Immune Response Effect on Dopamine Production During
Toxoplasma gondii Infection

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Submission in accordance with the requirement for the degree of Doctor of philosophy

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Faculty of Biological Science
University of Leeds
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2016

University of Leeds

Isra Mohammed Alsaady
Abstract

Many recent studies have developed our understanding of Toxoplasma gondii and host interactions whilst in the brain as well as the mechanisms that the parasite uses to change the behaviour of the host, since the parasite was found to increase dopamine production in catecholaminergic cells (PC12). Indeed, in vivo staining of the parasite cyst found dopamine accumulation inside the cysts. However, a detailed mechanism of dopamine production and the immune response involvement to dopamine levels is not yet clear. Several approaches have been used in this study to gain a better understanding. Firstly, immunostaining and mRNA expression have revealed that culturing T. gondii in tryptophan free media induces tachyzoite to bradyzoite differentiation. Secondly, analysis of the effect of kynurenic acid (KYNA) on dopamine levels showed that the parasite blocks the suppression of dopamine levels by KYNA through interfering with KYNA-induced changes in phosphorylation of tyrosine hydroxylase. Finally, host-parasite dynamics were examined by RNA sequencing (RNA-Seq) of T. gondii infected neurotransmitter-expressing cell. Our data found that the parasite does not increase dopamine production alone, but also modifies catecholamine metabolism to increase dopamine production and decrease norepinephrine and epinephrine; these modifications together with changes in expression of genes encoding dopamine receptors, neuronal function, neurodevelopmental, and NMDR suggest mechanisms of host neurotransmission modification. RNA-Seq data also revealed changes in the immune response of infected neural cells with subversion in expression of host cell cytokines and chemotaxis i. RNA-Seq data also show the simultaneous change in T. gondii expression during neural cell infection and showed that this profile is distinct from the expression profile in other host cells.
Acknowledgements

First, and above all, I praise God the Almighty for providing me with this opportunity and granting me the capability to proceed successfully.

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This thesis is dedicated to the memory of my beloved grandmother, whom I wish could share in the joy of finishing my PhD. May her soul rest in peace and May Allah be merciful to her.
### Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>3-HK</td>
<td>3-hydroxykynurenine</td>
</tr>
<tr>
<td>5-HT</td>
<td>Serotonin</td>
</tr>
<tr>
<td>a7nACh</td>
<td>α-7 nicotinic acetylcholine</td>
</tr>
<tr>
<td>AADC</td>
<td>Aromatic L-amino acid Decarboxylase</td>
</tr>
<tr>
<td>ADHD</td>
<td>Attention Deficit and Hyperactivity Disorder</td>
</tr>
<tr>
<td>APC</td>
<td>Antigen-presenting cells</td>
</tr>
<tr>
<td>ASD</td>
<td>Autism Spectrum Disorder</td>
</tr>
<tr>
<td>ATD</td>
<td>Acute Tryptophan depletion</td>
</tr>
<tr>
<td>BAG1</td>
<td>Bradyzoite Antigen1</td>
</tr>
<tr>
<td>BE (2)-M17</td>
<td>Human Caucasian Neuroblastoma</td>
</tr>
<tr>
<td>CCL</td>
<td>Chemokine Ligand</td>
</tr>
<tr>
<td>CNS</td>
<td>Central Nervous System</td>
</tr>
<tr>
<td>CPM</td>
<td>Counts Per Million</td>
</tr>
<tr>
<td>CREB</td>
<td>Cyclic AMP-Responsive Element Binding</td>
</tr>
<tr>
<td>CSF</td>
<td>Cerebrospinal Fluid</td>
</tr>
<tr>
<td>CXCR</td>
<td>Chemokines receptors</td>
</tr>
<tr>
<td>DA</td>
<td>Dopamine</td>
</tr>
<tr>
<td>DBH</td>
<td>Dopamine beta Hydroxylase</td>
</tr>
<tr>
<td>DE</td>
<td>Differential expression</td>
</tr>
<tr>
<td>DEPC</td>
<td>Diethyl Pyrocarbonate</td>
</tr>
<tr>
<td>DMEM</td>
<td>Dulbecco's Modified Eagle's medium</td>
</tr>
<tr>
<td>DRD</td>
<td>Dopamine Receptor</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
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<td>--------------</td>
<td>-------------</td>
</tr>
<tr>
<td>E</td>
<td>Epinephrine</td>
</tr>
<tr>
<td>ELISAs</td>
<td>Enzyme-linked immunosorbent assays</td>
</tr>
<tr>
<td>EMEM</td>
<td>Eagle's Minimum Essential Medium.</td>
</tr>
<tr>
<td>FBS</td>
<td>Foetal Bovine Serum</td>
</tr>
<tr>
<td>FRET</td>
<td>Fluorescence Resonance Energy Transfer</td>
</tr>
<tr>
<td>GO</td>
<td>Gene Ontology</td>
</tr>
<tr>
<td>GPCR</td>
<td>G-protein coupled receptor</td>
</tr>
<tr>
<td>GPI</td>
<td>Glycosylphosphatidylinositols</td>
</tr>
<tr>
<td>GRA</td>
<td>Dense Granule Protein</td>
</tr>
<tr>
<td>HFF</td>
<td>Human Foreskin Fibroblast</td>
</tr>
<tr>
<td>HPLC</td>
<td>High Performance Lipid Chromatography</td>
</tr>
<tr>
<td>HS70</td>
<td>Heat Shock Protein 70</td>
</tr>
<tr>
<td>IDO</td>
<td>Indoleamine 2, 3-dioxygenase</td>
</tr>
<tr>
<td>IFAT</td>
<td>Indirect fluorescence antibody tests</td>
</tr>
<tr>
<td>IFN-γ</td>
<td>Interferon gamma</td>
</tr>
<tr>
<td>IL</td>
<td>Interleukin</td>
</tr>
<tr>
<td>iNOS</td>
<td>Inducible nitric oxide synthesis</td>
</tr>
<tr>
<td>ISAGAs</td>
<td>Immunosorbent agglutination assays</td>
</tr>
<tr>
<td>KATs</td>
<td>Kynurenine Aminotransferases</td>
</tr>
<tr>
<td>KMO</td>
<td>Kynurenine 3-monooxygenase</td>
</tr>
<tr>
<td>KYN</td>
<td>Kynurenine</td>
</tr>
<tr>
<td>KYNA</td>
<td>Kynurenic acid</td>
</tr>
<tr>
<td>MHC</td>
<td>Major Histocompatibility Complex</td>
</tr>
<tr>
<td>MIC</td>
<td>Microneme protein</td>
</tr>
<tr>
<td>MyD88</td>
<td>Myeloid Differentiation factor 88</td>
</tr>
<tr>
<td>NE</td>
<td>Norepinephrine</td>
</tr>
<tr>
<td>Acronym</td>
<td>Description</td>
</tr>
<tr>
<td>---------</td>
<td>-------------</td>
</tr>
<tr>
<td>NF-κB</td>
<td>Nuclear Factor-κB</td>
</tr>
<tr>
<td>NGF</td>
<td>Nerve Growth Factor</td>
</tr>
<tr>
<td>NK</td>
<td>Natural killer</td>
</tr>
<tr>
<td>NMDR</td>
<td>N-methyl-D-aspartate receptor</td>
</tr>
<tr>
<td>PAH</td>
<td>Phenylalanine hydroxylase</td>
</tr>
<tr>
<td>PC12</td>
<td>Rat Pheochromocytoma</td>
</tr>
<tr>
<td>PFC</td>
<td>Prefrontal Cortex</td>
</tr>
<tr>
<td>PNMT</td>
<td>Phenylethanolamine N-methyltransferase</td>
</tr>
<tr>
<td>PV</td>
<td>Parasitophorous vacuole</td>
</tr>
<tr>
<td>PVM</td>
<td>Parasitophorous vacuole membrane</td>
</tr>
<tr>
<td>QUIN</td>
<td>Quinolinic acid</td>
</tr>
<tr>
<td>RNA-Seq</td>
<td>RNA sequencing</td>
</tr>
<tr>
<td>ROP</td>
<td>Rhoptry protein</td>
</tr>
<tr>
<td>RPMI</td>
<td>Roswell Park Memorial Institute medium</td>
</tr>
<tr>
<td>SAG</td>
<td>Surface Antigen</td>
</tr>
<tr>
<td>SOCS</td>
<td>Suppressor of Cytokine Signalling</td>
</tr>
<tr>
<td>STAT</td>
<td>Signal Transducer and Activator of Transcription</td>
</tr>
<tr>
<td>TDO</td>
<td>Tryptophan Dioxygenase</td>
</tr>
<tr>
<td>TgAaaH</td>
<td><em>T. gondii</em> Aromatic Amino Acid Hydroxylase</td>
</tr>
<tr>
<td>TH</td>
<td>Tyrosine Hydroxylase</td>
</tr>
<tr>
<td>TLR</td>
<td>Toll-like Receptor</td>
</tr>
<tr>
<td>TNF-α</td>
<td>Tumour Necrosis Factor-alpha</td>
</tr>
<tr>
<td>TRP</td>
<td>Tryptophan</td>
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Chapter One

1 *Toxoplasma gondii*: Biology, Immune Response and Host Manipulation
1.1 Abstract

*Toxoplasma* is arguably one of the most successful parasites infecting humans, causing severe encephalitis in immunocompromised patients; however, its pathogenesis in immunocompetent individuals remains unknown. Much evidence suggests that *T. gondii* may have behavioural effects on humans for three main reasons: first, it induces behavioural change in rodents. Second, a higher *T. gondii* seroprevalence has been found in schizophrenia patients compared to controls; and finally, the antipsychotic drugs used for the treatment of schizophrenia inhibit the growth of the parasite *in vivo* and *in vitro* and might induce behavioural change via proximate or indirect mechanisms. Two tyrosine hydroxylase enzymes that produce L-Dopa were discovered. This finding suggests that the parasite might be involved in the production of dopamine. Additionally, *T. gondii* may effect brain changes indirectly by inducing host-determined humoral and cytokine immune responses, whereby the *T. gondii* activates an immune response in the brain that leads to the release of different cytokines. Activated astrocytes will increase Indoleamine 2, 3-dioxygenase (IDO) activity, leading to decreased tryptophan and the production of kynurenic and quinolinic acid. Quinolinic acid is an agonist of the N-methyl-D-aspartate (NMDA) receptor, while kynurenic acid is an antagonist of NMDA; the net result of this may be an alteration in glutamatergic neurotransmission. In addition, kynurenic acid is an antagonist of α-7 nicotinic acetylcholine, resulting in a decrease in dopamine level. This chapter reviews *T. gondii* biology, and the proximate and indirect mechanisms of host behaviour manipulation.
Toxoplasma gondii is arguably the most successful intracellular parasites worldwide because of their ability to infect all warm-blooded animals (including humans) and birds. The parasite has been recorded to have 350 host species, the vast majority of which live in the wild (Weiss and Kim, 2007). It is estimated that 25-30% of the world’s human population are infected (Montoya and Liesenfeld, 2004). This prevalence varies widely between countries (10%-80%) and even within the same country and regions, and between different communities within these regions (Pappas et al., 2009). On the other hand, in North America, in South East Asia, in Northern Europe, and in Sahelian countries of Africa the seroprevalence is relatively low (10-30%). While in the countries of Central and Southern Europe, the seroprevalence is moderate (30-50%) and high prevalence has also been found in Latin America and in tropical African countries (Robert-Gangneux and Darde, 2012).

1.2 Life cycle of T. gondii

The definitive hosts of the parasite are cats and other members of the Felidae family, where the parasite undergoes its sexual life cycle in the host’s small intestines. The result of this sexual mating is millions of oocysts shed in cat stool. These oocysts are highly infectious and resistant to environmental conditions (Frenkel, 1970). The T. gondii oocyst is released unsporulated in the cat stool, but sporulate later and form an isospora-like oocyst with two sporocysts, each containing four sporozoites (Dubey et al., 1970). The ingestion of this oocyst by the intermediate host will lead to the release of the sporozoites in the intestine; the parasite first moves to the nearest secondary lymphoid tissue (Sumyuen et al., 1995) and then to the site of infection by using the dendritic cells as a Trojan horse (Channon et al., 2000). Inside the intermediate host, the parasite undergoes asexual replication, this stage is known as tachyzoite stage, the stage that allows the parasite to rapidly increase in number and spread throughout the body. Later, due to the immune response, the parasite will develop into hundreds of slowly dividing semi-dormant parasites within tissue cysts; this is called the bradyzoite stage. In chronic infection of the brain, bradyzoites are found in cysts. These cysts are located inside a viable cell surrounded by a thin layer of the host cytoplasm. Inside the cyst, there are hundreds of mature bradyzoites, which appear more elongated than the tachyzoites, and have a posteriorly located nucleus with the presence of polysaccharide granules (Weiss and Kim, 2007). Ingestion of tissue cyst from the intermediate host by a cat
will lead to the release of the merozoite stage, allowing this parasite to undergo the sexual life cycle again (Dubey, 1998).

All three *T. gondii* stages are involved in the infection: tachyzoites, bradyzoites and oocysts. Bradyzoite-containing tissues infect cats via ingestion, while herbivores are infected by the ingestion of *T. gondii*–sporulated oocysts contaminated food or drinking water. Transplacental infection occurs when tachyzoites are transmitted from infected mothers to her foetus during pregnancy. In addition, tachyzoites can be transmitted to humans during blood transfusion and organ transplantation (Weiss and Kim, 2007). The uniqueness of the life cycle of *T. gondii* among its group is the ability of the parasite to transmit between different intermediate hosts or even between definitive hosts (Afonso et al., 2006)(Figure 1-1)

![Figure 1-1: The life cycle of *T. gondii.*](image)

### 1.3 *T. gondii* biology

*T. gondii* is a crescent-shaped cell, around 2 μm wide and 5 μm long, with one pointed apical end and a rounded posterior end. It is surrounded by a complex membrane, called the pellicle, and a cytoskeleton, which is involved in the structural integrity and motility of the cell. *T. gondii* possesses many organelles, including a nucleus, ribosomes, an endoplasmic reticulum, a mitochondria, a Golgi complex and a unique plastid-like organelle called the apicoplast.
formed of multiple membranes. The apicoplast was hypothesized to formed due to a possible acquisition by the parasite via a secondary endosymbiosis of a free-living red alga (Roos et al., 1999). Similar to other members of the phylum Apicomplexan, the apicoplast is found specialized cytoskeletal structure in the apical part, called the conoid, together with numerous secretory organelles (rhoptries [ROPs], dense granules, and micronemes) this part is involved in cell invasion (Dubey et al., 1998; Weiss and Kim, 2007).

1.4  *T. gondii* genotyping and strains

*T. gondii* has a large clonal population structure, which consists largely of three main genotypic lineages (Strain I, II, and III), each with distinct properties in terms of virulence, epidemiological pattern of infection and effects on host cell signalling (Sibley and Ajioka, 2008; Weiss and Kim, 2007). Recently a new fourth clonal lineage has been described to share lineage with types I and II (Khan et al., 2011). Strain I am the most virulent during the acute stage of the infection, and can be found in congenital infection. Strains II and III are less virulent and have higher tendency to establish chronic infections (Sibley and Ajioka, 2008; Sibley and Boothroyd, 1992). Strain II is involved in congenital infection and infections involving AIDS patients. Strain III was isolated from animals (Weiss and Kim, 2007).

Genetic crosses were used to map loci involved in strain specific virulence differences in *T. gondii* and host cell signalling and two rhoptry kinases were identified: ROP16 and 18. These mediate key functional differences between the genotypes and secreted during host invasion (Taylor et al., 2006; Saeij et al., 2006; Saeij et al., 2007; Khan et al., 2009) as further discussed in (Behnke et al., 2011)

1.5  Mechanism of cell invasion

The invasion of the host cell is a highly coordinated process of attachment and penetration, Two apical secretory organelles are involved, the micromeres and rhoptries (Carruthers and Boothroyd, 2007).

The invasion processes starts with the attachment of the parasite to the host cell membrane through the calcium-dependent secretion of adhesions from micronemes, like the MIC2 microneme protein, this protein recognises host cell receptors and establishes parasite
attachment and reorientation. The invasion process is initiated by the gliding motility; a complex actin-myosin interaction between host cell surface and the parasite, besides a dynamic rearrangements of the parasite cytoskeleton (Carruthers and Boothroyd, 2007). After that, the entry of the parasite is established by the formation of the moving junction. A tight association is formed by moving junction between the parasite apical end and the host cell membrane. As this compound moves from the apical end to the posterior end of the parasite, the internalization of the parasite into a parasitophorous vacuole (PV) is established. During the establishment of the moving junction, micronemes secret apical membrane antigen (AMA1), that disrupted over the entire surface of the parasite, Rhopty (ROP) proteins are secreted into the host cell membrane (Dubremetz, 2007) and they are involved in the formation of the nascent parasitophorous vacuole membrane (PVM). For example, ROP18 is associated with the cytosolic side of the PVM and has protein kinase activity, which – as mentioned previously – has a massive effect on parasite growth and virulence (El Hajj et al., 2007).

Dense granular proteins beside rhopty proteins contribute to PVM formation. During the PVM formation and invasion process, the parasite modifies the biochemical characteristics of the PVM by stripping most of the host’s transmembrane proteins and preventing fusion with lysosomes or any cytoplasmic vesicle. In addition, dense-granule proteins are involved in the development of a complex network of membrane tubules that extend from the PVM into the vacuolar lumen (Mercier et al., 2005).

This network of membranes is involved in the exchange between the host and the parasite importing in nutrients from the host cell cytosol to the parasite, and exporting proteins or lipids from the parasite PVM or the host cell. In addition, the PVM and the host cell mitochondria are also closely associated, which contributes to parasite metabolism (Sinai et al., 1997; Schatten and Ris, 2004; Crawford et al., 2006).

1.6 Medical importance

*T. gondii* infection can be asymptomatic or symptomatic (toxoplasmosis). The medical importance of the parasite is discussed here in the setting of both immunocompetent and immunocompromised patients.
1.6.1 Immunocompetent patients

The majority of *T. gondii* infections are considered asymptomatic, although a minority of individuals with acute infection may present signs and symptoms. The symptoms are mainly mild and non-specific (flu-like symptoms), but sometimes symptoms may be severe, including prolonged fever, fatigue (Remington, 1974) and retinochoroiditis. The most common symptom is cervical lymph-adenopathy, where lymph nodes are discrete and non-tender, and measure only a few centimeters in diameter (McCabe et al., 1987). Chorioretinitis with visual impairment may rarely be associated with primary infection, although it was thought that chorioretinitis is due to congenital infection. (Delair et al., 2008) found that almost 25% of infections are acquired in immunocompetent individuals.

Congenital infection occurs if a seronegative mother is infected during pregnancy. The relationship between the duration of pregnancy and the clinical manifestation of infection is an inverse relationship. This means that an infection acquired during the last trimester is the most serious (Dunn et al., 1999). During congenital toxoplasmosis, the central nervous system is affected mostly; non-specific signs include retinochoroiditis, blindness, epilepsy, psychomotor or mental retardation, encephalitis, microcephaly, intracranial calcification, hydrocephalus, anaemia, jaundice, rash and petechiae due to thrombocytopenia. The severity of these clinical manifestations is high if infection is acquired before week 26 of gestation (Remington et al., 2001).

1.6.2 Immunocompromised patients

*Toxoplasma* encephalitis is the most common presentation of reactivated toxoplasmosis in AIDS patients, although toxoplasmosis in AIDS patients could be due to acute acquired infection (Luft and Remington, 1992). However, reactivated toxoplasmosis in AIDS patients might involve other organs e.g., heart, lungs, eyes, liver, pancreas, bone marrow, bladder, lymph nodes, kidney, spleen and skin (Robert-Gangneux and Darde, 2012).

The symptoms of toxoplasmosis in transplant patients are similar to those in AIDS patients. The infection might transmit from transplanted organs of seropositive donors to the seronegative patients. Otherwise, transplant-related immunosuppression may cause reactivation of latent infection of seropositive transplant recipients (Botterel et al., 2002; Rogers et al., 2008; Martina et al., 2011).
1.7 Diagnosis

The main method of diagnosing infection in clinical laboratories is through the detection of circulating antibodies during the acute stage of infection. First, IgM is present during acute infection, and, after eight weeks, IgG production starts with or without IgM (Jenum and Stray-Pedersen, 1998). Therefore, the acute stage is diagnosed through the presence of IgM, and serial specimens will demonstrate an increasing IgG titre. In the chronic stage, only IgG is present, without changing the titre in serial specimens. During pregnancy, the serological response is the same as in acute infection, but it is necessary to know whether the infection was acquired during pregnancy: IgM can be detected for months after the infection and the parasite can only be transmitted to the foetus if infection occurred during the pregnancy. However, the time of infection during pregnancy could be defined by the use of IgG-avidity (low in acute infection). This test is needed to determine and evaluate *T. gondii* transmission *in utero* (Robert et al., 2001).

Serological techniques rely on detecting the circulating antibodies in patient serum. The Sabin-Feldman dye test is one of the earliest serological techniques used to detect toxoplasmosis. The Feldman dye test nowadays has been replaced by wide varieties of techniques including indirect fluorescence antibody tests (IFATs), hemagglutination, enzyme-linked immunosorbent assays (ELISAs), capture ELISAs, and immunosorbent agglutination assays (ISAGAs). All these tests are suitable for IgM, IgA, or IgE detection. However, for low titres of IgG, sensitive Western blot (WB) assay might be used to reveal specific IgG interaction to several *T. gondii* antigens, including the SAG-1 tachyzoite major surface protein; a kit is available commercially (WB Toxo GII; LDBio). This method has been shown to have 100% specificity and 99.2% sensitivity compared to the dye test (Franck et al., 2008). Finally, PCR is a very good diagnostic tool and has employed to detect the presence of the parasite during congenital infection using amniotic fluid sample (Thalib et al., 2005). However, results from multicentric studies showed an absence of reproducibility of parasite quantification, especially with low parasite number, and showed that standardization of the techniques is required. Rep529 DNA target is more adequate and recommended for this diagnosis than the widely used B1 gene (Sterkers et al., 2010).
1.8 Treatment

Toxoplasmosis can be treated by combined therapy with sulfadiazine and pyrimethamine (Eyles and Coleman, 1953) or spiramycin (Beverly, 1985). Atovaquone may be given to pyrimethamine- and sulfonamide-intolerant patients (Kovacs, 1992). However, atovaquone and pyrimethamine resistance in patients has been reported with long-term treatment (Jacobson et al., 1996; Baatz et al., 2006). For *T. gondii* infections during pregnancy, the antibiotic spiramycin treatment is used (Wong and Remington, 1994) to minimize the possibility of transplacental transmission (McAuley et al., 1994).

These drugs are effective in the treatment of the acute stage, congenital and eye infection because they have antiparasitic effects on the tachyzoite stage but they are not effective against the bradyzoite stage or chronic infection.

1.9 Prevention and control of *Toxoplasma* infection

Knowledge of *T. gondii* biology and life cycle suggests that hygienic measures can be implemented to avoid infection. Direct contact with cat faeces increases the risk of infection with oocyst. Careful hand washing after handling a cat, wearing gloves when changing cat litter box, as well as washing the tray with hot water (60°C), coupled with avoiding putting cat litter box in the kitchen, and feeding cats dried or canned food are the key preventative measures necessary to avoid infection. Thorough washing of the hands and nails after any outdoor activities in contact with soil, and wearing gloves for gardening are advised to avoid infection with oocysts in the environment. Moreover, bottled mineral water is preferred to tap water (in countries where the water network is supplied mainly by surface water) so as to avoid infection through contaminated water. In addition, one has to be careful to wash thoroughly vegetables, fruits, and herbs that are eaten raw, especially if they grow close to the ground.

Finally, infection through consumption of tissue cysts in meat could be avoided by thorough cooking of meat; avoiding microwave cooking; keeping meat frozen in -20°C or lower for at least 15 days; and washing hands, knives, any containers, and tables thoroughly after meat manipulation or cutting (Robert-Gangneux and Darde, 2012).
1.10 Immune response to *T. gondii*

Usually *T. gondii* infection is acquired due to the ingestion of the tissue cyst. After surviving the gastric processes, the parasite crosses the intestinal epithelium and continues its propagation by regulating migratory capacity (Barragan and Sibley, 2002). *T. gondii* is an intercellular parasite; this is advantageous as it protects the parasite from soluble, humoral, or cellular antimicrobial factors (Foureau et al., 2010).

During *T. gondii* infection of enterocytes, the parasite causes physiological and morphological disturbances. Enterocytes might release cytotoxic molecules such as nitric oxide (NO) (Yap and Sher, 1999). In addition, chemokines and cytokines are released from enterocytes as a response to the infection; these secretions neutrophils and dendritic cells (DC). Parasite replication inside an enterocyte causes host cell lysis, parasite egress and tachyzoite dissemination throughout the host is circulating macrophages (Da Gama et al., 2004; Courret et al., 2006) or CD11c+ dendritic cells (DC). This may act as a ‘Trojan horse’ to spread the infection (Courret et al., 2006; Lambert et al., 2006).

The immune response to *T. gondii* is cell-mediated by the release of interferon gamma (IFN-γ). The first immune response is by macrophages, neutrophils and dendritic cells; these cells release IL-12 that stimulates the release of IFN-γ from T-cells, while NK cells kill *T. gondii*-infected cells (Gazzinelli RT et al., 1993; Scharton-Kersten TM et al., 1996; Pfefferkorn and Guyre, 1984).

1.10.1 Cells involved in the innate immune system

1.10.1.1 Neutrophils

Neutrophils have a role in the release of pro-inflammatory cytokines such as IL-12 and TNF-α, as well as several chemokines. when exposed to *T. gondii* antigen and lead to the recursion of other immune cells (Denkers et al., 2004b); additionally, neutrophils have an immune regulatory role (Bliss et al., 2001) and have reactive oxygen intermediate independent
antimicrobial functions (Denkers et al., 2004b). The neutrophils are recruited at the site of the infection by chemokines released from the enterocyte and by other chemotactic material released from the CD4 T-cells and neutrophils like IL-17, a granulopoiesis cytokine. These chemokines induce CXCR chemokines receptors, leading to the recruitment of neutrophils to the site of infection during early infection (Kelly et al., 2005). The receptor for these chemokines is CXCR2. The expression of the CXCR2 receptor is essential for the neutrophils trafficking (Del Rio et al., 2001).

1.10.1.2 Dendritic cells

Dendritic cells play a major role in innate immunity and as a connection between the innate and adaptive immune response (Aliberti et al., 2003). Furthermore, dendritic cells act as antigen-presenting cells (APC) for the T-cells through the expression of CD80 and CD86 (Weiss and Kim, 2007). They have an antimicrobial function via the release of IFN-γ and triggering oxygen-dependent inhibition of *T. gondii* (Aline et al., 2002). Moreover, dendritic cells are known for their ability to carry different pathogens to the lymph node.

1.10.1.3 Macrophages

Macrophages play a key role in the innate immunity during *T. gondii* infection. During *T. gondii* infection, macrophages function as APC and antimicrobial agents. These functions are activated by IFN-γ. Macrophages function as an antimicrobial by tryptophan starvation or stimulation of inducible nitric oxide synthesis (iNOS). Tryptophan depletion will prevent the parasite growth as *T. gondii* are dependent on tryptophan for growth (Pfefferkorn and Guyre, 1984). Tryptophan degradation occurs due to induction of indoleamine 2, 3-dioxygenase (Murray et al., 1989). While nitric oxide synthesis leads to the production of reactive nitrogen intermediates, these intermediates are toxic to the parasite (Liesenfeld et al., 1999).
1.10.1.4 Natural killer cells
Natural killer (NK) cells also play a critical role in innate immune response in the acute stage of *T. gondii* infection and they are the early source of IFN-\(\gamma\) (Sher et al., 1993).

During the early phase of infection with *T. gondii*, CCR5-binding chemokines mediate the recruitment of NK cells to the infection site (Khan A et al., 2006). In the site of the infection, NK cells have several functions: first, IFN-\(\gamma\) production by NK cells is induced by IL-12 released from infected DC or macrophages (Guan et al., 2007; Korbel et al., 2004; Sher et al., 2003). Moreover, it can regulate cytotoxic CD8 T-cell immunity to *T. gondii*, even in the absence of CD4 T-cells (Combe et al., 2005). Finally, *T. gondii*-infected target cells can be killed by NK cells (Subauste et al., 1992, while during the chronic stage, perforin-dependent cytolytic NK cell activity has been found to be important in protecting mice (Denkers et al., 1997).

1.10.2 Innate sensing of *T. gondii*

*T. gondii* is sensed as foreign by the innate arm of the immune system via several unique protozoan parasite different molecules.

Host CCR5 interacts with cyclophilin-18 and stimulates DC IL-12 production (Aliberti et al., 2003). In addition, TLR11 interacts with actin-binding molecule profilin and the interaction leads interleukin-12 production, though the role of this interaction is not clear, as TLR11 is non-functional in humans, and the profilin is a cytosolic protein that only exposed to immune system after the death of the parasite (Yarovinsky et al., 2005). TLR12 also recognise profilin; this TLR may increase host resistance by triggering NK and pDC cell function (Koblansky et al., 2013).

