Neurophysiological Changes During Chronic *Toxoplasma gondii* Infection

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IP

The candidate confirms that the work submitted is her own and that appropriate credit has been given where reference has been made to the work of others.

"The roots of education are bitter, but the fruit is sweet."

Aristotle

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Abstract

Mammalian neuronal function is regulated by a complex matrix of neurochemical signals, cellular effectors and cell-cell communication. Many of the mechanisms governing neurobiology are poorly understood. The obligate intracellular parasite *Toxoplasma gondii* forms a chronic neuronal infection lasting the lifetime of the mammalian host. In this thesis, changes in catecholaminergic signalling during *Toxoplasma gondii* infection were investigated. Preliminary RNAseq data was used to identify catecholaminergic genes exhibiting the highest expression changes during chronic *Toxoplasma gondii* infection. Reduced expression of dopamine β-hydroxylase mRNA was found during *Toxoplasma gondii* infection in rat catecholaminergic and human neuronal cells. This down-regulation of dopamine β-hydroxylase was shown in newly synthesised RNA, suggesting that regulation occurs at the transcriptional level. Using methylation sensitive restriction enzyme quantitative polymerase chain reaction, hypermethylation in the 5' upstream region of the dopamine β-hydroxylase promoter was shown in infected rat catecholaminergic cells, human neuronal cells and chronically infected mouse nuclei.

Surprisingly, dopamine β-hydroxylase mRNA down-regulation and methylation in the 5' promoter were globally altered *in vivo*, despite the fact that only a small number of cysts can be identified in the host brain. In order to delineate the mechanism(s) responsible for this global change, extracellular communication during chronic *Toxoplasma gondii* infection was examined. Dopamine β-hydroxylase silencing was observed only in cells exposed to infected cells. Extracellular vesicles purified from infected rat catecholaminergic cells induced

transcriptional silencing of dopamine β-hydroxylase. This effect could also be generated *in vivo* when rats received intracerebral injections with purified extracellular vesicles from infected cells. This represents a new perspective of the host-pathogen interaction. Through this mechanism, *Toxoplasma gondii* may be able to induce many of neurophysiological changes associated with chronic infection. In addition, *Toxoplasma gondii* is a unique model to study mammalian neurological function. By examining the influence the parasite is able to exert over cell-cell communication, we may be able to further understand the mechanisms governing CNS function and dysfunction.

Abbreviations

5-AC	5-Azacytidine
AGO	Argonaute
ADRs	Adrenoreceptors
ATP	Adenosine triphosphate
BBB	Blood brain barrier
BDNF	Brain-derived neurotrophic factor
M17	BE(2)-M17 cells
cAMP	Cyclic adenosine monophosphate
CMV	Cytomegalovirus
CNS	Central nervous system
DA	Dopamine
DAT	Dopamine active transporter
DBH	Dopamine β-hydroxylase
DDC	Dopamine decarboxylase
DNMT	DNA methyltransferase
DOPA	3,4-dihydroxyphenylalanine
DPBS	Dulbecco Phosphate-Buffered Saline
DRD1	Dopamine receptor-1
DRD2	Dopamine receptor-2
ERE	Oestrogen response element
ESCRT	Endosomal sorting complex
ESR1	Oestrogen receptor 1
EV	Extracellular vesicle
FACS	Fluorescence-activated cell sorting

GLT	Glutamate transporter
HRP	Horseradish peroxidase
JAK	Janus kinase
IDO	Indoleamine 2,3-dioxygenase
IFN-γ	Interferon-γ
IL-12	Interleukin-12
LC	Locus coeruleus
LncRNA	Long non-coding RNA
IP	Intraperitoneal
IP3	Inositol 1,4,5-triphosphate
GAD	Glutamic acid decarboxylase
GABA	γ-Aminobutyric acid
GFP	Green fluorescent protein
HDAC	Histone deacetylase
HPLC	High performance liquid chromatography
HPA axis	Hypothalamic-pituitary-adrenal axis
MA	Methamphetamine
MaoA	Monoamine oxidase A
MAP2	Microtubule-associated protein 2
MBD	Methyl-binding domain
MDP	Methyl-binding protein
MeCP2	Methyl-CpG binding domain protein 2
MHC	Major histocompatibility complex
MiRNA	Micro RNA
MOI	Multiplicity of infection
mPFC	Medial prefrontal cortex
MSRE	Methylation sensitive restriction enzyme

NAc	Nucleus accumbens
NE	Norepinephrine
NET	Norepinephrine transporter
NF-κB	Nuclear factor κ-light-chain-enhancer of activated B cells
NMDA	N-methyl-D-aspartate
N-SMase	Neutral spingomyelinase
PAH	Phenylalanine hydroxylase
PAMP	Pathogen-associated molecular pattern
PCA	Perchloric Acid
PKA	Protein kinase A
PKC	Protein kinase C
PLC	Phospholipase C
Pru	Prugniaud strain
PTGS	Post-transcriptional gene silencing
PV	Parasitophorous vacuole
RISC	RNA-induced silencing complex
RT-qPCR	Real time quantitative polymerase chain reaction
SAM	S-Adenosyl methionine
SCID	Severe combined immunodeficiency
STAT1	Signal transducer and activator of transcription 1
TH	Tyrosine hydroxylase
TNF-α	Tumor necrosis factor-α
TNF-β	Tumor necrosis factor-β
TLR	Toll-like receptor
TSA	Trichostatin A
UV	Ultra violet
VTA	Ventral tegmental area
WT	Wild type

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Chapter 1

Introduction

1.1 Overview

The influence of the central nervous system reaches throughout the body, affecting a huge variety of systems, organs and cellular pathways. Monoamines play an important role in many of these functions and dysregulation of these pathways can cause widespread disruption, affecting many neurological functions such as motor control, cognition and memory. *Toxoplasma gondii* is an obligate intracellular parasite able to infect almost any mammal. The initial acute infection typically lasts 3 weeks, after which time a chronic neuronal infection is established, potentially lasting for the host's lifetime. Although *T. gondii* is among the most pervasive neurotropic pathogens in the world, little is known about the host-parasite interactions during chronic central nervous system (CNS) infection. In rodents, chronic infection is associated with the loss of innate fear of cat odour. Historically considered asymptomatic in humans, there is mounting evidence that the chronic stage of infection is correlated with an array of neurological diseases and host behavioural change. *T. gondii* has evolved into a specialist of mammalian neurophysiology. Delineating how this single-celled parasite is able to co-opt normal neurological function can provide new insights into the mechanisms governing neuronal function and dysfunction within the CNS.

1.2 Neurophysiology

In the 4^{th} century BCE Aristotle wrote that the human heart was the centre of intelligence, cognition and emotion; he attributed little function to the brain, except noting it was cold, suggesting that it played a role in blood temperature regulation[1]. Today these ideas survive only metaphorically; the brain has been established as the centre of neurological mechanisms since the 2^{nd} century C.E[2]. The central nervous system (CNS), consisting of the brain and spinal cord, is now recognised as the centre of cognition, memory, motor control and much besides.

Neurons, of which there are many subtypes, account for approximately 50% of all cells in the brain. Typically, the average adult brain contains approximately 86.1 billion neurons, with glial cells making up the majority of other cells[3–5]. Neuronal development is a protracted process, beginning at embryonic day 42 and lasting into late adolescence. However, the process of neurogenesis continues throughout adulthood[6]. The majority of CNS neuronal cell bodies are located in the cerebral cortex and the subcortex. Neurons have a distinct morphology consisting of many dendritic arbours extending from a cell body; from here long axons project throughout the CNS. These axons carry electrochemical signals to the axon terminal, where synapses are formed with other neuronal dendrites. Compounds such as neurotransmitters are released into the synapse at the axon terminal. There they can bind to receptors and transporters found on both the pre- and post-synaptic neurons. The binding of a neurotransmitter to a receptor can alter the ion permeability across the plasma membrane. If the change in electrical charge is great enough, an action potential is induced, which travels along the neuronal axon. An electrical potential can be carried over 1 meter in the human brain at a speed of over 100 meters a second[7]. Neurons are able to make both local and long distance connections throughout the brain. A single axon can make connections with up to 100 other cells and these complex networks form the CNS. The cellular reactions induced by this process are determined by the signalling molecule, the receptor to which it binds and the cell types involved[8]. Much of CNS function is mediated by neurotransmitters. The concentration and duration of neurotransmitters found in the synaptic cleft determines the type and duration of the signal in target neurons. Broadly, there are 6 categories

of neurotransmitters in the mammalian brain: amino acids, gaseous signalling molecules, monoamines, trace amines, peptides and purines.

These neuronal interactions form the infrastructure of the CNS allowing information to be processed from external stimuli. This can induce homeostatic control, movement and many other complex behaviours. In this thesis the host-pathogen interaction is examined, specifically how *T. gondii* may alter the mechanisms of host catecholaminergic neuronal signalling and gene regulation. Here I will provide a brief overview of the neuronal signalling pathways examined.

1.2.1 Catecholamine signalling

The catecholamines are a group of monoamines containing a catechol group. They include important neurotransmitters such as dopamine, norepinephrine and epinephrine; these are involved in a wide variety of CNS functions such as motor control, memory and cognition. The cell bodies of monoamine containing neurons are typically found in the mid-brain region, however their axons are widespread, modulating neuronal activity throughout the brain. Dopamine and norepinephrine are synthesised in the pathway: L-tyrosine $\rightarrow$ L-DOPA $\rightarrow$ DA $\rightarrow$ NE (Figure 1.1). Although catecholamines all have a very similar structure, their functions within the CNS can be divergent.

Dopamine

Groundbreaking work by Carsson *et al* first identified dopamine as a neurotransmitter in 1957. Previously, it was thought to be merely a precursor to norepinephrine, with little independent neurological function [9]. Over the last 60 years dopamine has arguably become the most intensively studied of the catecholamines due to its role in diverse diseases. At a cellular level, L-DOPA synthesised in the cytosol is metabolised to dopamine while being concordantly transported into synaptic vesicles by vesicular monoamine transporter 2 (VMAT2) where it is stored until release. Typically, dopamine is released via exocytosis into the synaptic cleft, where it binds to a variety of dopamine receptors and elicits an action potential in the post-synaptic neuron. Dopamine is then rapidly released

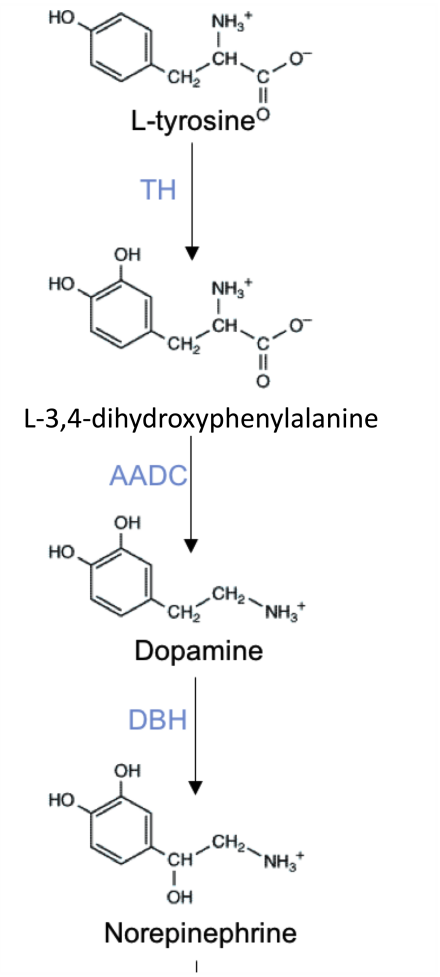


Figure 1.1: Schematic illustrating the synthesis of the catecholamines. L-tyrosine is converted to 3,4-dihydroxyphenylalanine (L-DOPA) by tyrosine hydroxylase (TH); L-DOPA is then converted to dopamine (DA) by aromatic L-amino acid decarboxylase (AADC). Dopamine is converted to norepinephrine (NE) by dopamine β-hydroxylase (DBH); norepinephrine can then be synthesised to epinephrine (E) by phenylethanolamine N-methyltransferase (PNMT).

from receptors and molecules can be recycled via re-uptake by dopamine active transporters (DAT).

Table 1.1: A summary of the major functions and mechanisms of the D1, 2, 3, 4 and 5 dopamine receptors[10].

Receptor Class	Subtype	Brain Region	Mechanism	Function in the Brain
DR1-like	D1	Caudate/putamen; nucleus accumbens; olfactory tubercule; hypothalamus; thalamus; frontal cortex.	Activate adenylyl cyclase inducing cAMP release and PKA activation; inhibit potassium channels, activate calcium channels	Motor function; development; reward pathway; fear; working memory and learning.
	D5	Hippocampus; thalamus; lateral mamillary; cerebral cortex.		Pain; learning and memory; endocrine signalling.
DR2-like	D2	Caudate/putamen; nucleus accumbens; olfactory tubercule; cerebral cortex; islands of calleja.	Inhibit cAMP release; block calcium channels; open potassium channels.	Memory; reward pathway; motor control; body temperature regulation.
	D3	Nucleus accumbens; olfactory tubercule; cerebral cortex; islands of calleja.		Endocrine pathway; cognition.
	D4	Frontal cortex; midbrain; amygdala; hippocampus; hypothalamus; thalamus; medulla; retina.		Vasodilation; blood pressure; cognitive function; body temperature regulation.

Dopamine is synthesised in the substantia nigra and ventral tegmental area (VTA) of the mid-brain where the majority of dopaminergic neuronal bodies are located[11]. Many of these neuronal projections reach the prefrontal cortex. Classically, there are three major dopaminergic pathways in the brain: the nigrostriatal, mesolimbic and mesocortical[12]. There is also the tubuloinfundibular pathway which links the hypothalamus to the pituitary gland. Each pathway has been associated with particular neurological functions and dysfunction-associated diseases: motor control, reward response, cognition and, endocrine regulation respectively[13–16]. However, absolute demarcation of pathways in this way does not account for the complexity of neurological circuitry.

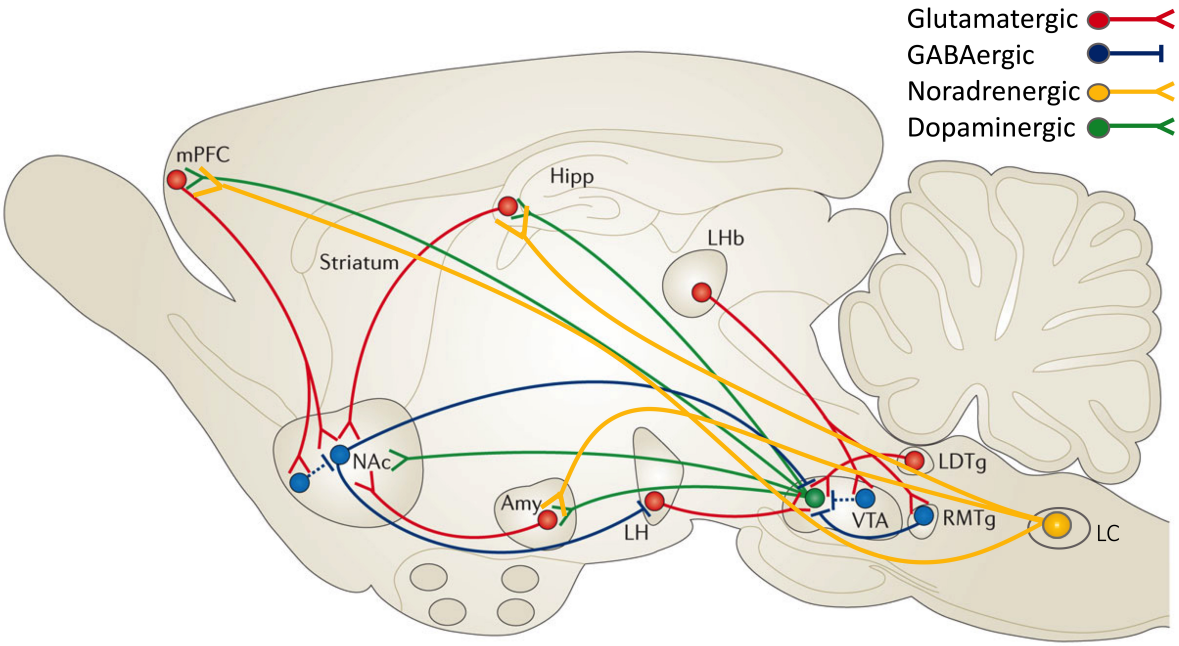


Figure 1.2: Schematic illustrating the major glutamatergic, GABAergic, noradrenergic and dopaminergic neuronal pathways. The glutamatergic pathways extend from the lateral dorsal tegmentum (LDTg), lateral habenula (LHb) and lateral hypothalamus (LH) and reach the ventral tegmental area (VTA). Additionally, the NAc receives glutamatergic input from the hippocampus (Hipp), amygdala (Amy) and medial prefrontal cortex (mPFC). Inhibitory GABAergic neurons project from the nucleus accumbens (NAc), lateral hypothalamus (LH) and to the ventral tegmental area (VTA). The latter is also reached by GABAergic neurons from the rostromedial tegmental nucleus (RMTg). Noradrenergic projections originating from the locus coeruleus to the amygdala (Amy), hippocampus (Hipp) and medial prefrontal cortex (mPFC). These regions are also reached by the dopaminergic pathways extending from the ventral tegmental area (VTA). Adapted from[17].

Dopamine receptors mediate many of the physiological effects of dopamine. There are 5 subtypes of dopamine receptor - 1, 2, 3, 4 and 5; all of which belong to the transmembrane G-protein coupled receptor family[18]. Type 1 dopamine receptors are the most abundant in the CNS and along with the type 5 receptors. The post-synaptic neuron determines whether these receptors are involved in excitation of the target neuron via sodium channels or inhibition via the opening of potassium channels. Types 2, 3 and 4 are primarily implicated in inhibition of the target neuron[19]. Type 2 dopamine receptors are implicated in movement control, mood and emotion[20]. Type 2-like receptors are the targets of many antipsychotic drugs. This has allowed limited elucidation of dopamine receptor functions; however, receptor agonists or antagonists typically lack receptor subtype specificity. This can lead to conflation of receptor roles[21]. Dopamine receptor knock-out mouse models have revealed much about the role of dopamine in the brain and these findings are summarised in (Table 1.1).

The impact of dopamine on various neurological pathways can be observed in dopamine deficient mice. These *DA-/-* mice lack tyrosine hydroxylase activity in dopaminergic neurons only; although these animals can survive into early-adulthood they exhibit hypoactivity and a reduced feeding behaviour which is lethal without intervention[22]. L-DOPA treatment in adulthood can rescue these behaviours suggesting that dopamine is not necessary for normal neuronal growth and development. *DA-/-* knockout mice also exhibit impaired active avoidance, a paradigm used to measure memory and learning[23]. Taken together this suggests dopamine plays an essential role in motor control, emotion and learning.

Norepinephrine

Cerebral dopamine concentration can also be affected by norepinephrine signalling. In noradrenergic neurons, once dopamine is packaged in vesicles the enzyme dopamine β-hydroxylase (DBH) synthesises its conversion to norepinephrine, also known as noradrenaline. Norepinephrine was first described in the 1960s by Ulf von Euler who, along with Julius Axelrod and Sir Bernard Katz shared the Nobel Prize in Physiology or Medicine for their pioneering study of neurotransmitters[25,26]. Norepinephrine neuronal cell bodies are predominately located in

Table 1.2: A summary of the major functions and mechanisms of the α1, α2 and β -ARs[24].

