % reduction in brain cyst number (Doggett et al., 2012) was active in the assay with an IC50 of circa 59 μM (Figure 4.2 and Table 4.1). While pyrimethamine, which is not

active against the cyst stages, had no activity (Figure 4.2 and Table 4.1). The TD-DMEM differentiation method (developed in Chapter 3) used in the assay will allow the parasite to differentiate without the change in pH that has previously shown to affect the MIC of drugs that target malaria and *Leishmania* (Yayon et al., 1985; Jiang et al., 2002). The newly-developed screening assay allows continued monitoring of the parasite growth without interfering with parasite growth and the use of external cytotoxic chemicals. In the future, this method can be used as a high throughput assay to screen chemicals that might be active against the bradyzoite stage, because it only measures the bradyzoite-specific GFP. TD-DMEM based bradyzoites drug screening assay is fast, easy, and take less time and effort compared to currently used animal model testing, which are time consuming and laborious (Sabin and Warren, 1942; Araujo et al., 1988; Araujo et al., 1992). Hence this method can be used as an initial throughput assay to select compounds to test *in vivo*; also as a great advantage in keeping with the 3R's of animal experimentation. Finding a drug that is active against latent *T. gondii* infection is the first step toward finding a cure against toxoplasmosis. After finding a chemical that is active against the latent stage, the drug toxicity and ability to pass the blood-brain barrier would need to be tested *in vitro* and in an *in vivo* animal model. After establishing the drug safety, efficacy in animals, and receiving ethical approval, the drug safety in humans can be tested (Phase I). Finally, if the drug were found to be safe and effective, an application would be made to the drug authorities (IND), such as the Food and Drug Administration to license the drug and be allowed to be given to patients. For this large investment, pharma partners are needed.

5.4   Conclusions

The aim of this thesis was to characterise DA synthesis in *T. gondii* and develop a bradyzoite specific drug screening assay. Characterisation of DA synthesis in *T. gondii* during infection established that the parasite can synthesis L-DOPA from phenylalanine. Nonetheless, *T. gondii* was not able to synthesis DA in non-dopaminergic cells, and for synthesising DA *T. gondii* require host DDC. The fact that *T. gondii* only forms DA in dopaminergic cells show a level of specific regulation of DA synthesis in host cells without the detrimental effects of unpackaged cytosolic DA.

Furthermore, *T. gondii* is unlikely to convert DOPA to dopaquinone unless the host cell provides a dopa-oxidase enzyme activity.

Learning more about DA synthesis, the parasite-encoded tyrosine hydroxylase enzyme (rate limiting enzyme for DA synthesis) was found to be membrane bound and was transported outside the parasite within the PV. Future studies are needed to define how DA affects the molecular mechanisms involved in the behavioural responses of rodents during *T. gondii* infection, and how these may be relevant to the associations with human neurological disorders. More studies are required to understand the link between *T. gondii* infection and mental disorders, including a long-term clinical investigation to establish whether the infection is acquired before or after the appearance of mental symptoms, and to compare *T. gondii* seropositivity between groups of mental disorder patients. The research would establish whether mental disorder patients have a higher risk of infection, or if the infection might be the cause of the disorder.

After characterising DA synthesis, bradyzoite specific drug screening assay was designed based on TD-DMEM differentiation methods and the detection of bradyzoite specific GFP of *T. gondii* ΔKU80-GFP strain. TD-DMEM differentiation method were successful in inducing tachyzoites to bradyzoite conversion, and keeping the parasite at that stage. The good news that this assay can be used as a high throughput screening assay to identify drugs active against the bradyzoite stage.

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