TLR4 is activated by both glycosylphosphatidylinositol (GPI) (Debierre-Grockiego et al., 2007), heat shock protein 70 (HS70) (Mun et al., 2005) and TLR2-activated glycosylphosphatidylinositol (GPI) (Debierre-Grockiego et al., 2007).

Such TLR signalling is mediated by a series of adaptor proteins of which myeloid differentiation factor 88 (MyD88) is the most influential. MyD88 is important for host resistance (LaRosa et al., 2008). MyD88-deficient mice that still express IL-1 or IL-18, which also signals through MyD88, showed an impaired IL-12 and IFN-\(\gamma\) response and could not control the infection (Sukhumavasi et al., 2008). This indicates effective activation of immunity through MyD88 signalling. TLRs must first recognise *T. gondii* molecules.
However, mice deficient in individual TLRs, including TLR2, 4, and 11, showed slightly more susceptibility to *T. gondii* infection when compared with wild-type mice. MyD88 in relation with TLR receptors during *T. gondii* infection was reviewed in (Egan et al., 2009).

Although the activation of TLR/MyD88 signalling clearly has a major impact on the *T. gondii* infection, the parasite is capable of suppressing the TLR/MyD88 signalling pathway in the infected cells. The mechanism of the blocking is unclear, though this may be due to the blocking of the nuclear accumulation of NF-κB, which has a short-term effect (Shapira et al., 2005). Alternatively, by targeting the chromatin modification machinery rather than gene-specific transcription factors (Leng et al., 2009), which might explain the parasite’s ability to simultaneously suppress a large panel of pro-inflammatory mediators.

### 1.10.3 Adaptive immune response

Adaptive immune response is also dependent on the production of IFN-γ. However, in adaptive immune response the IFN-γ is produced from T-cells and NK

#### 1.10.3.1 T-lymphocytes

Both CD4 and CD8 participate in the adaptive immune response. Proliferation of resting T-cells in response to parasite-infected cells is dependent on both CD80 and CD86, and IL-12 by monocytes, which require CD40 in antigen-processing cells (APCs) to interact with CD40 ligand on activated T-cells (Subauste et al., 1998).

While CD4 is needed for long-term protection, the decline of CD4 numbers during HIV infection (Luft et al., 1984; Israelski and Remington, 1988) or the lack of CD4 in mouse model (Johnson and Sayles, 2002) increases susceptibility during the chronic stage of infection. During the early stage of infection, CD4 cells optimise the response of the B-cell and CD8 T-cells (Johnson and Sayles, 2002; Lutjen et al., 2006) by producing IFN-γ or their expression of CD40L, leading to activation of macrophage-effector mechanisms (Gazzinelli et al., 1992; Reichmann et al., 2000; Andrade et al., 2005; Subauste and Wessendarp, 2006; Subauste et al., 2007; Portillo JA et al., 2010). However, during acute infection, CD4 plays a smaller role to that of the CD8 cytolytic effect (Casciotti et al., 2002).

#### 1.10.3.2 CD8+ T cell response

The involvement of CD8 cells in an anti-*T. gondii* immune response is not surprising, as CD8 cells are those that recognise and destroy cells infected with intracellular viral, bacterial
and parasitic organisms. Similar to CD4, the treatment of chronically infected mouse with Anti-CD8 mAb increases the susceptibility to toxoplasmosis, and the mice succumbing approximately 60 days post-infection (Gazzinelli et al., 1992). CD8 controls the infection through the production of INF-γ and through the perforin-mediated cytolysis of infected host cells (Gazzinelli et al., 1992; Montoya et al., 1996; Denkers et al., 1997).

1.10.3.3 Natural killer T cells
These cells are found to help CD8 cells by producing IL-12 (Denkers et al., 1996). However, NK cells can take part in CD8 priming in the absence of CD4 cells (Intlekofer et al., 2005). Moreover, they help in the production of the antibodies, have a cytotoxic effect, regulate Th1/Th2 differentiation, help in the parasite clearance, and contribute in GIT immunopathology by shifting the cytokine profile toward a Th1 pattern (Ronet et al., 2005).

1.10.3.4 B-cell and humoral immunity
As mentioned previously, CD4+ T-cells are necessary to activate and regulate optimal B-cell response. The absence of B-cells during *T. gondii* infection leads to the host’s death within 3-4 weeks following challenges, associated with high parasite burdens in the CNS (Kang et al., 2000). Antibodies produced by B-cells can mediate protective effects through several mechanisms. Antibodies can activate the classical complement pathway, block invasion and can opsonize parasites for phagocytosis based on *in vitro* studies (Nakao and Konishi, 1991; Erbe DV et al., 1991; Hammouda et al., 1995; Vercammen M et al., 1999; Schreiber and Feldman, 1980).

1.10.4 *T. gondii* antigens involved in immune response activation
A few *T. gondii* antigens activate the immune response. The B-cell is activated by SAG1 (Rachinel et al., 2004), SAG2A (Prince et al., 1990), GRA4 (Mevelec MN et al., 1994), while T-cells are activated by GRA6 (Blanchard et al., 2008), GRA4 and ROP7 (Frickel et al., 2008) in mice. However, the major *T. gondii* antigens and the T-cell antigens that interact with them are yet to be identified in humans.

1.10.5 Effector mechanism controlling *T. gondii* infection
Cellular immunity mediates protection through the production of pro-inflammatory cytokines e.g., IFN-γ, TNF-α and CD40 ligation; these pathways are integrated to trigger specific effector mechanisms needed to control infection with *T. gondii*. 
First, IFN-γ controls the infection by altering host cell metabolism, leading to tryptophan degradation in fibroblasts (Pfefferkorn and Guyre, 1984), and iron starvation in enterocytes (Dimier and Bout, 1998). Phagocytes is also stimulated by IFN-γ leading to the production reactive oxygen and nitrogen intermediates, resulting in parasite damage and impeding the growth in macrophages (Murray et al., 1985; Adams et al., 1990). The replication of *T. gondii* in macrophages and other cell types is inhibited by NO (Scharton-Kersten et al., 1997). This is in addition to induction of the recently described p47 GTPases, including IGTP, IRG-47, and LRG-47 (Taylor et al., 2004).

TNF-α is another cytokine that can prevent the replication of the tachyzoite *in vitro*, while TNF-α activated cells generate NO by inducible NO synthase (iNOS) and inhibit the tachyzoite intracellular replication (Adams et al., 2004). Nonetheless, it is important to note that IFN-γ alone is sufficient to control the acute infection in mice (Scharton-Kersten et al., 1997).

However, other interleukins play a less important role in controlling the *T. gondii* infection such as IL-4 (Suzuki et al., 1996), IL-6 (Suzuki et al., 1997), and IL-5 (Zhang and Denkers, 1999), while IL-10 (Wilson et al., 2005) IL-4 (Roberts et al., 1996) and LXA4 (Aliberti et al., 2002) are important for down regulation of pro-inflammatory responses during the infection and prevent immunopathology.

### 1.10.6 Inhibition of host cell signalling cascades

The binding of IFN-γ to its IFN-γ receptor at the cell surface promotes IFN-γ actions, leading to the initiation of a signalling cascade; the JAK family of tyrosine kinases and STAT family of transcription factors are involved in these signalling cascades (Cerávolo et al., 1999). *T. gondii* subverts the host immune response by inhibiting the signalling pathways initiated by this response for example by blocking the transcription factors signal transducer and activator of transcription 1 (STAT1) (Luder et al., 2001) and nuclear factor-κB (NF-κB) (Luder et al., 2001; Shapira et al., 2005; Shapira et al., 2002).

STAT1 transcription is inhibited by *T. gondii* subverting the IFN-γ signalling (Zimmermann et al., 2006). In fact, *T. gondii* infection causes a decrease in the response to IFN-γ-induced up regulation of many genes, including MHC Class II, iNOS, and the p47 GTPases (Luder et al., 2001). Besides, the down-regulation of the nuclear factor-κB (NF-κB) leads to down-
regulation of immune signals that use NF-κB e.g.: tumour necrosis factor (TNF) and CD40 and their effector mechanisms (Denkers et al., 2004a; Mason et al., 2004).

Additionally, *T. gondii* prevents LPS-triggered IL-12 and TNF-α production via up regulation of host STAT3 (Butcher et al., 2005) while the suppressor of cytokine signalling (SOCS) proteins SOCS1 (Zimmermann et al., 2006; Stutz et al., 2012) and SOCS3 (Whitmarsh et al., 2011) are anti-inflammatory pathways that are also up-regulated during the infection, potentially compromising host mechanisms of parasite control. The ability of *T. gondii* to subvert the immune system allows it to be a most successful parasite.

The parasite subverts the signalling pathway in the infected cells to evade the immune response by secreting molecules e.g., Profilin, GPI; Cyclophilin, Lipoxygenase; ROP16, ROP18 and HSP70 (Pollard et al., 2009).

### 1.11 Immune response in the brain

The immune response in the brain is similar to the immune response in different body parts; it is a cell-mediated immune response involving the release of IFN-γ. In the brain, IFN-γ is produced by dendritic cells, which play a major role in innate immunity and as a connection between innate and adaptive immune response. In innate immunity, dendritic cells release interleukin (IL)-12, which induces the release of IFN-γ from T-cells and NK cells (Aliberti, 2003). In addition, dendritic cells are known for their ability to carry different pathogens to the lymph nodes. In *T. gondii* infection, dendritic cells facilitate the *T. gondii* dissemination to different organs in the body (Dubey, 1997). The adaptive immune response is the immune response against the bradyzoite cyst during chronic infection by T-cells.

The means by which parasites cross the blood brain barrier (BBB) and how the immune system controls the parasites within the brain remain unclear (Masocha and Kristensson, 2012; Feustel et al., 2012). After entering the central nervous system (CNS), tachyzoites infect astrocytes, microglia and neurons. Parasite infiltration is followed by CD4+ and CD8+ T-cell recruitment, which is important for control of CNS infection; CD28 or ICOS stimulatory pathways are activated to control the infection (Parker et al., 1991; Villegas et al., 2002; LaRosa et al., 2008; Jordan and Hunter, 2010; Harris et al., 2010). This lymphocyte infiltration causes structural modification in the CNS based on two photon image observations (Wilson et al., 2009).

Microglia are the most important effector cells. They release a tumour necrosis factor (TNF)-α following infection, and this production of the TNF-α is mediated by IFN-γ (Deckert-
Schluter et al., 1999) Furthermore, the activation of the microglia leads to the production of IL-1a, IL-12 and IL-15, and the expression of MHC class I and II, LFA-1 and ICAM-1. This indicates that microglia work as antigen-presenting cells (Schluter et al., 2001).

Astrocytes function as immune effector cells that are involved in different immune responses and act as host cells. Activated astrocytes regulate T-cell trafficking by producing chemokines such as IL-10. In addition, *T. gondii*-infected astrocytes resist the development of *T. gondii* encephalitis by producing the proinflammatory cytokines IL-1, IL-6 and TNF-α. Moreover, the expression of MHC class II and co-stimulatory molecules on the surface of IFN-γ-activated astrocytes may indicate that these cells function as antigen presenting cells (Wilson and Hunter, 2004). It is also important to point out that some cytokines that have a minor role in *T. gondii* infection resistance play a more important role in the control of the TE, such as IL-1 (Wilson and Hunter, 2004), IL-6 and TNF-α (Chao et al., 1994; Däubener et al., 1996).

Finally, the significant antimicrobial effector mechanisms for controlling *T. gondii* in the brain are through IDO-induced tryptophan starvation and NO synthesis, as well as p47 GTPases. Microglia control the parasite via NO synthesis (Chao et al., 1993) While astrocytes control the infection by inducing NO synthesis and IDO-induced tryptophan starvation (Oberdorfer et al., 2003) and activating the genes encoding for the p47 GTPases in mice (Halonen et al., 2001).

During chronic infection, parasites are found in the cyst stage and disseminate through the brain, predominantly in the cerebral cortex, hippocampus, basal ganglia, and amygdala (Melzer et al., 2010). In addition, cyst-bearing cells burst open regularly (randomly) in immune-competent individuals and infect nearby cells (Ferguson et al., 1989).

It is important to realize that, during chronic infection, the cyst-bearing cells are not visible to the CD8 T-cells (Schaeffer et al., 2009). Besides, MHC I expression by neurons is low and the behaviour of T-cells in the CNS is dependent on antigen availability (Wilson et al., 2009), suggesting that such intracellular cyst structures are an effective means of immune evasion.

1.12 *T. gondii* and behavioural change

The manipulation hypothesis states that parasites change the host’s behaviour (phenotype) for their own benefit, generally to increase their transmission rate to a definitive host (Thomas et al., 2005). In *T. gondii*, the parasite manipulates the rat’s behaviour by decreasing the innate
fear of cats, which leads to an increase in the transmission rate. A number of types of
evidence support the theory that *T. gondii* is involved in behavioural change in humans. First,
the observed rodent behaviour found to change during chronic *T. gondii* infection. Second, a
higher *T. gondii* seroprevalence was found in schizophrenia patients compared to controls.
Finally, the antipsychotic drugs used for the treatment of schizophrenia inhibit the growth of
the parasite *in vivo* and *in vitro*. These evidences are discussed below.

1.12.1 Rat behavioural change due to *T. gondii* infection

A number of behaviours have been found to change during the chronic phase of *T. gondii*
infection. First, *T. gondii* infection blocks the innate fear of cats (Berdoy et al., 2000; Vyas et
al., 2007b; Webster, 2007) decreases neophobicity and enhances the willingness to approach
a novel object or odour in infected rodents (Webster, 1994). Furthermore, these changes were
found to be specific and not related to sickness behaviour (Vyas et al., 2007a). Moreover, the
spatial memory of the rats remained intact as their ability to compete for mates and social
status changes (Berdoy et al., 1995). In addition, the rodents did not lose their olfactory
sense; they only lost their fear towards a middle range of cat odour strength (Vyas et al.,
2007b). Finally, they did not lose their ability to learn aversion to unfamiliar food (Vyas et
al., 2007a). Rodent attraction to cat odours is not equal: infected rats had a stronger
preference for wild cat odour over domestic cats (Kaushik et al., 2014). Further evidence is
the fact that the intensity of some of the observed behavioural changes increased over time of
the infection (Flegr et al., 1996; Havlicek et al., 2001). Therefore, the observed behavioural
patterns cannot be side effects of the acute form of infection; otherwise, the intensity of the
behaviour change would decrease with the length of the time after the infection.

These observations suggest that the behavioural changes in the infected rodent are specific
and not related to loss of sensory perception or generic malaise. Another study (Evans et al.,
2014) showed that attenuation of predator odour aversion and changes in anxiety-related
behaviour are associated with *T. gondii* cyst presence in specific forebrain areas. Epigenetic
change in arginine vasopressin promoter DNA methylation in the medial amygdala of *T.
gondii*-infected male rats was also associated with rodent odour aversion. Furthermore, the
loss of fear in the infected animals can be inverted by systemic hypermethylation in the
1.12.2 Schizophrenia and seroprevalence of *T. gondii* in humans

It has been found in 40 studies that schizophrenia patients have a higher *T. gondii* seroprevalence than healthy controls (Torrey and Yolken, 2003; Torrey et al., 2007; Mortensen et al., 2007; Yolken and Torrey, 2008). Furthermore, the strong association between schizophrenia and the detection of *T. gondii* antibodies, summarised in terms of odds ratio (OR) is higher (OR = 2.73) than in any human gene in a genome-wide linkage analysis or environmental study (OR ≤ 1.40) (Purcell et al., 2009). and recent meta-analyses estimating potential associations between schizophrenia and different infectious agent found a prominent significant association with *T. gondii* (OR=2.70; CI 95%: 1.34–4.42; P=0.005) (Arias et al., 2012). It was also found that individuals with early life infection with *T. gondii* developed schizophrenia in later life; this includes both *in utero* exposure and early postnatal exposure (Torrey and Yolken, 2003).

Other evidence of the *T. gondii* effect on humans is the prolonged reaction times in infected individuals, measured by a test of simple reaction times (Havlíček et al., 2001) with psychomotor performance reduce with prolonged infection. The performance of the individuals in the 3-minute simple reaction time test indicates that toxoplasmosis decreases long-term concentration ability and not maximum performance. Interestingly, these performance changes were also associated with the host rhesus factor (RhD) genotype, while negative subjects have the lowest performance in the test, while the performance of RhD-positive heterozygotes was not changed by the infection (Novotna et al., 2008; Flegr et al., 2010). The decrease in psychomotor performance of infected individuals may explain the results of four retrospective studies (Flegr et al., 2002; Yereli et al., 2006; Kocazeybek et al., 2009; Alvarado-Esquivel et al., 2012) and one prospective study (Flegr et al., 2009) that found an increased risk of traffic accidents and work accidents observed in *T. gondii* seropositive subjects. In addition, the risk of traffic accident is increased in RhD-negative drivers compared to RhD-positive drivers (Flegr et al., 2009). Changes in personality profile and phenotype in humans with latent toxoplasmosis were also reported and have been reviewed elsewhere (Flegr, 2013).

1.12.3 Antipsychotic drugs

Antipsychotic drugs used for the treatment of schizophrenia inhibit the growth of the parasite in cell culture. The antipsychotic haloperidol and the mood stabiliser valproic acid most
effectively inhibit *T. gondii* growth *in vitro* (Jones-Brando et al., 2003). Furthermore, antipsychotic drugs prevent the development of behaviour alteration in infected rodents (Webster JP et al., 2006). Moreover, *T. gondii* antibody levels in treated individuals with a recent onset of schizophrenia are intermediate between the control groups and untreated patients, while patients undergoing current drug treatment showed a significant reduction in antibody levels (Leweke et al., 2004). Recently diagnosed bipolar disorder patients with positive serum antibodies against *T. gondii* presented more lifetime depressive episodes (P= 0.048) when treated using psychiatric drugs having no anti-Toxo activity, compared to patients having received drugs with anti-Toxo activity (Fond et al., 2015). These findings suggest that antipsychotic treatment may affect *T. gondii* infection levels, and this is another piece of evidence for the relation between behaviour change and *T. gondii* infection.

**1.13 Mechanisms of manipulation**

The exact mechanisms whereby *T. gondii* induces changes in the behaviour of the host are unknown, but there are some clues as to proximate and indirect mechanisms that might interact to induce such change. These are described below.

**1.13.1 The proximate effect**

The ideal mechanism for manipulating the host’s behaviour is changing neurotransmitter levels. Stibbs (Stibbs, 1985) was the first to study the level of neurotransmitters during *T. gondii* infection, finding that the level of norepinephrine showed a 28% decrease, while homovanillic acid (HVA) showed a 40% increase during acute infection. On the other hand, in chronic infection, dopamine showed a 14% rise above uninfected mice. Later, many studies showed that the dopamine level is modulated during *T. gondii* infection (Flegr et al., 2003).

Other evidence was found by measuring novelty seeking using the hole-board test. The principle of this test is to measure the length of time mice spend sniffing and head dipping at holes in order to test levels of novelty seeking. Infected mice recorded a longer compared to control, head dipping and sniffing. Furthermore, treating *Toxoplasma* infected BALB/c mice with (GBR 12909 1-(2-(bis (4-fluorophenyl) methoxy)-ethyl)-4-(3-phenylpropyl) piperazin),
a dopamine selective uptake inhibitor, significantly altered mice behaviour. This suggests a
direct relation between dopamine and *T. gondii* behavioural changes (Skallova et al., 2006).

A piece of evidence which suggests that *T. gondii* changes the host’s behaviour by dopamine
manipulation, and this manipulation is the effect antipsychotics have on the *T. gondii*
behavioural changes in rats. Haloperidol, which is a dopamine D2 antagonist, was found to
reduce the suicidal feline attraction and altered behaviour described in *T. gondii* infection.
The ability to normalise the behaviour of infected rats may involve controlling the dopamine
level (Webster JP et al., 2006).

All of this evidence, besides the relation between schizophrenia and dopamine known as the
“dopamine hypothesis” mentioned above and the similarity between *T. gondii*-infected rodent
behaviour and the hyperactivity caused by increased levels of dopamine in the mesolimbic
and nigrostriatal regions, has increased speculation that dopamine could be the key in *T.
gondii*-induced behavioural change. Furthermore, Gaskell et al. (2009) found that the
parasites have the ability to produce dopamine itself. Tyrosine hydroxylase was found in the
genome of the parasite. This enzyme represents the rate-limiting step in dopamine synthesis.
Tyrosine hydroxylase catalyses tyrosine to L-Dopa, which is then metabolised to dopamine
by the aromatic L-amino acid decarboxylase (AADC) in dopaminergic neurons, and
packaged into vesicles. Interestingly, *T. gondii* orthologues to the enzyme had the same
activity as the mammalian counterpart, and this was not found in any other Apicomplexan
parasite except *Neospora caninum*. Furthermore, two copies of this enzyme gene were found
in *T. gondii*. The genes were nearly identical in their sequence and kinetics, but different in
their expression time. One copy is expressed throughout the life cycle of the parasite, while
the other is up regulated during the bradyzoite stage. An explanation for this might be that the
enzyme is required to supply the parasite with tyrosine from phenylalanine during parasite
growth, while, during the bradyzoite stage, it is used in the production of L-Dopa. These
findings suggest that *T. gondii* might be involved in the synthesis of dopamine itself.
Furthermore, brain sections containing *T. gondii* cysts stain with commercial antibody
specific to dopamine showed accumulation of dopamine inside *T. gondii* cysts. Infection of
neural cells that produce, package and release catecholamine (PC12) cells led to increase in
the production and release of dopamine several fold. This result indicates that the large
amount of the produced dopamine is properly packaged for release from infected neurons. On
the one hand, tissue cysts *in vivo* contain a hundred fold more parasites than *in vitro* cysts,
which suggests that infected neurons might release several hundred times more dopamine
than uninfected dopaminergic neurons. This indicates that the physiological implication is massive, and, through this mechanism, *T. gondii* could discreetly alter dopamine levels from specific (i.e. infected) neurons (Prandovszky et al., 2011).

Recently Xiao et al. (Xiao et al., 2014) found that miRNA was up-regulated during *T. gondii* infection of neuroepithelioma cells (20h) (*in vitro*) and mice (5d) (*in vivo*). This miRNA is MiR-132, a cyclic AMP-responsive element binding (CREB)-regulated miRNA. *T. gondii* MiR-132 targets were identified by pathway enrichment analysis in the transcriptome of *T. gondii*-infected mice; the strongest affected pathways were 20 genes and dopamine receptor-signalling pathway. Furthermore, decreased expression of D1-like dopamine receptors (DRD1, DRD5), metabolizing enzyme (MaoA) and intracellular proteins associated with the transduction of dopamine-mediated signalling (DARPP-32 phosphorylation at Thr34 and Ser97) were detected by examination of striatum of *T. gondii*-infected mice.

Additionally, this study showed an increase in dopamine, serotonin (5-HT) and 5-hydroxyindoleacetic acid concentrations measured by HPLC analysis in five-day infected mice brains although the metabolism of dopamine was decreased while the 5-HT metabolism was unchanged.

In summary, these studies clearly identify dopamine with a key role in *T. gondii* cellular modification mechanisms and behaviour change. HPLC and immunohistochemistry had shown an increase in dopamine production *in vivo* and *in vitro*. Increase in dopamine production may be due to the expression of the rate-limiting enzyme in dopamine production tyrosine hydroxylase by the parasite, in addition to the parasite’s ability to manipulate the host’s dopamine metabolism.

Another mechanism that might be involved in the pathogenesis of the parasite is the localisation in the brain. Although gross pathology was found in the brain of immunocompromised individuals, this effect is rare in immunocompetent individuals, which shows that physical change is not the cause of the behavioural change. In addition, infected rats did not lose other brain functions; social status and mating success is still intact (Webster, 2007). The specificity of the behavioural change suggests that the effect of *T. gondii* might be due to the localisation in a certain region in the brain.

Cysts containing *T. gondii* can be found in different regions in the brain. However, such cysts have been found to be more frequent in limbic structures like the amygdala and nucleus accumbens (Vyas et al., 2007a; Gonzalez et al., 2007). The amygdala is important in the
expression of emotion, while nucleus accumbens is used in the processing of reward and pleasure (Gonzalez et al., 2007). This suggests that the presence of the *T. gondii* cyst in these brain areas may play a role in the pathogenesis of schizophrenia. However, the odour dose specificity and the attraction to the definitive host mentioned above cannot be explained by the presence of cysts in a certain region in the brain. Moreover, the small number of *T. gondii*-infected cells compared to the number of brain cells and the similarity of the behavioural change between different studies necessitates a specific localisation in the brain. All of these observations indicate that direct damage of the brain structure alone might not be the main cause of the behavioural change.

1.13.2 The indirect effect

*T. gondii* might manipulate the host’s behaviour indirectly through the involvement of the immune system. The immune system could change neurotransmitter concentrations, e.g. by changing the concentration of kynurenines. Kynurenines are tryptophan metabolites that are naturally found in the brain. Tryptophan is an essential substrate in the synthesis of serotonin. It is metabolised by two enzymes: tryptophan hydroxylase (TPH) and amino acid decarboxylase (AADC). The TPH-mediated reaction is the rate-limiting step in serotonin pathway (Walther et al., 2003).

Tryptophan starvation is one of the mechanisms that are induced by IFN-γ to control infections. IFN-γ activates indoleamine 2, 3 dioxygenase (IDO), which metabolises tryptophan through the kynurenine pathway (Pfefferkorn, Eckel, and Rebhun, 1986). IDO is present in macrophages, dendritic cells and all body organs (Wirleitner et al., 2003). It metabolises tryptophan to kynurenine, which is then degraded through catabolic pathways to either 3-hydroxykynurenine (3-HK) and quinolinic acid (QA) or kynurenic acid (KYNA). In the brain, microglia preferentially produce QA, whereas astrocytes produce KYNA (Figure 1-2)(Schwarcz, 2004).

The neuropsychiatric implications of IDO are not only related to with serotonin depletion, but also the metabolic products of tryptophan degradation. Kynurenic acid is an N-methyl-D-aspartate receptor (NMDA) and α-7 nicotinic acetylcholine (α7nACh) receptor antagonist (Schwarz and Pellecchiari, 2002). Actually, KYNA acts as a competitive blocker of the glycine coagonist site of the NMDA receptor. However, both receptors are involved in physiological processes underlying learning, memory and other manifestations of synaptic
plasticity. On the other hand, QA is an NMDA receptor agonist. However, 3-HK is not expected to participate in the kynurenine effect, because it does not show any direct interaction with glutamatergic or dopaminergic processes (Olsson et al., 2009).

Furthermore, kynurenine 3-monooxygenase (KMO), which converts L-kynurenine to 3-HK, was found to have a lower expression level and enzymatic activity in schizophrenic patients (Sathyasaikumar et al., 2009). However, this branch of the metabolic pathway is localised in microglial cells and does not interact with KYNA production under normal conditions. The effect of the up- or down-regulation of the microglial branch pathway is still under investigation, especially during the upstream activation of L-kynurenine production. On the other hand KYNA and kynurenine (KYN) have been reported to be higher in the cerebrospinal fluid (CSF) (Erhardt et al., 2001) and central nervous system (CNS) regions of schizophrenics as compared with controls, reported an evaluated level of KYNA in the post-mortem prefrontal cortex. These increases were probably unrelated to treatment with antipsychotic medications. Evidence from studies in rats suggests that treatment with antipsychotic drugs reduces endogenous concentrations of KYNA, while KYN levels are unaffected. In addition, the level of L-kynurenine, KYNA’s immediate bio-precursor, was found to increase with an increase in KYNA (Vecsei et al., 1992). Furthermore, KYNA was found to decrease dopamine level, suggesting that astrocyte-derived KYNA may exert functionally significant local control over dopaminergic activity (Wu et al., 2007).

In addition, KYNA was found to interfere with dopaminergic neurotransmissions as Nano molar concentration of KYNA were capable of significantly reducing dopamine level in vitro (Rassoulpour et al., 2005). Furthermore, Amori et al. (2009) found that the inhibition of kynurenine synthesis increases the extracellular level of dopamine. This means that the relationship between KYNA and dopamine is bidirectional. KYNA levels in the prefrontal cortex (PFC) caused a decrease in the extracellular levels of two other neurotransmitters known for their effect on cognitive functions, i.e., glutamate and acetylcholine. This effect is also bidirectional, which means that a decrease in the KYNA level causes an increase in the neurotransmitter level, as studies on rats have shown (Zmarowski et al., 2009; Wu et al., 2010). In schizophrenia, a blunted type-1 immune response and activated type-2 immune response both were linked to the imbalanced activation of astrocyte and in the tryptophan-kynurenine metabolism, resulting in increased production of KYNA in schizophrenia (Miller et al., 2006).
As mentioned above, astrocytes are the cell type that produces KYNA in the brain. Astrocytes are able to produce KYNA due to the presence of kynurenine aminotransferases (KATs) that convert L-kynurenine to KYNA. The produced KYNA is released rapidly from the astrocytes to the extracellular milieu, where it has access to neuronal α7nAChRs and NMDRs. Furthermore, KYNA cannot be degraded enzymatically or removed by reuptake from the brain. It only can be removed slowly by nonspecific acid transporters. KYNA synthesis in astrocytes is driven by the availability of substrate (L-kynurenine) and co-substrate (2-oxoacids), and further regulated by cellular energy status (Schwarcz and Pellicciari, 2002). All of this information suggests that astrocytes are the main producer of KYNA, and that they control the extracellular concentration of KYNA, thereby influencing cognitive functions. During *T. gondii* infection, the astrocytes are activated as one of the effector cells to control the infection (Oberdorfer et al., 2003). In addition, the IDO mRNA expression level in the brain of *T. gondii*-infected rats is higher than the normal level, with increased KYNA and a decreased tryptophan level (Silva et al., 2002).