Receptor Class	Subtype	Major localisation	Mechanism	Function in the Brain
α1	α1A, α1B, and α1D	Neurons, blood vessels	PLC and IP3activation, PKC is induced causing cAMP activation	Vasodilation, glucose metabolism and smooth muscle contraction
α2	α2A, α2B and	Neurons	cAMP inhibition	Inhibits norepinephrine release, glucose metabolism, memory and attention
β	β1, β2 and β3	Neurons, smooth muscle cells	Activate adenylyl cyclase inducing cAMP release and PKA activation;	Heart rate, muscle and motor control

the brain stem and from here widespread projections reach virtually all areas of the brain. The norepinephrine neurons of the locus coeruleus were first identified in 1965 and represent the first anatomically delineated monoamine neurological pathway[27].

The physiological effect of norepinephrine is primarily regulated by post-synaptic receptors as well as by the norepinephrine active transporter (NET). In 1948 Ahlquist *et al* first identified norepinephrine's affinity for three major classes of G protein-coupled receptors found in the mammalian brain - α1 (α1A, α1B, and α1D), α2 (α2A, α2B and α2C) and β (β1, β2 and β3) adrenergic receptors (ADRs) (Table 1.2)[28]. Typically, α1-ARs, located on the post-synaptic neuron, are coupled to Gq proteins; once norepinephrine binds the receptor is activated, inducing phospholipase C (PLC) and inositol 1,4,5-triphosphate (IP3) intracellular signalling, PKC is then activated and intracellular calcium is released stimulating the neuron[29,30]. α2-AR can be found on both post and pre-synaptic neuronal membranes; action via these receptors can reduce the amount of intracellular cAMP inhibiting signal transmission. Norepinephrine has the

highest affinity for the α2-AR, this produces autoregulation of norepinephrine signalling[31].

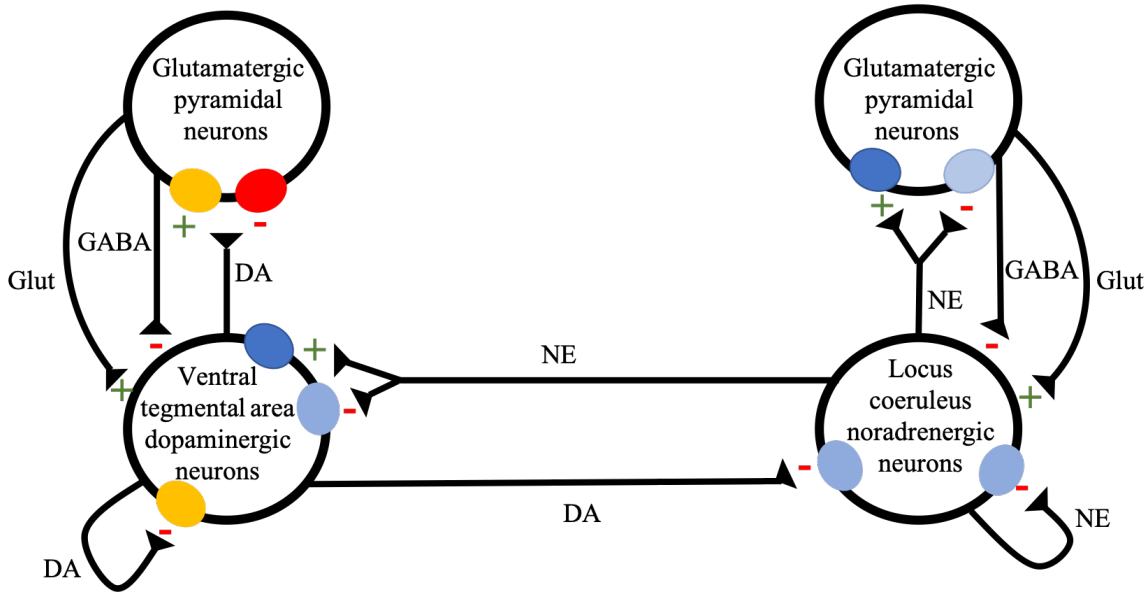


Figure 1.3: Schematic representation of the reciprocal interaction between dopamine, norepinephrine and glutamatergic signalling pathways. Schematic shows the complex nature of signalling pathways and regulation of glutamatergic, dopaminergic and noradrenergic activity. Dopamine-1 like receptors are shown in orange and dopamine-2 like receptors are shown in red. Dark blue denotes α1-adenoreceptors and light blue represents α2-adenoreceptors. (+) signs indicate an agonism or stimulatory effect and (-) signs indicate an antagonism or an inhibitory effect.

Norepinephrine signalling plays an important role in cognitive functions such as memory, learning and attention, as well as neuroendocrine and autonomic regulation[32]. Norepinephrine and dopamine also act in synergy as modulators of catecholaminergic signalling (Figure 1.3)[33]. Several publications have reported that treatment of noradrenergic receptors with dopamine inhibited norepinephrine release[34–36]. Guiade and colleagues observed selective depletion of dopaminergic neurons increased activity of LC noradrenergic neurons by 47%[37]. Conversely, selective loss of noradrenergic neurons in the LC enhanced the firing activity

of VTA DA neurons by 70%. Mouse models unable to synthesise dopamine and norepinephrine do not survive postnatally, primarily because of cardiovascular abnormalities[38]. Compared to healthy controls low norepinephrine mice are slower to navigate a maze they have previously been exposed to. This behaviour can be rescued by treatment with desipramine a reuptake inhibitor[39]. Furthermore, mouse models with impairments in either dopamine or norepinephrine signalling exhibit impaired working memory. Taken together these results suggest that both systems must work in synergy[40–46].

Norepinephrine is also important for the process of neurogenesis in the adult brain. Neurogenesis, the process of forming new neurons from primitive stem cells, persists throughout adulthood. Contrary to early dogma, it was observed by Reynolds and Weiss that cells isolated from the subventricular zone of the adult mouse brain are able to differentiate in culture to become astrocytes and neurons[47,48]. It is now known that neuronal stem cells can be isolated from the dentate gyrus of the hippocampus and many other areas[49–52]. It has been observed that neurogenesis is correlated with norepinephrine content of the brain suggesting norepinephrine increases proliferation of neuronal stem cells and hippocampal neurogenesis[53,54]. *DBH-/-* mice, unable to synthesis norepinephrine exhibit decreased adult hippocampal neurogenesis[55]. Jhaveri and colleagues observed that treatment with $\alpha 2$ and β-ARs agonists can induce increased neuronal stem cell proliferation[56,57]. However, the exact mechanisms governing neuronal stem cell proliferation and regulation remain poorly delineated.

Norepinephrine was identified as the major sympathomimetic neurotransmitter in 1948[58]. CNS derived norepinephrine plays a crucial role in physiological arousal pathways, such as the stress response, immune activation and the sympathetic nervous system[59–61]. The hypothalamic-pituitary-adrenal (HPA) axis regulates the physiological response to stressors; HPA axis activation is mediated by a complex feedback network of immunological, hormonal and neurotransmitter molecules. Norepinephrine is an important activator of this response. Norepinephrine release, by noradrenergic neurons originating in the locus coeruleus, binds to $\alpha 1$-ARs expressed at the surface of corticotropin-release factor neurons[62]. This can induce the post-synaptic neuron to release the neurohormone corticotropin-release factor; inducing the HPA axis signalling cas-

cade[63]. Corticotropin-release factor stimulates adrenocorticotropic hormone to be released by the anterior pituitary, which in turn causes the adrenal cortex to release glucocorticoid. Glucocorticoid release can induce immune and sympathetic system activation[64].

Epinephrine

Norepinephrine is the precursor to epinephrine, also known as adrenaline. Both norepinephrine and epinephrine are essential within the sympathetic nervous system. Epinephrine is exclusively synthesised in the adrenal glands, it is stored in the chromaffin cells of the adrenal medulla prior to release into the blood. This adrenergic signalling regulates the fight-or-flight response. External stimuli such as HPA axis mediated glucocorticoid release can induce rapid release of epinephrine into the blood[59]. This can induce changes in many mammalian systems including digestion, heart rate, vasodilation as well as blood oxygen and glucose concentrations. A comprehensive review of the role of monoamines in the sympathetic nervous system is beyond the scope of this thesis[61,65].

1.3 *Toxoplasma gondii*

This thesis examines the neurophysiological changes induced during chronic parasitic infection. *Toxoplasma gondii* belongs to the diverse Apicoplexan phyla of parasites which includes *Plasmodium falciparum*, the malaria parasite. Apicoplexans are characterised by apicoplasts, a unique organelle evolved from plastids found in plants, however, apicoplasts contain no pigment and are incapable of photosynthesizing. *T. gondii* seropositivity is present throughout the world, prevalence is estimated at 9% in the UK population[66] (Figure 1.4). Indeed, *T. gondii* was recently ranked as the second most important food-borne pathogen in Europe[67].

1.3.1 Life cycle through the human host

Toxoplasma gondii is ubiquitous throughout the animal kingdom. As a parasite it is unique in its ability to infect virtually any nucleated vertebrate cell. However, a

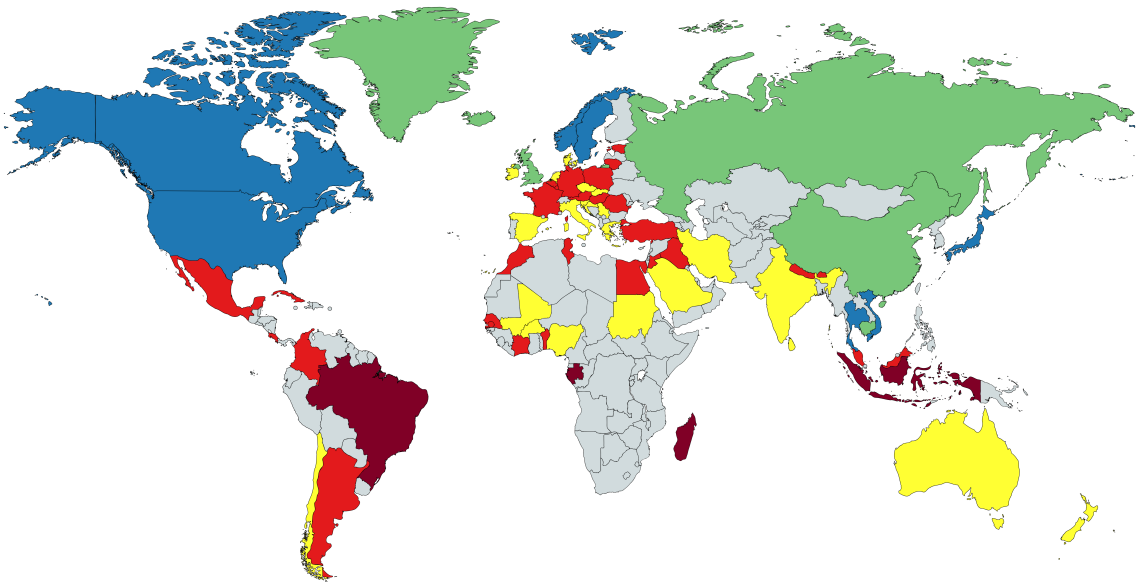


Figure 1.4: Seroprevalence of *T. gondii* throughout the world. Dark red, prevalence above 60%; red, 40-60%; yellow, 20-40%; blue 10-20%; and green, <10%. Grey indicates absence of data. Adapted from [66]

dichotomy exists between the diversity of *T. gondii*'s intermediate hosts wherein asexual reproduction can occur and the specificity of the sexual life cycle, which is only able to occur within the Felidae, the definitive host[68,69] (Figure 1.5). The mechanisms governing host recognition by the parasite, particularly how *T. gondii* identifies Felidae, remain poorly delineated. After a feline has ingested the parasite, typically as a bradyzoite tissue cyst, the parasite migrates to the gut and invades enterocytes. After asexual replication microgametes and macrogametes are formed. These macrogametes fuse to create a zygote which develops into an oocyst[70]. The parasite then undergoes endogenous sporogony and is finally shed in the faeces of the cat. These cysts can survive in the environment for up to 80 days and propagate the spread of the parasite[71]. As well as contaminating the soil, oocysts are widely found in food and drink, leading to intermediate host infection.

Intermediate host infection initially comprises a transient, acute stage characterised by the presence of rapidly replicating tachyzoites, found throughout the host. Toxoplasmosis, only observed in the immunocompromised or those infected while in utero, is caused by unchecked, rapidly dividing tachyzoites[72,73]. Recently published data has demonstrated tachyzoites are able to cross the blood-brain barrier via infection and lysis of barrier endothelial cells[71]. Ocular toxoplasmosis is common amongst immunocompromised patients, either due to infection or reactivation of bradyzoites and can result in chorioretinitis[74]. In *T. gondii* endemic areas of Europe and the United States of America it is the leading cause of vision loss[75]. During ocular toxoplasmosis tachyzoites enter the retina, here they divide rapidly creating lesions surrounded by scar tissue and inflammation. Without treatment this can lead to vision loss[76]. Toxoplasmic encephalitis, rapidly dividing tachyzoites throughout the CNS, is typically caused by caused by reactivation of latent *T. gondii* infection[77]. Despite advances in highly active antiretroviral therapy Toxoplasmic encephalitis is still the leading cause of morbidity and mortality in AIDS patients[78]. Patients can present with a broad range of symptoms including seizures, dementia and lethargy making diagnosis difficult[79]. Additionally, congenital toxoplasmosis is caused by infection during pregnancy and can have a wide spectrum of clinical symptoms; from the

subclinical to still birth depending on time of infection and virulence of the *T. gondii* strain[80].

The immune system of an immunocompetent host will begin to control tachyzoite growth within a week, by which time intracellular bradyzoites have formed in the brain and muscle tissue, establishing a chronic infection which may last for the lifetime of the host[81,82]. Bradyzoites are distinguished from tachyzoites by the presence of a cyst wall, which can contain hundreds of parasites. Historically considered a dormant infection in the immunocompetent host, recent data has demonstrated that bradyzoites continue to replicate[83]. As understanding of the host parasite interaction has improved, the chronic stages of infection are no longer considered asymptomatic. Indeed, the neurophysiological changes and how they affect host behaviour and health have become increasingly clinically significant.

1.3.2 Bradyzoite structure

This thesis focuses on chronic neuronal infection. A thorough review of the literature regarding *T. gondii* cell biology is beyond the scope of this thesis and can be found at[73,84,85]. Bradyzoites are encased by a glycan-rich cyst wall. The mechanisms governing the formation of this wall remain poorly understood: one hypothesis is that it is made via the secretion of dense granules by the tachyzoite stage during differentiation[86]. The cyst walls can encase hundreds of bradyzoites, tissue cysts can be as large as 100μm in size. The bradyzoite nucleus can be found at the centre, rhoptries at the apical end and dense granules throughout.

Historically bradyzoites were considered to be a non-replicative phase of infection, "brady" is taken from the Greek meaning slow[87]. As laboratory techniques have advanced, it has become clear that bradyzoites are constantly replicating by endodyogeny, and not all tissue cysts are alike in size nor density[83]. This suggests that bradyzoites are dynamic, able to adapt cellular processes to suit their environment. *T. gondii* is a parasite, and as such it is able to alter host cell function to facilitate its own survival. Bradyzoite cell biology remains under researched, partly because they were considered an asymptomatic stage of infection

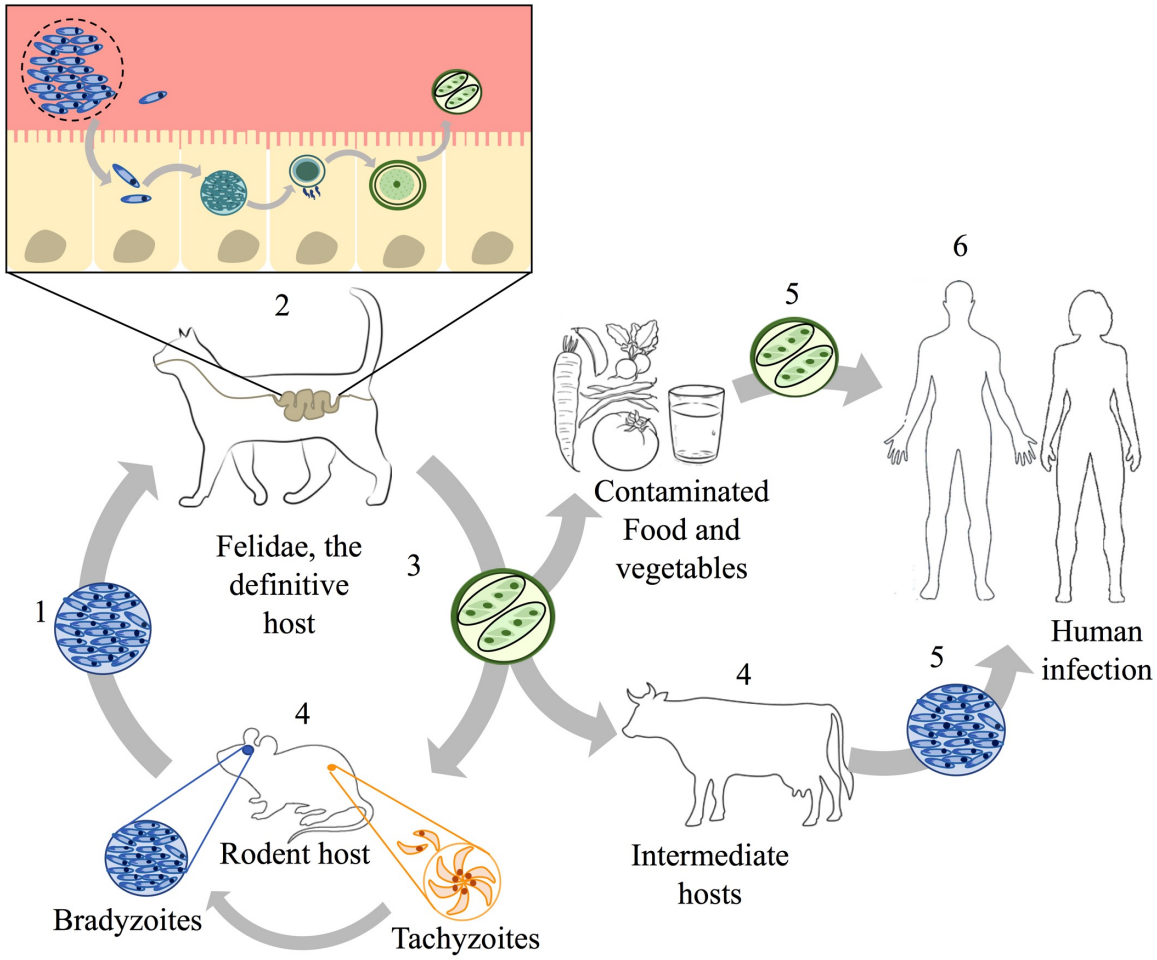


Figure 1.5: Schematic showing the life cycle of *T. gondii*. 1) Typically the Felidae, the definitive host, ingests the parasite as a bradyzoite tissue cyst. 2) Here the sexual life cycle stages can occur. Tissue cysts rupture and trophozoites invade the gut endothelial cells. Schizogony then occurs; gametocytes are formed and fuse to form a zygote; after this the oocyst develops. 3) Oocysts are shed by the cat in faeces where they can remain viable in the environment. 4) Intermediate hosts such rodents and livestock are infected by consuming these oocysts in the environment. 5) Human infection can originate from consuming uncooked fruits, vegetables or alternatively undercooked meat, or direct ingestion from the environment (e.g. contaminated sandboxes). 6) Infection can be passed between human hosts congenitally.

and also due to the difficultly of *in vitro* culture. The possible contribution of parasite effectors to host neurophysiological change is discussed in Section 1.5.5.

1.4 Host behavioural consequences

The concept of the "extended phenotype" describes how expression of an organism's genes may have a wide-reaching effect beyond that of the organism itself[88]. In the case of *T. gondii*, parasitic genes are able to alter host neuronal function leading to behavioural changes, which can facilitate the life cycle of the parasite. This thesis aims to further our understanding of what neuronal mechanisms may be affected by infection and the mechanisms involved. Here the various behavioural phenotypes associated with *T. gondii* infection will be discussed.

1.4.1 The rodent host

Classically, *T. gondii* infection is associated with the loss of innate fear phenotype. First identified by Berdoy *et al*, chronically infected rodents no longer exhibit a fear response when presented with feline urine[89]. Indeed, infected rodents respond to the odour with attraction, in a Y-shaped maze when presented with either rabbit (non predator) or feline urine, infected animals spent more time in the cat urine arm (Figure 1.6). This has been compared to sexual attraction in the rodent hosts[90]. Uninfected rodent fear of feline odour, even in laboratory bred animals never before exposed to predation, is evident through various behavioural and physiological markers. Rodents exhibit aversion to the odour as well as greater corticosterone, a hormone associated with the stress response, and increased c-Fos throughout the CNS[91]. It has also been observed that infected animals spend less time grooming, normally a conflict-avoidance behaviour which signals anxiety[92].

The loss of fear phenotype has been identified in both wild and laboratory raised mice and rats in various studies spanning almost 20 years[89,93–96]. This phenotype appears feline specific - infected rodents do not lose their aversion to odours of other non-definitive predators such as fox, dog or mink[49,94,97,98]. Kaushik and colleagues found that infected rats prefer wild-cat scent over that

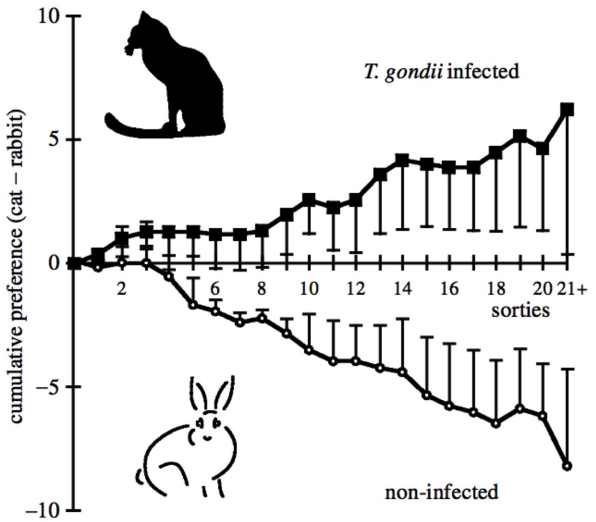


Figure 1.6: Experimental data showing chronically infected rats exhibit a loss of fear phenotype. Graph shows number of sorties made by either chronically infected or uninfected rats in a Y shaped maze with rabbit odour in one arm and feline odour in the other. Infected rats were more likely to enter to the arm scented with feline odour than uninfected controls. ±SEM shown, n=10, p=0.002. Reproduced from[89].

of domesticated cats. As well as the specific loss of fear phenotype, there is also evidence that infected rodents exhibit a generalised reduction in neophobic behaviours, such as novel object approach[99]. Interestingly, this behaviour can be observed in laboratory animals that have completely cleared the parasite and present no neuroinflammatory response[100]. The loss of fear phenotype is not observed during acute infection, appearing to be a bradyzoite specific "extended phenotype".

It is surmised that due to these behavioural changes in the wild there may be increased predation of infected rodents. This would allow *T. gondii* to infect its definitive host, facilitating the sexual life cycle of the parasite, however, this has never been definitively shown. Despite extensive investigation of the loss of fear phenotype the mechanisms underlying *T. gondii* induced behavioural changes are still poorly delineated; this is further discussed in Section 1.5.

The first behavioural studies undertaken with *T. gondii* infected mice found that infected mice demonstrated poor learning capacity compared to uninfected controls, a finding that has since been widely reproduced[101–103]. Ihara and colleagues found that chronic infection altered the learned fear response in infected mice[104]. However, there has been little evidence of this behaviour in infected rats[101]. Infected mice exhibit more sickness phenotypes than rats, such as circling and head bobbing[105]. Mice can develop upwards of 1000 neuronal cysts, whereas rats and humans typically have less than 10 neuronal cysts[106,107]. The large number of cysts may result in some generalised symptoms of pathology due to toxoplasmosis rather than host behaviour changes. This may be a result of the aggressive nature of *T. gondii* infection in mouse models, where behaviour due to toxoplasmosis may be falsely attributed to host behavioural manipulation. Therefore, rats may be a preferred chronic disease model due to their innate resistance and similarity to human infection.

1.4.2 Human behavioural consequences

Chronic *T. gondii* infection is correlated with human behavioural change. In a large cohort study, response times were reduced in participants with *T. gondii* seropositivity compared to controls. Seropositive participants also appeared to

lose concentration faster (Mante-Haenszel test for age-stratified data, chi-square = 21.45, p<0.0001)[108]. Indeed, several studies have found *T. gondii* seropositivity was increased in drivers involved in traffic accidents. It is surmised that this may be due to reduced response time, lower concentration on a task or increased risk-taking behaviour[109–111]. In more limited studies, chronic infection has also been associated with altered sexual preferences, promiscuousness and increased aggression in men in more limited studies[112,113]. Moreover, it has been proposed that these small behavioural changes may mean *T. gondii* holds influence over human culture[114]. However, *T. gondii* seropositivity is correlated with low socioeconomic status, which is likely to have a confounding effect on behaviour. Furthermore, behavioural data is primarily gathered by self-reporting behaviour or preferences. There is evidence that this self-reporting of behaviour may lead to under-reporting, causing reduced reliability and accuracy[115,116]. Additionally, seropositivity is an indirect method of detecting *T. gondii* parasites, prone to inaccuracies[117]. Parasites have been found in seronegative animals, and conversely no parasites detected in seropositive animals[118,119].

Chronic *T. gondii* infection and disease

Chronic infection has been associated with a diverse range of diseases, chiefly mental health disorders. Schizophrenia is characterised by an inability to distinguish thoughts from reality. Often schizophrenia is associated with auditory and visual hallucinations[120]. The aetiology of this chronic disease is poorly characterised and its onset is believed to be induced by a complex interplay between environmental and genetic factors. Many effective schizophrenia drug treatments are dopamine antagonist, therefore early research focused on increased dopamine signalling during schizophrenia. However, the role of dopamine in schizophrenia appears to be subtle, indeed altered dopaminergic signalling can be observed in patients before the onset of symptoms[121]. Abnormalities in almost every neurotransmitter pathway have been observed in the brain of schizophrenia sufferers[121–124]. There is increasing evidence that altered glutamatergic signalling, induced by *N*-methyl-D-aspartate (NMDA) receptor hypofunction may contribute to the disease[122,125,126]. The association between schizophrenia and infection is

the most extensively studied correlation. Two meta-analyses collectively spanning 70 studies over 55 years concluded that there is a positive correlation between *T. gondii* seropositivity and schizophrenia; identifying odds ratios of 2.54 and 1.81 ($p<0.0001$ and $p<0.0001$ respectively)[127,128]. Despite the large number of studies conducted no causal link between infection and schizophrenia has ever been found.

T. gondii infection has also been correlated with depression, psychosis and self-directed harm[128–132]. However, countries with higher *T. gondii* prevalence do not report increased incidence of mental health disorders[117]. Understanding changes induced by *T. gondii* to normal neurological function is essential to understanding how infection may predispose some to mental health issues, and may further our understanding of neurological dysfunction.

1.5 Host-parasite interaction during chronic *T. gondii* infection

Host-parasite interactions during *T.gondii* infection have been studied for some 40 years. Yet, despite this, the mechanisms governing host behavioural change remain poorly delineated. In this section the current state of research into CNS changes induced by *T. gondii* will be discussed, and the contribution of these to parasite associated phenotypes is evaluated.

1.5.1 The neuroimmune response to infection

The CNS is considered an immune-privileged organ, entry to which is protected by the blood-brain barrier (BBB). The BBB describes the unique microvasculature structure protecting the CNS from cellular mediators of inflammation, pathogens and toxins circulating in the blood. This allows CNS homeostasis to be maintained and protects the CNS from damage[133]. Tight capillary junctions between endothelial cells, and the specific polarized transport systems at the BBB exert tight restrictions on ion and molecule transport to the CNS[134,135]. Furthermore, astrocyte endfeet processes link endothelial blood flux to neurons, regulating the maintenance of the physical barrier to the brain[136]. Thus, for parasites that

have entered the CNS, the BBB provides *T. gondii* with limited protection from the host immune system. That said, the CNS is able to mount a neuroimmune response and has an, albeit limited, ability to regulate infection. As discussed in Section 1.3 chronic infection can last for the lifetime. This suggests that bradyzoites are able to exert long-term host immune evasion.

The host immune response to chronic infection of neurons is present and found to be essential throughout bradyzoite infection, preventing reactivation of tachyzoites and toxoplasmosis[137–139]. T lymphocytes are essential for this process. Lymphocyte recruitment spikes at 30 days post-infection and then decreases. When infected with a virulent strain of *T. gondii*, mice previously exposed to infection exhibited higher survival rates that naive mice. This suggests that memory T cell activity during neuronal infection is important during infection[140]. T cell infiltration to the brain is required during infection to prevent reactivation of tachyzoites[141,142]. However, $CD8^+$ cells cannot clear chronic infection and are unable to recognise bradyzoites[143]. It is clear that neurons also play an active part in the neuroimmune immune response to infection. Transgenic mice lacking neuronal gp130, the signal-transducing receptor for IL-6 cytokines, are unable to control toxoplasmosis[144].

The neuroimmune response is present throughout infection. Dendritic cell migration into the brain during infection has been well documented in mouse models[145–147]. Indeed, migration of infected dendritic cells to the CNS was thought to be the major mechanism used by *T. gondii* to pass the BBB[145,148,149]. In mouse models of chronic *T. gondii* infection, cells expressing $CD11^+$, a dendritic cell marker, were found to be the major producers of interleukin-12 (IL-12) in the brain. IL-12 is able recruit natural killer (NK) cells, as well as induce T cell differentiation to Th1 cells. Dendritic cells and macrophages express many Toll-like receptors (TLRs) including TLR11, able to recognise pathogen-associated molecular patterns[150]. *TLR11-/-* mouse models chronically infected with *T. gondii* were unable to recruit dendritic cells to the brain and therefore, could not mount an adaptive immune response to infection. Furthermore, these mice exhibited greater neuropathology and an inflammatory response. They also had an increased tissue cyst burden, suggesting this signalling pathway plays an important

role in regulating chronic infection[151]. TLR11 is able to bind *T. gondii* profilin inducing immunoactivation and the recruitment of many pro-inflammatory cytokines such as interferon-γ (IFNγ), tumor-necrosis factor-α (TNF-α), IL-1β and IL-12[152]. Many of these proinflammatory cytokines are increased in those suffering from major depressive disorder[153–156]. MyD88, an adapter molecule for all TLR recognition, excluding TLR3, is essential for the neuroimmune response during *T. gondii* infection[157,158]. *MyD88-/-* mice were found to have reduced sera IL-12 and interferon-γ (IFNγ) resulting in increased toxoplasmosis[158].

Chronic infection is characterised by increased inflammation. Host transcriptome analysis of chronically infected mice has identified increased IFN-inducible GTPase families, granzymes, major histocompatibility complex class II (MHC II) and IL-10. Microglia are important producers of IFNγ, essential for cell mediated protection during chronic infection. IFNγ is able to diffuse throughout the brain, it can bind to IFN-γ receptors 1 and 2, activating the JAK/STAT1 signalling pathway. JAK/STAT1 signalling induces up-regulation of immunity-related GTPases; as well as the transcription factor IRF1, able to activate IFN-β and MHC II molecules. During neuroimmune activation microglia and, to a lesser extent macrophage are the major producers of IFNγ. This Th1 cytokine is essential for pro-inflammatory activity. IFNγ plays a central role during the neuroimmune response. *IFNγ -/-* mouse models are unable to prevent tachyzoite reactivation, succumbing to infection[142].

Increased IFNγ is found throughout the course of infection. As well as recruitment of lymphocytes it stimulates the release of indoleamine 2,3-dioxygenase (IDO) the rate-limiting enzyme in the kynurenine pathway, catabolising tryptophan. *T. gondii* requires host tryptophan and by increasing IDO IFNγ is able to limit parasite growth. Given that tryptophan scavenging is the precursor to serotonin, constant immune activation can lead to reduced serotonin throughout the brain during infection[159]. Indeed, chronic IFNγ expression in the brain is associated with anxiety and depression[160,161]. Commonly, patients with hepatitis C virus treated with IFN develop depression. IFN-induced depression has been found in $\sim$30% of those suffering from chronic hepatitis C[162,163]. IFNγ also induces macrophage to produce nitric oxide synthase (NOS)[164]. Kynurenic acid

and quinolinic acid, products of the kynurenine pathway, can also induce oxidative stress in the brain, leading to neuronal damage and apoptosis. Kynurenic acid is an antagonist for glutamate ionic receptors and is also able to inhibit $\alpha 7$ nicotinic acetylcholine receptors, altering glutamatergic and catecholaminergic neurotransmission[165,166]. Increased kynurenic acid is associated with mental health disorders such as schizophrenia, bipolar disorder and depression[167–170]. *T. gondii* has been shown to increase kynurenic acid concentration in the brain of a Huntington's disease mouse model[171]. This may contribute to the observed correlation between chronic infection and mental health disorders.

The cerebral immune response described alters neurophysiology, and is likely a compounding factor during chronic *T. gondii* infection, particularly in those already genetically, or otherwise, predisposed to mental health disorders such as schizophrenia or depression. However, other pathogens such as cytomegalovirus and meningitis which induce a comparable chronic neuroinflammatory response are not associated with the same behavioural phenotypes as *T. gondii* infection[172,173]. Indeed, the loss of fear phenotype is typically observed 60-days post infection when the immune response is at its lowest level. Furthermore, the loss of fear phenotype can be observed in mice that have cleared the parasite, with no detectable immune response. This suggests that although the immune response may be a compounding factor, particularly important in *T. gondii*'s association with mental health disorders it does not provide a comprehensive narrative for host behavioural change.

Given the complex nature of signalling within the CNS, changes in one system can have far reaching implications. As discussed in Section 1.2.1 neurotransmitters such as norepinephrine and glutamate play an important role in the neuroimmune response. *T. gondii* has evolved many specialised mechanisms to circumvent the host immune response, further discussed in Section 1.5.5. Mechanisms which have evolved to aid in immune evasion may also affect many other aspects of the CNS because of the complex nature of these biological systems.

1.5.2 Hormonal changes during chronic infection

Endocrine changes have been associated with chronic *T. gondii* infection. Sex differences during infection have been observed during rodent and human behaviour. Male and female subjects report disparate behavioural changes compared to control groups[174]. Both male and female rodents exhibit the loss of fear phenotype, however, it has been observed that the loss of fear was less significant in female rodents[175]. Golcu and colleagues observed that the loss of fear is dependent upon the oestrus cycle, not widely acknowledged in the literature[176]. Notably, the majority of *T. gondii* rodent studies were performed with males, biasing much of the available data.

Testosterone is the principal male sex hormone, primarily synthesised in the testes from the substrates cholesterol and acetate. It is released into the bloodstream where it is able to travel throughout the body, including to the CNS. It plays an important role in androgenises, physiology and behaviour[177]. In the brain testosterone is able to alter dopaminergic signalling, Purves-Tyson *et al* observed that reduced testosterone can induce increased gene expression of dopaminergic regulatory genes and therefore, turnover of dopamine in adolescent male rats[178]. Altered testosterone during chronic *T. gondii* infection has been observed. Chronically infected castrated male rats do not exhibit the loss of fear phenotype, suggesting that the mechanism of loss of fear may be different in males and females[179]. Increased testosterone induced by infection in male rodents may induce the observed sexual arousal pathways, directed towards feline odour[95,112]. Individual testosterone levels have also been identified as a variability factor when analysing increased risk-taking behaviour of infected rodents. Testosterone supplementation was found to mimic the risk-taking behaviour observed in male rats[180]. However, there is a lack of consensus in the literature regarding how testosterone is changed during *T. gondii* infection. Studies of chronically infected men have found both increased and decreased testosterone with infection[181].

Testosterone release is regulated by and regulates the HPA axis[182]. As described in Section 1.2.1, the HPA axis modulates the stress response; activation can be triggered by chronic immune activation, such as the neuroimmune response induced by *T. gondii* infection. Increased activation of the HPA axis

increases blood glucocorticoid concentration[183,184]. This elevation is associated with many neurological disorders including depression and psychosis[185–187]. Increased testosterone production and HPA axis activation is associated with the release of arginine vasopressin, a key regulator of the stress response. This neuropeptide hormone is produced in the pituitary[188]. Arginine vasopressin is associated with a variety of functions in the CNS including aggression, reproductive behaviour and homeostasis[189]. Dass *et al* observed that arginine vasopressin expression was altered during chronic *T. gondii* infection. Indeed, it was observed that infection can induce hypomethylation of the arginine vasopressin promoter region, reducing gene expression[95,190].

As discussed in Section 1.4.2, *T. gondii* infection is associated with schizophrenia. Schizophrenia affects significantly more males than females, possibly due to the role of testosterone in altered dopaminergic signalling[191,192]. Given that *T. gondii* is able to alter testosterone signalling, this may contribute to the role of infection, increasing an individual's risk of developing schizophrenia. However, given that females also exhibit altered behaviour during infection testosterone is likely to augment the effect of *T. gondii* infection rather than inducing it. Alteration of testosterone levels alone can replicate risk-taking behaviour and aggression observed with infection, however, it is not able to induce the loss of fear phenotype. HPA axis activation is correlated with many of the same mental health disorders as *T. gondii* infection. However, chronic HPA activation is found in many disorders, such as insomnia, fibromyalgia and irritable bowel syndrome and these are not associated with specific behavioural changes[193–196]. Additionally, disorders associated HPA axis dysregulation are more common in females than males. However, there is no sex effect in *T. gondii* associated schizophrenia or depression[127,197]. The complete role of hormones during infection has yet to be delineated, our understanding is hampered by a bias towards male animal models. Furthermore, many studies with female subjects do not control for the oestrus cycle. Although it is probable hormonal changes play a role in susceptibility to behavioural changes, there is little evidence they are the major mechanism of neurophysiological change.

1.5.3 Distribution of bradyzoites in the CNS

Chronic *T. gondii* infection can persist for the lifetime of the host as tissue cysts form and grow within host cells. A model of *T. gondii* tropism for a particular brain region, such as the amygdala, has historically appealed as a straightforward explanation for host behavioural change. When exposed to sedated cats, unlike healthy rats, those with lesions in the amygdala exhibit no fear response; instead they approach and interact with the cats[198]. This behaviour replicates the loss of fear phenotype exhibited by *T. gondii* infected rodents. However, there is a lack of consensus throughout published data reporting *T. gondii* cyst distribution in the CNS (Table 1.3).

Although several publications find a higher density of cysts in the mid-brain region, this tropism is only mild. In the largest study to date distribution of bradyzoite cysts the CNS was analysed in 109 rats, only a mild tropism for the olfactory bulb, frontal cortex and hippocampus was identified[107]. Taken together this paucity of evidence for specific tropism suggests that *T. gondii* does not target a particular region.