This evidence suggests that *T. gondii* might manipulate the host behaviour via the immune system. *T. gondii* infection activates the astrocytes, which induces IDO and leads to the production and release of kynurenine. Kynurenine will block the NMDR and α-7 nicotinic acetylcholine receptors, leading to the decrease of dopamine in the brain in individuals who are genetically susceptible to developing schizophrenia i.e., the increase expression of the tryptophan dioxygenase (TDO).

KYNA production starts with the opening of the oxidative ring of tryptophan by IDO and/or TDO. However, the mRNA for tryptophan 2,3-dioxygenase (TDO2) is elevated in the brain of schizophrenic patients; furthermore, a concomitant increased density of TDO2-immunopositive astroglial cells is seen in the patients’ white matter (Miller CL et al., 2004). This evidence suggests that TDO2 is involved in the pathophysiology of schizophrenia. It has actually been speculated that TDO2 is the enzyme responsible for the pathophysiology of the schizophrenia rather than IDO. The evidence for this hypothesis is that in schizophrenia, type-2 immune response is activated and the IDO is inhibited by the type 2 cytokines (Miller et al., 2006). In addition, the brain’s IDO expression level appears normal in schizophrenia patients (Miller CL et al., 2004).

These results suggest the following hypothetical sequence: *T. gondii* activates the astrocytes, which leads to an increase in the formation of KYNA in the brain. This effect is augmented in individuals with elevated brain TDO activity and reduced KMO levels and activity, i.e.,
individuals with a genetic predisposition for schizophrenia. In addition, increased brain KYNA levels contribute to the excessive reduction in glutamatergic and nicotinergic neurotransmitters, which is believed to play an important role in the pathogenesis of schizophrenia.
1.14 Objectives

This thesis includes a number of studies that investigate *T. gondii*’s proximate and indirect mechanism(s) that may cause behaviour changes in the host. In terms of the proximate mechanism, the transcriptome of nerve growth factor NGF-activated *T. gondii*-infected PC 12 cells...
cells was studied. These cells were infected with induced bradyzoite-stage parasites to mimic chronic infection conditions. Bioinformatics analysis were performed for the host and the parasite transcriptome to identify the changes induced by the infection on both the parasite and the host. This also allowed me to identify the infection effect on genes involved in dopamine metabolism. Moreover, whether the parasite produces the dopamine itself or activates the PC-12 cells to produce it.

In terms of the indirect mechanism, the effect of tryptophan metabolites on *T. gondii* was studied. *T. gondii* was cultured in tryptophan-free media in the presence of different concentrations of three tryptophan metabolites: KYNA, kynurenine and QUIN. Then, the dopamine levels were measured by high-performance liquid chromatography (HLPC) also the tyrosine hydroxylase expression levels were measured. The aim of this study was to determine whether these metabolites affect dopamine production during *T. gondii* infection.

Besides the above, the effect of tryptophan starvation on *T. gondii* differentiation was also investigated by using histoimmunochemistry and mRNA expression levels.
Chapter Two

2 Effect of Tryptophan Starvation on Host and *Toxoplasma gondii*
2.1 Abstract

*T. gondii* infection induces IFN-γ secretion by the immune system and one of IFN-γ effector mechanisms is tryptophan (TRP) degradation; this effect has been implicated as controlling the tissue cyst (bradyzoite stage) in the host. Therefore, the effect of TRP starvation on host and parasite was analysed *in vitro*. Initial results found that human fibroblasts and myotubes survive in TRP-free media, while neural PC12 cells do not survive. *T. gondii* survived in TRP-free media. Indeed, the absence of TRP induced *T. gondii* differentiation to bradyzoites as monitored by immunostaining and detection of mRNA expression of stage-specific markers for vegetative tachyzoite and bradyzoite stages. *T. gondii* TRP starvation was found to induce bradyzoite differentiation and cystogenesis, but not autophagy of *T. gondii* or host mitochondria as seen during starvation of multiple amino acids as monitored by assessing the membrane potential of the *T. gondii* mitochondria under TRP starvation. Therefore, TRP starvation as an immune effector controls the parasite growth, but does not have a microbicidal effect on *T. gondii*. Suggesting, TRP-free media may also be used as a method for culturing bradyzoites *in vitro*. 
2.2 Introduction

During *T. gondii* infection, the host immune response induces interferon gamma INF-γ, a cytokine involved in innate and adoptive immune response (Green et al., 1969). INF-γ plays a major role in the immune response and induces immune cells (dendritic cells, NK cells) and effectors (Weiss and Kim, 2007). These anti-*T. gondii* effector mechanisms include nitric oxide (NO) production, TRP starvation, and generation of reactive oxygen species, iron deprivation, and finally induction of the p47 GTPases, including IGTP, IRG-47, and LRG-47.

2.2.1 Tryptophan starvation

TRP starvation as an IFN-γ effector mechanism to inhibit *T. gondii* growth was first described by Pfefferkorn (Pfefferkorn, 1984) and treatment of human fibroblast with IFN-γ 24 hours prior to infection were found to inhibit *T. gondii* growth significantly. The mechanism of the inhibition is due to the induction of indoleamine 2, 3-dioxygenase (IDO). IDO is an enzyme that catalyses the initial, rate-limiting step of TRP conversion to N-formylkynurenine and kynurenine (Pfefferkorn and Guyre, 1984; Pfefferkorn et al., 1986). TRP starvation leads to *T. gondii* growth inhibition, as TRP is an essential amino acid and required for parasite growth. Furthermore, adding excess TRP to cultures treated with IFN-γ, abrogated the IFN-γ toxoplasmastatic effect. TRP starvation mediated by IDO has been described in a variety of host cell species including humans, rats and mice. And different cell lines including epithelial cells, endothelial cells, and tumour cell lines (Dimier et al., 1992; Nagineni et al., 1996; Daubener and MacKenzie, 1999). TRP degradation as a toxoplasmastatic effect was detected in pigmented epithelial cells and microvascular endothelial cells in vivo. This pathway has also been found in the central nervous system immune response to infection with researchers (Gazzinelli et al., 1993; Silva et al., 2002) finding decreased levels of TRP and increased of IDO expression in the lungs and brains of infected mice. Different human cells express IDO when stimulated by IFN-γ; and IL-4 and IL-13 regulate IDO expression (Chaves et al., 2001).

2.2.2 Tryptophan starvation effect on brain

The question remains: how does TRP starvation affect brain functions, especially in the chronic stage of the infection? Several studies have been carried out on TRP depletion. TRP
enters the brain by active transport for the synthesis of serotonin. Acute TRP depletion (ATD) leads to serotonin reduction and consequently mood lowering (Moore et al., 2001) but a large decline in TRP is needed to see this effect on serotonin. TRP depletion has not been observed to affect dopamine levels also, acute phenylalanine/tyrosine depletion does not modulate DA levels (Young, 2013).

In addition, patients who receive immunotherapy have low TRP plasma levels; which was correlated with patient depression scores (Capuron et al., 2002). The fall in plasma TRP level could be due to the activation of enzymes involved in TRP catabolism (Wirleitner et al., 2003). Despite the TRP level, IDO activation might affect brain functions through the increase in the production of kynurenic pathway products (KYNA, QUIN) (for a detailed discussion, see the next chapter). These findings indicate that TRP starvation during the chronic stage of infection might influence the functions of the brain.

2.2.3 Tryptophan starvation effect on *T. gondii*

TRP starvation controls *T. gondii* growth and might induce differentiation via induction of a stress response (similar to that with altered pH or serum starvation). Indeed, this is important especially when we consider that TRP is an essential amino acid and *T. gondii* lacks the enzymes to make TRP (Pfefferkorn, 1984). Likewise, arginine depletion during *T. gondii* infection has been found to induce differentiation of tachyzoites to bradyzoite-containing cysts *in vitro* due to natural arginine auxotrophy, although arginine is a non-essential amino acid (Fox et al., 2004).

Although differentiation is a programmed response, bradyzoite differentiation is stress induced. Stress conditions were used to induce differentiation of *T. gondii* to bradyzoites *in vitro*, these conditions result in more bradyzoites under such conditions than simple inhibition of tachyzoite replication. Conditions that induce bradyzoite formation within host cells are temperature stress (43°C; (Soete et al., 1994)), pH stress (pH 6.6–6.8 or 8.0–8.2 (Soete et al., 1994); chemical stress (Na arsenite (Soete et al., 1994)) and arginine starvation (Fox et al., 2004).

However, these systems result in heterogeneity in bradyzoite differentiation in *vitro*; the presence of tachyzoites in the cultures will lead to complications in analysis of bradyzoite populations, and the tachyzoite overgrown bradyzoite culture, though using more extreme differentiation conditions, may be toxic and therefore detrimental to long-term culture
maintenance. In addition, other studies (Fouts and Boothroyd, 2007) looked at the difference between bradyzoite and tachyzoite by using alkaline induction of bradyzoite, and different experiment controls included the uninfected cells with or without alkaline media. Comparing controls shows that alkaline media itself induced numerous transcriptional changes in host cells. Therefore, the hypothesised TRP effect on bradyzoite differentiation was investigated, as was the suitability of TRP starvation as a bradyzoite induction system that may provide longer culturing period without being toxic to the parasite.

2.3 Aims

TRP starvation is a phenomenon that occurs during *T. gondii* infection due to the immune response. However, the direct effect of this starvation on *T. gondii* is poorly characterised. The effect of TRP starvation on host cell and parasite growth and development were investigated to find an optimum condition to induce bradyzoite differentiation and model-exclusive bradyzoite cultures. This could provide an *in vitro* system for the analysis of neurochemical effects of bradyzoite infection.
2.4 Experimental design

2.4.1 Growth of the parasite and cell culture

Low passage number HFF (human foreskin fibroblast) cells (ECACC) were maintained in Dulbecco's Modified Eagle's medium (DMEM) (Invitrogen Paisley, UK) supplemented with 10% Foetal bovine serum (FBS) (Invitrogen) and 100 units/ml penicillin/streptomycin (Sigma, Poole, UK). This cell line was grown as monolayers at 37°C in an atmosphere of 5% CO2. HFF cells were passaged every four to five days. For HFF passaging, cells were washed in pre-warmed phosphate buffer saline (PBS) and then 0.5 ml trypsin was added, and, after 90 seconds, the cells were forced to detach. Afterwards, 30 ml of fresh DMEM was added and then the newly passaged cells were moved to three new flasks.

Rat pheochromocytoma (PC12 cells) cells from ECACC were maintained in Roswell Park Memorial Institute medium RPMI (Invitrogen Paisley, UK) supplemented with 10% horse serum (Invitrogen), 5% FBS (Invitrogen) and 100 units/ml penicillin/streptomycin (Sigma, Poole, UK). PC12 was passaged by pipetting up and down to break the cell clusters, then centrifuged at 800 rpm for 10 min and re-suspended in 30 ml of new fresh RPMI media and then removed to three new flasks and incubated at 37°C in an atmosphere of 5% CO2.

The parasite was grown by infecting the HFF cell monolayer. The parasite was passaged by first trypsinization of the HFF cells, the cells were then centrifuged at 2500 rpm for 10 minutes, after that the media was discarded and the pallet was re-suspended in PBS and transferred through a 27-gauge needle to release the intracellular parasite. Then the suspension was centrifuged at 2500 rpm for ten minutes; finally, the parasite pellet was re-suspended in fresh media and used to infect a 75-95% confluent HFF.

Human myotubes were extracted, cultured and differentiated by Dr Patrick Booms, Leeds Institute of Molecular Medicine, UK Malignant Hyperthermia, Investigation Unit Level 8, Clinical Sciences Building St. James's University Hospital lab and were a kind gift.

2.4.2 Induction of bradyzoite differentiation

For the induction of parasite differentiation to bradyzoites, free, released tachyzoites were re-suspended in alkaline DMEM (pH 8.2) and incubated in 5% CO2 for 18 hours. Then the alkaline media was removed by centrifugation at 2500 rpm for 10 minutes and the parasites were re-suspended in fresh DMEM or RPMI depending on the type of the infected cells.
2.4.3 Poly-D-lysine coating

500 µL of 0.1 mg/ml Poly-D-lysine (P6407 Sigma) solution in PBS was used to coat cover slips in 24 well plates. Then the plates were incubated at 37°C for two hours. After this, the wells were rinsed twice with PBS.

2.4.4 Preparing Tryptophan-free DMEM

2.4.4.1 Dialysed serum

To prepare the dialysed serum, a strip of Bio Design Dialysis Tubing was used. The tube was prepared by soaking it in distilled water (H₂O) for one hour, and then for three hours to get rid of the glycerol. To remove heavy metals, the tube was soaked in EDTA for one hour and then a further three hours. Finally, the tube was placed in a beaker with distilled H₂O and autoclaved before use.

Fifty ml of FBS was dialyzed by placing the FBS in the tube tied by a knot, and the FBS-filled tube was placed in an HHBS x 10 filled autoclaved beakers. The HHBS was changed after one hour, three hours and overnight.

2.4.4.2 TRP-free media

Five hundred ml of DMEM-TRP medium was prepared by mixing 50ml prepared HBSS (10X), 5ml MEM vitamin (100X)(Invitrogen), 5 ml MEM Non-Essential Amino Acid Solution 10 mM (100X)(Invitrogen), 10 ml prepared amino acid mix (50X), 282 mg solid L-Glutamine 282 mg, and 1.75g glucose. The pH was adjusted to 7.2 with 10% NaHCO₃, and then sterilized dH₂O was added to a total volume of 500 ml. The prepared DMEM medium was then filtered, sterilized and supplemented with 50 ml dialysed FBS and 1% Penicillin-Streptomycin antibiotic and stored at 4°C. HBSS (10X) stock solution was created by dissolving the following inorganic salts in 500ml sterilized ddH₂O:

1.32 g CaCl₂.2H₂O, 0.5 ml Fe (NO₃)₃.H₂O stock solution (Fe (NO₃). 3H₂O was prepared by dissolving 0.05 g Fe (NO₃)₃.H₂O in 500 ml sterilized dH₂O as a stock solution), 2 g KCl, 1g MgSO₄.7H₂O, 32 g NaCl, 18.5 g NaHCO₃, 0.7 g NaH₂PO₄.2H₂O. In addition to adding other compounds such as 22.5 g of D-Glucose and 0.075 g Phenol Red, the mix stock was stored at 4°C.

100X amino acid mix stock solution were prepared by dissolving the following amino acids in 100ml sterilized dH₂O:
420 mg L-Arginine, HCl, 240 mg L-Cystine, 29 g L-Glutamine, 150 mg Glycine, 210 mg L-Histidine HCl, 525 mg L-Isoleucine, 522 mg L-Leucine, 730 mg L-Lysine, HCl 150 mg L-Methionine, 330 mg Phenylalanine, 210 mg L-Serine, 475 mg L-Threonine, 360 mg L-Tyrosine, 470 mg L-Valine and (with and without) 80 mg L-tryptophan. It was then filtered, sterilized and stored at 4ºC. All chemicals were analytical grade and provided from (Sigma-Aldrich).

2.4.5 Induction of tryptophan starvation

A confluent HFF cell monolayer in T25 flask or cover slip, PC12 cells T25 flask or differentiated myotubes in T25 flask were cultured with TRP-free media (TM) or normal homemade DMEM. Before adding the TM to the cultured cells, cells were washed twice with 1XPBS each time for 20 minutes. The media was changed every three days.

If the cells were infected, the parasites were also washed before the infection twice with 1XPBS for 20 minutes. 5X10^5 parasites were used to infect cells cultured in T25 flask and 5X10^4 were used to infect cells cultured on cover slip.

2.4.6 Impact of starvation on cell viability

The viability of a confluent HFF cell monolayer cultured in 96 well plates, the medium of these cells was changed to TRP reduced media (10%, 1%, 0.1% TRP), TRP-free media and DMEM with TRP. At week one, two and three after induction of TRP, a starvation effect on viability of the cells was detected by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) viability assay (CT02 Millipore). The assay reflects the viable cells number by measuring NAD (P) H-dependent cellular oxidoreductase enzymes ability to reduce MTT dye. A 10 µl of 5mg/ml MTT solution was added to each well of 96 well plates cultured with HFF cells in 100 µl media. After four hours of incubation in a CO₂ incubator, the medium was removed via a 21 g needle and a 10 ml syringe, and 100 µl of DMSO was added to dissolve the formazan precipitate. After 10 minutes on the shaker, the result was obtained by reading the absorbance by a spectrophotometer at 670 nm and 570 nm. The result was the subtraction of the 670 nm reading from the 570 nm.

This assay was also performed on PC12 cells. The first assay was performed on cells cultured in RPMI, homemade DMEM and TRP-free media and a second on PC12 cells cultured in TRP-free DMEM with and without a gradual amount of added TRP.
2.4.7 Immunofluorescence analysis and image processing

Confluent HFF cells growing on sterile 12 mm glass cover slips in 24-well plates were infected with freshly harvested parasite, and starvation was induced, as mentioned above. At various time points noted in the figure legends, the cells were fixed and permeabilized by 4% paraformaldehyde, and 0.5% 10x Triton in PBS solution was then blocked with streptavidin biotin-blocking kit Streptavidin/Biotin Blocking Kit (SP-2002)(vector lab) USA. The cells were stained by incubating overnight at 4 C° with rabbit anti-BAG1 antibodies 1:1000. After that, it was incubated with anti-rabbit biotin1:500(BA-1000) vector lab USA, then streptavidin TRITC conjugated 1:200 (SA-5006) vector labs USA for 30 minutes each at RT. Finally, the slides were washed-stained with Hoestch stain and mounted by vectashield. Images were acquired by using a Zeiss confocal Fluorescence Microscope with a 40X, 63X oil immersion objective. ImageJ software was used to merge images that were generated by pseudo-colourings. In all images and experiments, uniform adjustments for brightness and were used.
2.4.8 RNA extraction

The cells were scraped by using a small scraper to detach the cells from the flask surface. Then the media and cell mixture was collected and moved to a conical tube and centrifuged for 45000 g for 10 minutes. Afterwards, the media was discarded and the cells were re-suspended with leftover media. Then the RNA extraction was performed following the manufacturer’s instruction. Briefly, the cells were lysed by 350 µl adding Buffer RLT, homogenated by pipetting several times, and then 350 µl of 70% ethanol was added and the mixture was left in RT for five minutes. After that, the mixture was transformed to a spin column; the column was placed in 2 ml collection tubes and centrifuged at 15s at 8000 x g (10,000 rpm) and the flow-through was discarded. After this, the column was washed several times with 700 µl of RW1 buffer and 500 µl RPE buffer (twice); during the washing step the column and collection tube ware centrifuged at 15s at 8000 x g (10,000 rpm) and the flow-through was discarded. Lastly, the column was placed in 1.5 Eppendorf tube, and 20 µl of Diethyl Pyrocarbonate (DEPC) treated H2O was added then the column and the collection tube were centrifuged for 1 min at 8000 x g (10,000 rpm). RNA quantity was examined using Nano Drop 2000.

2.4.9 cDNA synthesis

First, a single strand of cDNA was constructed by adding 100 ng of mRNA and 8.3 µl of DEPC H2O. Then, 1 µl random hexamers (50 ng/µl) (Promega) and 1 µl dNTPs (0.5 µg/µl) (Promega) were added and incubated at 65°C for five minutes. In the second step, a 4 µl reaction buffer, 2 µl 0.1 M DTT and 1ul RNAsin (Promega) were added and incubated at 25°C for two minutes. Finally, 1µl superscript of RT enzyme (Invitrogen) was added and incubated at 25°C for 10minutes, 42°C for 50 minutes and 70°C for 15 minutes.

2.4.10 RT-PCR

Each sample contained 50ng cDNA, 1 µl Forward primer(100 ng/µL), 1 µl Reverse primer (100 ng/µl), 1 µl dNTPs (0.5 µg/µl)(Promega), 4 µl Green Buffer, 0.2 µl GoTaq polymerase (Promega) and DEPC-H2O in a final volume of reaction 20 µl as described by the manufacturer. These were mixed together, incubated for two minutes at 95°C and 35 cycles of one minute at 95°C, one minute at 55°C and one minute at 72°C. The final step was five minutes at 72°C with H2O included instead of cDNA as a negative control. After this, the product was run in 2% agarose gel with 1µl of ethidium bromide for 20 minutes at 70 volts.
2.4.11 Detection of stage specific genes by quantitative PCR

For each sample, a 12.5 µl SYBR® reen PCR master mix (4309155 Applied Biosystems) was added and a 0.5 forward primer (100 ng/µL), a 0.5µl reverse primer (100 ng/µL) with each initial concentration at 20µM then 11µl of DEPC treated H2O and 5ng of the template. This was then incubated at 95°C for 10 minutes and followed by 45 cycles of 95°C for 15 seconds, 55°C for 15 seconds, and 60°C for 45 seconds. It was finally followed by a melt curve.

The primers that were used are *T. gondii* Glyceraldehyde 3-phosphate dehydrogenase(GAPDH), surface antigen 1(P30) (SAG1) as tachyzoite marker (Cheng Xiong et al., 1993) and surface antigen 4 (SAG4) as bradyzoite marker (Odberg-Ferragut et al., 1996). Values are expressed using the delta-delta Ct method to derive relative fold change, ΔΔ Ct= ΔCt (SAG sample-GAPDH sample)- ΔCt (SAG control-GAPDH control).

| *T. gondii* GAPDH | Forward | GTA TTG GCC GTC TGG TGT TC |
| | Reverse | CGT GGA CCG AGT CGT ATC TC |
| SAG1 | Forward | GAC GAC GCA CAG AGT TGT AT |
| | Reverse | ACA ACT TGA CAG GAC CAA GAG |
| SAG4 | Forward | TGG ACC TAC GAT TTC AAG AAG GC |
| | Reverse | GCT GCG AGC TCG ACG GGC TCA TC |

2.4.12 Mitochondrial membrane potential assay

For mitochondria membrane potential detection, Mitotracker ® Red CM – H2X Ros (Molecular Probes; Invitrogen detection technologies, Carlsbad CA) dye was used as previously described (Sinai et al., 1997). Infected cells were fixed and permeabilized, as mentioned before. After this, cover slips were incubated with 1:500 diluted dyes for 15-30. A Hoechst stain (33342, Thermo Fisher) was used to detect the host and parasite nucleic acid.
2.5 Results

2.5.1 Impact of starvation on cells

In order to investigate the effect of tryptophan on HFF cells survival, the HFF cells were cultured in normal DMEM, different concentrations of TRP and TRP-free media, and the viability was measured by MTT assay after weeks 1, 2 and 3. A reduction in the viability of HFF cells that were cultured in TRP-free DMEM was showed in (Figure 2-1), and this reduction did not change after two and three weeks. Moreover, there is no difference between the reduced and the TRP-free media. Hence, HFF cells were used to culture *T. gondii* in TRP-free DMEM.

![Figure 2-1: The effects of DMEM, TRP-reduced DMEM, and TRP-free DMEM on viability of HFF cells. TRP values expressed as percentage of normal medium.](image_url)

The viability of PC12 cells in RPMI, DMEM and TRP-free DMEM was measured. PC12 cells were cultured with these media and, after three days, the viability of PC12 cells in each culture was measured by MTT assay. The spectrophotometer readings were converted to percentages, comparing to the reading of the cells cultured in RPMI media. TRP-free media is not appropriate for PC12 cell culturing (Figure 2-2). The RPMI is slightly better for
viability of PC12 cells than DMEM, although the difference between the two media is not significant.

Figure 2-2: Viability of PC12 cells in RPMI, DMEM and TRP-free DMEM. The
The optimum concentration of TRP for PC12 cells growth was investigated by growing the cells in seven different TRP concentrations. After three days the viability of these PC12 cells cultures was measured by the MTT assay, with the spectrophotometer readings then presented as a percentage of the cells cultured in 100% TRP media. Direct relationship between the TRP concentration and PC12 cell growth was showed in (figure 2-3). This test clearly shows the necessity of TRP for PC12 growth. Therefore, PC12 cells do not present a good model for investigating TRP-free media effect on *T. gondii* ability to increase dopamine production.

**Figure 2-3:** Viability of PC12 cells in different concentrations of TRP in DMEM media. The
2.5.2 Impact of TRP starvation on *T. gondii*

The impact of TRP starvation on *T. gondii* structure was investigated by immunofluorescent staining with anti-BAG1 antibody, BAG1 is a bradyzoite specific protein. Immunofluorescent staining was used to study *T. gondii* cultured in HFF cells in TRP-free DMEM on cover slips. The staining of *T. gondii* bradyzoite with BAG1 shows intact, healthy bradyzoites in TRP-free media (Figure 2-4). The restricted media did not deform the parasite and TRP starvation induced the differentiation of *T. gondii* from tachyzoite to bradyzoite.
Moreover, the effect of TRP-free media on *T. gondii* was further investigated by detecting mRNA expression levels of stage-specific genes. Induced *T. gondii* Prugniaud tachyzoites were used to infect HFF cells. Normal DMEM was used in some cultures, while TRP-free DMEM were used in other cultures. Gene expression of differentiation marker SAG1 and
SAG4 were detected on day three for *T. gondii* cultured in normal media and TRP-free media; these markers were detected at day six for *T. gondii* cultured in TRP-free media. The gene expression of mRNA-encoding SAG1 (tachyzoite marker) (Figure 2-5) had a positive result in normal media only while gene expression of mRNA-encoding SAG4 (bradyzoite marker) had positive results in TRP-free media at both day three and day six. These results confirm the conversion of the parasite from tachyzoite to bradyzoite in TRP free media.

![Figure 2-5: Photograph of an agarose gel (2%) showing RT-PCR products with SAG1 and SAG4 specific primers for T. gondii grown in HFF cells for different times.](image)

The previous experiment was repeated using qPCR to confirm the previous results, (Figure 2-6) shows a fold change decreases in SAG1 expression and fold change increase in SAG4 expression, confirming the ability of TRP free media to induce the bradyzoite differentiation in HFF cells.
Figure 2-6: Quantitative PCR showing stage-specific mRNA expression of SAG4 and SAG1 in parasites cultured in TRP free media.

Myotubes were cultured and infected with *T. gondii* in TRP-free media and bradyzoite conversion and viability was monitored by measuring mRNA expression levels of stage specific markers (SAG1 and SAG4). The expression level of SAG1 and SAG4 in myotubes in the absence of TRP (Figure 2-7). Culturing the *T. gondii* in myotubes and TRP-free media increased the expression of SAG4 and absence of the expression of SAG1, thus indicating differentiation of *T. gondii*.
To further confirm the previous result, qPCR was used to detect mRNA levels of *T. gondii* cultured in myotubes and TRP free media. Gene expression of mRNA-encoding SAG1 during infection was down-regulated (Figure 2-8), while gene expression of mRNA-encoding SAG4 was up-regulated in TRP-free media in days three, six and nine. The gene expression of mRNA encoding the differentiation markers illustrate that TRP-free DMEM and myotubes induce *T. gondii* differentiation from tachyzoite to bradyzoite.
Figure 2-8: Bradyzoite conversion in myotubes in TRP-free media. Quantitative RT-PCR
2.5.3 Mitochondrial membrane potential

The possibility that the absence of TRP induces autophagy was investigated. Previous studies found that the absence of amino acids causes starvation of the parasite and that this starvation causes autophagy and mitochondrial depolarization which was evident in *T. gondii* (Ghosh D et al., 2012), similar to yeast and mammalian systems upon nutrient starvation (Rodriguez-Enriquez et al., 2006; Zhang et al., 2007; Rodriguez-Enriquez et al., 2009; K and Kondo-Okamoto, 2011).

Mitotracker, a membrane potential-sensitive dye, was used to dye intracellular *T. gondii* after six days of infection in TRP-free DMEM; (Figure 2-9) shows that using TRP-free media for culturing did not affect the membrane potential. In addition, no difference is observable between the TRP-free media and the normal media, which shows that absence of TRP does not have a starvation effect on *T. gondii* and the absence of TRP specifically induces differentiation and not starvation of the parasite.
a
Normal DMEM

b
TRP-free DMEM

b
TRP-free DMEM

b
TRP-free DMEM

b
TRP-free DMEM

Day 3

Bradyzoites

Bradyzoites

Single infected cell

Day 6
Figure 2-9: The effect of TRP-free media on the induction of autophagy. Mitochondrial potential was measured as a marker of autophagy using MitoTracker.
2.6 Discussion

TRP is an essential amino acid; TRP starvation is one of the methods that the immune system uses to control *T. gondii* infection in the brain and peripherally (Pfefferkorn, 1984; Pfefferkorn et al., 1986). The effect of TRP starvation was examined on the host cells with extremely different results. Three different types of cells were used: HFF, PC12 and myotubes. First, the effect of TRP starvation was examined on HFF cells (Figure 2-1), HFF cell viability was reduced in TRP-free media, but the reduction was within an acceptable range. In particular, the examined HFF cells were found to be confluent and contact inhibited, and excess TRP was not needed for cell survival.

However, the investigation of TRP absence on the PC12 cells shows that the cells were able to grow in homemade DMEM but not in TRP-free DMEM (Figure 2-2). and adding TRP to TRP-free media recovers the TRP-free media effect and increases the viability of these cells, indicating that the reduction of the PC12 cell growth is a direct effect on the of TRP starvation(Figure 2-3). Therefore, PC12 cells cannot be used for studying *T. gondii* without TRP. Finally, myotubes were found to differentiate and survive in TRP-free DMEM. Moreover, TRP-free media was found useful for survival and differentiation of myotubes (result not shown as the experiment was carried out by P. Booms Laboratory, Leeds Institute of Molecular Medicine).

To investigate the effect of the absences of TRP on *T. gondii*, *T. gondii*-infected HFF were cultured in TRP-free media. Interestingly, absence of TRP was found to induce the differentiation of *T. gondii* to the bradyzoite stage; moreover, the majority of *T. gondii* cultured in TRP-free media was at the bradyzoite stage with the tachyzoite stage in a minority.

The figures (Figure 2-4) of the *T. gondii*-infected HFF stained with anti-BAG1(Figure 2-4), a bradyzoite-specific antigen (Bohne et al., 1995; Parmley et al., 1995), show a parasite stained with BAG1 at different time points with cysts and single parasites stained with BAG1, indicating that *T. gondii* parasite differentiated to bradyzoites, mostly formed cyst-like structures. Indicating that TRP-free media induces tachyzoite differentiation to bradyzoite, and cyst formation and maturation efficiently.

Furthermore, to confirm the differentiation of tachyzoite to bradyzoite in the absence of TRP, RT-PCR was performed with stage-specific primer SAG1 (tachyzoite) and SAG4
(bradyzoite). The first conventional RT-PCR showed that both SAG1 is expressed in *T. gondii* cultured in normal DMEM at day three, while SAG4 expression was also not detected (Figure 2-5). On the other hand, *T. gondii* cultured in TRP-free media showed an increase in SAG4 expression and diminished in SAG1 expression at day three. Comparing days three to day six in terms of *T. gondii* cultured in TRP-free media, similar results were seen, indicating that the TRP media does not eradicate the parasite and suitable for the parasite survive and differentiate.

Furthermore, quantitative PCR confirms these results. The expression levels for normal media at day three (Figure 2-6) showed that SAG1 is expressed more than SAG4 and that most of the parasites are tachyzoites. On the contrary, SAG4 expression is much higher than SAG1 for the parasites that were cultured in TRP-free media, confirming the dominant TRP-free media effect on *T. gondii* differentiation. However, the day six results showed an increase in the SAG4/SAG1 ratio (result not shown) indicating that the parasite continued to differentiate. Concluding, PCR confirms the ability of TRP-free media to induce *T. gondii* differentiation to bradyzoite, and the parasites survive and grow in this media.

In order to demonstrate the effect of the host cell and to validate that the effect of TRP-free media does not occur in HFF cells only, differentiated myotubes were used to culture parasite in TRP-free media. RNA was extracted at days three, six and nine and conventional and qualitative PCR were carried out by using SAG1 and SAG4 primers.

Myotubes have been used to culture *T. gondii*, and were found to be an appropriate cell type for *T. gondii* stage conversion and tissue cyst formation (Guimaraes et al., 2008; Guimaraes et al., 2009). This conversion is spontaneous and does not need any exogenous stress systems such as heat or alkaline media (Ferreira da Silva Mda et al., 2008). Indeed, other studies (Swierzy et al., 2014) have also reported that the differentiated myotubes of the murine SkMC cell line C2C12 induces expression of bradyzoite-specific genes and formation of a carbohydrate-rich tissue cyst wall. In contrast, the undifferentiated myoblasts did not efficiently induce *T. gondii* differentiation, which indicates that differentiation of myotubes is essential for bradyzoite differentiation and cyst formation. (Blader and Saeij, 2009) have also suggested a model in which tachyzoite growth is preferred inside of growing cells, suggesting that bradyzoite development initiate when tachyzoites are unable to manipulate the host’s cell cycle.
When TRP-free media was used with *T. gondii*-infected myotubes, the RNA expression profiling showed that SAG4 expression increased while SAG1 expression decreased (Figure 2-7, 8). This indicates that parasites were mostly if not completely at the bradyzoite stage; moreover, it indicates that the bradyzoite continues to grow in these media. Indeed, this further indicates that TRP starvations induction of differentiation might be performed using different cell types, as long as the host cell can survive in the TRP-free media. Therefore, *T. gondii*-infected myotubes cultured in TRP-free media might be a good model for studying *T. gondii* bradyzoite and cystogenesis. Considering that *T. gondii* bradyzoite, induction methods by stress or host cell increased the bradyzoite numbers but did not achieve a bradyzoite exclusive and tachyzoite free culture.

However, amino acid starvation triggers the loss of mitochondrial integrity. Mitochondrial depolarization is evidence of autophagy in both yeast and mammalian systems induced by nutrient starvation (Rodriguez-Enriquez et al., 2009; Rodriguez-Enriquez et al., 2006; Zhang et al., 2007). Mitochondrial morphology is associated with the loss of the membrane potential (ΔΨm); therefore, membrane potential-sensitive dye MitoTracker was used. Accumulation of MitoTracker detected both the parasite and host mitochondria. MitoTracker stain clearly shows that TRP starvation did not affect mitochondrial depolarization and the induction of autophagy of host or parasite (Figure 2-9). This indicates that TRP absence is different from the absence of other amino acids, and TRP starvation triggers parasite differentiation to bradyzoite not parasite autophagy. Therefore, TRP starvation as an immunological method controls the parasite growth and induces diffraction, but does not induce autophagy or kill the parasite.

Previously, it has been reported that TRP degradation may be neither sufficient nor required for antimicrobial activity (Murray et al., 1989). whereas, during *Chlamydia sp* infection, TRP degradation mediates persistent growth of *Chlamydia trachomatis* (Beatty et al., 1994) and inhibits the growth of *Chlamydia psittaci* replication (Byrne et al., 1986), indicating that the TRP starvation effect may not have antimicrobial activity and the effect of TRP starvation varies with different organism and species.

Moreover, another amino acid was found to trigger cystogenesis, *T. gondii* lacks the enzyme required for de novo arginine, and arginine starvation induces bradyzoite differentiation cyst formation (Fox et al., 2004). Inducible nitric oxide synthase causes local depletion of arginine and bradyzoite differentiation and thus the host’s immune system controls parasites replication. Both TRP and arginine auxotrophy were linked to host immune response by
increasing bradyzoite differentiation during the host’s immune response. These findings are consistent with a model of host-parasite evolution: as the host reduces the parasite virulence by bradyzoite induction, its increases the parasite persistence.

Additionally, developing a bradyzoite exclusive culture might be very useful for future researchers, where these cultures are required to study bradyzoite stage without tachyzoite involvement. Such culture might be helpful for studying the bradyzoite-switching mechanism and drug development for bradyzoite stages.

**Future work:**

TRP-free media is suitable for understanding the *T. gondii* mechanism of differentiation and for comparing the tachyzoite and bradyzoite transcriptome during different time points. This is a good and easy method to understand the *T. gondii* differentiation mechanism. Moreover, the bradyzoite culture provided by TRP-free media is an easy and cheap method that could be used for *in vivo* investigation of the *T. gondii* chronic stage.

On the other hand, further investigations are required on the effect of other amino acids on *T. gondii* growth and differentiation – especially phenylalanine and tyrosine, which will give further clues about the function of the parasite aromatic amino acid hydroxylase functioning as a phenylalanine hydroxylase.
Chapter Three

3 *Toxoplasma gondii* blocks the effect of KYNA on Dopamine
3.1 Abstract

Latent infection with *Toxoplasma gondii* involves the induction of the immune response that degrades tryptophan, producing kynurenic acid and quinolinic acid. As changes in kynurenic acid are associated with dopamine levels and dopamine has been found to accumulate in brain tissue cysts in mice and to be released at several-fold higher levels in infected dopaminergic cells, this study sought to understand the relationship of kynurenic acid with dopamine levels during infection. Kynurenic acid decreased dopamine synthesis and release from dopaminergic PC12 cells with a direct correlation between kynurenic acid concentration and dopamine level paralleling published *in vivo* studies of kynurenic acid effect on dopamine levels. In contrast, there were no changes in dopamine released from infected cells at any kynurenic acid concentration tested. Kynurenic acid reduced tyrosine hydroxylase (TH) activity at nano-molar concentrations in uninfected cells. Further analysis found that this is associated with a decrease in phosphorylation of ser19, a key residue for enzyme activation. In contrast, TH activity remained high in infected cells and phosphorylation of key residues in enzyme activation was unchanged. Hence, *T. gondii* infection mitigated the effects of kynurenic acid on dopamine. This is the first description of a mechanism for kynurenic acid suppressing dopamine expression and its subversion by *T. gondii* infection.
3.2 Introduction

Immune response to infection may be involved in the behaviour changes observed during latent *T. gondii* infection. As an indirect effect, this involvement may be mediated by the continuous production of proinflammatory cytokines (Aliberti, 2005; Miller et al., 2009). Likewise, a critical cytokine of the body’s defenses against parasites is interferon-γ produced by immune cells (Denkers and Gazzinelli, 1998), through activation of macrophages and lymphocytes. Subsequently, indoleamine 2, 3-dioxygenase (IDO) expression is increased leading to tryptophan levels being reduced and induction of tryptophan starvation. Tryptophan starvation is a critical immune defence effector mechanism against *T. gondii* proliferation (Pfefferkorn, 1984; Pfefferkorn et al., 1986). Tryptophan starvation will lead to the production of tryptophan-degradation products i.e., kynurenine, kynurenic acid and quinolinic acid. *T. gondii* -infected mice show an increase in KYNA levels and activation of astrocyte (Guidetti et al., 2006).

3.2.1 Synthesis and elimination of tryptophan catabolic products

Tryptophan is degraded mainly by the kynurenine pathway in mammals. The pathway starts by transforming TRP into kynurenine (KYN) with this reaction being catalysed by two enzymes: indoleamine 2, 3-dioxygenase (IDO) and tryptophan 2, 3-dioxygenase (TDO). After tryptophan conversion into kynurenine by (IDO), kynurenine is then metabolized by the action of enzymes within the kynurenine pathway to tryptophan degradation products (Stone and Darlington, 2002). Kynurenine 3-hydroxylase (kynurenine 3-monooxygenase) converts KYN to toxic 3-hydroxykynurenine (3-HK) (Stone, 1993). One of the end products of this pathway arm is quinolinic acid (QUIN), which is an agonist of the N-methyl- D-aspartate (NMDA) sensitive glutamate receptors (Stone and Perkins, 1981).

However, kynurenic acid (KYNA) is produced in the astrocyte by an arm of the kynurenine pathway; KYNA is an endogenous antagonist of NMDA receptors (Swartz et al., 1990; Vecsei et al., 1992; Stone, 1993). The synthesised KYNA is released into the extracellular milieu and it is not removed by reuptake or degraded enzymatically; however, KYNA is slowly eliminated from the brain by a nonspecific acid transporter (Schwarz and Pellicciari, 2002). The slow eliminating process places the metabolite in an excellent position to influence surrounding neurons.
3.2.2 Tryptophan catabolic products function in brain physiology and pathology

KYNA was first described as a neuroinhibitory compound (Perkins and Stone, 1982). At high non-physiological concentrations, KYNA is a broad-spectrum antagonist of ionotropic excitatory amino acid receptors. Therefore, it was used to block excitatory neurotransmission in vitro and in vivo experiments. Also high concentrations of KYNA are anticonvulsant and protect from excitotoxic injury (Foster et al., 1984). KYNA is a non-competitive antagonist at the glycine site of the NMDA receptor (IC50 ~8 μM) (Parsons et al., 1997) and an orphan G protein-coupled receptor GPR35 (Wang et al., 2006) and aryl hydrocarbon receptor (DiNatale et al., 2010), KYNA does have a controversial ability to inhibit alpha7 nicotinic receptors (IC50 ~7 μM (Hilmas et al., 2001; Dobelis et al., 2012). The difference in the observed inhibitory effect on nicotinic receptors might be due to experimental differences between groups and the age of the examined rat (Alkondon et al., 2011). as well as to the fact that the inhibition of the a7nAChR by KYNA is a complicated phenomenon that depends on many factors, i.e., cell maturation, receptor expression on interneurons versus pyramidal neurons, compartmentalization of receptors to dendritic versus somatic locations, and additional variables such as modification of subunits and association with other proteins and intracellular regulatory factors (Albuquerque and Schwarcz, 2013). Moreover, KYNA does not only influence dopaminergic neurotransmission, it also influences glutamatergic and cholinergic neurotransmission i.e., in vivo studies on the rat brain striatum shows that reduction in KYNA levels enhance vulnerability to an excitotoxic insult (Poeggeler et al., 1998) and there is an inverted relationship between KYNA level and glutamate release (Carpenedo et al., 2001).

KYNA is capable of altering dopamine levels, and even nano molar increases in the brain levels of KYNA are capable of decreasing interstitial dopamine levels in anesthetized rats in vivo by using micro dialysis technique. KYNA reduce dopamine in a dose-dependent manner , resulting in a more than 50% reduction at 500 nM. Dopamine levels return to control value after KYNA was removed by perfusion solution (Rassoulpour et al., 2005). Furthermore, Amori et al (2009) found that the level of dopamine was increased by the inhibition of kynurenic acid synthesis. KYNA was inhibited by kynurenine aminotransferase II (KAT II) knock out or by using the selective KAT II inhibitor (S)-4-(ethylsulfonyl) benzoyl alanine (S-ESBA); these results suggest that the relationship between KYNA and dopamine is bidirectional(Amori et al., 2009). Finally, behavioural studies in rats have found that
increased cortical KYNA levels affect sensory gating, and startle reflex prepulse inhibition (Wonodi and Schwarcz, 2010)

QUIN is an agonist of the N-methyl-D-aspartate (NMDA) sensitive glutamate receptors (Stone and Perkins, 1981). The over-activation of NMDA by the increased levels of quinolinic acid may possess antidepressant properties (Schwarcz et al., 2012). Moreover, it has been demonstrated that increased QUIN levels are associated with increased depressive symptoms (Maes et al., 2011). The kynurenine pathway has a regulatory function for both innate and adaptive immune responses; for that reason, tryptophan metabolism and kynurenine pathway reveal an important border between immune and nervous system (Mandi and Vecsei, 2012). During T. gondii infection, tryptophan degradation might favour immunosuppression and systemic tolerance (Engin et al., 2012).

3.2.3 T. gondii and schizophrenia: linkage through kynurenic acid?

During T. gondii infection, T. gondii modulates biochemical pathways associated with schizophrenia, this evidence together with the other evidence, including the hypothesised T. gondii and schizophrenia relationship; the fact that T. gondii activates astrocytes during the infection; KYNA levels have been linked to schizophrenia; KYNA is produced by astrocytes; KYNA is an antagonist for NMDA and a7nACh receptors; and finally the link between infection and TDO. The following sequence of events was hypothesised: T. gondii infection increases the KYNA in the brain by astrocyte activation. This effect is increased in persons with elevated brain TDO activity, i.e., in individuals with a genetic tendency towards schizophrenia (Schwarz and Hunter, 2007). Further, increased brain KYNA during infection might contribute to glutamatergic and nicotinergic neurotransmission- and these neurotransmitters play an important role in the cognitive impairments seen in schizophrenia.

KYNA is associated with decreased dopamine in rats whereas large amounts of dopamine were observed with T. gondii infection in infected mouse brains (Prandovszky et al., 2011). The finding that T. gondii increases dopamine level is not coherent with the fact that T. gondii infection increases KYNA level and KYNA reduces dopamine levels. Therefore, the aim of this chapter is to investigate the effect of KYNA on dopamine during T. gondii infection.
3.3 Aims

The aim of this chapter is to investigate the effects of tryptophan catabolic products (KYN, QUIN, and KYNA) during *T. gondii* infection on dopamine levels. In particular, we are looking at the effect of tryptophan catabolic products on dopamine production at the molecular level and how this is affected by *T. gondii* infection. In order to understand how dopamine levels are altered during infection, a cell-based model was developed to investigate the effect of KYNA during *T. gondii* infection. Initial experiments investigated the effect of KYN, QUIN and KYNA on dopamine in PC12 cells to determine whether these cells show parallel observations to *in vivo* findings. After this, the effects on *T. gondii* infected cells were investigated.
3.4 Experimental design

3.4.1 Parasite and cell culture
As described in chapter 2.

3.4.2 Induction of bradyzoite differentiation
As described in chapter 2.

3.4.3 Growth and differentiation of *T. gondii* in the presence of tryptophan catabolic metabolites

Prugniard Δku80 Δhxgprt strain (kind gift of David Bzik) was used to assess the effect of tryptophan metabolites on *T. gondii* differentiation. In this strain, GFP is expressed under the control of a bradyzoite-specific promoter (Fox et al., 2011). HFF cells cultured in black 96 well plate were infected with this strain. The fluorescence was measured every three days using a POLAR star OPTIMA microplate reader at excitation 488 nm, emission 508 nm. To detect the effect of TRP metabolites (KYNA, KYN and QUIN) on *T. gondii* growth, the RH-YFP strain (kind gift of B. Striepen) was used and the fluorescence was measured after 24 and 72 hours. YFP in expressed by this strain, and YFP intensity is relative to parasite number. The readings were converted to a percentile by comparing them with the control reading for each time point (n=9).

3.4.4 Kynurenic acid treatment

The dopaminergic cell line PC12 (ECACC) was infected with the Prugniard strain of *T. gondii*. Tachyzoites were alkaline shocked to induce bradyzoite differentiation. The cell: parasite ratio was 1:1 and culture proceeded for five days prior to assay. The percentage of infected cells was 40–50%.

Stock of KYNA (K3375 Sigma) was dissolved in sterile distilled H₂O to a concentration of 500 mM. Dilutions of 1000, 500, 200, 100 and 50 nM were added to cultures and incubated 37°C in CO₂ incubator for three hours prior to experimentation. The concentration used in the experiments was within the physiological range of each component (Linderholm et al., 2012; Fujigaki et al., 2002; Guillemin et al., 2007; Guillemin et al., 2001; Rassoulpour et al., 2005; Braidy et al., 2009; Espey et al., 1997).

The same above experiment design was used for QUIN (Q104 Sigma) and KYN (K8625 Sigma) treatments.
3.4.5 MTT Assay

The cells were cultured in a 96 well plate and treated with different concentrations of KYN, QUIN and KYNA. After three days, an MTT assay was carried out, as described in chapter 2 for samples (n=9).

3.4.6 Dopamine release assay

The dopaminergic cell line PC12 (ECACC) was infected with Prugniard strain tachyzoites that had been alkaline shocked to induce bradyzoite differentiation; the cell to parasite ratio was as described above and cultures proceeded for five days prior to assay. First, PC12 cells cultures were washed with PBS and then equilibrated with wash buffer with low KCl concentration (140 mM NaCl, 4.7 mM KCl, 1.2 mM MgCl2, 2.5 mM CaCl2, 11 mM dextrose, 10 mM HEPES, pH 7.4) for 30 minutes, with a 250µl aliquot taken and supplemented by 125 µl of (0.1M) perchlorate (PCA), followed by incubation with two volumes high KCl-containing buffer (40 mM NaCl, 100 mM KCl, 1.2 mM MgCl2, 2.5 mM CaCl2, 11 mM dextrose, 10 mM HEPES, pH 7.4) for two minutes and then supplemented with125 µL PCA. After that, cultures were harvested by adding 500µl trypsin for five minutes at 37C; the trypsin was deactivated by fresh RPMI and collected, centrifuged for five minutes at 2000 and re-suspended in 1ml of PBS; in addition, 100µl of suspension was centrifuged at 2000 for five minutes in 4c and used later for normalization assay. The 900µl of cell suspension was centrifuged at 2000 for five minutes and then resuspended in 350µl of PCA. After that, it was lysed by sonication and centrifuged at 13000 for 15 minutes in 4°C and finally the supernatant was used for dopamine measurement by HPLC with electrochemical detection, and dopamine levels were detected by HPLC with electrochemical detection(HPLC-ED) using Dionex HPLC system, 20µl of sample was injected on to a C18 Acclaim 120 column (5µm, 4.6 x 150 mm). The mobile phase consisted of 90% 57 mM citric acid, 43 mM sodium acetate trihydrate, 0.1 mM EDTA (Sigma Aldrich), 1 mM sodium octanesulphonate at pH 4, and 10% methanol (All reagents were HPLC grade or above). Flow rate was 0.4 ml/min. Peaks were detected via an ESA Coulochem III (Dionex, Camberley, UK) detector with the potential set at 275 mV. Data were collected using the Chromeleon software (Dionex) and processed as previously described (Prandovszky et al., 2011). Statistical analysis was carried out by a graph pad prism; first, the concentration of the dopamine was obtained by using standard curve, then the result was divided by the
number of the cells obtained from the normalization assay and the results were presented as a percentage compared to the control (n=9).

### 3.4.7 Normalization assay

To normalize the result, the dopamine concentration was divided by cell count. Cyquant assay (Invitrogen) was used to obtain cell count. First, the cells were lysed by using 100µl of 1:20 diluted lysing buffer, then 25µl the samples were plated in 96 well plate. Then 75µl of the lysing buffer were added to the samples; after this, 100µL of 1:400 diluted GR dye was added and the plates were read on a fluorescence plate reader with excitation =485nm and emission=520nm. The cell counts were calculated from sample reading by using a standard curve of known PC12 cell count.

### 3.4.8 Tyrosine hydroxylase activity assay

PC12 cells were cultured in T25 flasks of 1x10^6 cells. Cells were infected with 1x10^6 differentiation-induced Prugniaud tachyzoites and treated with different concentrations of KYNA. After five days, Tyrosine hydroxylase enzymatic activity was measured using the method described (Naoi et al., 1988).

First, the PC12 cells were sonicated after they were washed with PBS and resuspended in 50µL 10mM potassium phosphate buffer (pH 7.4); at this point, a Bradford assay was performed after which the (25 µg) cell lysate was incubated at room temperature with 26µL 200mM sodium acetate-acetic acid buffer (pH 6.0), then 1µL from 2mM carbidopa (Sigma Aldrich) in sodium acetate-acetic acid buffer, 0.01M glacial acetic acid and 1mg/ml catalase (Sigma Aldrich) was added and incubated at 37°C for five minutes. Lastly, 1µL of 20mM L-tyrosine solution and 20µL of 10mM tetrahydrobiopterin (Schicks Laboratories, Jona, Switzerland) in 1M β-mercaptoethanol was added and reactions incubated at 37°C for 10 minutes. The reaction was terminated by adding 100µl (0.1M) PCA perchloric acid. After this, the L-Dopa was quantified by using HPLC-ED as descried previously. Statistical analysis was carried out by using prism graph pad software; first, the concentration of the L-Dopa was obtained by using standard curve, and then the result was divided by the number of the cells calculated from the normalization assay and the results were presented as a percentage compared to the control (n=6).
3.4.9 Immunoblotting

PC12 cells plated in 6-well plates and treated as described above were centrifuged at 2000 rpm for five minutes. Cell pellets were then re-suspended in a radio-immunoprecipitation assay (RIPA) buffer (Caymen Chemicals, Ann Arbor, MI). Protein was quantified and five mg of total protein was loaded in each well for 12% SDS-polyacrylamide gel electrophoresis and subsequent transfer to a nitrocellulose membrane. Following the transfer, the membrane was incubated at room temperature for 1 h in 5% non-fat dried milk. The membrane was then washed three times for five min with PBS at pH 7.4 containing 0.05% Tween 20 at room temperature. After overnight incubation with the primary antibody at 4°C, the membrane was washed as above and then incubated with goat anti-mouse HRP conjugate (1:10,000; Abcam) secondary antibody and then washed and visualised with SuperSignal West Pico Chemiluminescent Substrate (Pierce, Loughborough, U.K.). Primary antibodies were rabbit anti-tyrosine hydroxylase phosphor ser19 (Millipore, Watford, U.K.), rabbit anti-tyrosine hydroxylase phosphor ser40 (Millipore) and rat tubulin (1:25,000; Sigma). Different concentrations of antibodies were used to adjust the experiment (1:5000, 1:10000 and 1:25000).

3.4.10 Bradford Assay

One µl of each sample was placed in a well of 96 well plate then the samples were diluted by 4µl of dH2O. Then, 250 µl of Bradford assay reagent (B6916 sigma Aldrich) was added and incubated for 30 seconds. After this, the absorbance was read by spectrophotometer at 595 nm wavelength. Finally, the final protein concentration was calculated by using standards prepared from descending concentrations of bovine serum albumin BSA.
3.5 Results

The PC12 cell line was used as a neuronsecretory model as in numerous studies (Westerink and Ewing, 2008). Firstly, the use of a dopaminergic cell line as an \textit{in vitro} model of dopamine regulation by KYNA was verified. \textit{In vivo} studies have found that nano molar concentrations of KYNA decreased interstitial dopamine (Rassoulpour et al., 2005). The effect of KYNA on cell viability was measured. Moreover, the effects of other tryptophan metabolites quinolinic acid (QUIN) and kynurenine (KYN)) on PC12 cells were also investigated.
The effect of tryptophan catabolism metabolites on PC12 cell viability was tested by the MTT assay at different concentrations of tryptophan metabolite compounds in the cultures, whilst the pH was maintained at 7, and incubated for three days. The measurements are the averages of three biological replicates with each three technical replicate presented as a percentage of control media with error bars representing standard deviation. Kynurenic acid (KYNA), kynurenine (KYN) and quinolinic acid (QUIN) did not affect PC12 cell viability at the concentrations tested (0.01nM to 1 mM) (Figure 3-1). Thus, these compounds do not effect PC12 growth.

**Figure 3-1:** Effect of tryptophan metabolites on the viability of PC12 cells. Prior studies have observed that KYNA protects the brain parenchyma by acting on the NMDA receptor glycine site and other different molecular targets (Urenjak and Obrenovitch, 2000), but the neuroprotective effect of KYNA was not tested here. QUIN is an agonist in the NMDA receptors, and it produces axon-sparing lesions on the brain by generating free radicals and, to a certain extent, creating mitochondrial damage (Stone, 2001). PC12 cells used here do not express functional NMDA receptors (Edwards et al., 2007).

The use of dopaminergic cells as an *in vitro* model to study the effect of KYNA on the dopamine level was tested, and compared to prior studies using an *in vivo* model (Rassoulpour et al., 2005). Moreover, other effects of tryptophan catabolism metabolites (QUIN and KYN) on dopamine levels produced from PC12 cells were also investigated.
Infected and uninfected dopaminergic PC12 cells were treated with different concentrations of KYNA, QUIN and KYN, and the amount of dopamine released from PC12 cells after three hours of treatment with a range of concentrations were measured via HPLC-ED. Dopamine release was induced with a high concentration of potassium (Yamboliev et al., 2009). KYNA reduced the dopamine content of PC12 cells, resulting in more than a 40% reduction at 200 nM cells (p =0.01, paired t-test, n=9) (Figure 3-2). This result verifies that PC12 cells can be used as a cell-based model of the relationship between KYNA and dopamine. The PC12 cells showed a similar response as those in rat brains, except that they responded to KYNA at a lower concentration than in the in vivo model. However, QUIN and KYN did not affect the dopamine production in PC12 cells.
Figure 3-2: Dopamine levels in dopaminergic cells incubated with tryptophan metabolites. The effect of tryptophan degradation products (KYNA, QUIN and KYN) on *T. gondii* growth was investigated by culturing RH-YFP in different concentrations of tryptophan metabolites. This parasite strain expresses YFP, thus the parasite growth was detected by measuring the YFP fluorescence.

In comparison with untreated samples, TRP metabolites treated samples had no significant effect on parasite growth measured on days 1 (dark grey bars) and day 3 (light grey bars). (ANOVA p>0.97) (Figure 3-3), The results (averaging of three biological replicates each three technical replicates) was plotted as percentage compared to untreated for each time point and shows that tryptophan metabolites have no effect on the growth of the parasites.
Figure 3-3: Effect of tryptophan metabolites on the growth of *T. gondii*. Strain

*T. gondii* Prugniaud Δku80 Δhxgprt parasites were cultured in DMEM with different concentrations of tryptophan metabolite. The fluorescence emitted by the parasites differentiated into bradyzoite, monitored every three days. No significant difference at any tryptophan metabolite concentration (Figure 3-4) (ANOVA, p>0.94), indicating that these TRP metabolites do not effect *T. gondii* differentiation.
**Figure 3-4:** Effect of tryptophan metabolites on *T. gondii* differentiation.

To observe the effect of *T. gondii* infection on the ability of KYNA to reduce PC12 dopamine production, PC12 cultures were infected with alkaline-induced *T. gondii* tachyzoites. On day 4 after infection, concentrations of 50-1000 nM KYNA were added to the infected and uninfected PC12 cells.