Although *T. gondii* brain region tropism remains unsupported, there is strong evidence of *T. gondii* tropism for neurons *in vivo*. Since bradyzoites were characterised it has been widely observed that bradyzoite cysts are exclusively found in neurons[199–202]. It is unlikely that this is due to selective invasion as *in vitro*, induced bradyzoites can be formed in a wide variety of cell lines, primary cells cultures and explants. The mechanism governing this neuronal predilection remain poorly delineated; there may be, as yet unidentified, neuronal properties promoting bradyzoite growth. Emerging techniques of primary and 3-D cell culture may shed light on these interactions[203,204].

Tissue cysts are able to physically alter neuronal morphology, bradyzoites can be as large as 100μm in size and continue to grow throughout infection. 3-D image analysis has revealed that tissue cysts are predominantly found in neuronal processes[208]. Reduced dendritic spine length and density of chronically infected neurons have been observed[202,205]. Interestingly, loss of dendritic spine integrity and function is associated with schizophrenia and other mental health disorders[209]. This suggests that infection is likely to disrupt neuronal signalling,

Table 1.3: A summary of published data examining *T. gondii* tissue cyst distribution throughout the rodent brain.

Region with highest tissue cyst density	Species	Sample size	P value	Reference
Amygdala	Mouse	7	ns	[96]
Cerebral cortex	Mouse	5	ns	[205]
Basal ganglia	Mouse	6	<0.01	[206]
Forebrain cortex	Mouse	58	<0.001	[106]
Orbital cortex	Rat	6	ns	[207]
Olfactory bulb	Rat	109	ns	[107]

however, little research has been undertaken to establish the functionality of infected neurons *in vivo*. Rapidly dividing tachyzoites are able to induce extensive neuronal damage and inhibit neuronal function[210,211]. However, given that behavioural changes are not observed during acute infection, physical disruption alone is unlikely to be the cause of behavioural changes, particularly when taken together with the evidence that cysts can be located throughout the CNS the hypothesis of local neuronal distribution cannot be reconciled with the specific behavioural phenotypes observed during chronic infection.

1.5.4 Neuronal changes induced by chronic infection

The ability of chronically infected neurons to continue to function within the CNS of an animal remains largely unknown. The hypothesis that *T. gondii* is able to co-opt neuronal function, thereby altering host behaviour remains appealing. Changes in neuronal signalling during infection have been widely observed. Groundbreaking work by Stibbs and colleagues first reported changes in catecholamines during infection; a reduction in serotonin and norepinephrine, and an increase in total dopamine was observed in the brain tissue of drug-treated chronically infected mice[159]. Since then many publications have observed changes in catecholamine signalling during infection[212–218]; these findings are discussed at length in Section 3.2.

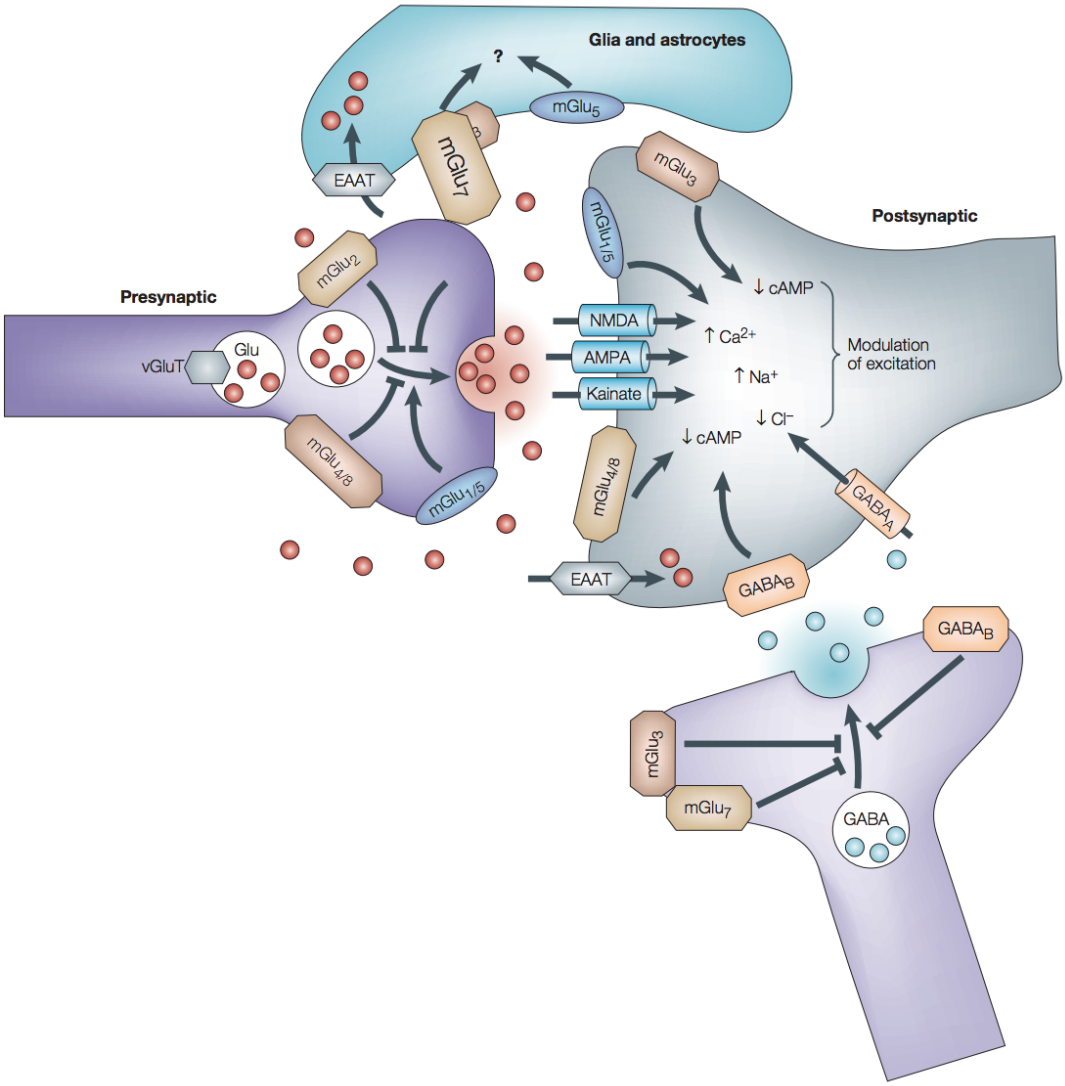


Figure 1.7: Glutamatergic signalling at a neuronal synapse. Glutamate is packaged into vesicles and released via exocytosis. Once released into the synapse glutamate can bind to a number of receptors and transporters including NDMA, AMPA and karinate; binding to these receptors induces excitation of the post-synaptic neuron. Unbound glutamate is then able to induce autoregulation, inhibiting further synaptic release. Glutamate is synthesised into GABA by GAD, GABA acts as a regulator of glutamatergic signalling, decreasing cAMP and Cl^- in the post-synaptic neuron and, therefore, inhibiting excitation. Scheme is reproduced from[219]

Changes in glutamate signalling have also been observed during chronic infection. Amino acids are responsible for the majority of neuronal signalling in the mammalian brain and glutamate is the major excitatory neurotransmitter of the CNS[220]. Glutamatergic signalling is regulated by γ-aminobutyric acid (GABA), an inhibitory neurotransmitter which may also play an important role during infection (Figure 1.7). David and colleagues found that during chronic infection there was a 2-fold down-regulation of GLT-1, a glutamate transporter expressed in glial cells[221]. Interestingly, despite only a small number of cysts being found, global changes in intraneuronal localisation of the enzyme glutamic acid decarboxylase 67 (GAD67), also known as GAD1, have been observed *in vivo*[222].

Transcriptome analysis of chronically infected mouse brain tissue showed GAD67 was significantly down-regulated during infection[223]. GAD67 catalyses the synthesis of GABA from glutamate and disruption of this pathway has been associated with neurophysiological consequences such as seizures also associated with *T. gondii* infection[224–227]. Recently published data identified global down-regulation of several essential components of glutamate synapses including the glutamate transporters: EAAT2, NDMA, and AMPA; as well as the glutamate receptors GluA1, GluA2[228]. Additionally, there is mounting evidence that disrupted glutamate signalling plays an important role in the development of schizophrenia[125,126]. However, the mechanism by which *T. gondii* alters glutamate signalling remains unknown. HPA axis activation, particularly increased glucocorticoids have been observed to alter glutamatergic signalling[229]. Global changes can be observed, despite only a small number of cysts being found in the brain. This suggests that these changes may be due to the generalised immune response, or the parasite is able to affect distant, uninfected cells in some way.

1.5.5 Parasite secreted effectors

Apicoplexans are in part characterised by the presence of rhoptry and dense granule proteins. These mediate much of the cellular host-parasite interaction. A comprehensive review of all parasite effector is beyond the scope of this thesis and can be found at[230]. Communication is initiated by rhoptry (ROP) proteins

injected during tachyzoite infection to facilitate cellular invasion, establishment of infection and formation of the parasitophorous vacuole. During infection host-parasite communication is partly mediated by effector molecules secreted from parasite dense granules (GRA proteins), that are trafficked to the host cell cytosol and nucleus[231]. Soares-Silva and colleagues recently reported GRA24 was able to subvert the host cell MAP kinase pathways. This altered host neuroimmune signalling and facilitated parasite to survival[232].

As previously discussed, host INFγ signalling is essential during *T. gondii* infection. Early studies observed that infected cells exhibited altered IFNγ response[233]. Additionally, inhibition of STAT1 pro-inflammatory cytokine signalling during infection[234]. Recently, Gay *et al* identified *T. gondii* inhibitor of STAT1 transcriptional activity (TgIST), a parasite derived protein that localises to the host nucleus[235]. It was observed that TgIST is able to sequester host STAT1 as well as induce epigenetic regulation by recruiting the nucleosome remodelling deacetylase transcriptional repressor.

Although not all rhoptry proteins are characterised, many are crucial during the chronic stage of *T. gondii* infection. *T. gondii* knock-out strains unable to produce deletion of ROP5, ROP17, ROP18 or ROP35, exhibit severely reduced cyst burdens *in vivo*[236]. Parasite derived proteins are able to regulate the host immune response. Schneider *et al* observed that *T. gondii* ROP16 was able to dampen INF-γ stimulated JAK/STAT1 responses and induced STAT3/6 phosphorylation[237,238]. This inhibited a parasite induced inflammatory response from infected cells. Furthermore, it has been observed that ROP16 can altered IL-12 mediated responses of infected macrophage[239]. Another rhoptry protein ROP18 is released into the host cytosol by tachyzoites during cellular invasion[240]. Du and colleagues reported that ROP18 phosphorylated p65, a subunit of NF-κB, targeting it for cellular degradation and suppressing the host immune response[241]. Furthermore, it has been demonstrated that ROP18 interacts with host endoplasmic reticulum-bound transcription factor (ATF6β)[242]. In mice models this inhibited dendritic cell antigen presentation to T lymphocytes.

Groundbreaking work by Koshy *et al* has demonstrated that *T. gondii* is able to inject rhoptry proteins into cells it does not infect[243]. Through this mechanism, termed 'kiss and spit', ROP proteins were observed in uninfected neuronal

cells during tachyzoite infection. However, live imaging of this mechanism has yet to be reported and it remains possible that these neurons were able to clear the parasite. Given that tissue cysts are only found in a limited number of neurons; a parasite secreted factor would provide a possible mechanism to explain global changes observed during infection. The injection of parasite proteins by circulating tachyzoites may play a role in some global neurophysiological changes observed in the CNS. However, it is unknown how long these parasite injected proteins may persist in uninfected cells and none have been detected during chronic infection.

1.6 Conclusion

The CNS is comprised of billions of neurons with a seemingly infinite number of connections, able to interpret and process external stimuli and effect change in every organ of the body. The brain is responsible for cognition, movement, emotion and much besides; it provides us with our own identity. The CNS is extremely complex, where a synapses function is characterised by neurochemical nuances. Neuronal function is governed by a tremendously diverse and complex network of neurotransmitter, hormones and cellular effector systems. Subtle changes in one system can have far reaching implications. Neurological dysfunction is associated with a plethora of clinical symptoms and diseases. Although treatment for many neurological disorders exist, mechanisms governing the aetiology of disease remain poorly understood.

T. gondii bradyzoites have evolved as specialists of human neurobiology. The parasite induces many specific neurophysiological changes to the CNS of the host, that appear to be designed to facilitate parasite survival and proliferation. Chronic *T. gondii* infection impacts many of these CNS pathways (Figure 1.8). Changes in neuroimmune, hormonal and neurotransmitter regulation contributes to altered CNS neurophysiology during infection. By understanding the cellular processes of this change we can further understand the mechanisms governing these complex networks of the CNS and how they contribute to disease.

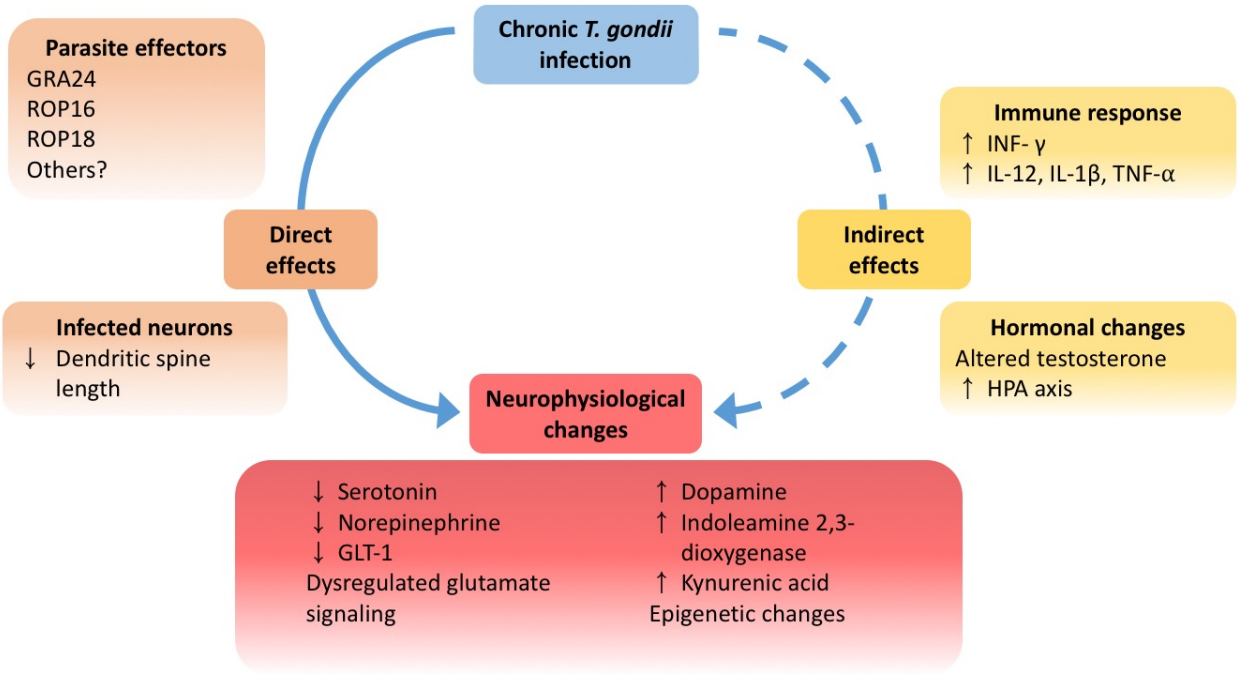


Figure 1.8: Flow chart summarising the possible mechanisms of neurophysiological change directly or indirectly induced by *T. gondii* infection. Adapted from[244].

1.7 Aims

1.7.1 Identify the possible cause of changes in catecholamine concentrations during chronic *T. gondii* infection

As discussed catecholamine changes can be observed in the brain of infected animals. I will attempt to characterise these changes and identify altered gene expression in the catecholaminergic regulator genes. I will use our previously conducted RNAseq data to identify target genes that may be altered during chronic *T. gondii* infection[245].

1.7.2 Identify the mechanism governing DBH down-regulation during *T. gondii* infection

Once a mechanism for catecholamine change is established, I will investigate the mechanism responsible for the observed change(s) in gene expression. The level of regulation (post-transcriptional vs transcriptional) will be investigated. Changes at the genome level (ie. epigenetic) will be interrogated.

1.7.3 Investigate the nature of spreading observed during parasite-induced changes in catecholamine levels

How can a specific behavioural phenotype be observed if cysts are able to form anywhere in the brain? Further, how can one explain the changes with a very limited number of neurons infected *in vivo*? I will investigate cell-cell communication during *T. gondii* infection to assess whether induced changes in gene expression are spread throughout the brain. Here, *T. gondii* infection can provide a model system to investigate the nature of neuronal gene regulation.

Chapter 2

Materials and Methods

2.1 Cell culture

2.1.1 Parasite and Cell Culturing

T. gondii wild-type Prugniaud strain, isolated from mouse neuronal cysts, was used unless otherwise stated. Parasites were maintained in monolayer cultures of human foreskin fibroblasts (HFFs) in Dulbecco's Modified Eagles Medium (DMEM, GIBCO, USA) containing 10% foetal bovine serum (FBS) and 20 µg ml^{-1} penicillin/streptomycin (Sigma, USA). Differentiation of tachyzoites to bradyzoites was induced by the incubation of free tachyzoites in pH 8.2 DMEM with 5% FBS, overnight at 37°C. Expression of *T. gondii* differentiation markers over 5 days of infection confirmed bradyzoite conversion (Figure S1). All infections were performed with induced bradyzoites unless otherwise stated.

Rat adrenal phaeochromocytoma (PC12) cells were maintained at a density of 2x10^5 cells/ml in Roswell Park Memorial Institute (RPMI) 1640 Medium (GIBCO, USA) and supplemented with 10% horse serum (GIBCO, USA), 5% FBS (GIBCO, USA) and 20 µg ml^{-1} penicillin streptomycin (Sigma, USA). ME(2)-M17 cells were maintained in a 1:1 ratio of F12 Hams and OptiMEM (GIBCO, USA) media supplemented with 10% horse serum (GIBCO, USA), 5% FBS (GIBCO, USA) and 20 µg ml^{-1} penicillin streptomycin (Sigma, USA). If extracellular vesicles were being harvested or analysed exosome free-FBS was used

(GIBCO, USA). All cells were maintained at 5% carbon dioxide and 37°C and monitored by light microscopy throughout.

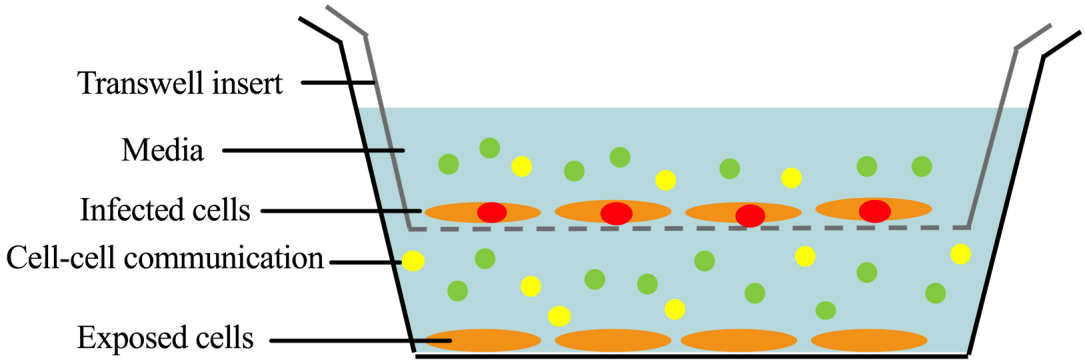


Figure 2.1: Scheme showing a cross-sectional view of a transwell culture. Cells are cultured on two layers separated by a 0.4μm membrane.