Dopamine release assays were performed to assess the effect of KYNA on dopamine signalling and to determine whether it changed over the course of the infection. Three hours after adding different concentrations of KYNA using the methods reported in previous studies, the uninfected cultures and those infected with alkaline-induced *T. gondii* were induced to release dopamine with K+, which causes the release of vesicle-packaged dopamine (Yamboliev et al., 2009). The dopamine levels were measured via HPLC-ED. The experiment found that KYNA no longer decreased dopamine when PC12 cells were infected (Figure 3-5). By contrast, the effect of KYNA in decreasing dopamine levels was maintained in uninfected cells (p =0.0003, paired t-test, n=9). The amount of dopamine released from the uninfected PC12 cells was around 50% of the total dopamine content. Also, adding KYNA to PC12 cells cultures did not affect the dopamine signalling, as dopamine release by high K buffer indicates. In addition, measurements of total dopamine content of cells found the same pattern of reduction in dopamine in response to KYNA as the measurements of released that was also reversed by infection. Hence, infection mitigates the reduction in dopamine induced by KYNA.
To investigate the mechanism by which KYNA reduces the dopamine content in uninfected PC12 cells and how infection disrupts this effect, a tyrosine hydroxylase (TH) activity assay was performed on the cultures. Infected and uninfected PC12 cells were assayed for TH activity three hours after adding different concentrations of KYNA. In this assay, the TH activity was assessed by measuring the production of L-Dopa using HPLC-ED.

The results (Figure 3-6) revealed that KYNA reduced the TH activity in PC12 cells by greater than 40%. This finding correlates directly with the decrease in amounts of dopamine with KYNA treatment (Figure 3-2).

The reduction in TH activity by KYNA was not observed in the infected cells (Figure 3-6). These data indicate that *T. gondii* infection maintains the dopamine content level by sustaining the TH activity. Hence, the infection may have mitigated the effect of KYNA on dopamine by stopping the KYNA from reducing the TH activity (p =0.0005, paired t-test, n=6).
Figure 3-6: Tyrosine hydroxylase (TH) activity assay from PC12 cells exposed to KYNA.

To understand how KYNA reduces TH activity, the TH mRNA expression level of infected and uninfected PC12 cells treated with different concentration of KYNA was measured. No change in the amounts of TH mRNA was detectable using RT-PCR (data not shown). The phosphorylation of TH was investigated as TH activity is tightly regulated by phosphorylation and KYNA effect the phosphorylation. Serine 19 and serine 40 are amino residues involved in the activation of the regulatory domain of TH (Fitzpatrick, 1999). Different concentrations of KYNA were added to PC12 cell cultures and PC12 cell lysates were probed with anti-ser19, anti-ser40 and, as a control, anti-tubulin. No change in Ser40 phosphorylation was observed, but Ser19 phosphorylation decreased in a dose-dependent manner (Figure 3-7). Detection of Tubulin expression was used as a control. The quantification of the Ser19 band intensity shows a direct relationship between the decrease in
phosphorylation and the increase in KYNA concentration. This may explain the decrease in
dopamine levels observed in vivo with KYNA (Rassoulpour et al., 2005; Fitzpatrick, 1999).
KYNA concentration (nM)

Tubulin

Ser19

Ser40

Ser19/tubulin band intensity

KYNA concentration (nM)
Figure 3-7: TH phosphorylation is inhibited by KYNA. To examine the effect of *T. gondii* infection on the relationship between KYNA and Ser19, lysates from *T. gondii* -infected PC12 cells treated with KYNA were probed with anti-ser19 and anti-tubulin. (Figure 3-8). We found that *T. gondii* -infected PC12 cells maintained the phosphorylation of serine 19 even in the presence of KYNA in contrast to the decrease observed in KYNA-treated uninfected cells. Hence, *T. gondii* infection mitigates the effect of KYNA on serine 19 phosphorylation.
**Figure 3-8**: Effect of *T. gondii* infection on TH regulatory phosphorylation with KYNA treatment.
3.6 Discussion

During the immune response to *T. gondii* infection, IFN-mediated degradation of tryptophan produces KYNA that alters neuromodulators (Pfefferkorn, 1984; Pfefferkorn et al., 1986). Intriguingly, one of the effects of elevated KYNA is a decrease in extracellular dopamine (Rassoulpour et al., 2005). Therefore, decreased dopamine levels in the brain might be observed in *T. gondii*-infected individuals. These conflicts with reports of excess dopamine associated with *T. gondii* cysts in infected mouse brains. This study set out to resolve these conflicting possibilities.

Initially, *T. gondii* PC12 cells were found to replicate *in vitro* the observations *in vivo* that KYNA decreases dopamine, although the cultured cells responded at a slightly lower concentration of KYNA than *in vivo* experiments (Rassoulpour et al., 2005) (Figure 3-2). The PC12 system is considerably simplified from *in vivo* with the brain containing many different cell types. The relationship between dopamine reduction and KYNA was previously explained as an antagonism of alpha7 nicotinic receptors by KYNA based on *in vivo* studies (Hilmas et al., 2001). However, recent studies have refuted this relationship (Alkondon et al., 2011), leaving the KYNA and dopamine correlation unexplained. This study found, for the first time, that KYNA directly caused reduction of TH activity and dopamine content in dopaminergic cells (Figures 3-5, 6).

We further dissected the process finding that KYNA caused a decrease in phosphorylation of serine 19 in TH that directly correlated be with KYNA concentration and level of phosphorylation. Phosphorylation of the Ser19 residues in the R domain increases the activity of the TH (Dunkley et al., 2004). In contrast, the phosphorylation status of the regulatory residue serine 40 in TH was unaffected by KYNA treatment. The results clearly suggest that KYNA causes a reduction in TH activity through reduced phosphorylation of serine 19.

To determine the effect of KYNA on dopamine levels during infection, dopaminergic cells were infected with *T. gondii* and treated with KYNA. In this study, the parasite-infected cell cultures did not respond to the added KYNA but maintained their dopamine levels (Figure 3-5). In contrast to the observations with uninfected PC12 cells, the TH activity in infected cultures was not only unchanged but was elevated in infected cultures relative to control PC12 cells (Figure 3-6), Additionally TH ser19 phosphorylation did not change in the infected cultures. Clearly, the parasites have the ability to obstruct the effect of KYNA on dopamine metabolism.
Two possible scenarios can explain how infection blocks the KYNA effect on dopamine. Firstly, TH activity in infected cells may be partially produced by the *T. gondii*-encoded TH replacing the blocked activity of the rat TH. However, this assumption suggests that the *T. gondii*-encoded TH differs from the mammalian TH such that it is not inhibited by KYNA.

Secondly, *T. gondii* infection may modulate the host proteins or pathways that interact with KYNA, leading to the interruption of the KYNA effect on TH activity. *T. gondii* modulates many different signalling pathways in host cells and subverts host cell signalling pathways by secreting effectors, e.g. ROP16, ROP18 and ROP5 (Blader and Saeij, 2009). ROP16 directly subverts the host STAT6 through tyrosine phosphorylation (Ong et al., 2010), and ROP18 is a Ser/Thr protein kinase important in acute virulence because it inhibits the host NF-κB pathway (Du et al., 2014). Blocking phosphorylation have been reported as a machinery for host altering, *T. gondii* blocks histone H3 Ser\(^{10}\) phosphorylation and Lys9/14 acetylation at the IL-10 promoter and TNF-α promoter, leading to reduced IL-10 and TNF production in infected microphages (Leng and Denkers, 2009; Leng et al., 2009).

This study illustrates the effect of KYNA on dopamine metabolism in the presence of *T. gondii*. However, when the brain is infected, the KYNA effect may be different. During brain infection, the produced KYNA is released into the extracellular milieu. It is not removed by reuptake or degraded enzymatically but is slowly eliminated from the brain by a nonspecific acid transporter (Schwarcz and Pellicciari, 2002). However, KYNA may have a pleiotropic effect and KYNA can modulate other neuromodulators than dopamine. For example, extracellular glutamate in the hippocampus is modulated by KYNA (Pocivavsek et al., 2011).

A recent study evaluated the KYNA levels in the brains of *T. gondii*-infected mice and found that KYNA increased several-fold during infection (Notarangelo et al., 2014). However drug treatment of infected mice from with anti-parasitic drugs pyrimethamine and sulfadiazine, retained the KYNA post-infection levels but did not reliably duplicate the abnormalities seen in the brain of individuals with schizophrenia (Notarangelo et al., 2014). In conclusion, we assume that the effect of KYNA on the TH of an infected brain is limited as the amount of increased KYNA during the infection is much lower than that required to induce the KYNA effect on TH activity or the physiological levels during psychiatric diseases.

*T. gondii* infection has the ability to modulate its microenvironment. Koshy (Koshy et al., 2012) reported that *T. gondii* had the ability to co-opt uninfected cells *in vivo* by injecting rhoptry proteins into the uninfected cells. IL-12 was found to be produced from the infected and uninfected cells, and that a soluble host or parasite factor was responsible for the bulk of
IL-12p40 production in vivo (Christian et al., 2014). These findings suggest that the parasites may affect the response of uninfected cells to KYNA and therefore stop the KYNA effect on the infected brain.

In sum, these studies show that KYNA reduces dopamine production by controlling the regulation of TH, the rate-limiting enzyme in the dopamine production. Moreover, *T. gondii* infection blocks the KYNA effect on the infected cells, and KYNA does not change the dopamine production or the TH activity in the infected cells.

In the future, to distinguish between the parasite-encoded and host TH activity, TH activity assays could be performed using a TH knockout strain of *T. gondii*, to further investigate the ability of *T. gondii* block the KYNA effect.
Chapter Four

4 Dual transcriptional profiling of PC12 cells and *T. gondii* reveal potential behaviour change modification mechanisms
4.1 Abstract

*T. gondii* induces behavioural changes in its intermediate host during its infection of neurons; therefore, it is of interest to obtain information about the global alteration to the neuronal cell transcriptome caused by infection. RNA sequencing was performed on *T. gondii*-infected differentiated PC12 cells at three time points in infection, and a collection of differentially regulated genes were identified. The most significantly changed gene in bradyzoite-stage *T. gondii* infection was down-regulation of dopamine beta hydroxylase mRNA expression. qPCR of *T.gondii*-infected rat’s brain samples found that dopamine beta hydroxylase (DBH) is reduced *in vivo* during infection and this reduction is modulated in female rats by the stage in the oestrogen cycle. Monitoring monoamine neurotransmitters during infection found reduced epinephrine and norepinephrine in *T. gondii* infected PC12 cells as measured by HPLC-ED. Gene ontology (GO) analyses of the sequencing data found that *T. gondii* infection manipulates gene expression of several neurological functions including genes involved in catecholamine secretion; olfactory sensory, cell signalling, neurodevelopment, behaviour and N-methyl-D-aspartate receptor (NMDR). Hence, parasitic infection alters several host neurological functions that may be involved in host behaviour changes particularly expression of genes involved in catecholamine metabolism pathways that decreased epinephrine and norepinephrine and result in an increase in dopamine production. Finally, our data showed the neuronal cell immune response to *T. gondii* infection regulates host genes in chemotaxis and cytokines secreted during infection as well as up regulation of immunoglobulin gene expression and T-cell interaction. Moreover, the parasite methods to down-regulate the immune response like steroids secretion and G-coupled protein receptors down-regulation were revealed.
4.2 Introduction

The information in the previous chapters has provided a valuable insight into host-parasite interaction during infection. In order to get a clearer global view of the changes that *T. gondii* induces in the infected cells and changes in the whole transcriptome profile, RNA sequencing (RNA-Seq) was performed to obtain expression data on the cell’s complete set of transcripts, and the quantity of these transcripts, at a physiological condition or specific developmental stage.

The transcriptome profiles of *T. gondii* and host cell have been studied several times before; however, most of the previous investigations have used microarray data. Microarray has a number of limitations: firstly, for reliability, several biological repeats are necessary; secondly, microarray detection is biased towards unusual transcripts; third, it has a narrow dynamic range as compared with RNA-Seq technology; fourth, RNA-Seq allows transcriptome profiling of any organism that have a sequenced genome; and, finally, RNA-Seq has high specificity and sensitivity and provides an easier detection of rare and low-abundance transcripts. The table below enumerates transcriptome analyses that have been done on *T. gondii* and its host.
<table>
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<th>Parasite strain</th>
<th>Stage</th>
<th>Host</th>
<th>Method</th>
<th>Reference</th>
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<td>Microarray</td>
<td>(Blader et al., 2001)</td>
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<td>I, II and III strain</td>
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<td>The human neuroepithelioma cell line SK-N-MC</td>
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**Table 4-1:** *T. gondii* and host transcriptome analysis
Previous studies (Table 4-1) that characterised the transcriptome of *T. gondii* and host provide valuable information about host-parasite interactions; however, there are several limitations of these studies. The study design of the *in vivo* studies did not demonstrate the changes that occur during chronic infection, and most of these studies involved only the tachyzoite stage. Additionally, host selection is critical as host-parasite interaction varies between different hosts or cell lines. Given that the main goal of these experiments to have comprehensive knowledge about the global changes that *T. gondii* induces in host brain during chronic infection, most of the published data are not relevant to the pursued information.

Two investigations focused on the host neurological transcriptome: (Xiao et al., 2011) and (Pittman et al., 2014). Xiao et al. (2010) studied the changes that different *T. gondii* strains induced in the human neuroepithelioma cell line SK-N-Mc through microarray. Although the work provided significant information on the differences between *T. gondii* strains, the use of microarray and limitation to tachyzoite infection restricted the type of information that can be obtained from this experiment. Pittman et al. (2014), on the other hand, reported differences in host-parasite interaction between the acute and chronic stages, suggesting that host-parasite interplay is contained during the chronic infection. Indeed, less than 0.01% of cells are infected (J.P. Dubey, personal communication); therefore, only indirect effects of infection are observable by RNA sequencing in *in vivo* studies. Analysis of the data obtained from different microarray analyses related to host-parasite interactome revealed that the pathogen interacts with ∼3000 host proteins or genes. These genes include susceptibility genes for Alzheimer’s disease, multiple sclerosis, schizophrenia, depression, bipolar disorder, Parkinson’s disease, childhood obesity and attention deficit hyperactivity disorder – all found to be differentially expressed during infection (Carter, 2013). However, the results of this study are very vague because the study did not consider the specific strain and infection stages.

To provide a comprehensive analysis of *T. gondii* and host changes, and host-parasite interactions during the chronic infection of the brain, RNA-Seq was performed on nerve growth factor (NGF) differentiated PC12 cells where *T. gondii* conversion to bradyzoites was induced and RNA was collected at days 0, 3 and 6 to monitor the changes over time. PC12 cells are an embryonic origin line, derived from a pheochromocytoma of male rat adrenal medulla (Greene and Tischler, 1976). These cells differentiate into neuron-like cells by NGF and have the ability to produce catecholamines, making them a good model for
neuronsecretion in vitro (Westerink and Ewing, 2008) and one of the best studied models of monoaminergic neurons. Finally, the analysis focused on the neurological and the alteration in catecholamine metabolism. Besides, immunological functions were also analysed to understand the immune response in infected non-immune host cells. Finally differential gene analysis was also performed on the T. gondii transcriptome results.

4.3 Aims

The aim of this study was to obtain information about the changes in host neural cells that the parasite induces during infection. Specifically, this study looked into the changes that occur during chronic stages of infection, as well as the alterations that the parasite induced that may lead to behavioural changes. Transcriptome analysis of T. gondii-infected PC12 cells was performed through RNA sequencing of the host and parasite transcriptome at several time points in order to observe the changes over time. Differential expression analysis between different time points was performed, with particular focus on genes involved in neurological and immunological functions.
4.4 Experiment design

4.4.1 Growth of the parasite and cell culture

The cells and parasites that were used in this experiment were cultured, as mentioned previously in section 2.4.1., and parasite differentiation was induced, as previously described in section 2.4.2.

4.4.2 Differentiation of PC12 cells by NGF

Three ml of PC12 cells were cultured in each well of a poly-D-lysine-coated 6-well plate, 20 plates were used with a cell density of $1 \times 10^5$ cells/ml and incubated at 37°C and 5% CO2 for 24 hours. Afterwards, the media was changed with low serum media HS 1%, FBS 0.5%.

After another 24 hours, 100 ng/ml of Nerve Growth Factor (NGF) (Sigma) was added. The addition of NGF was repeated once every 24 hours throughout the length of the experiment. After 72 hours from the initial addition of NGF, free wild-type Prugniard tachyzoites induced by high pH media were transferred to each well, maintaining a parasite density of $2.5 \times 10^4$ cells/ml (Day 0) (1:1 ratio). At this point, 33.3% of the plates were used for RNA extraction while the rest were extracted at day 3 and day 6 of infection. The cultures were monitored daily by light microscope. At day 6 of infection, the percentage of cells infected was estimated to be 60-70% by light microscope examination.

4.4.3 RNA extraction

The cells were detached from the well surface using a small scraper. Then the media with the suspended cells were transferred to a conical tube and centrifuged at 8000 g for 10 minutes. The media (supernatant) was discarded and the cells were resuspended in leftover media. TRI reagent solution (Invitrogen) (1 ml) was added to each tube and mixed by pipetting the mixture several times. This was followed by removing the insoluble material from the homogenate by centrifugation at 12,000 g for 10 minutes at 2-8 °C. The samples were then allowed to stand for five minutes at room temperature. After that, 0.2 ml of chloroform was added and the sample was shaken vigorously for 15 seconds and incubated for 2-15 minutes at room temperature. The resulting mixture was centrifuged at 12,000 g for 15 minutes at 2-8 °C. Subsequently, the aqueous phase of the separated mixture was transferred to a fresh tube and 0.5 ml of isopropanol was added. The sample was kept at room temperature for 5-10 minutes before it was centrifuged at 12,000 g for eight minutes at 4 °C. The supernatant was
removed and the RNA pellet was briefly dried for 5-10 minutes. Finally, 20 µl DEPC-treated 
H2O was used to resuspend the pellet. The RNA samples were stored in -80°C.

4.4.4 mRNA purification by Poly A selection

RNA was resuspended in nuclease-free H2O to a final concentration of 600 μg/ml and was 
vortexed vigorously. Then, an equal volume of 2X binding solution was added and the 
resulting solution was mixed thoroughly. mRNA was selected with a Poly A selection: Poly 
(A) Purist™ MAG Kit (Ambion AM1922). Briefly, an aliquot of Oligo (dT) was prepared. 
For each RNA sample, an equivalent mass of Oligo(dT) Capture the Oligo(dT) MagBeads 
was used by placing the tube on the magnetic stand and the tube was left on the stand until 
all of the Oligo(dT) MagBeads were arranged inside the tube near the magnet; then the buffer 
was carefully removed by aspiration. Then, Wash Solution 1 was added to the captured Oligo 
(dT) MagBeads at a ratio of 500 μl per mg of beads. The tube was removed from the 
Magnetic Stand and the beads were resuspended by inverting the tube several times. The 
Oligo (dT) MagBeads were recaptured with a magnetic stand and supernatant was discarded. 
The RNA in 1X binding solution was added to the Oligo (dT) MagBeads and the resulting 
solution was mixed by inversion to thoroughly resuspend the RNA. Then the mixtures were 
incubated for five minutes at 65-75°C. After this, the tube was gently rocked for 30-60 
minutes at room temperature. The Oligo (dT) MagBeads were then captured by putting the 
container on a magnetic stand to pull the Oligo (dT) MagBeads to the side of the tube while 
the supernatant was carefully removed by aspiration. The Oligo (dT) MagBeads were washed 
twice with Wash Solution 1 (as described above) and once with Wash Solution 2. 
The poly (A) RNA was eluted by adding 200 µl of pre-warmed RNA storage solution to the 
Oligo (dT) MagBeads and resuspended. The Oligo (dT) MagBeads were captured on a 
magnetic stand and the RNA Storage Solution was transferred to a fresh tube. The RNA 
eluting steps was repeated with the Oligo (dT) MagBeads once more. The RNA was 
precipitated by adding 0.1 volume 5 M NH4 Ac,1 µl Glycogen and 2.5x volumes ethanol to 
the eluted poly(A) RNA and the mixture was left in the in a -70°C freezer for 30 minutes. 
After this, the RNA were recovered by centrifugation at ≥12,000 x g for 20-30 min at 4° C. 
Finally, the RNA was resuspended in pre-warmed RNA storage solution and stored at -70° C.
4.4.5 mRNA purification by ribosomal RNA removal

One hundred μL hybridization buffer, 10 μl RiboMinus™ Probe (15 pmol/μL) and 1-10 μg total RNA were mixed and incubated at 70-75°C for five minutes to denature the RNA. After that, the tube was transferred to a 37°C water bath/heat block and incubated for 30 minutes. The mRNA was selected using RiboMinus™ (Ambion K1500-01). Prior to use, RiboMinus™ Magnetic Beads were thoroughly resuspended using a vortex, and 750 μl of bead suspension was pipetted into a sterile, RNase-free, 1.5-ml micro centrifuge tube. Then the tube with the bead suspension was placed on a magnetic separator for one minute and supernatant was gently aspirated and discarded. After this, 750 μl of sterile, nuclease-free H₂O was added to the beads and the previous preparing procedures were repeated; beads were re-suspended in 750 μl hybridization buffer. For use at a later step, 250 μl of the beads were transferred to a new tube and maintained at 37°C. Finally, the tube with a remaining 500 μl of beads was placed on a magnetic separator for one minute. The supernatant was aspirated and discarded, and the beads were resuspended in 200 μL Hybridization Buffer and kept at 37°C for later use.

After the RNA/RiboMinus™ Probe mixture had cooled to 37°C for 30 minutes, the sample was centrifuged briefly to collect the sample at the bottom of the tube. They were then transferred to the prepared 200μl RiboMinus™ Magnetic beads from the final step in the preparing procedure and mixed well by pipetting several times. After this was done, the mixture was incubated at 37°C for 15 minutes. During incubation, the content was gently mixed occasionally, and was then centrifuged briefly to collect the sample to the bottom of the tube. Lastly, the tube was placed on a magnetic separator for one minute to pellet the rRNA-probe complex, and the supernatant containing RiboMinus™ RNA was aspirated and kept.

The tube with 250 μl of beads from the previous subsection was placed on a magnetic separator for one minute and the supernatant was aspirated and discarded. Subsequently, the supernatant containing RiboMinus™ RNA (~320 μl) was added to the new tube of beads, mixed well by pipetting up and down, incubated at 37°C for 15 minutes and briefly centrifuged to collect the sample to the bottom of the tube. Finally, the tube was placed on a magnetic separator for one minute to pellet the rRNA-probe complex, and the supernatant containing RiboMinus™ RNA was aspirated and transferred to new tube.
The sample was then concentrated according to the same standard protocol mentioned above in section 9.4.5.1.4.

Sample processing was by The University of Liverpool Centre for Genomic Research.

The concentrated RNA samples were sent to the University of Liverpool Centre for Genomic Research where a cDNA library was prepared from the RNA using the Epicentre ScriptSeq v2 RNA-Seq Library Preparation Kit (Catalogue No. SSV21106) and eventually was sequenced using the Illumina Hiseq 2000.

4.4.6 Bioinformatic analyses

The generated data were mapped against the reference genomes using Tophat 2.0.8b and Bowtie 2.1.0, and the results were analysed using R version 2.15.2 and edgeR package version 3.0.4. Reference sequence and annotation for *Rattus norvegicus* and *T. gondii* were obtained using the Ensembl Genome Browser. The results were normalised for differential gene expression analysis, as the difference in library size might cause biased fold change values. The normalisation factor was calculated for handling the difference in library size. In RNA-Seq data analysis, the library size of a sample is the total number of reads of this sample counted across all features.

A set of tables and plots was generated from the analytical results. The main table contains all the information, including the data, annotation and results, and shows the log^2^ counts per million (CPM) mapped reads, log2 fold changes for each contrasts, p-value and adjusted p-value (FDR), raw counts and FPKM values. All further investigations were conducted based on this table (results not shown in this chapter). *T. gondii* and rat genes were separated and analysed independently from each other.

4.4.6.1 Rat gene analysis

The rat genes were divided into four groups based on their differential expression patterns. The first group was the genes that were up-regulated day 3 compared to day 0 (day 3/0). These genes had p-value<0.05 and log^2^ fold change higher than 1. After this, then the down-regulated gene at day 3/0 group was created, These genes had p-value<0.05 and log^2^ fold change lower than -1; the next group was the up-regulated gene at day 6 compared to day 0 (day 6/0). These genes had p-value<0.05 and log^2^ fold change higher than 1; the last group was the down-regulated gene at day 6/0. These genes had a p-value<0.05 and log^2^ fold change lower than -1; these groups of genes were referred to as segregated genes. In addition,
a list that combines all these groups was created; this list contains any gene that had significantly changed and was called the ‘pooled gene list’. Finally, the genes that had a p-value < 0.05 and the log^2 between <1 and >-1 were included in the control gene list.

The genes from the segregated gene list were submitted to PANTHER (http://www.pantherdb.org) to get GO slim annotations of these gene groups. The same genes were then submitted to GOrilla (http://cbl-gorilla.cs.technion.ac.il/) to obtain the GO enrichment analysis. PANTHER was used to interface the gene functions by using a list of genes, and the list was analysed graphically in the terms of sortable functional classes (Mi et al., 2013). GOrilla was used for identifying and visualizing enriched GO terms, by using ranked lists of genes, the search mode that was used in searching for enriched GO terms in a target list of genes compared to a background list of genes (Eden et al., 2009).

In addition, the pooled genes were analysed by DAVID to get the GO functional annotations; genes that were categorised in immunological or neurological GO term and had enrichment scores higher than 1 were further analysed by generating differential expression heat maps for each GO term by MeV. finally, the gene group was submitted to STRING to create pathway analysis maps. However, the neurological pathway analysis contained the gene found by DAVID analysis plus any gene was found in the pooled genes list and in schizophrenia database (www.szgene.org) (Allen et al., 2008).

DAVID is a bioinformatics resources consisting of an integrated biological knowledgebase and analytic tools aimed at systematically extracting biological meaning from large gene/protein lists (Huang da et al., 2009b; Huang da et al., 2009a). STRING (http://stringdb.org) is a database of known and predicted protein interactions. Including direct (physical) and indirect (functional) associations. STRING represents a comprehensive description of cellular mechanisms and function by quantitatively integrates data; these data are derived from different sources: Genomic Context, High-throughput Experiments, (Conserved) Co-expression and Previous Knowledge (Franceschini et al., 2013).

MeV Multiexperiment Viewer (http://www.tm4.org/mev.html) is an analysis tool that analyses algorithms used mostly for microarray data; this tool clusters, visualizes, classifies, and perform statistical analysis and biological theme discovery. MeV represents the analysis result in informative and interrelated displays of expression and annotation data from single or multiple experiments (Eisen et al., 1999).
Figure 4-1: Flow chart showing the procedures used in the RNA-Seq data analysis for rat and *T. gondii*. 
4.4.6.3  

*T. gondii* gene analysis

*T. gondii* genes were divided into four groups based on their differential expression patterns. The first group was the up-regulated gene at day 3 compared to day 0 (day 3/0); these genes were considered significantly differentially expressed because they had p-value<0.05 and log$_2$ fold change higher than 1; after this the down-regulated gene at day 3/0 group included genes with a p-value<0.05 and log$_2$ fold change lower than -1. The next two group were the gene at day 6 compared to day 0 (day 6/0) significantly differentially up and down-regulated, based on the criteria that stated previously. These groups of genes were referred to as segregated genes. In addition, a list that combined all these groups was created; this list contain any gene had significantly changed and was called pooled gene list. Lastly, the genes that have a p-value<0.05 and the log$_2$ between <1 and >-1 were included in a control gene list.

First, a manual search was carried out to find genes involved in differentiation and secretory organelle genes (microneme, rhoptry and dense granules). After this, GO terms grouping was carried out by using R project software, *T. gondii*. The GO term list was downloaded from ToxoDB (http://www.toxodb.org/toxo) (Gajria et al., 2008). The inbuilt GO annotation was used to identify over-represented GO terms in each of the gene sets and only the terms that belong to biological process are listed. The lesser the p-value, the more the likelihood of the GO term occurring in the gene set in comparison to the rest of the genes in *T. gondii*.

4.4.7  

Cell culture for DBH detection

PC12 cells were cultured in six well plates, with 1.25 x 10$^4$ PC12 cells in 2 mL in each well. The following day, the cell cultures were infected with differentiation-induced *T. gondii* (by high pH; shocked). The PC12 cells were counted and the number of parasites used in the infection was estimated to get these parasite/cell ratios: 2, 1; 0.5, 0.25; 0.125 and 0. After five days, the experiment was terminated by freezing down the cell pellet for RNA extraction or NE assays.

4.4.8  

Rat brain sections

A frozen (-80°C) rat’s brain sections were used for RNA extraction; these sections were provided by my lab colleagues (Dr. Greg Bristow and Mohamed Alsaad).

All parasites were cultured in HFF (Human foreskin fibroblast) cells by Dr. Greg Bristow, and then the *T. gondii* parasites were released as described previously from HFF cells in Leeds, counted, and then transported from Leeds to London in Dulbecco’s Modified Eagle’s
Medium (DMEM) solution. To infect three weeks old Lister-hooded rats from Harlan UK Ltd., by Maya Kaushik Dept. of infectious disease epidemiology, School of Public Health, Imperial College London. Rats were euthanized after six months.

The number of *T. gondii* cyst infected rat brain was determined by immunohistochemistry. The rat’s brain sections were lectin stained by Mohammed Alsaad. Brain sections were taken from both an infected and uninfected rat; the infected rats brain was selected to have a high number of cysts (mean = 8.7).