2.1.2 Infection of PC12 and M17 cells

Cells were cultured in multi-well plates at a density of 5x10^4 cells/ml. When stated, transwell plates were used, with cells plated at 2.5x10^4 cells/ml Figure 2.1. Following 24 hrs of incubation on a 6-well plate, induced Prugniard bradyzoites were transferred to each well, maintaining a multiplicity of infection (MOI) of 1. Cells were harvested immediately following infection (day 0) and after 3 and 5 days of infection for downstream processing. Mock-infection was performed using heat-killed *T. gondii* parasites subjected to 80°C for 10 minutes. The cultures were monitored daily by light microscopy.

2.1.3 Treatment of cells with purified extracellular vesicles

Extracellular vesicles were extracted and purified from rat catecholaminergic PC12 cells as described above. PC12 cells were plated on 6 well plates and grown to a density of 2x10^4 cells/ml. Cultures were treated with extracellular vesicles at a 10:1 culture media concentration (eg. 1ml of PC12 cells would be treated with extracellular vesicles purified from 10ml of culture). Cells were treated every 24hrs. DNA and RNA were harvested 24hrs after the last treatment.

2.1.4 Drug assays

Cells were cultured and infected as previously described. Drug was added an hour before the time of infection (day 0). Cultures were grown in media containing up to 100nM GW4869 (Sigma, US) and monitored using light microscopy.

De-methylation drug assays

Drugs were added an hour before infection (day 0). Cultures were grown in media containing up to 100nM Trichostatin A (TSA), 100nM RG108 or 50μM 5-Azacytidine (5-AC) (SIGMA, US). Drug treatment did not alter tachyzoite, nor bradyzoite growth, measured using fluorescent parasite strains (Figure S2). Human fibroblasts were 5-day infected with KU80-GFP bradyzoites or RH-YFP tachyzoites; growth was measured by fluorescence using a FLUOstar Omega plate reader (BMG, UK).

2.1.5 Fluorescence-assisted cell sorting (FACS) of infected human neuronal cells

Human neuronal cells were infected with the Pru Δhpt/GFP/FLUC *T. gondii* strain, (kind gift from Boothroyd)[49]. Five day infected and uninfected cell cultures were resuspended in PBS and sorted to isolate negative and GFP^+ fluorescent cells. Uninfected cells were also subjected to sorting. DNA was then extracted.

2.2 Animal Work

2.2.1 Triple slice co-cultures

All animal care and experimental procedures were approved by the Kyoto University Animal Research Committee. These experiments were performed with the help and guidance of the Kaneko Laboratory at Kyoto University. Sectioning was performed as described[246]. Sprague-Dawley rat pups at postnatal days 3-4 were anesthetized by hypothermia. Brain was removed and separated into two hemispheres. Coronal sections (350μm thickness) were prepared under sterile

conditions. Slices were dissected to include the ventral tegmental area (VTA), nucleus accumbens (NAc) and the medial prefrontal cortices (mPFC). Each slice containing the VTA, NAc or mPFC region was arranged so that they contacted each other. These triple slice co-cultures are placed on 30 mm insert membranes (Millicell-CM 0.4μm; Millipore). Slice culture medium contained RPMI and was supplemented with 10% horse serum (GIBCO, USA), 5% FBS supplemented with 6.5 mg/ml glucose and 2 mM l-glutamine. The triple slice co-cultures were cultured at 37°C in a 5% CO2 for 15 days after dissection then used in experiments.

2.2.2 Rodent Infections

Brain samples were supplied from animal experiments previously conducted at the University of Leeds research animal facility and procedures were approved by the University Animal Care and Use Committee and following Home Office, HSE, regulations and guidelines for Animals (Scientific Procedures) Act 1986 published in 2014 and with considerations of the replacement, reduction, and refinement in the use of animals for research.

Mice

Mice were housed 5 to a cage and maintained on a standard light-dark cycle with access to chow diet and water *ad libitum*. Throughout the experiments animals were monitored for illness or weight loss (more than 25%) and sacrificed 5-6 weeks after infection. Mice used were BALB/cAnNCrl x C57BL/6NCrl) F^1 generation mice. All mouse work prior to RNA extraction was undertaken by Dr Mohammad Alsaady[247].

Rats

All rat experiments were undertaken by Greg Bristow prior to tissue sampling. Lister Hood rats (Harlan Ltd, UK) were used for all experiments. Rats were housed in individual cages and maintained on a standard light-dark cycle with access to chow diet and water *ad libitum*. Animals were infected by intraperitoneal (IP) injection with tachyzoites. Throughout the experiments animals were

monitored for illness or weight loss (more than 25%) and sacrificed 5-6 months post-infection. Brains were snap-frozen for cryosectioning or RNA exaction.

Extracellular vesicle treatment

All animal care and experimental procedures were undertaken by Dr. Beatrice Maria Filippi[248]. Extracellular vesicles were extracted and purified from rat catecholaminergic PC12 cells as previously described. Eight-week-old male Sprague-Dawley rats weighing between 260 and 280g (Charles River Laboratories) were used. Rats were housed in individual cages and maintained on a standard light-dark cycle with access to chow diet and water *ad libitum*. Rats were stereotactically (David Kopf Instruments) implanted with indwelling bilateral cannula targeting the basal ganglia. After 1 week of recovery rats received a 2μl infusion twice a day for 3 days with 2mg/ml of purified extracellular vesicles isolated from infected or uninfected PC12 cells. On the fourth day rats were anaesthetized and received an injection 3 μl bromophenol blue through each side of the bilateral DVC cannula to verify their placement.

2.2.3 Fluorescence-assisted cell sorting (FACS) of mouse brain samples

Mouse brain samples were processed as described[249]. Briefly, brains were suspended in a lysis buffer (0.32M sucrose; 0.1mM Ethylenediaminetetraacetic acid; 5mM CaCl2; 3mM Mg(Acetate)2; 10mM Tris-HCl pH 8; 1mM Dithiothreitol; and 0.1% Triton X-100) and dounced using a 15ml glass dounce (Wheaton, UK) in 5-10 strokes. Samples were then centrifuged at 100,000xg with a sucrose gradient to purify nuclei. Nuclei were stained with primary antibody NeuN (1:250) and secondary antibody Alexa-488 (1:1000). All nuclei were also stained with Hoechst 33342. Samples were sorted using the Becton Dickinson FACSAria II system.

2.3 Catecholamine quantification

2.3.1 Dopamine Analysis

Cells were counted using a haemocytometer and normalised prior to infection. The cultures were incubated with 1 µmol KCl for 15 min and the buffer removed and supplemented with 125 µl of perchlorate (PCA) to provide a background dopamine measurement. The culture was then harvested and centrifuged for 5min at 2000rpm and resuspended in 1mL of PBS. 100 µl of suspension was centrifuged at 2000rpm for 5min at 4°C and using the Bradford Assay samples were normalised. The cell suspension of 900 µl was centrifuged at 2000rpm for 5 min and then resuspended in 350 µl of PCA, followed by sonication and centrifuged at 13000rpm for 15 min at 4°C. The supernatant was used for dopamine measurement by HPLC-ED.

2.3.2 HPLC Analysis

Before HPLC analysis, samples were normalised for cell count. Brain tissue was weighed and homogenised in a 1:1 ratio of PCA. HPLC analysis was performed with a Dionex HPLC system consisting of a P580 Pump (Dionex, USA) and Ultimate 3000 Autosampler Column Compartment with a C18 Acclaim 150 column and an ESA Coulochem III cell. The mobile phase contained 57mM anhydrous citric acid (Fisher Scientific, UK), 43mM sodium acetate (Dionex, USA buffer containing 0.1mM EDTA (Sigma, USA), 1mM sodium octanesulphonate monohydrate, and 10% methanol. The pH was adjusted to 4. The mobile phase was delivered at a flow rate of 0.8ml/min, and the column temperature was set at 40°C. Catecholamine standards were dissolved in 0.1M PCA for chromatography. The concentration of compounds was determined and analysed using Chromeleon software.

2.3.3 Bradford Assay

In order to determine protein concentration, a Bradford assay was performed as per manufacturer instructions. Briefly, Bradford reagent (Bio-rad, USA) was

added to the sample and BSA standards at a 5:1 ratio in triplicate, after a 5 minute incubation at room temperature, absorbance was measured at 595nm using a plate reader and protein concentration calculated using a standard curve.

2.4 Extracellular vesicle characterisation

2.4.1 Extracellular vesicle isolation

Extracellular vesicles were isolated by ultracentrifugation as described[250,251]. Briefly, 50ml of cell culture of uninfected or 5 day infected cells was harvested and centrifuged at 3000xg for 10 minutes at 4°C. The supernatant was isolated and centrifuged at 160,000xg for 2 hours in a Type 60 Ti fixed angle rotor at 4°C. The pellet was resuspended in 1ml of 90% sucrose. 11 layers of 1ml of sucrose 70%-10% w/v were then added and centrifuged at 70,000xg for 16 hours at 4°C in a Type 60 Ti fixed angle rotor. Supernatant was then collected in 2ml fractions. 8ml of PBS was added to the fraction with a density corresponding to that of extracellular vesicles and centrifuged at 160,000xg for 70 minutes at 4°C (Figure 2.2).

2.4.2 UV Ablation

Purified extracellular vesicles were isolated as described. Prior to UV treatment, DBH down-regulation was verified after a 24hr treatment. Extracellular vesicles were exposed or mock exposed to UV light at 290nm for 5 minutes at 4°C.

2.4.3 Western blotting

Western blotting was performed using the Exo-CheckTM Exosome Antibody Array (Cambridge Bioscience, UK) as per manufacturer's instructions (Figure S7). Briefly, 500μg of extracellular vesicles isolated from PC12 cells were lysed and ligated to horseradish peroxidase (HRP) overnight at 4°C. Ligated protein was then incubated with the antibody membrane for 2 hours at room temperature. After 3 washes SuperSignal West Femto Chemiluminescent Substrate kit (Thermo

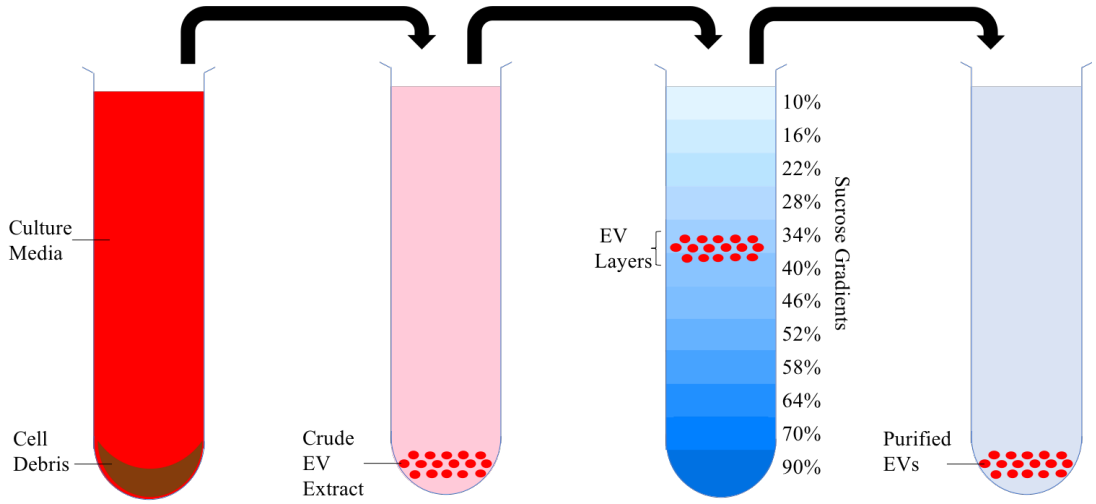


Figure 2.2: Scheme summarising of sequential ultracentrifugation steps undertaken to produce purified extracellular vesicles. Sucrose gradient densities range from 0.104 g/ml to 1.43 g/ml. The density of extracellular vesicles is between 1.15 and 1.19 g/ml on sucrose gradients. By comparison, vesicles purified from the endoplasmic reticulum float at 1.18 to 1.25 g/ml and vesicles from the Golgi at 1.05 to 1.12 g/ml. The density of protein aggregates is approximately 1.22 g/ml[252,253].

Scientific, UK) was used as per manufacturer's instructions. The blot was visualised via 90 second exposure to X ray film. Film was developed using the Konica SRX-101A Tabletop Processor.

2.4.4 Transmission electron microscopy

Extracellular vesicles were isolated as described above. Electron microscopy was performed as described[254]. Freshly isolated extracellular vesicles were resuspended in cold DPBS containing 2% para-formaldehyde. Exosomes were mounted on copper grids, fixed by 1% glutaraldehyde in cold Dulbecco Phosphate-Buffered Saline (DPBS) for 5 minutes at room temperature to stabilize the immunoreaction, washed in sterile distilled water, contrasted by uranyl-oxalate solution at pH 7 for 5 min, and embedded by methyl cellulose-UA for 10 min at 4°C. Excess cellulose was removed, and samples were dried for permanent preservation. All electron microscopy was performed by Martin Fuller, Electron Microscopy Technician using the Titan Krios 2 electron microscope. Analysis and extracellular vesicle diameter were measured using ImageJ software.

2.5 Molecular Techniques

2.5.1 DNA Extraction

Classic phenol-chloroform method extraction was performed as described in[255]. Briefly, tissue samples were first homogenised using a Micro Tissue Homogenizer (Fisher Scientific, UK). Homogenised tissue or cell pellets were incubated at 56°C overnight with $20\,\mathrm{mg\,ml^{-1}}$ of Proteinase K. An equal amount of Phenol/chloroform/isoamyl alcohol (PCI) solution (25:24:1) was then added, mixed and centrifuged. The aqueous layer was removed and an equal volume of chloroform:isoamyl (CI) alcohol (24:1) added. DNA was then precipitated using absolute ethanol and DNA resuspended in deionised water.

RNA extraction was performed using Direct-zol® (Zymo, USA) as per manufacturer's instructions.

2.5.2 PCR

Polymerase chain reaction was performed using GoTaq PCR master mix (Promega, USA) as per manufacturer instructions. Samples were run using 2720 thermal cycler (Applied Biosystems, USA). The PCR parameters were 94°C for 30 seconds; 35 cycles of 94°C for 30 seconds (denaturing step), 55°C for 15 seconds (annealing step) and 72°C for 10 seconds (elongation step); a final elongation of 72°C for 30 seconds.

Samples were visualised on a 2% w/v agarose gel with a Trackit 1kb Plus DNA ladder run at 80V until the DNA ladder and the sample products of interest had separated adequately.

2.5.3 RT-qPCR

RT-qPCR was performed using SYBR green master mix (Life Technologies, USA) as per manufacturer's instructions and run using a CFX Max Real Time PCR machine (Bio-rad, USA). Parameters were 95°C for 2min and followed by 45 cycles of 95°C for 15 seconds, (primer Tm-5) °C for 15 seconds, and 72°C for 10 seconds. Primer sequences are recorded in Table 2.1. A melt-curve was performed to check for expected products. All RT-qPCR was performed with four technical replicates which were averaged.

2.5.4 Nuclear run-on

Nuclear run-on was performed as described (Figure 4.5)[256]. Briefly, after 5 days of infection with induced bradyzoites cells were then incubated with lysis buffer containing 10 mM Tris-HCl pH 7.4, 10 mM NaCl, 3 mM MgCl2, 0.5% NP-40 at 4°C isolating nuclei. Nuclei are then suspended in transcription buffer containing 20 mM Tris-HCl pH 8.3, 5 mM MgCl2, 300 mM KCl, 4 mM DTT, RNase OUT 40 Units/μl, 0.5 mM BrUTP, 1mM ATP, 1mM GTP, 1mM CTP, 1mM UTP. Sample was incubated for 45min at 37°C prior to RNA extraction using MEGAclear Transcription Clean-Up Kit (Life Technologies) as per manufacturer's instructions. Bead based immunoprecipitation of Bromouridylated NRO-RNA was then

Table 2.1: Primer sequences used throughout.

Name		**Sequence**	**Tm (°C)**	**CG%**
Rodent DBH	F	5'-CCACAATCCGGAATATA-3'	54.5	41.2
	R	5'-GATGCCTGCCTCATTG-3'	62	55.9
Human DBH	F	5'-GAAACGACTCCTCAGGCAT-3'	62.4	52.6
	R	5'-TCGCAGAGTAGAGTGCACA-3'	62.1	52.5
Rodent GAPDH	F	5'-GTGGACCTCATGGCCTACAT-3'	64.2	55
	R	5'-TGTGAGGGAGATGCTCAGTG-3'	65.1	54.8
Human GAPDH	F	5'-AGGGCTGCTTTTAACTCTG-3'	59.2	47.4
	R	5'-CCCCACTTGATTTTGGAG-3'	63.3	50
Rat DDC	F	5'-CGGAGAAGAGGGAAGGAGAT-3'	64	55
	R	5'-GCCGTGGGGAAGTAAGCGAAG-3'	65.9	63.9
Rat PAH	F	5'-GGAACGGTGTTCAGGA-3'	58.5	56.3
	R	5'-CTTCACGGAAACCGCAGTA-3'	66.3	52.5
Rat TH	F	5'-CCCAAAGTCTCCATCCCCTTC-3'	68.1	57.1
	R	5'-GTTGAGAAGCAGTGTTG-3'	55.8	47.2
Rat DRD1	F	5'-AGTCCCCGGAAGTGTG-3'	60.2	62.5
	R	5'-GTGTCGAAACCGGATG-3'	61.7	55.9
Rat MaoA	F	5'-GTGGGAGGCAGGACTTAC-3'	60.2	61.1
	R	5'-CTGGCGAATCACCCTTCC-3'	68.1	60.5
Rat DRD2	F	5'-GCTGATACCATTGGGC-3'	57.9	56.3
	R	5'-GTTGGATCCCAGAACC-3'	59.4	55.9
Rat ESR1	F	5'-CTACGCTGTACGCGACAC-3'	61.3	61.1
	R	5'-ATTCTGGCGTCGATTG-3'	60.6	50
Rodent MAP2	F	5'-AAGGTGGTGGACGTGTG-3'	61.3	58.8
	R	5'-CTGGTTCATTGCCATTCTC-3'	63.5	47.5

Name		Sequence	Tm (°C)	CG%
TgActin	F	5'-CGAGCTGGTCAGTTCCTC-3'	61.8	61.1
	R	5'-GCTAGCATGTGGCAGGCC-3'	69.3	65.8
TgBAG1	F	5'-GACGTGGAGTTCGACAGC-3'	62.8	61.1
	R	5'-GACGTGGAGTTCGACAG-3'	61.1	58.3
TgSAG1	F	5'-CGACAGCCGCGGTCATT-3'	69.1	64.7
	R	5'-GCAACCAGTCAGCGTC-3'	62.3	61.8
TgSAG4	F	5'-GGACCTACGATTTCAAGA-3'	55.6	44.4
	R	5'-GCTGCGAGCTCGACGG-3'	70.1	73.5
Rat DBH MSRE	F	5'-CAGGACGTAGCCACAGATGG-3'	60	60
	R	5'-TGCCTTCCCCGGG-3'	61	75
Human DBH MSRE	F	5'-CAATACCCCAGAGGGGAGAG-3'	65	60
	R	5'-GCTTTCCCATCAGGACATGC-3'	67.2	55
Methylation standards	F	5'-GGTGTGAAAACTTTGAAGGA-3'	57.9	40
	R	5'-ATGGGGCCTATTGCTTGGTC-3'	60.1	55
Human Ascl1 MSRE	F	5'-TTGGCGCAGCCACCATC-3'	60.8	64
	R	5'-CACTATTCAAATTCCAACCC-3'	59	40
Rat Ascl1	F	5'-GGCTTGAGCCTACTTCTGGG-3'	60	50
	R	5'-GGTTGCTGAACTTGAGATACCG-3'	60.1	60
Human Ascl1	F	5'-GAGCAGGAGCTTCTCG-3'	57.8	62.5
	R	5'- GATGCAGGTTGTGCG-3	61.7	59.4

performed and RNA was extracted and RT-qPCR performed as previously described in Section 2.5.1.