### 4.4.9 Reverse transcriptase PCR and qualitative PCR

RT-PCR and qualitative PCR was performed as described previously, GAPDH primers (Qiagen PPR06557B-200) were used, beside the following primers, on mRNA extracted from PC12 cells or rats brain sections as described previously. The data were presented as ΔΔCt or fold change and was calculated as described previously. Values are expressed using the delta-delta Ct method to derive relative fold change, ΔΔ Ct = ΔCt (DBH sample-GAPDH sample)-ΔCt (DBH control-GAPDH control).

**DBH**

- Forward primers: CCACAATCCGGAATATA
- Reverse primers: GATGCCTGCCTCATTGGG

**ESR**

- Forward primers: CTACGCTGTACGCGACAC
- Reverse primers: CCATTCTGGCGTCGATTG

### 4.4.10 HPLC for monoamines

The catecholamines dopamine, norepinephrine, and epinephrine were measured by HPLC-ED following the method adopted from (Prandovszky et al., 2011). Briefly, cultures were harvested by scraping, pelleted and an aliquot taken for cell counting and normalization. The remaining amount was pelleted again and resuspended in 350 µL of PCA followed by sonication. The mixture was centrifuged at 13000 for 15 minutes in 4°C to remove particulates and an aliquot taken for HPLC analysis. Epinephrine was detected at 3.5 minutes, norepinephrine at 4.5 minutes and dopamine at eight minutes.
4.5 Results

Wild type Pruignaud II strain was used in this experiment and an initiation of differentiation procedure was done on the parasite to mimic the chronic model. An example of a gene that was induced during bradyzoite differentiation is TgAaaH2 (Gaskell et al., 2009).

The experiment was conducted three times to represent three biological replicates. Each replicate was evaluated for total RNA isolated at each time point, poly A selected RNA yield by using Poly (A) Purist™ MAG Kit (Ambion) following the manufacturers’ instructions, and results of RT-PCR.

Samples sent the University of Liverpool Centre for Genomic Research were analysed using a Bioanalyser for the amount and purity of the RNA samples. The QC results shows that these replicate samples were good quality and the amounts were enough, but the r-RNA percentage was 9-15%, therefore rRNA depletion was carried out by using a RiboMinus™ Eukaryote Kit for RNA-Seq (Ambion).

Afterwards, the samples were sent to the University of Liverpool Centre for Genomic Research, where the cDNA libraries were prepared from the submitted RNA using the Epicentre ScriptSeq v2 RNA-Seq Library Preparation Kit (Catalogue No. SSV21106), after this, it was sequenced using Illumina Hiseq 2000. The location of sequence data is:

http://www.cgr.liv.ac.uk:8088/illum/LIMS1239IsraAlsaady_0b6d450fee81c0ff6/

Furthermore, the University of Liverpool Centre for Genomic Research undertook the data analysis. Tophat2.0.8 were used to map the generated data, then these data were aligned with Bowtie2.1.0 after which the results were analysed using R version 2.15.2 and edgeR package version 3.0.4. The combination of the *R. norvegicus* and *T. gondii* annotated genomes were references for mapping sequence and annotation. The source of the references was downloaded from the Ensemble website; afterwards, the results were normalised for differential gene expression analysis. Then, the results from the two biological replicates were combined in the result analysis. The generated result is located at:

http://www.cgr.liv.ac.uk:8088/illum/ID2061_ecf2da8abc694_f26/results1/ for model 1

http://www.cgr.liv.ac.uk:8088/illum/ID2061_ecf2da8abc694_f26/results2/ for model 2
A set of tables and plots was generated from the analytical result (not included here); the information including the annotation, the log2 counts per million (CPM) mapped reads, log2 fold changes for each contrasts, p-value and adjusted p-value (FDR), raw counts and FPKM values were all included in a main table. All the further investigations were conducted based on this table.

### 4.5.1 Host gene analysis

The number of genes that were either up- or down-regulated grouped based on differential expression at each time point (Table 4-2). The results are presented as a fold change (differential expression) between day 3/0 and day 6/0.

<table>
<thead>
<tr>
<th></th>
<th>&gt;2 fold change</th>
<th>&gt;4 fold change</th>
<th>&gt;8 fold change</th>
</tr>
</thead>
<tbody>
<tr>
<td>Up-regulated day 3/0</td>
<td>180</td>
<td>117</td>
<td>61</td>
</tr>
<tr>
<td>Down-regulated day 3/0</td>
<td>710</td>
<td>544</td>
<td>312</td>
</tr>
<tr>
<td>Up-regulated day 6/0</td>
<td>643</td>
<td>425</td>
<td>286</td>
</tr>
<tr>
<td>Down-regulated day 6/0</td>
<td>192</td>
<td>131</td>
<td>98</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td><strong>1725</strong></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**Table 4-2:** The number of differentially expressed host genes at different time points.

Based on previous observations of changes in dopamine metabolism (Prandovsky et al., 2011), the investigation of *T. gondii*’s effect on catecholamine metabolism is one of the main aims of this experiment and, therefore, the differential expression for genes involved in catecholamine metabolism were analysed.

The differential expression of the genes that are involved in catecholamine metabolism. DBH, MaoB and PAH were down-regulated (Table 4-3), while Drd2 and Moxd2 were up regulated at day 6/0.
<table>
<thead>
<tr>
<th>Gene name</th>
<th>Description</th>
<th>Fold change at day 3/0</th>
<th>Fold change at day 6/0</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dopamine beta-hydroxylase</td>
<td>Dbh</td>
<td>-1.30</td>
<td>-244</td>
</tr>
<tr>
<td>Monoamine oxidase B</td>
<td>MaoB</td>
<td>-2.75</td>
<td>-3.65</td>
</tr>
<tr>
<td>Phenylalanine hydroxylase</td>
<td>PAH</td>
<td>-1.25</td>
<td>-2.58</td>
</tr>
<tr>
<td>Dopamine receptor D3</td>
<td>Drd3</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Dopamine receptor D5</td>
<td>Drd5</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Dopamine receptor D4</td>
<td>Drd4</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Aromatic L-amino acid decarboxylase</td>
<td>AADC</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Monoamine oxidase A</td>
<td>MaoA</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Tyrosine hydroxylase</td>
<td>Th</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Catechol-O-methyltransferase</td>
<td>Comt</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Solute carrier family 6</td>
<td>Slc6a3</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Dopamine receptor D1</td>
<td>Drd1a</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Solute carrier family 18</td>
<td>Slc18a2</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Dopamine receptor D2</td>
<td>Drd2</td>
<td>3.58</td>
<td>6.59</td>
</tr>
<tr>
<td>Monooxygenase, DBH-like 2</td>
<td>Moxd2</td>
<td>2.21</td>
<td>8.88</td>
</tr>
</tbody>
</table>

**Table 4-3:** Differential expression of genes involved in catecholamine metabolism in bradyzoite infected dopaminergic cells.

The segregated gene lists were submitted to PANTHER to create a visual presentation of the GO slim classification of the biological functions of these genes. The genes that did not significantly change were also submitted to this analysis and presented as a control. The pie chart in (Figure 4-2) shows the major biological function terms and the proportion of genes
that were classified under each term. Comparing between these charts shows that the infection modulates different functions in the PC12 cells such as metabolic process, response to stimulus and immune response. Moreover, these changes’ vary between times points indicates distinctive alterations along with the parasite differentiation.
**Figure 4-2:** Biological functional categories (GO slim) of rat genes that have changed between the different time points. In addition, significantly segregated data were submitted to GOrilla to perform GO-enriched analysis for each time point. The enrichment analysis found which GO terms were over-represented (or under-represented) using annotations for that gene set.

The analysis (Table 4-4) shows that at day 3/0 the up-regulated genes under specific GO term classifications with the lowest p-values were: positive regulation for catecholamine secretion and positive regulation of amine transport or regulation of epinephrine secretion. These GO terms of biological function might be involved with increased production of the catecholamine by the parasite. Metabolic functions as well as regulation of acute inflammatory response and T cell selection are immune functions that were down-regulated at day 0/3.

Moreover, (Table 4-5) list the up regulated genes GO terms at day 6, this list include more specific immune responses e.g., response to protozoan, response to chemical, response to...
external stimulus, response to stimulus, defines response, response to lipopolysaccharide, cellular hormone metabolic process, regulation of toll-like receptor 9 signalling pathway, cellular response to high density lipoprotein particle stimulus, cellular response to interleukin-6 and defence response to protozoan. Furthermore, neurological and signalling functions were up-regulated, like the cell surface receptor signalling pathway, response to external stimulus, detection of stimulus, cellular response to biotic stimulus, and response to stimulus, signal transduction, neuron-neuron synaptic transmission, signalling and single organism signalling. Gene clusters with the lowest p-values were involved in ion transmembrane transport and chloride transmembrane transport. In contrast, multiple organismal processes and positive regulation of neuron differentiation GO terms were found to be significantly enriched by GO-enriched analyses for the genes that were down-regulated at day6/0 (Table 4-7).

Genes associated with olfactory function, G-protein coupled receptor signalling pathway, detection of stimulus involved in sensory perception, detection of chemical stimulus involved in sensory perception and detection of stimulus were down-regulated at day 3/0 (Table 4-6), while those involved in sensory organ development and olfactory pit development were down-regulated at day 6/0 (Table 4-7).
<table>
<thead>
<tr>
<th>GO term</th>
<th>Description</th>
<th>P-value</th>
<th>FDR q-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>GO:0033605</td>
<td>positive regulation of catecholamine secretion</td>
<td>1.46E-06</td>
<td>1.76E-02</td>
</tr>
<tr>
<td>GO:0050433</td>
<td>regulation of catecholamine secretion</td>
<td>2.37E-06</td>
<td>1.43E-02</td>
</tr>
<tr>
<td>GO:0051954</td>
<td>positive regulation of amine transport</td>
<td>2.73E-05</td>
<td>1.10E-01</td>
</tr>
<tr>
<td>GO:0051952</td>
<td>regulation of amine transport</td>
<td>4.57E-05</td>
<td>1.38E-01</td>
</tr>
<tr>
<td>GO:0014060</td>
<td>regulation of epinephrine secretion</td>
<td>7.05E-04</td>
<td>1.00E+00</td>
</tr>
<tr>
<td>GO:0051047</td>
<td>positive regulation of secretion</td>
<td>8.92E-04</td>
<td>1.00E+00</td>
</tr>
<tr>
<td>GO:0002676</td>
<td>regulation of chronic inflammatory response</td>
<td>9.36E-04</td>
<td>1.00E+00</td>
</tr>
<tr>
<td>GO:0006700</td>
<td>C21-steroid hormone biosynthetic process</td>
<td>9.36E-04</td>
<td>1.00E+00</td>
</tr>
</tbody>
</table>

**Table 4-4:** Classifications enriched in GO analysis of rat genes that were up-regulated at day 3 using GOrilla.
<table>
<thead>
<tr>
<th>GO term</th>
<th>Description</th>
<th>P-value</th>
<th>FDR q-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>GO:0034220</td>
<td>ion transmembrane transport</td>
<td>2.08E-05</td>
<td>0.251</td>
</tr>
<tr>
<td>GO:1902476</td>
<td>chloride transmembrane transport</td>
<td>2.45E-05</td>
<td>0.148</td>
</tr>
<tr>
<td>GO:0007166</td>
<td>cell surface receptor signalling pathway</td>
<td>0.000108</td>
<td>0.435</td>
</tr>
<tr>
<td>GO:0001562</td>
<td>response to protozoan</td>
<td>0.000204</td>
<td>0.352</td>
</tr>
<tr>
<td>GO:0042221</td>
<td>response to chemical</td>
<td>0.000285</td>
<td>0.431</td>
</tr>
<tr>
<td>GO:0099605</td>
<td>response to external stimulus</td>
<td>0.000376</td>
<td>0.505</td>
</tr>
<tr>
<td>GO:0055090</td>
<td>acylglycerol homeostasis</td>
<td>0.000398</td>
<td>0.482</td>
</tr>
<tr>
<td>GO:0070328</td>
<td>triglyceride homeostasis</td>
<td>0.000398</td>
<td>0.438</td>
</tr>
<tr>
<td>GO:0051606</td>
<td>detection of stimulus</td>
<td>0.000432</td>
<td>0.436</td>
</tr>
<tr>
<td>GO:0071216</td>
<td>cellular response to biotic stimulus</td>
<td>0.000463</td>
<td>0.43</td>
</tr>
<tr>
<td>GO:0050896</td>
<td>response to stimulus</td>
<td>0.000506</td>
<td>0.437</td>
</tr>
<tr>
<td>GO:0045765</td>
<td>regulation of angiogenesis</td>
<td>0.000539</td>
<td>0.435</td>
</tr>
<tr>
<td>GO:0007165</td>
<td>signal transduction</td>
<td>0.000558</td>
<td>0.422</td>
</tr>
<tr>
<td>GO:0006952</td>
<td>defines response</td>
<td>0.000589</td>
<td>0.419</td>
</tr>
<tr>
<td>GO:0007270</td>
<td>neuron-neuron synaptic transmission</td>
<td>0.000606</td>
<td>0.407</td>
</tr>
<tr>
<td>GO:0070092</td>
<td>regulation of glucagon secretion</td>
<td>0.00064</td>
<td>0.407</td>
</tr>
<tr>
<td>GO:0032496</td>
<td>response to lipopolysaccharide</td>
<td>0.000661</td>
<td>0.399</td>
</tr>
<tr>
<td>GO:0034754</td>
<td>cellular hormone metabolic process</td>
<td>0.00072</td>
<td>0.415</td>
</tr>
<tr>
<td>GO:0071403</td>
<td>cellular response to high density lipoprotein particle stimulus</td>
<td>0.000734</td>
<td>0.404</td>
</tr>
<tr>
<td>GO:0034163</td>
<td>regulation of toll-like receptor 9 signalling pathway</td>
<td>0.000734</td>
<td>0.386</td>
</tr>
<tr>
<td>GO:0023052</td>
<td>signalling</td>
<td>0.000748</td>
<td>0.362</td>
</tr>
<tr>
<td>GO:0044700</td>
<td>single organism signalling</td>
<td>0.000748</td>
<td>0.377</td>
</tr>
<tr>
<td>GO:0071354</td>
<td>cellular response to interleukin-6</td>
<td>0.00075</td>
<td>0.349</td>
</tr>
<tr>
<td>GO:0055085</td>
<td>transmembrane transport</td>
<td>0.000793</td>
<td>0.355</td>
</tr>
<tr>
<td>-------------</td>
<td>-------------------------</td>
<td>----------</td>
<td>-------</td>
</tr>
<tr>
<td>GO:0040020</td>
<td>regulation of meiosis</td>
<td>0.000842</td>
<td>0.364</td>
</tr>
<tr>
<td>GO:0042832</td>
<td>defense response to protozoan</td>
<td>0.00096</td>
<td>0.4</td>
</tr>
</tbody>
</table>

**Table 4-5:** Classes of genes enriched in GO analysis of rat genes that were up-regulated at day 6 using GOrilla.
<table>
<thead>
<tr>
<th>GO term</th>
<th>Description</th>
<th>P-value</th>
<th>FDR q-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>GO:0007186</td>
<td>G-protein coupled receptor signalling pathway</td>
<td>2.61E-08</td>
<td>0.000315</td>
</tr>
<tr>
<td>GO:0050907</td>
<td>detection of chemical stimulus involved in sensory perception</td>
<td>4.63E-07</td>
<td>0.0028</td>
</tr>
<tr>
<td>GO:0050906</td>
<td>detection of stimulus involved in sensory perception</td>
<td>8.88E-07</td>
<td>0.00358</td>
</tr>
<tr>
<td>GO:0007166</td>
<td>cell surface receptor signalling pathway</td>
<td>1.37E-06</td>
<td>0.00276</td>
</tr>
<tr>
<td>GO:0051606</td>
<td>detection of stimulus</td>
<td>4.48E-06</td>
<td>0.00774</td>
</tr>
<tr>
<td>GO:0007165</td>
<td>signal transduction</td>
<td>0.000306</td>
<td>0.462</td>
</tr>
<tr>
<td>GO:0045058</td>
<td>T cell selection</td>
<td>0.000521</td>
<td>0.7</td>
</tr>
<tr>
<td>GO:0002673</td>
<td>regulation of acute inflammatory response</td>
<td>0.000543</td>
<td>0.657</td>
</tr>
<tr>
<td>GO:0009092</td>
<td>homoserine metabolic process</td>
<td>0.000641</td>
<td>0.597</td>
</tr>
<tr>
<td>GO:0019344</td>
<td>cysteine biosynthetic process</td>
<td>0.000641</td>
<td>0.705</td>
</tr>
<tr>
<td>GO:0019346</td>
<td>transsulfuration</td>
<td>0.000641</td>
<td>0.646</td>
</tr>
<tr>
<td>GO:0007155</td>
<td>cell adhesion</td>
<td>0.000718</td>
<td>0.621</td>
</tr>
<tr>
<td>GO:0022610</td>
<td>biological adhesion</td>
<td>0.000802</td>
<td>0.646</td>
</tr>
</tbody>
</table>

**Table 4-6**: GO enriched classes of rat genes that were down-regulated at day 3 using GOrilla
<table>
<thead>
<tr>
<th>GO term</th>
<th>Description</th>
<th>P-value</th>
<th>FDR q-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>GO:0007423</td>
<td>sensory organ development</td>
<td>9.04E-05</td>
<td>0.219</td>
</tr>
<tr>
<td>GO:0007166</td>
<td>cell surface receptor signalling pathway</td>
<td>5.58E-05</td>
<td>0.338</td>
</tr>
<tr>
<td>GO:0032501</td>
<td>multicellular organismal process</td>
<td>5.38E-05</td>
<td>0.651</td>
</tr>
<tr>
<td>GO:0045666</td>
<td>positive regulation of neuron differentiation</td>
<td>0.000989</td>
<td>0.797</td>
</tr>
<tr>
<td>GO:0007165</td>
<td>signal transduction</td>
<td>0.000868</td>
<td>0.75</td>
</tr>
<tr>
<td>GO:0001763</td>
<td>morphogenesis of a branching structure</td>
<td>0.00071</td>
<td>0.66</td>
</tr>
<tr>
<td>GO:0061138</td>
<td>morphogenesis of a branching epithelium</td>
<td>0.000566</td>
<td>0.571</td>
</tr>
<tr>
<td>GO:2000026</td>
<td>regulation of multicellular organismal development</td>
<td>0.00039</td>
<td>0.429</td>
</tr>
<tr>
<td>GO:0050678</td>
<td>regulation of epithelial cell proliferation</td>
<td>0.00029</td>
<td>0.351</td>
</tr>
<tr>
<td>GO:0048754</td>
<td>branching morphogenesis of an epithelial tube</td>
<td>0.000269</td>
<td>0.361</td>
</tr>
<tr>
<td>GO:0060166</td>
<td>olfactory pit development</td>
<td>0.000249</td>
<td>0.376</td>
</tr>
<tr>
<td>GO:0001654</td>
<td>eye development</td>
<td>0.000167</td>
<td>0.288</td>
</tr>
<tr>
<td>GO:0051239</td>
<td>regulation of multicellular organismal process</td>
<td>0.000129</td>
<td>0.26</td>
</tr>
<tr>
<td>GO:0044707</td>
<td>single-multicellular organism process</td>
<td>0.000076</td>
<td>0.23</td>
</tr>
<tr>
<td>GO:0014070</td>
<td>response to organic cyclic compound</td>
<td>0.000075</td>
<td>0.302</td>
</tr>
</tbody>
</table>

**Table 4-7:** GO enrichment analysis gene classes of rat genes that were down-regulated at day 6 using GOrilla.

The pooled genes were submitted to DAVID to categorise the genes according to biological functions. The genes were categorised to the following neurological GO terms: cell-cell signalling, neurodevelopmental, behaviour and neurotransmitter NMDR with enrichment scores of 4.25, 2.36, 1.24 and 1, respectively. The list of genes under each GO term was submitted to MeV to create a heat map. The genes in the lists were submitted along with log Fold Change (log FC) for each gene at day 0/3 and 6/0 and the heat maps were created according to the log FC of these genes.
These figures (Figure 4-3) show that the infection of differentiated PC12 cells infected with induced *T. gondii* had significantly modulated expression of genes involved in cell-cell signalling.

While most of the genes in the behaviour category were up-regulated, the DBH gene was down-regulated. The gene expression patterns were specific and changed throughout the infection as *T. gondii* differentiated from tachyzoite to bradyzoite stage.
Figure 4-3: Heat maps of neurological function genes modulated by infection.
To examine the relationship between the differentially expressed genes that fall under the neurological GO term category, these genes were submitted to STRING pathway analysis including the genes that were significantly changed and those associated with schizophrenia. In the resulting figure (Figure 4-4), each protein is represented by a coloured circle and the protein name, and the lines between the proteins represent the predicted interaction. The thickness of the lines is proportional to the strength of the associations. The small box in the lower right highlights the dopamine metabolism and receptors genes.

The highlighted group of genes (box) is involved in dopamine metabolism and GABA receptors; the figure shows a strong association between DBH, MaoB, SAT1, Aanat and Aldah1a3, which are genes involved in dopamine metabolism. Cacng4, Grik4, Gria4, Gabrb2, Gabrg2 and Gabrg3 are involved in the formation of GABA receptors. Meanwhile, other genes form a large cluster and, in this cluster, there is a strong association found between UQrc1 and Cox6a2; Bmp7 and Bmpr1b; Gdf9, Fgf9, Fgf2, Kitlg, CXCL12, LIF, CCL2, CCL5 and Il12b; Alexo12 and Alexo12e; Epha7, Efna5 and Epha4; and, finally, between Vav3, Syk and Apbb.
**Figure 4-4:** Network analysis of neurological genes differentially expressed in catecholaminergic cells with infection. The pooled genes were submitted to DAVID (http://david.abcc.ncifcrf.gov/) to categorise them according to biological functions. In (Figure 4-5) the genes were categorised according to the following immunological GO terms: cytokines, chemotaxis, immunoglobulin-like and T-cell selection with enrichment scores of 2.16, 1.74, 1.5 and 1.13, respectively. After this, MeV was used to create a heat map for each gene list for each GO term. The heat maps were created according to log Fold Change (log FC) for each gene at day 0/3 and 6/0.
**Figure 4-5**: Heat maps of genes involved in immunological function modulated by infection.

Similar to the neurological genes analysis, immunological genes were also submitted to STRING. The resulting figure shows a visual pathway analysis for the protein that was classified as immunological by DAVID. In figure (Figure 4-6), Thicker lines are indicative of greater evidence of association. All proteins are labelled by their gene name. The diagram shows the interaction between genes that are involved in the immune response to *T. gondii* infection in these neuron-like cells.
Figure 4-6: Network analysis of immunological genes differentially expressed in catecholaminergic cells with infection
4.5.1.1 Regulation of DBH during infection

As the most significant decrease observed in the RNA-Seq analysis is the down-regulation of DBH mRNA, therefore this finding will further confirmed by measuring mRNA levels and NE levels.

First, RT-PCR using DBH primers of samples from *T. gondii*-infected PC12 cells with different parasite/cell ratios was performed. The result (Figure 4-7) showed that DBH levels were decreased in infected PC12 cells as found with RNA sequencing of infected NGF-differentiated cells. It is a dose-dependant reduction, with an inverse relationship between the number of parasites and the reduction in DBH mRNA expression.

![Figure 4-7: Expression of DBH in *T. gondii*-infected PC12 cells. Furthermore, as a control to identify the effect of NGF on DBH expression, qPCR was done on uninfected NGF-differentiated PC12 cells at days 0, 3 and 6. (Figure 4-8) Quantitative RT-PCR with DBH primers for mRNA of NGF-differentiated uninfected PC12 cells showed no change in the expression of DBH mRNA because of NGF differentiation, confirming that the initially observed DBH expression was caused by *T. gondii* infection and not by NGF differentiation.](image-url)
Figure 4-8: DBH mRNA expression in NGF differentiated PC12 cells over time

The DBH down-regulation was examined *in vivo* by detecting the mRNA of DBH in infected rat brain RNA. qPCR was performed on RNA purified from frozen sections from infected and uninfected rat brains. A Quantitative PCR done on mRNA from rats’ brain homogenate showed a reduction in DBH in the infected male rat’s brain, while female brains did not show a detectable change in DBH expression, indicating that DBH reduction is gender specific *in vivo* (Figure 4-9). With this in mind, the oestrogen receptors ESR in the female rat brains were measured to investigate any link between oestrogen levels and DBH expression. Although no difference in ESR between female rats was observed, there was not a clear variation because of the high variability in the experiment.
Rat brain samples

Fold change compared to the uninfected control

P = 0.0003

ΔΔCT

C1 C2 F1 F2 F3 F4 F5

Rat brain samples
Figure 4-9: Expression in rat brain of DBH and ESR. As DBH is responsible for norepinephrine (NE) and epinephrine (E) synthesis, experiments were performed to monitor the effect of infection on NE and E levels. Down-regulation in DBH mRNA is expected to be reflected in decreases in NE and E in T. gondii-infected PC12 cells. Norepinephrine and epinephrine production was measured by HPLC-ED. (Figure 4-10) revealed a reduction in NE and E production whilst DA increases T. gondii reduces the production of NE and E by almost 90%, for NE (ANOVA, p value=1.91E-05) and for E (ANOVA, p value=2.02E-05). In addition, it was observed that an increase in the number of the parasite resulted in a decrease in the production of both NE and E in a dose-dependent manner.
Figure 4-10: Norepinephrine and epinephrine production in *T. gondii*-infected PC12 cells.
### 4.5.3 *T. gondii* gene analysis

The expression of housekeeping, bradyzoite-specific, and tachyzoite-specific genes were used as controls to monitor the differentiation of *T. gondii* to bradyzoites. *T. gondii* actin, tubulin and GAPDH served as housekeeping genes, whereas bradyzoite-specific genes are BAG1, ENO1, and SAG4, and tachyzoite-specific genes are MIC1 and SAG1.

The results (Table 4-8) shows housekeeping genes and tachyzoite genes commensurate with increased numbers of parasites in the infected culture. In addition, elevation of bradyzoite-specific genes confirming the bradyzoite differentiation was observed.

<table>
<thead>
<tr>
<th>Gene ID</th>
<th>Description</th>
<th>Fold change day6/3</th>
</tr>
</thead>
<tbody>
<tr>
<td>TGME49_009030</td>
<td>Actin</td>
<td>2.1</td>
</tr>
<tr>
<td>TGME49_116400</td>
<td>α-tubulin</td>
<td>2.1</td>
</tr>
<tr>
<td>TGME49_089690</td>
<td>GAPDH</td>
<td>1.9</td>
</tr>
<tr>
<td>TGME49_059020</td>
<td>HSP30/BAG1</td>
<td>6.61</td>
</tr>
<tr>
<td>TGME49_068860</td>
<td>ENO1</td>
<td>7</td>
</tr>
<tr>
<td>TGME49_080570</td>
<td>SAG4</td>
<td>5.1</td>
</tr>
<tr>
<td>TGME49_091890</td>
<td>MIC1</td>
<td>2.0</td>
</tr>
<tr>
<td>TGME49_033460</td>
<td>SAG1</td>
<td>2.1</td>
</tr>
</tbody>
</table>

**Table 4-8:** Fold change between day 6 and day 3 of infection for previously characterized *T. gondii* genes.
The table below (Table 4-9) presents the number of *T. gondii* genes that were either up or down-regulated at different time points.

<table>
<thead>
<tr>
<th></th>
<th>&gt;2 fold change</th>
<th>&gt;4 fold change</th>
<th>&gt;8 fold change</th>
</tr>
</thead>
<tbody>
<tr>
<td>up-regulated day 3/0</td>
<td>274</td>
<td>229</td>
<td>147</td>
</tr>
<tr>
<td>down-regulated day 3/0</td>
<td>133</td>
<td>121</td>
<td>89</td>
</tr>
<tr>
<td>up-regulated day 6/0</td>
<td>494</td>
<td>324</td>
<td>171</td>
</tr>
<tr>
<td>down-regulated day 6/0</td>
<td>131</td>
<td>120</td>
<td>79</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td><strong>1,032</strong></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**Table 4-9:** The number of *T. gondii* genes differentially expressed at different time points.

*T. gondii* secretory proteins that were differentially expressed during the experiment are presented in the following table.

Secretory proteins (dense granules, micronemes and rhoptry) were differentially expressed throughout the experiment (Table 4-10). Other secretory proteins were expressed during the experiment, i.e., ROP16 and ROP18, but not included in the table because no difference in their expression was found between different time points.

Dense granules proteins show a similar differential expression profile, the expression of these proteins increases at day 6/0, while MIC13 was the microneme that had the highest fold change during the experiment. ROP11, ROP15 and ROP41 were the most up-regulated at day 6/0.

<table>
<thead>
<tr>
<th>Gene ID</th>
<th>Description</th>
<th>Fold change day 3/0</th>
<th>Fold change day 6/0</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gene ID</td>
<td>Protein</td>
<td>Log2 Fold</td>
<td>Log2 Odds</td>
</tr>
<tr>
<td>----------------</td>
<td>----------</td>
<td>-----------</td>
<td>-----------</td>
</tr>
<tr>
<td>TGME49_091890</td>
<td>MIC1</td>
<td>1.25</td>
<td>2.50</td>
</tr>
<tr>
<td>TGME49_119560</td>
<td>MIC3</td>
<td>1.07</td>
<td>2.71</td>
</tr>
<tr>
<td>TGME49_008030</td>
<td>MIC4</td>
<td>-1.58</td>
<td>2.43</td>
</tr>
<tr>
<td>TGME49_018520</td>
<td>MIC6</td>
<td>1.53</td>
<td>2.30</td>
</tr>
<tr>
<td>TGME49_061780</td>
<td>MIC7</td>
<td>2.09</td>
<td>3.42</td>
</tr>
<tr>
<td>TGME49_004530</td>
<td>MIC11</td>
<td>-1.24</td>
<td>2.02</td>
</tr>
<tr>
<td>TGME49_060190</td>
<td>MIC13</td>
<td>4.88</td>
<td>13.5</td>
</tr>
<tr>
<td>TGME49_054430</td>
<td>MIC Putative</td>
<td>-1.19</td>
<td>-10.9</td>
</tr>
<tr>
<td>TGME49_070250</td>
<td>GRA1</td>
<td>1.12</td>
<td>2.05</td>
</tr>
<tr>
<td>TGME49_027620</td>
<td>GRA2</td>
<td>1.40</td>
<td>2.05</td>
</tr>
<tr>
<td>TGME49_027280</td>
<td>GRA3</td>
<td>1.50</td>
<td>2.44</td>
</tr>
<tr>
<td>TGME49_086450</td>
<td>GRA5</td>
<td>1.19</td>
<td>2.58</td>
</tr>
<tr>
<td>TGME49_003310</td>
<td>GRA7</td>
<td>1.86</td>
<td>2.07</td>
</tr>
<tr>
<td>TGME49_054720</td>
<td>GRA8</td>
<td>-1.41</td>
<td>2.50</td>
</tr>
<tr>
<td>TGME49_109590</td>
<td>ROP1</td>
<td>1.37</td>
<td>2.34</td>
</tr>
<tr>
<td>TGME49_108080</td>
<td>ROP5</td>
<td>1.20</td>
<td>3.19</td>
</tr>
<tr>
<td>TGME49_095110</td>
<td>ROP7</td>
<td>1.08</td>
<td>2.48</td>
</tr>
<tr>
<td>TGME49_015780</td>
<td>ROP8</td>
<td>-1.03</td>
<td>2.61</td>
</tr>
<tr>
<td>TGME49_027810</td>
<td>ROP11</td>
<td>-1.16</td>
<td>6.66</td>
</tr>
<tr>
<td>TGME49_011290</td>
<td>ROP15</td>
<td>-1.12</td>
<td>5.09</td>
</tr>
<tr>
<td>TGME49_062050</td>
<td>ROP39</td>
<td>1.05</td>
<td>2.51</td>
</tr>
<tr>
<td>TGME49_066100</td>
<td>ROP41</td>
<td>4.54</td>
<td>14.5</td>
</tr>
</tbody>
</table>

**Table 4-10**: Secretory *T. gondii* proteins differentially expressed in the RNA-Seq experiment.

After this, GO term enrichment grouping was carried out using R project software of each group of segregated genes.

The analysis shows (Tables 4-11, 12, 13, 14) that at day 3/0 the up-regulated genes classified under GO terms that had the higher significance (lowest P value) were translation and gene
expression. Moreover, pentose-phosphate shunt, NADPH regeneration, NADP metabolic process and pyridine nucleotide metabolic process were the GO terms that down-regulated genes at day 3/0 were grouped into.

At day 6/0, the up-regulated genes were grouped into several groups, the most significant of which were translation, cellular macromolecule biosynthetic process, macromolecule biosynthetic process and cellular protein metabolic process. In contrast, the down-regulated genes were grouped into ion transport; cations transport ATP catabolic process and establishment of localization.
<table>
<thead>
<tr>
<th>GO Term</th>
<th>Description</th>
<th>P-value</th>
<th>Fold enrichment</th>
</tr>
</thead>
<tbody>
<tr>
<td>GO:0006412</td>
<td>translation</td>
<td>0.0018</td>
<td>2.26</td>
</tr>
<tr>
<td>GO:0010467</td>
<td>gene expression</td>
<td>0.002</td>
<td>1.9</td>
</tr>
<tr>
<td>GO:0009101</td>
<td>glycoprotein biosynthetic process</td>
<td>0.02</td>
<td>11.16</td>
</tr>
<tr>
<td>GO:0043413</td>
<td>macromolecule glycosylation</td>
<td>0.02</td>
<td>11.16</td>
</tr>
<tr>
<td>GO:0006486</td>
<td>protein glycosylation</td>
<td>0.022</td>
<td>11.16</td>
</tr>
<tr>
<td>GO:0009100</td>
<td>glycoprotein metabolic process</td>
<td>0.022</td>
<td>11.16</td>
</tr>
<tr>
<td>GO:0034645</td>
<td>cellular macromolecule biosynthetic process</td>
<td>0.028</td>
<td>1.65</td>
</tr>
<tr>
<td>GO:0070085</td>
<td>glycosylation</td>
<td>0.029</td>
<td>9.3</td>
</tr>
<tr>
<td>GO:0009059</td>
<td>macromolecule biosynthetic process</td>
<td>0.029</td>
<td>1.64</td>
</tr>
</tbody>
</table>

**Table 4-11:** GO clusters over-represented for *T. gondii* gene up-regulation at day 3 of infection.
<table>
<thead>
<tr>
<th>GO term</th>
<th>Description</th>
<th>P-value</th>
<th>Fold enrichment</th>
</tr>
</thead>
<tbody>
<tr>
<td>GO:0006740</td>
<td>NADPH regeneration</td>
<td>0.008</td>
<td>17.56</td>
</tr>
<tr>
<td>GO:0006098</td>
<td>pentose-phosphate shunt</td>
<td>0.008</td>
<td>17.56</td>
</tr>
<tr>
<td>GO:0006739</td>
<td>NADP metabolic process</td>
<td>0.010</td>
<td>15.36</td>
</tr>
<tr>
<td>GO:0046496</td>
<td>nicotinamide nucleotide metabolic process</td>
<td>0.013</td>
<td>13.66</td>
</tr>
<tr>
<td>GO:0019362</td>
<td>pyridine nucleotide metabolic process</td>
<td>0.013</td>
<td>13.66</td>
</tr>
<tr>
<td>GO:0072524</td>
<td>pyridine-containing compound metabolic process</td>
<td>0.013</td>
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**Table 4-12:** GO clusters over-represented for *T. gondii* gene down-regulation at day 3 of infection.
<table>
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<th>Go Term</th>
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<td>GO:0044267</td>
<td>cellular protein metabolic process</td>
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<td>GO:0051276</td>
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**Table 4-13:** GO clusters over-represented for *T. gondii* gene up-regulation at day 6 of infection.
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<th>GO Term</th>
<th>Description</th>
<th>P-value</th>
<th>Fold enrichment</th>
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<td>cation transport</td>
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<td>4.5</td>
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<td>GO:0051234</td>
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<td>GO:0006304</td>
<td>DNA modification</td>
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**Table 4-14**: GO clusters over-represented for *T. gondii* gene down-regulation at day 6 of infection.
4.6 Discussion

4.6.1 Rat gene analysis and verification

The sequencing of RNA performed on the transcriptome of the *T. gondii*-infected PC12 cells and the *T. gondii* transcriptome during infection revealed numerous genes significantly altered in expression. NGF differentiated PC12 cells infected with Prugniard strain to mimic *T. gondii* chronic infection is the first attempt to analyse gene expression in a model of chronic neural infection.

Considerable regulation of genes involved in the catecholamine pathway were differentially expressed with infection (Table 4-3). DBH, MaoB and PAH were down-regulated with DBH exhibiting the extreme change in infected PC12 cells. Both DBH and MaoB encode enzymes that catabolize dopamine indicating that the parasite blocked catabolism of dopamine during infection of dopaminergic cells. This could help maintain increased levels of dopamine. Meanwhile, Drd2 and Moxd2 were up-regulated with the Moxd2 gene having similarity to the DBH gene. Moxd2 is strongly expressed in the medial olfactory epithelium (Su et al., 2004), raising the possibility of its overexpression being involved in olfactory function alterations. Moxd2 up-regulation could partly compensate for the down-regulation of DBH although further studies of the function of the Moxd2 gene product are needed.

The RNA-Seq experiment indicated that *T. gondii* infection caused a massive reduction in dopamine beta hydroxylase (DBH) mRNA expression. DBH synthesises norepinephrine (NE) from dopamine as a copper type-II, ascorbate dependent monooxygenase [EC 1.14.17.1] found in the noradrenergic neurons (Weinshilboum, 1978).

DBH is found within the synaptic vesicles of central and peripheral noradrenergic neurons. DBH is simultaneously released into the extra-cellular space during the secretion of the synthesized NE (Dunnette and Weinshilboum, 1976). Therefore, DBH can be measured easily in the cerebrospinal fluid (from the central nervous system) and plasma (from the peripheral nervous system). DBH activity in cerebrospinal fluid and plasma are controlled genetically (Goldin et al., 1982), although it varies among populations widely (Weinshilboum et al., 1973).

Down-regulation of DBH leads to a huge reduction in the production of norepinephrine (NE) and epinephrine (E). Epinephrine is synthesised by methylation of the primary amine of norepinephrine by phenylethanolamine N-methyltransferase (PNMT) (Bulbring and Burn, 1949). NE is synthesised mainly in the locus coeruleus (LC) in the brain. The LC
preferentially projects to the thalamus, hippocampus, the frontal and the entorhinal cortices, as well as to a minor extent most other brain regions (Freedman et al., 1975).

Norepinephrine and epinephrine are adrenergic catecholamines that play a major role in different brain functions including behavioural and physiologic processes. Several reviews had already covered the role of the LC noradrenergic system in cognitive processes, arousal and wakefulness (Berridge et al., 2012; Berridge and Waterhouse, 2003; Ramos and Arnsten, 2007; Robbins and Arnsten, 2009; Sara, 2009). Apart from its decrease with aging, changes in NE transmission has been related to major brain disorders in psychiatry (depression, attention deficit disorder, Tourette's, psychosis, post-traumatic stress disorder, epilepsy, Parkinson's, Alzheimer's disease (AD) and sleep) (Feinstein et al., 2002; Szot, 2012).

Previous studies on DBH knockouts (KO, mutant) animals have shown that DBH -KO rats were unable to synthesis both NE and E (Murchison et al., 2004). Mice that lack NE were shown to be susceptible to seizures (Szot et al., 1999) (Weinshenker et al., 2001) and exhibit reduced contextual-fear but intact cued-fear, indicating that adrenergic signalling is important for the retrieval of intermediate-term contextual and spatial memories, but is not essential for the consolidation or retrieval of emotional memories in general (Murchison et al., 2004). Finally, another study reported that the loss of NE itself impaired synaptic plasticity and cognitive performance (Hammerschmidt et al., 2013).

The DBH down-regulation was verified by RT-PCR of samples from infected PC12 cells that were infected cells with increasing parasite/cell ratios. The results showed that *T. gondii* infection decreases DBH expression with an increase with increasing dose supporting that the down-regulation of DBH relative to other host genes is directly caused by *T. gondii* infection.

In order to confirm that the down-regulation of DBH expression leads to a decrease in epinephrine and norepinephrine production, norepinephrine and epinephrine were measured from *T. gondii*-infected PC12 cells, where the cells were infected at increasing parasite/cell ratios. The results showed that *T. gondii* decreases norepinephrine and epinephrine production in infected cells, suggesting that the parasite reduced norepinephrine and epinephrine to mediate changes in host behaviour, providing more details of the behaviour-change mechanism. To confirm that norepinephrine and epinephrine production are reduced during infection *in vivo*, DBH mRNA levels were detected by qPCR from *T. gondii*-infected rats’ brains. The results showed DBH down-regulation in male rats but not in female ones, indicating that the DBH down-regulation is gender associated.
Plasma DBH activity in female rats has been reported to be higher than in male ones (Koudelova and Mourek, 1990) and injection of oestradiol benzoate (EB) to ovariectomised rats resulted in the elevation of DBH mRNA levels in the rat’s brain (Serova et al., 2002). Furthermore, oestrogen regulates DBH at the transcriptional level by inducing DBH promoter activity (Serova et al., 2002); (Sabban et al., 2010). Therefore, to detect if the oestrogen cycle is the reason for the lack of differences between DBH level in infected and uninfected female rat brains, the estradiol receptor alpha (ESR) mRNA expression levels were detected by qPCR; however, no difference was found between the infected and uninfected once. This indicates that ESR might controls the DBH levels, and blocks the *T. gondii* effect on DBH levels.

The difference between female and male expression of DBH indicates that *T. gondii* possibly has different effects regarding genders. Moreover, another consideration is that *T. gondii* behaviour change is sex-specific (Flegr et al., 2008; Xiao et al., 2012) as *in vivo* studies showed different expression pattern in the brains of infected male and female rats.

The massive decrease in DBH expression *in vitro* in male rat brains suggests that the reduction in DBH expression might occur in infected and non-infected cells, especially considering that the percentage of the infected cells was 60-70% in PC12 cultures, whereas the infected cells number is relatively small to the number of cells in the whole brain number. This phenomenon might be due to the ability of *T. gondii* to modulate uninfected cells and set up an appropriate microenvironment. For example, the parasite modifies infected cells and neighbouring cells inducing them to enter the S-phase (Lavine and Arrizabalaga, 2009). Moreover uninfected dendritic cells and monocytes cultured with *T.gondi*-infected dendritic cells and monocytes, secrete IL-12 implying that a soluble host or parasite factor is responsible for the bulk of IL-12 p40 production *in vivo* (Christian et al., 2014). The parasite modulates the uninfected cells by secretion of host or parasite low molecular weight factors or by injection of rhoptry proteins by the parasite without infecting the cells (Carruthers and Boothroyd, 2007; Boothroyd and Dubremetz, 2008; Koshy et al., 2010).

To eliminate the possibility that DBH reduction might be due to NGF differentiation, mRNA was extracted from NGF-differentiated PC12 cells at days 0, 3 and 6, and the DBH levels were detected by qPCR (Figure 4-8). The results showed that NGF did not affect DBH levels at the different time points, while the ascending parasite numbers used to infect undifferentiated PC12 cells reduced DBH levels; therefore, we can conclude that the changes in the DBH expression level is due to the infection not NGF differentiation.
These results clearly show that *T. gondii* decreases norepinephrine and epinephrine *in vitro* and in rats male brain *in vivo*. This reduction in production of NE and E, can lead to increase in the production of dopamine. Furthermore, the RNA sequencing results show that the parasite down-regulates monoamine oxidase (MaoB) an enzyme that catabolizes dopamine. It has already been reported that monoamine oxidase A type (MaoA) expression is decreased in human neuroepithelioma cells in response to infection with *T. gondii* with up-regulation of MiR-132 micro RNA (Xiao et al., 2014). In addition, dopamine metabolism changed during the infection based on pathway analysis for neurological genes. All the evidence indicates that *T. gondii* manipulates host catecholamine metabolism.

Earlier studies have suggested that elevated NE signalling plays a pathophysiological role in schizophrenia (Yamamoto et al., 1994; Yamamoto and Hornykiewicz, 2004; Lechin and van der Dijs, 2005). However, NE still contributes to the pathology of schizophrenia possibly by modulating dopamine and NMDR dysfunction. In addition, the drop in NE concentration was linked with the progression and extent of memory dysfunction and cognitive impairment during dementia (Matthews et al., 2002).

Norepinephrine and epinephrine are neurotransmitters involved a number of brain functions (Berecek and Brody, 1982). Norepinephrine affects parts that control the brain’s attention and responding actions; therefore, by decreasing the levels of NE and E, the parasite reduced the host’s response to threats e.g., in cats. Consequently, this facilitates the transmission of the parasite to the host and completes the life cycle. In humans, (Gale et al., 2014) found that *T. gondii* seropositivity is not associated with panic disorder or generalised-anxiety disorders, generally NE and E contributes to these disorders. However, a more recent study gave a contrasting result and an association was found between generalised-anxiety disorders and *T. gondii* infection seroprevalence (Markovitz et al., 2015). However, this emerging research area needs further investigations.

Dopamine receptor D\(_2\) was up-regulated. Previous studies have shown that D2R over-expression caused a behavioural hypersensitivity to D2R-like agonists, as well as enhanced electrophysiological responses to D2R activation in midbrain dopaminergic neuron (Kramer et al., 2011).

Finally, phenylalanine hydroxylase (PAH) was down-regulated; PAH is a class of monooxygenase with a non-heme iron for catalysis, similar to TH, that uses tetrahydrobiopterin (BH\(_4\), a pteridine cofactor) (Kaufman, 1993; Almas et al., 1996). The
down-regulation of PAH may be necessary to reduce the consumption of BH₄ and increase the availability of BH₄ to TH. PAH deficiency causes phenylketonuria (Hoang et al., 1996), a disease that causes neurological complications.

Reviewing the catecholamine pathway clearly shows that the pathway was modified to increase the production of dopamine and the reduction of E and NE, while the change in the expressions of D2R, Moxd2 and PAH provided insights into a new approach to alter the host’s neurological function and behaviour.

GO slim analysis was performed to give a general view of the changes that accrued during the experiment that was caused mainly by *T. gondii* infection and NGF differentiation (Figure 4-1). Each pie graph represents the GO slim analysis at each time point. The up-regulated and down-regulated genes were analysed separately. Finally, the genes that were significantly expressed (p-value <0.05) and the gene expression folds change were between two and two were analysed as a control.

By comparing the results of the differentially expressed up-regulated genes at day 3/0 with the differentially expressed up-regulated genes at day 6/0, we see that genes involved in reproduction had the highest increase while those involved in cellular component organization and biogenesis had the highest decrease. This might indicate that though the experiment period the change in the GO slim categories of the two up-regulated groups is minimal, indicating that the changes in these two up-regulated groups have the same tendency.

When the differentially expressed up-regulated genes at day 6/0 were compared with differently expressed down-regulated genes at day 6/0, there is an observed increase in genes involved in reproduction while most of the other genes had only minor changes.

However, major changes were observed when the differentially expressed down-regulated genes at day 6/0 were compared with differentially expressed down-regulated genes at day 3/0 and with differentially expressed up-regulated genes at day 6/0. Specifically, genes involved in the metabolic processes that were increased and those involved in biological regulation and immune response were decreased the most. This indicates that biological regulation and immune response genes were the most up-regulated through the experiment due to *T. gondii* infection, along with the increase in the differentiation of *T. gondii*.

However, the changes in the PC12 gene expression might also be due to NGF activation. A previous transcriptome study on NGF-activated PC12 cells reported GO slim analysis results
that were different from our study (Dijkmans et al., 2008). This might be due to the difference in experiments’ time points, as day 0 of infection is day 4 on NGF activation while in the published study, day 4 in the latest time point in which the experiment was conducted. Furthermore, NGF differentiation can be divided into two processes: an initial stimulation-driven latent process and an extension stimulation-driven extension process (Vaudry et al., 2002). Given the FPKM values of genes that have been reported to have a high fold change as a result of NGF differentiation i.e., Emp2, dusp5, PVR, Rrad, Loc499660 and Mmp13 (Chung et al., 2010), these genes were also expressed in our study samples. However, the fold change and the p-value did not show any significant differential expression between these time points. In sum, the changes that might be due to NGF activation and investigated by this study are not similar to what have investigated before. Due to the difference in time points in the study design, and the fact that our study was performed late in the NGF activation process, suggesting that most of the NGF activation process had occurred already.

In addition, to have a detailed and specific functional analysis of the changes induced by *T. gondii* infection in PC12 cells, GO enrichment analysis was performed to identify overrepresented GO terms in each gene set annotation. Therefore, GO enrichment analysis was performed on each gene group of the segregated data according to the time point and the regulation direction (up or down).

At day 3/0, up-regulated genes involved in positive regulation of catecholamine secretion and C21 steroid hormone biosynthetic process had the most significant P-value. This indicates that *T. gondii* infection not only increases dopamine production (Gaskell et al., 2009; Prandovszky et al., 2011) and decrease epinephrine and norepinephrine production in the infected cells, but it also modulates the host protein to regulate the secretion of these catecholamines from the infected cell.

While C21 steroid hormone biosynthetic process GO term is involved with pregnane compounds that contain 21 carbons (C21 steroids) which include progesterone and corticosteroids, both of these hormones groups have anti-inflammatory effects that may be associated with infection. In addition, injection of cortisone to *T. gondii* infected mice results in an increased the number of tachyzoites and cysts in the brain of the infected mice (Hulinska et al., 1990). This may explain the increase in C21 steroid hormones as a means to modulate the host’s immune response in favour of *T. gondii*. 
In the group of up-regulated genes at day 6/0 that represent the infection with bradyzoite stage and mimic the chronic infection, genes involved in chloride transmembrane transport and ion transmembrane transport had the most significant p-value, 2.45E-05, 2.08E-05 respectively, showing that the parasite is modulating the host transport system. In addition, the GO enrichment shows that the infection up-regulates the immune response to protozoa and some neural functions.

However, the down-regulated genes GO enrichment analysis showed the G-protein coupled receptor (GPCR) signalling pathway down-regulated. G-protein coupled receptor signalling pathway is involved in transmitting the external stimuli such as neurotransmitters, chemokines, hormones, inflammatory mediators, proteinases, odorants, and light (Gilman, 1987). Immune cells express GPCRs for classical, chemokines, chemoattractant neuropeptides, and neurotransmitters (Lombardi et al., 2002). In addition, the injection of soluble extract of T. gondii induces G-protein-coupled signalling through the chemokine receptor CCR5, and consequently have a key role in the induction of IL-12 from these cells (Aliberti and Sher, 2002). Therefore, the down-regulation of this signalling pathway might reduce host resistance and response to the external stimulus.

Moreover, GO enrichment analysis for the group of down-regulated genes shows a down-regulation of detection of stimulus involved in sensory perception and detection of chemical stimulus involved in sensory perception. This finding corresponds with a previous finding that the fatal fear attraction in T. gondii-infected rodents is specific and not related to loss of sensory perception or generic malaise as detailed previously in the Introduction. RNA sequencing results shows transcriptional changes in the genes related with the detection of stimulus involved in sensory perception and up- and down-regulation of olfactory genes that might lead to odour aversion. However, the ability of T. gondii infection to modulate the olfactory sense and block innate fear might be a combination of both transcriptional and epigenetic changes and the outcome of neuroanatomical location of T. gondii cyst (Evans et al., 2014).

Once more, GO enrichment analysis of down-regulated genes for day 6/0 revealed a down-regulation of genes involved in sensory organ development and cell surface receptor signalling pathway, which correspond with the earlier changes that were observed in day 3/0 indicating that these changes are found through the chronic stage of the infection.
To analyse the pathways and networks of genes that were differentially expressed with infection regardless of whether the genes were up- or down-regulated, the segregated genes of each time point were pooled in one list and this list was used in network analysis focussing on neurological and immune functions.

Cell-cell signalling is major function of any neuron. The heat map (figure 4-3) shows that *T. gondii* infection modulates the genes involved in cell-cell signalling by up-regulating 16 genes and down-regulating 41 genes. This suggests that the infection does not exclusively increase or decrease cell-cell signalling; instead, the parasite modifies gene expression to its advantage. Furthermore, *T. gondii* might interrupt major neuronal functions. Indeed, an earlier study by (Haroon et al., 2012) regarding live cell calcium (Ca2+) imaging *in vitro* studies showed that tachyzoites actively manipulate Ca2+ signalling upon glutamate stimulation causing either hyper- or hypo-responsive neurons. Moreover, the endoplasmic reticulum Ca2+ stores were depleted by *T. gondii*, and *in vivo* studies revealed that the activity-dependent uptake of the potassium analogue thallium was reduced in cyst-harbouring neurons indicating functional diminishment; moreover, over time the percentage of non-functional neurons were increased. However, further *in vitro* and *in vivo* investigations are needed to understand these processes, magnitude and the effect of this modulation on neurons and the nervous system.

The neurodevelopment GO term was also revealed as overrepresented by the GO categorization. The effect of *T. gondii* during congenital infection on foetal neurodevelopment is widely recognised. Congenital infection can cause a range of neurological diseases such as retinochoroiditis, blindness, epilepsy, psychomotor or mental retardation, encephalitis, microcephaly, intracranial calcification and hydrocephalus (Dunn et al., 1999; Remington et al., 2001). Moreover, schizophrenia is a neurodevelopmental disease; in fact, elevated maternal *T. gondii* IgG antibody was associated with a 2.5 fold increase in risk of schizophrenia (Brown et al., 2005). *T. gondii* infection may also cause other neurodevelopmental diseases such as autism spectrum disorder (ASD) and attention deficit and hyperactivity disorder (ADHD) with the link between these diseases and *T. gondii* infection already having been hypothesized (Prandota, 2010; Carter, 2013).

Another GO term category that was found to be over-represented in the change with infected PC12 cells is the behaviour GO term. The effect of *T. gondii* infection on behaviour having been previously discussed, the RNA sequencing result reinforces genes’ involvement in behaviour altered by *T. gondii* infection.
Network mapping of all the genes that were grouped in any neurological GO term plus any gene that was differentially expressed between different time points and found in the schizophrenia gene database (Figure 4-4), found a network of catecholamine-related genes differentially expressed. In addition, the map showed a connection between dopamine metabolism pathway genes and GABA receptor genes. This suggests that the changes in the genes involved with GABA receptors expression might be due to the change in catecholamine metabolism by the parasite.

It has also been observed that some GABA receptor subunit genes that are classified under the four neurological GO terms were found significant in this study, and most of these subunits are overexpressed. However, over activation and an increase in the secretion of GABA receptors have been noticed before in infected dendritic cells, and this activation has led to exhibition of hyper migratory phenotype (Fuks M et al., 2012). Overall, the GO term classification revealed that *T. gondii* infection influences host neuropsychology directly and indirectly.

The DAVID analysis for the pooled genes also showed clustering under four significant immunological GO terms: cytokines, chemotaxis, immunoglobulin like and T-cell selection. This illustrates the immunological response of infected neuronal cells. Gene clusters related to cytokines had the highest enrichment score and therefore the highest significance. The results showed that the infection manipulates the innate immune response to the parasite’s advantage by down-regulating key innate immune mediators like CXCL12, CXCL11 and IL-15. However, the key regulator for pro-inflammatory and anti-inflammatory immune response during *T. gondii* infection such as IL-12 and IL-10 were highly up-regulated during the infection, especially at Day 6/0.

Additionally, the chemotaxis GO term genes showed an up-regulation in most of these genes, and indicated that chemokine ligand 2 (CCL2), chemokine ligand 5 (CCL5), chemokine ligand 20 (CCL20) and chemokine ligand 24 (CCL24) were the major chemotaxis involved in recruiting immune cells to the infected cell. It has been reported that different immune cells produce these chemokines during *T. gondii* infection, *T. gondii* triggers neutrophil production of CCL2 (Del Rio et al., 2004) and CCL20 (Denkers et al., 2003), as well as neutrophil and monocyte up-regulation of CCL20 and CCL24 mRNA during *T. gondii* infection (Ju et al., 2009). Finally, CCL5/RANTES is induced in the brains of mice during chronic infection with *T. gondii* (Wen X et al., 2010).
While the Immunoglobulin GO term genes show that, the genes involved in immunoglobulin-like fold Go term were up-regulated. This term is represent domains with an immunoglobulin-like (Ig-like) fold, Ig-like domain is found in many proteins including, receptors and MHC class I and II MHC II expression; the MHC class that presents antigens of intracellular pathogens. Previous studies have shown *T. gondii*’s ability to down-regulate surface major histocompatibility complex (MHC) class II on infected macrophages by inhibition of the STAT1 signalling pathway (Luder et al., 2001). This reduction was also found in astrocytes and microglia (Luder et al., 2003). Previous studies show a down-regulation contradicting our results; possibly the parasite has a different effect on APC than normal cells, whereas the effect of the parasite on different cell type MHC expression from both classes should be further investigated.

However, the T-cell selection class shows an up-regulation for most genes at Day 6/0, indicating that this function is activated after day 3. Surprisingly, the results here indicate that the parasites are involved in T-cell selection and activation, which might allow the destruction of infected cells. Nonetheless, further lab investigations are needed to have a clear understating of T-cell interaction with the *T. gondii*-infected neural cells.

Cluster analysis of the genes under immune response indicates a host response to infection inducing chemokines and cytokines that recruit and activate different immune cells. Interestingly, genes that are involved in catecholamine metabolism are also categorized in the immune response and differentially expressed. As dopamine, norepinephrine and epinephrine have immunomodulator effect, the relation between catecholamines and the immune system has been previously reviewed (Sarkar et al., 2010; Madden et al., 1995).
4.6.2 *T. gondii* gene expression analysis

One of the aims of this study is to understand the molecular alterations of neuronal cells induced by *T. gondii* and the mechanism of these inductions. Therefore, bioinformatics analysis of the parasite transcriptome was also performed to investigate host-parasite interactions. The results confirm bradyzoite differentiation and mimicking of the chronic stage of infection (Table 4-8).