2.5.5 Methylation sensitive restriction enzyme (MSRE) quantitative PCR

DNA was extracted from cell cultures and isolated as previously described. DNA concentrations were confirmed using a Nanodrop spectrophotometer. Samples were divided into reference and test samples (Figure 2.4). Test samples were digested with the high fidelity methylation sensitive restriction enzymes (MSRE) HpaII, MaeII and SmaI (TaiI) in the Tango buffer. These MSREs were chosen based on the restriction enzymes sites present in the DBH promoter (Figure S4). For Ascl1 MSRE qPCR MaeII and HpaII were used as they cut the promoter sequence with the highest frequency (Figure S5). Reference samples were mock digested. Digestion was performed by heating to 25°C for 30 minutes, 37° for 30 minutes and 65° for 30 minutes. RT-qPCR was then performed. Percentage methylation calculated based on the difference in Ct values between test and reference samples. Digestion was confirmed with methylated and unmethylated human DNA standards provided with the OneStep qMethyl kit (Zymo) as per manufacturer's instructions (Figure 2.3A). Additionally, primers and MSRE digestion was confirmed with MSRE-PCR (Figure 2.3B). qPCR was performed using SYBR®Green Real-Time PCR Master Mix (Thermo Fisher).

2.5.6 Global Methylation

Global methylation was measured using a colourimetric, ELISA adapted method using the MethylFlash Methylated DNA 5-mC Quantification Kit (Epigentek, US) as per manufacturer's instructions. Samples used were the same as those analysed in Figure 4.7 and Figure 4.9. 5ng of DNA was used for quantification, absorbance at 405nm was read using the Molecular Devices SPECTRA MAX PLUS Plate Reader Ver. 3.05 Spectrophotometer.

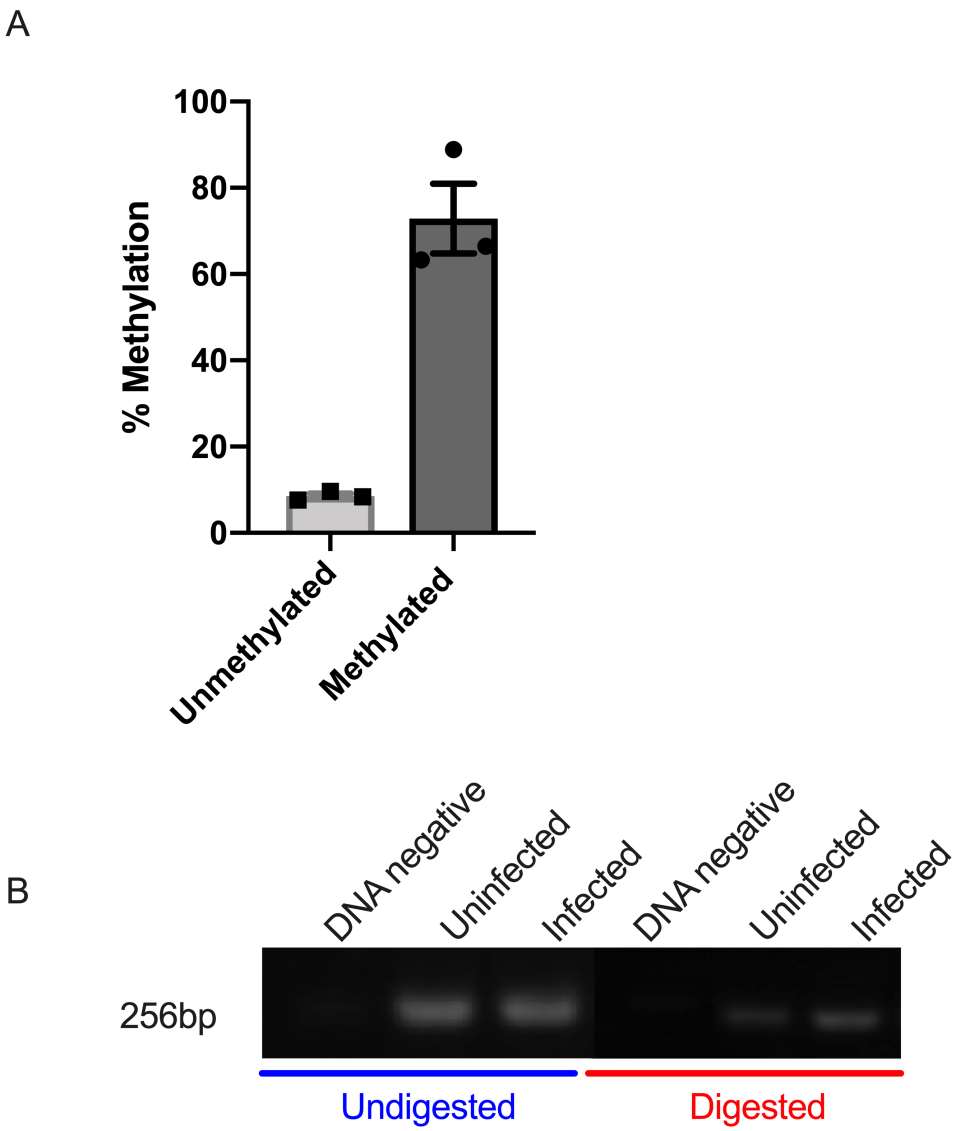


Figure 2.3: Representative percentage methylation of MSRE standards used for QC. A) Plot shows percentage methylation of unmethylated and methylated standard DNA. B) A representative gel showing MSRE-PCR of uninfected and 5-day infected PC12 cells. Samples were either undigested (blue) or digested (red) with MSREs and then amplified with rat MSRE DBH primers.

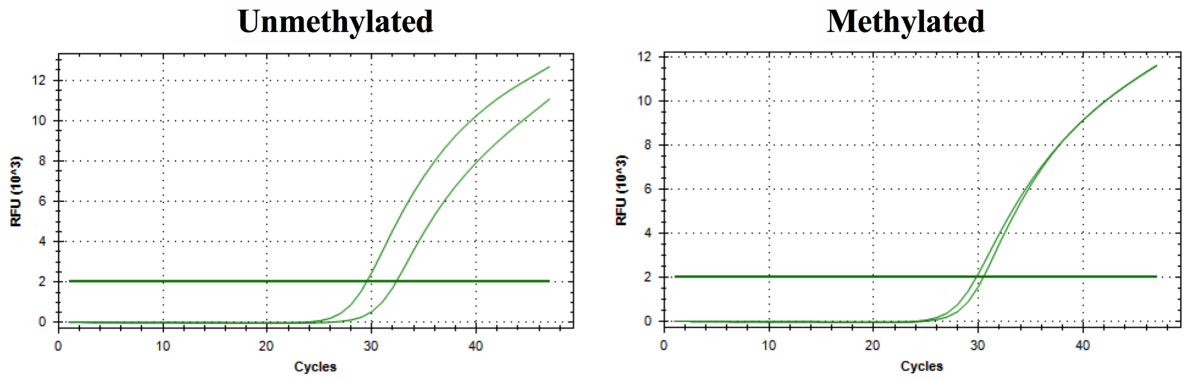


Figure 2.4: Representative MSRE-qPCR plot showing relative fluorescence for methylated and unmethylated samples. Left panel shows amplification of a test and reference sample of unmethylated DNA; because MSREs are able to cut at unmethylated sites the difference in Ct values is large. The right panel shows test and reference sample of methylated DNA; as MSREs are unable to cut DNA at methylated sites the difference in Ct values is small.

2.5.7 Whole-genome bisulphite sequencing

Whole-genome sequencing was undertaken with the help and guidance of the Next Generation Sequencing facility at St James Hospital. PC12 cells were either uninfected or 5-day infected with bradyzoite induced WT-Pru. DNA was collected using phenol-chloroform extraction as previously described. 5 biological replicates were pooled for uninfected and control samples. Library was prepared using the TruSeq DNA Methylation Kit (Illumina, UK). Briefly, samples were bisulphite treated using the EZ DNA Methylation-Gold^{TM} Kit (Zymo). Treated DNA was then amplified and 5' adapters ligated. 3' indexing adapters were then ligated to the ssDNA and 12 rounds of PCR performed. QC analysis was then performed (Figure S3). Sequencing was performed using the HiSeq 4000 Systems (Illumina). Initial data analysis was performed by Dr Agne Antanaviciute, St James Hospital. Dr Antanaviciute performed genome alignments and calculated differential methylation across the genome. For further analysis all differentially methylated bases within 2kb of a gene were isolated. Of these, promoter sequences with more than three differentially methylated bases were selected. Differentially

methylated sites with a read depth of at least 10 were then selected. Finally, only those with a p value <0.05 were analysed. The proportion of methylated bases at each site was then calculated and a sum of these was used to calculate GO terms. GO terms analysis was performed using PANTHER[257].

2.6 Statistical Analysis

All statistical analysis was performed using GraphPad and SPSS. For each data set, Levene's test of equal variance was performed. Unless otherwise stated, a student t test was performed for all equally distributed data.

Chapter 3

Altered catecholamine signalling during chronic *Toxoplasma gondii* infection

3.1 Overview

This chapter addresses the mechanism of catecholamine change during *T. gondii* infection. Previously, published work has identified an increase in cellular dopamine content during chronic *T. gondii* infection. In this chapter, high performance liquid chromatography with electrochemical detection (HPLC-ED) analysis revealed that dopamine content was increased during chronic infection both in cell cultures and in the brain of chronically infected rodents. The expression of catecholamine regulator genes was analysed by real-time quantitative polymerase chain reaction (RT-qPCR), and dopamine β-hydroxylase was found to be down-regulated during infection. It was observed dopamine β-hydroxylase was down-regulated beginning at 3 days of infection in rat catecholaminergic and human neuronal cells. This down-regulation was specific to *T. gondii* as cytomegalovirus, which also forms a chronic neuronal infection, did not induce dopamine β-hydroxylase expression changes. For the first time, a possible mechanism for dopamine changes during infection has been identified.[258]

3.2 Introduction

Since the 1970s, changes in catecholaminergic signalling have been observed during chronic *T. gondii* infection[159]. The catecholamines are a group of neurotransmitters, including dopamine, norepinephrine and epinephrine. Dysfunction of catecholamine signalling has been associated with several neurological conditions including movement disorders, schizophrenia, and depression[11,19,21,259,260]. The neurobiology of catecholamine signalling is discussed further in Section 1.2.1.

3.2.1 Changes in catecholamine signalling during chronic *T. gondii* infection

Altered catecholamine synthesis, during *T. gondii* infection, has been widely investigated since seminal work by Stibbs *et al* in 1985[159]. Prandovszky and colleagues observed a 2.7 fold increase in total dopamine concentration, as well as an increased concentration of dopamine metabolites during *in vitro* bradyzoite infection[212]. During infection, expression of dopamine receptors was also found to be altered. Transcriptomic analysis of chronically infected mouse brain samples, revealed that DRD1 and DRD5 expression was down-regulated *in vivo*[223]. Signalling via these type 1 dopamine receptors typically induces activation of the post-synaptic neuron, and are implicated in motor function, memory and learning; this is fully discussed in Section 1.2.1[10]. Recently published data has also found an increase in dopamine metabolites, suggesting dopamine turnover is increased during chronic infection. Although the concentration of DOPA decarboxylase (DDC) was not altered during chronic infection, relocation to the tissue cyst parasitophorous vacuole (PV) was observed[213].

It was found that chronically infected rats treated with dopamine antagonists, inhibiting catecholaminergic signalling, did not develop the loss of fear phenotype. Chronically infected mice also exhibit an altered response to amphetamine treatment, suggesting catecholamine dysregulation[261,262]. However, it has not yet been demonstrated that disrupting catecholamine synthesis can also reverse behavioural changes. Interestingly, dopamine supplementation during parasite culture increased growth and proliferation of *T. gondii* although any

tyrosine present in the supplement could explain the observations. As discussed in Section 1.5.3, loss of dendritic spine integrity can be observed during infection. If dopamine is improperly packaged into vesicles, cell damage can occur via reactive species generation, this can cause oxidative damage to DNA and proteins; this may account for the neurodegeneration damage observed during infection[214,263,264].

3.2.2 Possible mechanisms of catecholamine change

The mechanisms governing these changes during infection remain poorly delineated. Altered miRNA-132 expression has been observed during chronic infection[265]. This microRNA may play a role in catecholamine regulation during infection[266–269]. However, changes in miRNA-132 have not been directly linked to catecholamine changes. Expression changes of catecholaminergic regulators such as tyrosine hydroxylase, vesicular monoamine transporter (VMAT) or phenylethanolamine N-methyltransferase (PNMT) have not been identified during infection[223]. Gaskell *et al* identified that the *T. gondii* genome contains two copies of the aromatic amino acid hydroxylase gene (AAH1 and AAH2)[270]. These genes encode for enzymes with tyrosine hydroxylase activity, the rate limiting enzyme in the synthesis of dopamine[271]. This may provide a possible mechanism by which dopamine is increased in the brain, particularly as no changes of host tyrosine hydroxylase have been observed. Taken together with DDC relocation to cysts it is possible that this may affect catecholamine synthesis. A double knock-out of both genes was recently achieved and current data suggests that TgAAH plays a role in feline transmission[217,272]. The effect of AAH1 and AAH2 knock-outs on catecholamine synthesis have yet to be assessed.

Changes in host norepinephrine have been widely observed, but the mechanism responsible has not been investigated[159]. Even in papers reporting little to no change in catecholamine concentrations, a significant reduction in total norepinephrine in animal models was observed[215,273]. Given that dopamine is the precursor to norepinephrine, any alterations in regulation of the catecholamines may impact other steps of the metabolic pathway. Despite mounting evidence that catecholamine synthesis is altered during infection, the underlying mechanisms

have yet to be delineated. This chapter investigates changes in catecholamine synthesis during chronic infection. For the first time a possible mechanism of catecholamine change is identified.

3.3 Results

3.3.1 Catecholamine synthesis gene changes during chronic *T. gondii* infection

In order to first establish if catecholamine changes can be observed during chronic infection. Rat catecholamine PC12 cells were infected with bradyzoite-induced *T. gondii*, after 5-days of infection the catecholamine concentration was measured using HPLC-ED (Figure 3.1). Dopamine (DA) was increased from 1.38μM $\pm$0.27 in uninfected cells to 5.2μM $\pm$0.6 (p=0.0043) during infection. This finding is in line with previously published work[213]. In addition, norepinephrine (NE) content was reduced from 511.5nM $\pm$20.6 to 318.7nM $\pm$19.4 (p=0.0024) in the same 5-day infected PC12 cells compared to uninfected controls.

To establish if this can be observed *in vivo* the catecholamine content of rat brain samples was analysed by HPLC-ED. In the infected samples NE content was decreased from 355.5 ng/g $\pm$35.0 to 176.1ng/g $\pm$24.3 (p=0.0019) in the brains of chronically infected rats (Figure 3.2A). DA content of chronically infected rat brain samples was found to be increased from 1.33μg/g $\pm$0.38 to 3.30μg/g $\pm$0.73 (p=0.043) compared to controls (Figure 3.2B). This suggests that *T. gondii* either directly or indirectly is able to alter the synthesis of catecholamines.

In order to investigate if catecholamine changes were due to altered expression of related genes a RT-qPCR screen was performed. Genes were selected based on transcriptomic data obtained by Alsaady *et al*[274]. Preliminary experiments with a genome scan of infected rat catecholaminergic cells for gene expression levels identified that the most significantly altered expression was downregulation of the dopamine β-hydroxylase (DBH) gene (p=7.2x10^{-13}). All catecholaminergic genes identified as differentially expressed were further investigated. From this, the catecholaminergic genes most changed were selected. RNA of 5-day infected PC12 cells was collected and RT-qPCR was performed to identify any changes

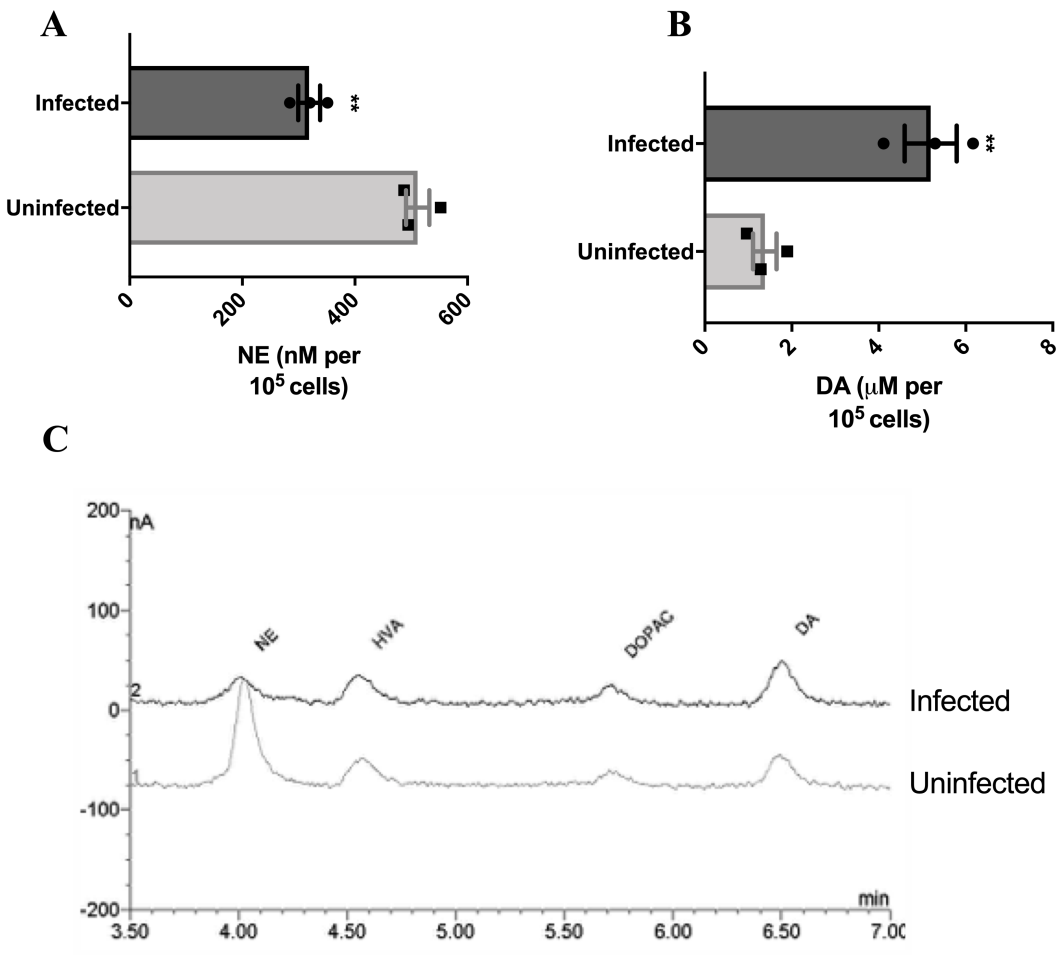


Figure 3.1: Dopamine content is increased during chronic *T. gondii* infection in rat catecholaminergic cells; MOI=1; ±SEM shown; n = 10, Student t test ** $p<0.01$. A) Plot of normalised norepinephrine content of uninfected and infected PC12 cells. B) Plot showing normalised dopamine (DA) content of uninfected and infected PC12 cells measured by HPLC-ED; MOI=1; ±SEM shown; n=5, Student t test ** $p<0.01$. C) A representative HPLC-ED chromatograph showing catecholamine levels of uninfected and infected PC12 cells. Plot shows monoamine signal (y-axis) against retention time (x-axis). Left to right peaks identified are norepinephrine (NE), homovanillic acid (HVA), 3,4-dihydroxyphenylacetic acid (DOPAC) and dopamine (DA). Plot represents raw data and is not corrected for sample protein content.

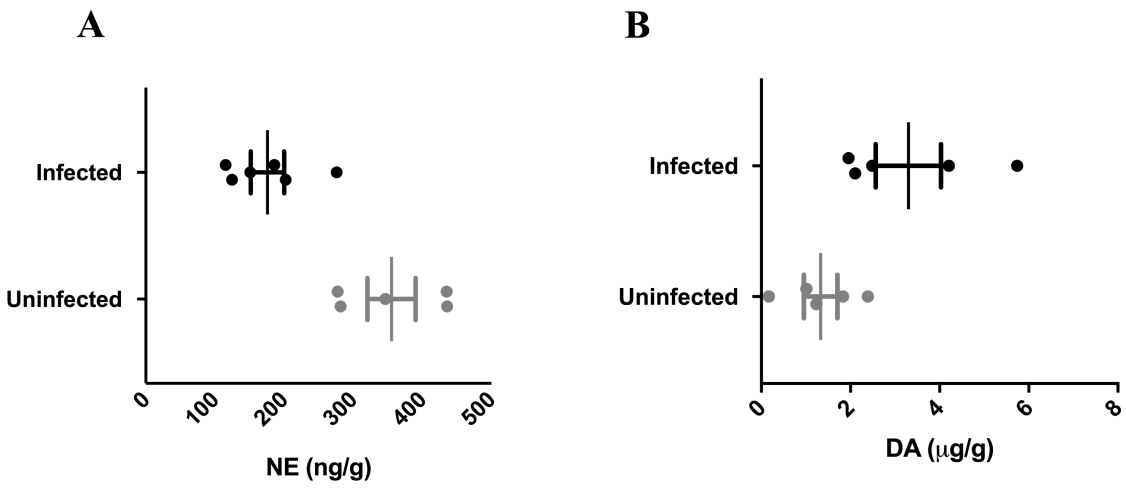


Figure 3.2: Dopamine content is increased during chronic *T. gondii* infection in rat brain. A) Plot showing normalised HPLC-ED analysis of rat brain homogenate. Norepinephrine (NE) content of uninfected (grey, n=5) and chronically infected (black, n=5) rat brain samples is shown; ±SEM shown, biological repeats, student t test $p<0.01$. B) Plot showing normalised dopamine (DA) content of uninfected (grey, n=5) and chronically infected (black, n=6) rat brain samples, ±SEM shown, student t test $p<0.05$.

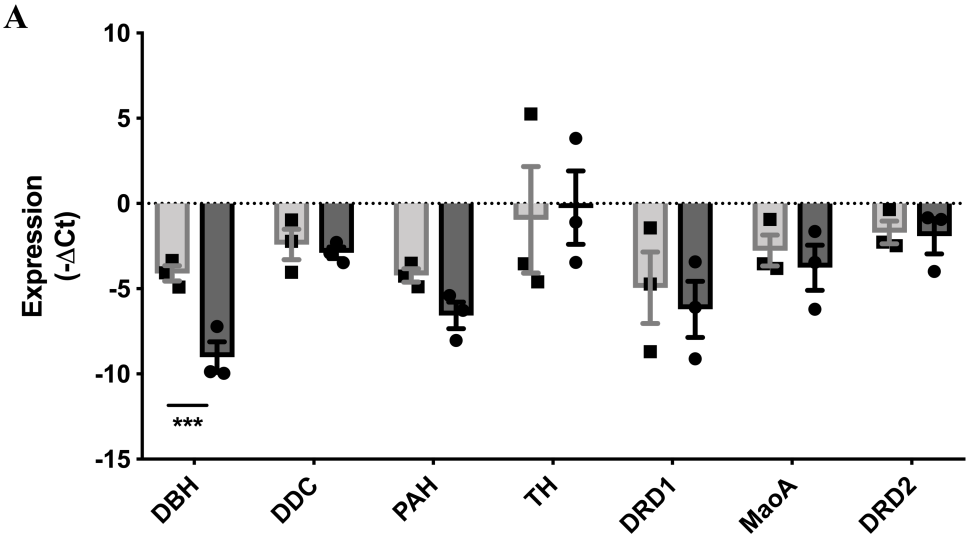
in expression relative to glyceraldehyde 3-phosphate dehydrogenase (GAPDH). It was found that dopa decarboxylase (DDC); phenylalanine hydroxylase (PAH); tyrosine hydroxylase (TH); dopamine receptor 1 (DRD1); dopamine receptor 2 (DRD2) and monoamine oxidase A (MOA-A) were not significantly changed during infection (Figure 3.3A). Although PAH appears down-regulated this was not significant (p=0.056). Gene expression of TH and DDC was unchanged during infection which is consistent with previously published data[213]. The only gene significantly down-regulated was DBH. A 40-fold ±1.6 (p=0.008) reduction in expression was observed after 5 days of infection (Figure 3.3B).

3.3.2 Changes in dopamine β-hydroxylase expression during *T. gondii* infection

In order to identify a time course of DBH down-regulation during infection RNA was collected from PC12 and BE(2)-M17 (M17) cells at 0, 3 and 5 days post infection (Figure 3.4). DBH was decreased after 3 days, although not significantly (p=0.056); and further decreased 18-fold ±5.3 at 5 days of infection (p=0.0009), relative to the housekeeping gene GAPDH (Figure 3.4A). The expression level of DBH in uninfected cells, relative to GAPDH was unchanged during this time course (one-way ANOVA, p=0.58). In order to assess the generality of this down-regulation during infection of catecholaminergic cells, a time course was also used to examine gene expression of DBH in the human neuronal cell line BE(2)-M17 (M17). Similarly, DBH was found to be down-regulated 4.6-fold ±0.63 at 3-days (p=0.001) and 70-fold ±17.8 at 5-days (p=0.00032) post infection (Figure 3.4B).

The effect of chronic infection on DBH expression *in vivo* was then investigated. DBH is 22.8-fold ±10.1 (p=0.0032) down-regulated in mRNA collected from whole mouse brain homogenate (Figure 3.5A). Although this change was significant it is possible that down-regulation of DBH is centred in the areas of DBH synthesis and activity, and so by homogenising the whole brain regional changes in gene expression can be overlooked. Therefore, the brain was sectioned in order to isolate the frontal lobe containing the prefrontal cortex (pFC); the mid-brain containing the hippocampus, thalamus and hypothalamus (H); and the posterior section containing the locus coeruleus (LC). DBH was 17.1-fold

A



B

Gene	Uninfected		Infected		P Value
	Average Expression (ΔCt)	SEM	Average Expression (ΔCt)	SEM	
DBH	-4.11	±0.45	-9.02	±0.89	0.01
DDC	-2.41	±0.90	-2.89	±0.34	0.64
PAH	-4.22	±0.40	-6.57	±0.78	0.05
TH	-0.96	±3.12	-0.24	±2.15	0.86
DRD1	-4.95	±2.10	-6.21	±1.64	0.66
MaoA	-2.76	±0.91	-3.77	±1.33	0.56

Figure 3.3: Expression of catecholamine regulation genes during chronic *T. gondii* infection. A) Plot showing expression of various catecholamine genes measured using RT-qPCR shown with respect to the housekeeping gene GAPDH. Uninfected (grey) or 5-day infected with *T. gondii* (black) PC12 cells. Only DBH gene expression was significantly altered by infection; ±SEM shown, n=3, student t test ** $p<0.01$). B) Summary table showing average -ΔCT, ±SEM and p value of each gene analysed

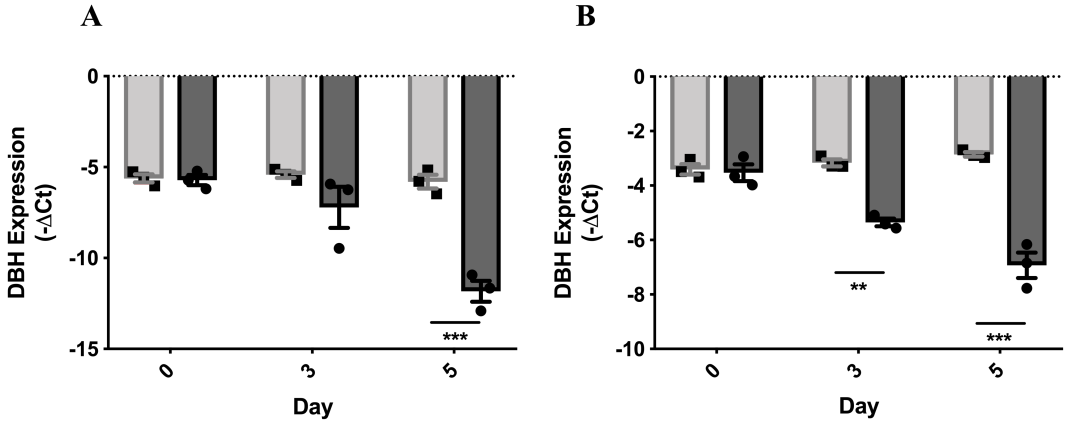


Figure 3.4: Down-regulation of dopamine β-hydroxylase during chronic *T. gondii* infection *in vitro*. Plot showing DBH expression is down-regulated using RT-qPCR shown with respect to the housekeeping gene GAPDH over a time course of 0, 3 and 5 days. A) Rat catecholaminergic PC12 cells were either uninfected (grey) or infected (black) with bradyzoite induced *T. gondii* and RNA was collected at 0, 3 and 5 days. ±SEM shown; n=5; student t test, *** $p<0.001$. B) Human neuronal BE(2)-M17 cells were uninfected (grey) or infected (black) with *T. gondii* and RNA was collected at 0, 3 and 5 days. ±SEM shown; n=5; student t test ** $p<0.01$, *** $p<0.001$.

±3.5 down-regulated in the PFC (p=0.0033) and 24.8-fold ±6.1 down regulated (P=0.012) in the LC (Figure 3.5B). It is possible that down-regulation of DBH may be because of neuronal damage due to infection, which can be particularly aggressive in mice[210]. In order to assess neuronal damage, the expression of microtubule-associated protein 2 (MAP2) was analysed, relative to GAPDH. No difference was found between infected and uninfected mouse homogenate, suggesting that there is not neuronal damage in infected samples.

RNA was isolated from the brain tissue of chronically infected rats and DBH expression analysed using RT-qPCR. DBH was found to be down-regulated 32 ±2.1-fold (p=0.0032) in infected rats compared to uninfected controls (Figure 3.6A). As with mouse data, MAP2 expression was analysed to identify if there was any neuronal damage during chronic *T. gondii* infection (Figure 3.6B). MAP2 was not significantly changed relative to GAPDH in infected animals compared to controls suggesting that down-regulation of DBH is specific.

Interestingly, DBH expression was also analysed in female rats and infection did not alter DBH expression (Figure 3.7A). RT-qPCR revealed that DBH expression was not significantly altered by chronic *T. gondii* infection (p=0.45). However, the data set exhibited high variance in both infected and uninfected brain samples. DBH is known to be regulated by oestrogen via oestrogen receptor binding to ER-response elements (ERE), identified in the 5' flanking region of the DBH gene. Thus, expression of the oestrogen receptor 1 (ESR1) levels were analysed within the female rat brains (Figure 3.7 B). ESR1 and DBH expression were found to be positively correlated, R=0.87. This suggests that the effects of chronic *T. gondii* infection are being obscured by the oestrus cycle.

3.3.3 DBH down-regulation is specific to *T. gondii* infection

Cytomegalovirus (CMV) is also able to establish a chronic infection in the brain of the host. In order to assess whether DBH down-regulation was parasite specific, RNA was collected from CMV infected human neuronal M17 cells at 6, 24 and 48 hours and *T. gondii* human neuronal M17 cells at 0, 2, 3 and 5 days (Figure 3.8). CMV did not cause down-regulation of DBH (p=0.053) relative to the

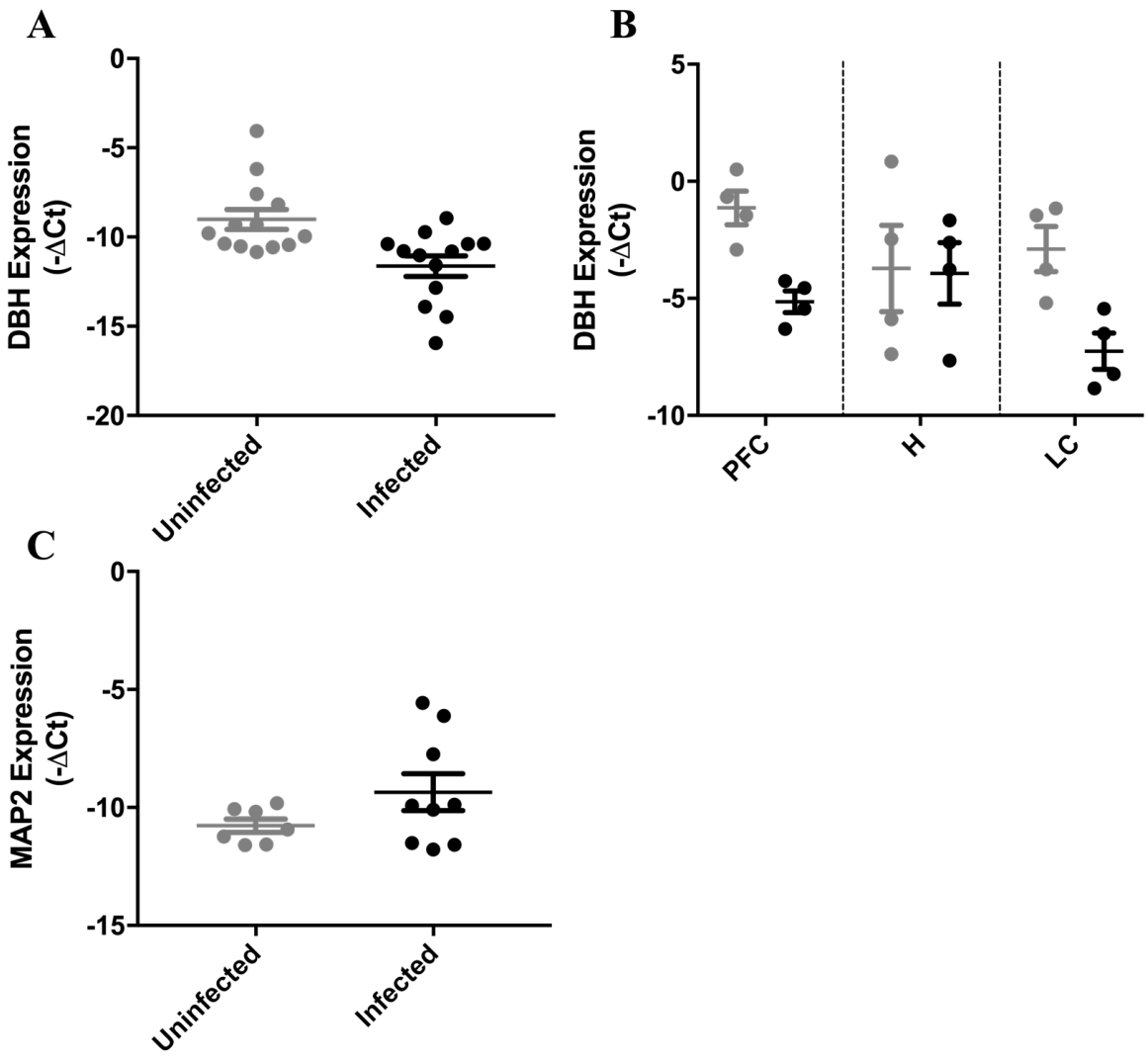


Figure 3.5: DBH is down-regulated in the brains of male mice chronically infected with *T. gondii*. A) Plot showing DBH expression, relative to the housekeeping gene GAPDH, in whole brain homogenate isolated from uninfected (grey, n=18) and chronically infected (n=18) mice; student t test p=0.0032, n=36, ±SEM shown. B) Plot showing expression of DBH, relative to GAPDH, in the frontal lobe (PFC), mid brain (H) and posterior region (LC), in uninfected (grey, n=4) and chronically infected (black, n=4) mouse brain sections. DBH was down-regulated in the PFC, Student t test p=0.003, and the LC, Student t test p=0.01, ±SEM shown, n=8. C) Plot shows MAP2 expression, with respect to the housekeeping gene GAPDH, in brain tissue of uninfected (grey, n=6) and chronically infected (black, n=8) mice; ±SEM shown, student t test p=0.14, n=14.

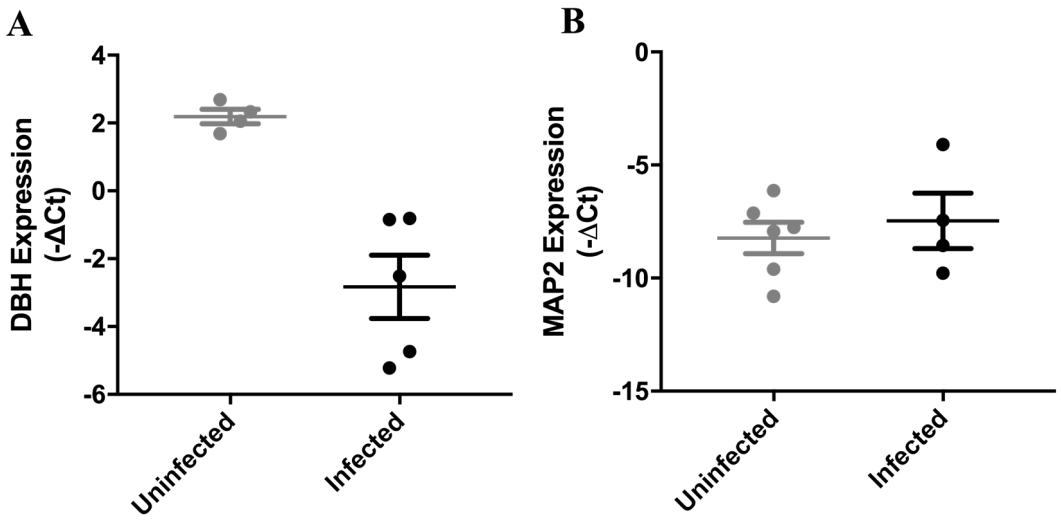


Figure 3.6: DBH mRNA is down-regulated in the brains of chronically infected male rats. A) Uninfected (grey, n=4) and chronically infected (black, n=5) rat brain homogenate was analysed by RT-qPCR. DBH expression, relative to the housekeeping gene GAPDH, shown; student t test p=0.003, n=9, ±SEM shown. B) lot shows MAP2 expression, with respect to the housekeeping gene GAPDH, in brain tissue of uninfected (grey, n=6) and chronically infected (black, n=4) male rats; student t test p=0.14, n=14, ±SEM shown.