Dense granules proteins (GRA) are secreted to alter the PV and acquisition of nutrients. Actually, GRA1, GRA3, GRA7, GRA2, GRA4, GRA6, GRA9, GRA12, and GRA14 are involved in the vacuolar network membranes. GRA2, GRA4 and GRA6 is associated with the formation of a multimeric protein complex, while GRA3, GRA5, GRA7, GRA8 and GRA10 are specially detected as PVM associated proteins (Nam, 2009). Recently, GRA proteins were found to be exported outside the PV and reach host nucleus and alter host gene expression (Bougdour et al., 2014). However, the results show that the GRA proteins expressed (Table 4-10) have a similar DE profile that is similar to housekeeping genes. This indicates that these GRA functions continued throughout the experiment and did not have a specific expression pattern in neuronal cells.

Rhoptry organelles are the most unique organelles found in apicomplexan parasites (Sam-Yellowe, 1996); these organelles secret their rhoptry proteins (ROP) after the invasion and also inject these proteins into uninfected cells (Koshy et al., 2012). In this study, ROP11, ROP15 and ROP41 were highly up-regulated, indicating that these ROP proteins may have a major role during neuronal cell infection. ROP11 and ROP41 were previously suggested to be active kinases (Bradley et al., 2005; Peixoto et al., 2010), while the function of ROP15 is unknown. In addition, ROP5 was moderately up-regulated, indicating an increase in the activity of ROP18, as ROP5 is a pseudokinase that acts as a cofactor for ROP18 that facilitate the phosphorylation of the target by the kinase (ROB18) (Fleckenstein et al., 2012). Furthermore, the expression profile of these ROP in PC12 cells is very different from their expression profile in HFF (www.toxoDB.org) indicating that the parasite might have a distinct expression in different hosts and cell types.

Micronemes are adhesion proteins involved in parasite motility and host cell invasion. (Table 4-10) shows the microneme differentially expressed during the infection. First, MIC1 ,MIC4 and MIC6 expression is up-regulated; these micronemes form a complex MIC1/4/6 that is involved in gliding motility and cell invasion by being an essential part of the actin–myosin
driven propulsion system (Opitz and Soldati, 2002). The up-regulation of this MIC complex indicates that the parasite undergoes new host cell invasion.

Moreover, MIC7 and MIC13 were excessively up-regulated (3.4) and (13.5) respectively; MIC7 and MIC13 are bradyzoite specific (Buchholz et al., 2011). This result further confirms bradyzoite differentiation. Moreover, MIC13 expression was previously reported to increase during mouse brain infection (Pittman et al., 2014) suggesting that this protein might have a specific neurological function. Finally, TGME49_054430, a putative MIC, was down-regulated; the putative GO biological functions of this protein are proteolysis and blood coagulation (toxoDB). This protein was reported in the oocyst wall fraction (Fritz et al., 2012) and was down-regulated during the merozoite stages (Behinke et al., 2014). In this study, TGME49_054430 was down-regulated during the bradyzoite stage infection of neuronal cells.

One of the interesting GO terms that was found to be associated with down-regulated genes is the cation transport and ion transport GO term at day 6/0; cation transport genes are related to neuronal functions, suggesting that certain aspects of neurological function may have diminished during *T. gondii* infection. This result was reported previously, as cation transport was also associated with down-regulated genes in *T. gondii*-infected mice brain transcriptome (Tanaka et al., 2013).

At day 3/0, the up-regulated genes GO enrichment score revealed that the *T. gondii* glycosylation function is significantly increased. *T. gondii* glycosylation is important for host parasite interaction, inhibition of glycosylation of RH stain *T. gondii* with tunicamycin loses the ability to invade host cells and the few parasites that invade the host were found to be incapable of replicating and accumulating with a distended endoplasmic reticulum, deformed nuclei, and without recognizable late secretory organelles (Luk et al., 2008). Moreover, the proteomics and glycan analyses of RH stain glycoproteins identified components involved in gliding motility, moving junction, and other additional functions implicated in intracellular development (Fauquenoy et al., 2008). Furthermore, glycosylation is important for the formation of the bradyzoite cyst wall (Zhang et al., 2001) and N-glycosylation and O-glycosylation activity was demonstrated from *T. gondii* extract (Dieckmann-Schuppert et al., 1994; Stwora-Wojczyk et al., 2004b; Stwora-Wojczyk et al., 2004a).
4.6.3 Conclusion

Overall, the analysis of the RNA sequencing results for infected PC12 cells have shown that the parasite induces a high number of changes in the infected cells, influencing a number of neurophysiological functions. These suggest that the parasite has a specific effect on the neuropsychological state of the host through different mechanisms, yet the outcome of the infection on the psychological state of the host is a result of the interaction of *T. gondii* - induced changes with other psychological factors. In addition, the analysis provided a model of the non-immunological (neuronal) host cells innate immune response to *T. gondii* infection.

Our experiments were performed on rat cells, a *T. gondii* intermediate host. While humans are an accidental intermediate host, it is important to note that the effect of *T. gondii* on humans might be different and the results should be confirmed on human cells. Moreover, *in vivo* investigations and behaviour studies confirm *T. gondii* mechanisms of neurological alterations are necessary to understand the outcome of these transcriptional changes. Further studies to understand the relationship between *T. gondii* and other neurodevelopmental diseases, while measuring cytokines and chemotaxis released from infected cells media, will be needed to confirm the innate immunity results.

In addition, several experiments may be performed to help understand the mechanism of *T. gondii* down-regulation of DBH. In addition, the special secretory protein (ROP, MIC and GRA) profile of *T. gondii* might be confirmed by the construction of *T. gondii* knockouts for these proteins, to reveal the function of these protein and their contributions in host altering.

Finally, all these results are transcriptomic and need to be further confirmed by proteomic analysis to assure that these transcriptome modifications affect the protein expression of these genes (Xia et al., 2008). Moreover, it might be helpful to repeat with *in vivo* infected brains, though isolation of infected cells, e.g., laser capture, is required.
Chapter Five

5 Discussion

Immunological and neurological host parasite interaction during neural infection: the mechanisms of manipulation
The previous chapters’ experimental results have led to expanding our knowledge and understanding of *T. gondii* and its host interactions, particularly investigating mechanisms of neurophysiological changes that may be involved in host behavioural changes.

It was hypothesized that the parasite changes the host’s behaviour via several mechanisms, for example, changing neurotransmitter levels, specifically dopamine, based on the observation of increased dopamine during *T. gondii* infection *in vivo* and *in vitro* (Gaskell et al., 2009; Prandovszky et al., 2011; Xiao et al., 2014). Further influences might be imposed, for example, by the location of the *T. gondii* cysts in the host brain or host response to infection.

One indirect mechanism is based on this hypothetical sequence: first, *T. gondii* activates astrocytes, which leads to an increase in the formation of kynurenic acid (KYNA) in the brain. Increased brain KYNA levels, in turn, contribute to an excessive reduction in glutamatergic and nicotinergic neurotransmitters, which is believed to play an important role in the pathogenesis of schizophrenia.

Advancements in our knowledge related to both mechanisms will be discussed in this chapter and will be occasionally interjected with previous information to acquire an extensive understanding of the host behaviour change mechanism by *T. gondii*. 
5.1 Proximate mechanisms

An important change in the neural system is the influence of the parasite on catecholamine metabolism. The components involved in catecholamine metabolism start with the hydroxylation of phenylalanine to tyrosine by the enzyme phenylalanine hydroxylase. The synthesised tyrosine, in addition to tyrosine acquired from the diet, is metabolised to L-Dopa by tyrosine hydroxylase. L-Dopa is converted to dopamine by aromatic L-amino acid decarboxylase (AADC). Finally, dopamine is converted to norepinephrine and epinephrine by dopamine beta hydroxylase (DBH) and phenylethanolamine N-methyltransferase (PNMT). Also catecholamines are degraded by several monoamines. Figure 5-1 illustrates this pathway.

During T. gondii infection various modifications in the catecholamine metabolism occur. These modifications include up- and down-regulation of (host) enzymes that are involved in the anabolism or catabolism of catecholamines, involvement of T. gondii enzymes in catecholamine metabolism directly (chapter 4) and increase the secretion of the synthesised metabolites. Figure 5-1 summarises the changes with infection. Based on the findings of the RNA-Seq analysis and prior work the changes in catecholamine metabolism include major increases in DA and L-dopa whilst catabolism of DA to NE, E and DOPAC are substantially decreased.
Figure 0-1: Catecholamine metabolism during *T. gondii* infection.
Phenylalanine hydroxylase was down-regulated although the reasoning for this down-regulation is not clear, although it is intriguing that TgAaaH and, to a lesser degree, host TH convert phenylalanine to tyrosine and a consequence of decreasing PAH would be to liberate more of the shared co-factor tetrahydrobiopterin for TH activity.

Moreover a recent study of micro RNA (Xiao et al., 2014), showed that *T. gondii* down-regulates MaoA and MaoB. This might be an additional mechanism to increase the level of dopamine by modifying the dopamine static system, via down-regulation of enzymes involved in breaking down dopamine as a part of a negative feedback circuit for excess produced dopamine.

Furthermore, the parasite also increases dopamine synthesis via massive down-regulation of dopamine beta hydroxylase (DBH) (chapter 4), i.e., the enzyme that catalyses dopamine to norepinephrine. This finding was further confirmed by RT-PCR for DBH mRNA from *T. gondii*-infected PC12 cells, which showed a direct relation between the number of the parasites and the reduction in DBH expression. This effect would be restricted to neurones expressing DBH. Finally, DBH levels were detected *in vivo*; mRNA from rat brain sections homogenate confirmed the *in vitro* results. In contrast to male rat results, the female rat results did not show a reduction. Hence, *T. gondii* infection does not only increase dopamine production, but also decreases NE and E production too. This observation fits well with observations that *T. gondii* behaviour change is sex-specific (Flegr et al., 2008, Xiao et al., 2012) since *in vivo* study showed different expression patterns of DBH in the infected male and female rat brains. NE and E were also decreased in *T. gondii*-infected PC12 cells corresponding with decreases in DBH mRNA. The effect correlated with numbers of *T. gondii* parasites.

Another modification in the catecholamine pathway is the elevated expression of monooxygenase DBH-like 2 (Moxd2), an enzyme that might have a DBH like function and is principally expressed in the medial olfactory epithelium (Su et al., 2004). This may indicate a direct modification by *T. gondii* on the olfactory function and imply that the infection could induce different modifications in different brain regions with Moxd2 at least partially compensating for the decrease in DBH. Finally, dopamine receptor D2R was over-expressed during infection, while D1R expression was previously reported to decrease during infection (Xiao et al., 2014). D1R and D2R exert opposing intracellular effects on cAMP signalling; Drd1 activates it, whereas Drd2 inhibits it (Sibley et al., 1993).
GO enrichment analysis of RNA-Seq data showed that G-protein coupled receptor signalling pathway genes are down-regulated. Further, dopamine receptors are G-protein coupled receptors, this observation combined with the modification of specific dopamine receptors, might suggest that these changes contribute to the behaviour change pathology. Especially as D2R-enhanced sensitivity was postulated in schizophrenia pathology (Seeman et al., 2005, Seeman et al., 2007) and the over-expression of D2R led to enhanced electrophysiological responses to D2R activation in midbrain dopaminergic neurons and behavioural hypersensitivity to D2R-like agonists (Kramer et al., 2011). In addition, the GO enrichment results showed that the parasite up-regulated catecholamine secretion genes in the infected cells suggesting that the increased amounts of dopamine were secreted from the infected cells.

In summary, modifications in the catecholamine pathway are indicative of many neurological changes that can lead to behavioural changes. T. gondii’s proximate effects are not based on a single modification, the parasite influences (host) neurotransmitter and receptor expression to induce specific changes in the host's psychological status.

Other effects on host neurology were revealed by RNA-Seq data analysis (chapter 4). Gene ontology grouping revealed that the parasite modulates cell-cell signalling, in line with observations that bradyzoites and tachyzoites can functionally silence infected neurons (Haroon et al., 2012). In addition, gene ontology grouping showed that the parasite modulates neurodevelopmental genes. In fact, the effect of congenital toxoplasmosis on foetal neurodevelopment is the major clinical manifestation of toxoplasmosis. Clinical manifestation includes a range of neurological diseases such as retinochoroiditis, blindness, epilepsy, psychomotor or mental retardation, encephalitis, microcephaly, intracranial calcification and hydrocephalus that may be due to parasite proliferation or effects of cyst stages (Dunn et al., 1999; Remington, 2001). In addition schizophrenia is a neurodevelopmental disease; and the risk of schizophrenia is 2.5 fold increased in those with elevated maternal T. gondii IgG antibodies (Brown et al., 2005).

However, these studies were conducted with PC12 cells which are embryonic cells extracted from a pheochromocytoma of the rat adrenal medulla, and have an embryonic origin from the neural crest (Greene and Tischler, 1976); therefore, the infection of adult brain might elicit different modifications. In addition, the infection location in the brain might be critical for the infection outcome. The expression profile of different cells in different brain regions widely varies. Indeed, effects on dopamine would not be observed in non-catecholaminergic
brain cells when infected. Neuronal cell bodies in the brainstem produce dopamine; the two areas are the substantia nigra and the ventral tegmental area, while melanin-pigmented cell bodies in locus ceruleus produce norepinephrine.

Moreover, dopamine receptors are expressed in many brain areas. D1 dopamine receptors are highly expressed in nigrostriatal, mesolimbic, and mesocortical areas, such as the caudate-putamen ( striatum), nucleus accumbens, substantia nigra, olfactory bulb, amygdala, and the frontal cortex whereas lower expression levels are found in the hippocampus, cerebellum, thalamic areas, and hypothalamic areas. D2R dopamine receptors meanwhile are found at a high density in the striatum, nucleus accumbens, and the olfactory tubercle. D2 receptors are also expressed at significant levels in the substantia nigra, ventral tegmental area, hypothalamus, cortical areas, septum, amygdala, and hippocampus (Missale et al., 1998; Beaulieu and Gainetdinov, 2011). Hence, the site of the infection might induce different effects according to the type of the infected cells. Although *T. gondii* parasites do not have any preference for specific brain regions in their secondary hosts, the amygdala may be more consistently infected (Vyas et al., 2007a)

Another example of a site-specific modification is the effect of the parasite on olfactory function: if the olfactory epithelium was infected, this will lead to the overexpression of Moxd2. GO enrichment analysis revealed that genes associated with olfactory function such as G-protein coupled receptor signalling pathway, detection of stimulus involved in sensory perception, detection of chemical stimulus involved in sensory perception, detection of stimulus sensory organ development and olfactory pit development were down-regulated. These results may suggest that the parasite directly controls the olfactory functions in the brain, although further investigation is needed on olfactory cells.

Lastly, another mechanism that might contribute to the host behaviour change is NMDR manipulation by the parasite. RNA-Seq analysis showed that the infection affects host NMDR expression. Previous pharmacologic, genetic, and biochemical evidence supports the idea that NMDR hypofunction is a key etiological component of schizophrenia (Krystal et al., 1994; Lahti et al., 2001; Dalmau et al., 2007; Allain et al., 1998; Dalmau et al., 2011; Kirov et al., 2012; Fromer et al., 2014; Purcell et al., 2014). NMDR involvement may also function as an indirect mechanism through KYNA, as discussed in the next section.

All the changed mechanisms that could affect behaviour are summarised in Figure 5-2.
Figure 0-2: Flow chart summarises the host’s neurological changes induced by *T. gondii*. 
5.2 The indirect mechanisms of neurological change

Indirect mechanisms of altering neurological function through the immune system have been suggested. The hypothesis stated that infection activates astrocytes, which then induce IDO leading to the production and release of KYNA (Schwarcz and Hunter, 2007). KYNA blocks the NMDR and α-7- nicotinic acetylcholine receptors which then lead to a decrease of dopamine in the brain.

An investigation on the mechanisms in KYNA and *T. gondii* infection was carried out for the first time in this study (chapter 3). First, the KYNA effect on dopamine was confirmed *in vitro*: KYNA decreases dopamine production from PC12 cells, replicating observations *in vivo*. Surprisingly, *T. gondii* blocked the KYNA effect on PC12 cells and no reduction in dopamine levels was detected. KYNA was found to reduce Ser19 phosphorylation in the regulatory domain of TH; that will lead to TH activity reduction and therefore reduced dopamine production. *T. gondii* blocked this activity by KYNA on Ser19 phosphorylation.

These results contradict an indirect mechanism hypothesis of behaviour modification. Mainly, the relationship between the host immune system and *T. gondii* showed that the parasite controls the host system mostly and therefore the parasite can subvert mechanisms for the parasite advantage.

Another KYNA effect is the relationship between KYNA and NMDR. KYNA is a non-competitive antagonist at the glycine site of the NMDA receptor (IC50 ~8 µM) (Kessler et al., 1989)(Parsons et al., 1997). RNA-Seq bioinformatics analysis of GO grouping showed that the parasite infection of PC12 cells moderated NMDR expression. However, further investigations on the effect of *T. gondii* on the glycine-binding site and the receptor response to agonist and antagonist like KYNA are needed. Yet, it is important to study KYNA levels during infection as KYNA has pleiotropic effects such as, interactions with orphan G protein-coupled receptor GPR35 (Wang et al., 2006) or the aryl hydrocarbon receptor (DiNatale et al., 2010).

5.3 *T. gondii* interaction with the host immune system during *in vitro* infection

During *T. gondii* infection, parasites persist as intra-neuronal cysts that are controlled but not eliminated. The immune system controls the infection via brain-resident cells including leukocytes, astrocytes and microglia while neurons contribute to the brain immune response via the production of cytokines, chemokines and expression of immune-regulatory cell surface molecules, such as major histocompatibility (MHC) antigens. The parasite avoids
elimination by several approaches to subvert the immune system. In this study, the neurons’ immune response to the infection and the parasite methods to avoid host elimination were investigated.

During *T. gondii* infection, the host’s immune response initiates tryptophan starvation by increasing the expression of IDO (Pfefferkorn and Guyre, 1984; Pfefferkorn et al., 1986). In this study, experiments on the effect of tryptophan starvation on parasite survival have shown that tryptophan starvation did not eliminate *T. gondii in vitro* (chapter 3). Tryptophan starvation induced bradyzoite differentiation and cystogenesis. Amino acid starvation can trigger the loss of mitochondrial integrity. Mitochondrial depolarization is evidence of autophagy in both yeast and mammals caused by nutrient starvation (Rodriguez-Enriquez et al., 2009; Rodriguez-Enriquez et al., 2006; Zhang et al., 2007). Tryptophan starvation was found to differ from amino acid starvation as the parasite mitochondria remained intact and no sign of autophagy was detectable using the Mitotracker stain.

The kynurenine pathway has an immune-regulatory role. As stated previously, *T. gondii* blocks KYNA effects on dopamine production. Yet the effect of KYNA and other kynurenine catabolites on the immune system during *T. gondii* infection and the relationship with the immune system are unknown. The IDO-mediated tryptophan catabolism plays a significant counter-regulatory role in down-regulation of the immune system, as IDO suppresses T cell response by induction of regulatory T cells. IDO-kynurenine pathway can serve as a negative feedback loop for TH1 cells. By down-regulating the immune response, IDO and the kynurenines have an anti-inflammatory effect. Furthermore, the close connection between the cytokine and kynurenine systems, and any imbalance in the cytokine activation, with a consequent change in the kynurenine cascade, might cause neurologic or psychiatric disorders (Mándi and Vécsei, 2012).

*T. gondii* infection induces host immune responses in neuronal cells. Transcriptome analyses of infected PC12 cells have provided precise details of this immune response (chapter 1). Grouping the significant genes into GO terms revealed that the cytokines GO term had the highest enrichment score and therefore the most significant immunological activation. The results also showed that the infection down-regulated key innate immune mediators like CXCL12, CXCL11 and IL-15, thus reinforcing that the parasite manipulates the innate immune response to the parasites advantage. On the other hand, the key regulators for pro-inflammatory (IL-12) and anti-inflammatory immune response (IL-10) during *T. gondii* infection are highly up regulated during the infection, especially at day 6.
Another immunological function found to be modified by GO analysis of the RNA-Seq data is the chemotaxis GO term. The results showed an up-regulation in most of the genes involved in this GO term, and shows that chemokine ligand CCL2, CCL5, CCL20 and CCL24 are the major chemotaxis induced and potentially involved in recruiting immune cells to the *T. gondii*-infected cell.

GO term analysis also showed that the Immunoglobulin-fold like genes were also differentially expressed during neural cell infection. This protein domain is found in many proteins including immunoglobulins, receptors, adhesion molecules and MHC class I and II antigens. The analysis showed that the up-regulated genes are involved in MHCI expression, the MHC class that presents antigens of intracellular pathogens to CD8 T cells. On the hand, the parasite altered the expression of some receptors that were involved in immune regulation, such as CD3. Previously, *T. gondii* was found to down-regulate surface major histocompatibility complex (MHC) class II on infected cells by inhibition of the STAT1 signalling pathway (Luder et al., 2001) and infected neurons showed low MHC class I expression (Wilson et al., 2009).

T-cell selection genes showed an up-regulation for most genes at day 6, indicating that this function is activated after day 3; surprisingly, the results show that the genes are involved in T-cell selection and activation, which might allow the host cell to control the infection via the T-cell. Further lab investigations are need to clarify the role of these altered genes *T. gondii*.

Network analysis of genes categorized under immunological GO terms during *T.gondii* infection suggests that infection induces chemokines and cytokines together that will recruit and activate different immune cells. Interestingly, the genes involved in catecholamine metabolism were also included as these have an immunomodulator effect as previously reviewed (Sarkar et al., 2010; Elenkov, 2008). The involvement of catecholamine metabolism genes in immune response, illustrates the interaction between the nervous system and immune system and the parasite, by manipulating catecholamine metabolism, is manipulating the immune response.

In addition, the GO enrichment analysis revealed that the parasite also affects another hormone that has an anti-inflammatory effect. Pregnanes contain 21 carbons (C21 steroids); these GO term genes were found to be up-regulated during infection. C21 steroids, including progesterone and corticosteroids, have anti-inflammatory effects. The injection of cortisone in *T. gondii* infected mice increased the number of tachyzoites and cysts in the brain of the
infected mice (Hulinska et al., 1990). This indicates that the increase in C21 steroid hormone might be another method to modulate the host immune response to the *T. gondii* advantage.

Finally, the G-protein coupled receptor (GPCR) signalling pathway that was observed to be down-regulated in GO enrichment analysis is also involve in immune modulation. GPCRs are expressed by immune cells and involved in several immune functions such as chemoattractant and chemokines (Lombardi et al., 2002). In addition, the injection of soluble extracts of *T. gondii* induced G-protein-coupled signalling through the chemokine receptor CCR5, and consequently, plays a major role in the induction of IL-12 from these cells (Aliberti and Sher, 2002). Therefore, the down-regulation of this signalling pathway might reduce host resistance.

In summary, the RNA-Seq data have provided detailed transcriptome information about the cytokine, chemotaxis, MHC expression and T-cell selection genes involved in the immune response, as well as the secretion of anti-inflammatory steroids and down-regulating of the G-protein coupled receptors to subvert the immune response and the effect of the increased dopamine on immune response.
5.4 *T. gondii* host manipulation mechanisms and effector proteins

Although the parasite is enclosed inside the parasitophorous vacuole, the parasite delivers effector protein to the host cell; these proteins interface with the host response by modifying the host-signalling pathway, co-opt the host transcription factors, and eventually modulate the gene expression. Secretory proteins (micronemes, rhoptry and dense granules) are the main effectors; the dense granules proteins deliver products that remain confined in the vacuolar space or export to the host cell nucleus and contribute to altered host gene expression.

RNA Seq simultaneously provided the transcriptome of *T. gondii* during the infection of neuronal cells (PC12) (chapter 4). First, the analysis showed that the experiment succeeded to mimic the chronic stage of the infection by showing an up-regulation of different bradyzoite specific markers, both structural (BAG1, ENO1 and SAG4) and functional (MIC7, MIC13). Furthermore, the *T. gondii* genes involved with cell invasion and PV formation are moderately up-regulated (similar to the housekeeping genes). MIC1, MIC4 and MIC6 expression showed this expression pattern; these micronemes form a complex MIC1/4/6 that were involved in gliding motility and cell invasion by being an essential part of the actin-myosin driven propulsion system (Opitz and Soldati, 2002). GRA1, 2, 3, 5, 7 and 8 also had this expression pattern, dense granules proteins (GRA) are secreted to alter the PV and acquire nutrient. The expression of the MIC complex and GRA protein indicates that the parasite is to undergo new host cell invasion and PV formation, but these functions are limited compared to bradyzoite differentiation and cystogenesis.

The results have shown that *T. gondii* transcriptome during neuronal infection is unique, and different from the transcriptome of bradyzoites infecting human foreskin fibroblast cells (toxoDB). For example, ROP proteins have a special expression pattern during PC12 infection. This indicates that the parasite might have a distinct expression in different host and cell types. In this study, ROP11, ROP15 and ROP41 were highly up-regulated, indicating that these ROP proteins may have a major function during neuronal cell infection. ROP11 and ROP41 were previously suggested to be active kinases (Bradley et al., 2005; Peixoto et al., 2010), while the function of ROP15 is unknown. Other ROP proteins were expressed throughout the experiment, but no differential expression was found between different time points. This indicates that the modifications in host gene expression might be caused by the secretion of ROP11, 15 and 41. However, a moderate up-regulation of ROP5 might indicate an increase in the activity of ROP18, as ROP5 is a pseudokinase that facilitates the
phosphorylation of the target by the kinase (ROP18) by acting as a cofactor for ROP18 (Fleckenstein et al., 2012).

As mentioned previously, we found that the parasite blocks the KYNA effect on dopamine levels by blocking the down-regulation of tyrosine hydroxylase ser19 phosphorylation by KYNA. Previous studies have shown that T. gondii phosphorylates host proteins and thereby activates host transcription factors. For example ROP16 phosphorylates serine to activate STAT1 (Jensen et al., 2013), and phosphorylates tyrosine to activate STAT3 and STAT6 proteins (Saeij et al., 2007). While GRA24 triggers an unusual and sustained p38a auto-phosphorylation (Braun et al., 2013), ROP18 is another known serine/threonine protein kinase that phosphorylates host protein to modulate virulence. It phosphorylates immunity-related p47 GTPases (IRG) proteins and impairs their accumulation on the PV membrane (Fentress et al., 2010; Steinfeldt et al., 2010) and phosphorylates the host endoplasmic reticulum–bound transcription factor ATF6β leading to ATF6β proteasome-dependent degradation and interface with host immune response (Yamamoto et al., 2011). And, finally, it inhibits the host NF-κB pathway (Du et al., 2014). T. gondii is also able to block the host phosphorylation directly, for example: T. gondii prevents histone H3 Ser10 phosphorylation and Lys9/14 acetylation at the IL-10 promoter and TNF-α promoter too, leading to reductions in IL-10 and TNF production in infected microphages (Leng and Denkers, 2009; Leng et al., 2009).

Moreover, T. gondii down-regulates cytokines by targeting the host cell chromatin remodelling machinery. This suggests that DBH might be down-regulated via similar machinery; however, this hypothesis needs to be further investigated alongside other down-regulating machinery like signalling pathway activation (Zhou et al., 2013).

RNA-Seq revealed that parasite glycosylation genes are modulated during the early stages of bradyzoite conversion (day 3). Glycosylation is an important mechanism for host-parasite interactions as inhibition of glycosylation of RH strain parasites with tunicamycin led to a loss in the ability to invade host cells and the few parasites that invaded the host were found to be incapable of replicating, accumulating with a distended endoplasmic reticulum, deformed nuclei, and without recognizable late secretory organelles (Luk et al., 2008). Proteomic and glycan analyses of RH glycoproteins identified components involved in the moving junction and gliding motility, and additional functions involved in intracellular development (Fauquenoy et al., 2008). Furthermore, glycosylation is important for the formation of the bradyzoite cyst wall (Zhang et al., 2001) and N-glycosylation and O-
glycosylation activity has already been demonstrated in *T. gondii* extracts (Dieckmann-Schuppert et al., 1994; Stwora-Wojczyk et al., 2004b; Stwora-Wojczyk et al., 2004a). In sum, glycosylation is important in the host-parasite machinery during the early stages of infection.

The parasite may down-regulate neuronal functions directly through *T. gondii* gene expression; for example, cation transport genes were down-regulated, and, since cation transport genes are related to neuronal functions, this suggests that certain aspects of neurological function may be diminished during *T. gondii* infection. A similar result was previously reported in transcriptome analysis of *T. gondii* infected mice with an association between the cation transport GO term and the down-regulated genes (Tanaka et al., 2013).

Further confirmation of the biological impact of our results would be provided by examining transcriptomic changes from *in vivo* infected neurones, on both genders. Also the findings here show RNA changes and parallel experiments should include proteomic analysis to confirm the transcriptome data. In addition, it might be useful to conduct these experiments on human cell lines due to the importance of *T. gondii* infection in humans. Finally, further investigation of the parasite mechanism of down-regulating DBH expression is needed.

RNA-Seq is an ideal method to understand tachyzoite-bradyzoite differentiation, and the TRP free media described in this thesis could by used to perform this experiment. Moreover, expression patterns in different cell types could by investigated for a better global understanding of *T. gondii* host manipulation.
6 Bibliography


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