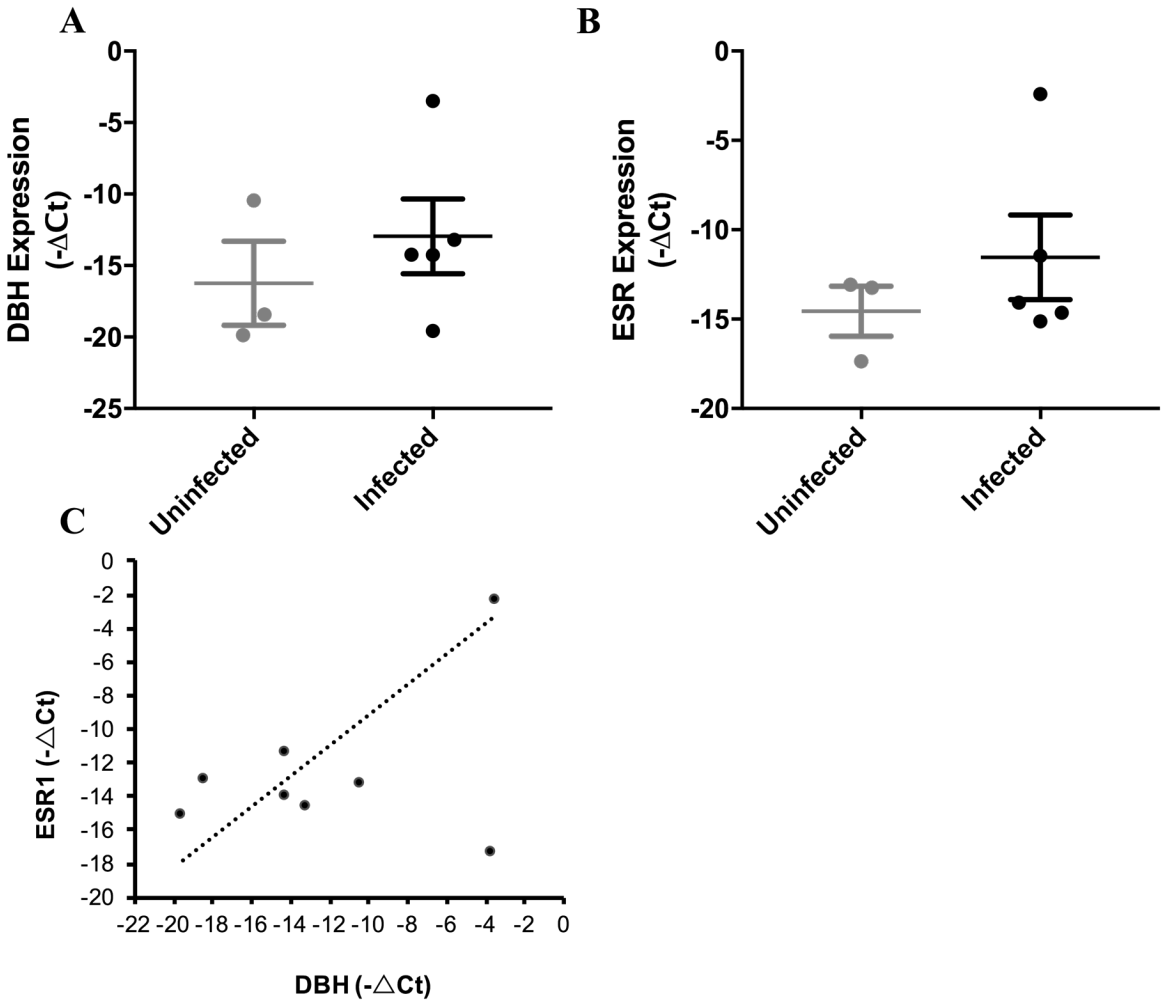


Figure 3.7: DBH in chronically infected female rats is linked to the oestrus cycle. A) DBH expression of uninfected (grey, n=3) and chronically infected (black, n=5) female rat brain homogenate. DBH is expressed relative to the house keeping gene GAPDBH; ±SEM shown, student t test p=0.42, n=8. B) Plot shows ESR1 expression relative to GAPDH in uninfected (grey, n=3) and chronically infected (black, n=5) female rat brain homogenate; ±SEM shown, student t test p=0.398, n=8. C) Plot showing correlation between DBH and ESR1 expression, Pearson's coefficient R=0.87

housekeeping gene GAPDH (Figure 3.8A). Indeed, DBH appeared slightly up-regulated at 48 hours although not significantly (p=0.19). DBH was 16-fold ±7.1 down-regulated (p=0.0015) with respect to GAPDH in human neuronal M17 cells infected with bradyzoite-induced *T. gondii* after 3 days. After 5-days of infection DBH was down-regulated 82.7-fold ±3.6 (p=0.00078), suggesting that DBH down-regulation is parasite specific (Figure 3.8C). Expression of Immediate-Early (IE) CMV gene and *T. gondii* actin demonstrates that infection levels in both experiments were comparable (Figure 3.8B and Figure 3.8D).

3.4 Discussion

Dysregulation of neurotransmitter signalling and concentration of the catecholamines and their metabolites has been previously identified; however, the mechanism for this change remained elusive[104,159,212,213,261]. For the first time, DBH down-regulation has been observed, providing a possible mechanism for changes in catecholamine signalling during infection. This suppression may reduce the metabolism of dopamine to norepinephrine in the brain of chronically infected mammals, causing dopamine to be stockpiled and norepinephrine concentrations to be decreased.

An increase in dopamine and associated metabolites has been previously reported in both cellular and animal models of infection[104,159,212,213,261]. Some publications have reported no change or a decrease in dopamine, however, differences in method and animal models used prohibit comparison. As discussed in Section 1.2.1 dopaminergic signalling can be disrupted by physiological stress and neuronal injury; the aggressive nature of mouse *T. gondii* infection may result in generalised pathology rather than resulting directly from host behavioural change, as discussed in Section 1.4.1[105]. Indeed, classical measures of mouse sickness behaviour may not be evident in infected animals despite them having extensive neuronal damage. Hence, other measures of neuronal health, such as MAP2, should be used to confirm neurophysiological changes are not due to toxoplasmosis.

Norepinephrine concentration during chronic infection has not been widely investigated, although publications reporting norepinephrine concentration during infection consistently report a decrease in concentration[104,159,213]. Mahmoud *et*

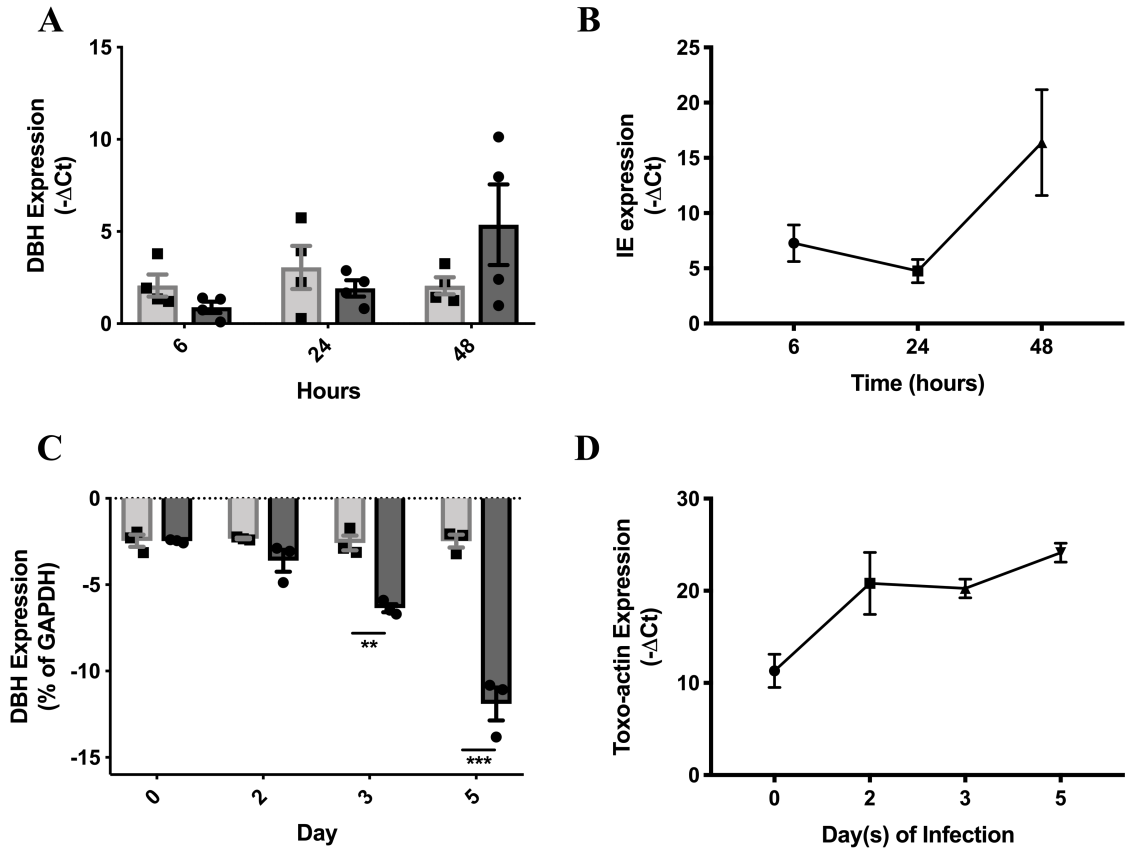


Figure 3.8: Down-regulation of DBH is parasite specific. A) Plot showing DBH expression relative to GAPDH measured using RT-qPCR in uninfected (grey) and cytomegalovirus (CMV) infected (black) human neuronal M17 cells; ±SEM shown, n=3, student t test showed no significance. B) Plot shows expression of a CMV Immediate-Early (IE) gene marker relative to GAPDH for infected samples only; n=3, ±SEM shown. C) Plot showing DBH expression relative to GAPDH in uninfected (grey) and *T. gondii* infected (black) human neuronal M17 cells over a time course of 5 days; ±SEM shown, n=5, Student t test, ** $p<0.01$, *** $p<0.001$. D) Expression of *T. gondii* actin, in infected samples, expressed relative to GAPDH over the course of 5 days, n=5, ±SEM shown.

al observed a decrease in norepinephrine during infection but no corresponding change in norepinephrine turnover. DBH down-regulation provides a possible explanation for this observation[275]. As DBH down-regulation was most significant in the LC and PFC is it possible that down-regulation occurs selectively in norepinephrine producing neurons. Given norepinephrinergic neurons represent about 0.01% of neurons in the brain, changes in gene expression in this small number of cells can be nullified when whole brain homogenate is analysed. This may account for contradictory published data. Published RNAseq data conducted of the mouse forebrain shows no evidence of DBH down-regulation, likely due to the fact that the locus coeruleus, the centre of norepinephrine synthesis was not included in the analysis[276]. This work demonstrates that the area of the brain isolated affects the amount of change observed (Figure 3.5). It was observed that DBH was down-regulated in the prefrontal cortex and locus coeruleus regions but not the hippocampus. Glanzer and colleagues first demonstrated that RNA splicing could occur in live neuronal dendrites isolated from the neuronal cell body[277]. Since then many publications have reported evidence of local RNA translation at the synaptic cleft[278,279]. Noradrenergic neuronal bodies are predominantly located in the region of the locus coeruleus, from there neurons project throughout the brain. Interestingly, down-regulation of DBH mRNA in the prefrontal cortex observed here also demonstrates that DBH RNA is translated locally at the synaptic cleft.

Due to the problems discussed above catecholaminergic cell lines such as PC12 and BE(2)-M17 cells allow catecholaminergic changes during infection to be studied independently of the host immune response. DBH was found to be the most altered catecholaminergic regulator, consistent with PC12 RNAseq data[245,274] (Figure 3.3). As no other catecholaminergic genes are altered, this suggests that the increase in dopamine and corresponding decrease in norepinephrine is a result of DBH suppression caused by chronic infection. Indeed, there is evidence of chronic infection altering other neuronal signalling pathways, Brooks *et al* found that chronic infection altered GABA signalling in the brain by dysregulation of GAD67 expression and localisation[222].

DBH down-regulation is evident at 3 days of induced-bradyzoite infection in both rat and human catecholaminergic cells but becomes significant at 5 days of

infection (Figure 3.4). This timecourse correlates with bradyzoite marker formation; beginning at day three morphological markers such as cyst wall formation and nucleus relocation are observed, as well as expression of various bradyzoite specific genes such as BAG1[81,280–283]. This down-regulation can also be observed *in vivo*. Homogenised brain tissue from chronically infected rats exhibited global down-regulation of DBH mRNA (Figure 3.6). This is particularly striking because, as discussed in Section 1.5.3, chronic *T. gondii* infection in rats typically forms fewer than 10 cysts in the whole brain. Therefore, it is likely there is a mechanism to facilitate this global change. Koshy *et al* observed a 'kiss and spit' wherein *T. gondii* tachyzoites were able to inject rhoptry proteins into uninfected cells. However, this has not been observed during bradyzoite infection. Given that DBH down-regulation is only observed during this chronic stage of infection another mechanism may facilitate this global change.

MAP2 expression was unaltered during chronic rat and mouse infection (Figure 3.6 and Figure 3.5). MAP2 is a sensitive marker of neuronal death, these results suggest that DBH down-regulation is not due to neuronal damage caused by a neuroimmune response to infection[284]. Moreover, down-regulation was found to be parasite-specific, as CMV infection did not induce DBH down-regulation (Figure 3.8). DBH suppression was not observed after 3 days of infection, by which time host gene expression changes are identified[285]. This further indicates that down-regulation is a targeted mechanism of *T. gondii* infection, rather than a consequence of the neuroimmune response to a pathogen.

Interestingly, down-regulation of DBH and the corresponding reduction in norepinephrine was only observed in male rats (Figure 3.7). Regulation of DBH by oestrogen has been demonstrated in PC12 cells, with two oestrogen response element (ERE) sites being identified[318]. Given that DBH expression was strongly correlated with ESR1 expression in female rats it is likely that the oestrus cycle is a confounding factor when trying to assess the effect of *T. gondii* infection. There are sex differences observed during chronic *T. gondii* infection; as discussed in Section 1.5.2. However, the majority of animal behavioural studies are carried out on same gender subjects, typically males, thus behavioural differences between sexes are not yet fully characterised. Some reported sex differences such as sexual arousal towards feline odour in male but not female rodents may be due to

Table 3.1: A summary of literature reporting *T. gondii* infection and movement disorders. Of the 79 patients listed 87% are male and 13% are female[286–313].

Movement Disorder	**Number of Patients**	**Sex**	**Reference**
Involuntary movement	1	Male	[314]
Chorea	1	Male	
Hypokinesia, involuntary movements	1	Female	[302]
Dystonia	1	Female	[315]
Parkinsonism	1	Male	
Dystonia	3	Male	
Ballism	1	Male	
Chorea	1	Male	
Rigidity, Tremor	1	Male	[290]
Holmes tremor	1	Male	[301]
Holmes tremor	1	Male	[312]
Holmes tremor	1	Male	[309]
Holmes tremor	1	Male	[303]
Parkinsonism	14	Male	
Chorea	6	Male	
Myoclonus	4	Male	
Pain during Movement	2	Male	
Dystonia	1	Male	
Chorea	1	Male	[294]
Hemiparesis	1	Male	[304]
Dystonia	1	Female	[305]
Hemiparesis	4	Male	[298]
Nystagmus	1	Female	

Movement Disorder	Number of Patients	Sex	Reference
Hemiparesis, Tremors	1	Male	[316]
Ataxia	1	Male	[295]
Ballism	1	Male	[299]
Ballism	1	Female	[306]
Chorea	1	Male	
Chorea	1	Male	
Chorea	1	Male	[308]
Psychomotor retardation	1	Male	
Psychomotor retardation	1	Male	
Psychomotor retardation	1	Male	
Dysmetria	1	Male	
Ballism	1	Female	[311]
Dystonia	1	Male	[313]
Akathisia	1	Male	[289]
Paroxysmal involuntary movement	1	Male	[287]
Dysonia	1	Male	[317]
Dysonia	1	Male	[300]
Focal paralysis	1	Female	
Involuntary movement	1	Male	
Hypokinesia	1	Female	[292]
Involuntary movement	1	Female	[286]
Guillain-Barre syndrome	1	Male	[296]
Rubral Tremor	1	Male	[297]

sex differences in catecholaminergic signalling[95,319]. Down-regulation of DBH in male but not female mice provides a possible hypothesis for these changes. This also provides a possible explanation for inconsistent dopamine changes during infection of animal models[215,273].

The large suppression of DBH and norepinephrine observed may cause disruption of catecholaminergic signalling, impacting a wide variety of neuronal mechanisms ranging from emotional response to movement. Reduced norepinephrine, and a corresponding loss of neurons in the locus coeruleus is associated with motor impairments[320,321]. Indeed, *DBH-/-* mouse models exhibit spontaneous dyskinesias and are a model for Parkinson's disease. Toxoplasmosis has also been associated with movement disorders. Chronic infection with T. gondii in mice has also been associated with coordination difficulties, and loss of coordination is a common symptom of human toxoplasmosis[72]. In an analysis of case studies of *T. gondii* infected subjects who also experience movement disorders (Table 3.1). 87% of the 79 patients were male, despite some movement disorders such as chorea affecting 3 women to every man[322,323]. Altered DBH expression in men but not women, induced by *T. gondii* infection, may explain these observations. However, GABAergic signalling is also associated with movement disorders and is altered during *T. gondii* infection[222]. Effects of altered GABA metabolism with *T. gondii* infection in promoting seizures would be compounded by a lack of anticonvulsant effect promulgated by norepinephrine. Therefore, it is likely that *T. gondii* infection alters many neuronal pathways associated with movement disorders.

Norepinephrine also plays a role in the host neuroimmune and HPA axis activation. Activation of microglia via β-2 adenosine receptor binding induces further microglial recruitment and proliferation[324,325]. Interestingly, DBH was up-regulated during CMV infection, although not significantly, which may be due to the cellular immune response (Figure 3.8). By down-regulating DBH, and therefore, norepinephrine the parasite may be able to evade the host whilst maintaining the required immune balance to prevent reactivation of tachyzoites growth which ultimately can prove fatal for the host. This is important for successful parasite propagation and survival. DBH and norepinephrine are also important

during adult neurogenesis, the process of replacing neurons throughout the mammalian brain. As discussed in section 1.2.1 this process cannot occur without norepinephrine. Indeed, DBH knockout mice exhibit decreased adult hippocampal neurogenesis[55]. *T. gondii* may selectively down-regulate this process in order to inhibit clearance of infected neurons thus facilitating parasite survival.

DBH down-regulation, and the resulting decrease in norepinephrine concentration, is also associated with many disorders. Given the diverse nature of norepinephrine's biological functions, many mammalian systems can be affected by a change in DBH expression. It remains unclear why *T. gondii* may have evolved a mechanism to specifically down regulate DBH, and consequentially norepinephrine. Although catecholamine dysregulation is associated with behavioural change and toxoplasmosis exhibits pathologies in systems involving norepinephrine (e.g. movement), to date this *T. gondii*-induced disruption has not been directly linked to any parasite associated behaviour phenotype.

3.5 Conclusion

The changes observed in dopamine and norepinephrine concentrations during chronic *T. gondii* infection are consistent with previously published work, however, a causal link had not previously been identified[104,159,212,213,261]. For the first time DBH suppression has been observed, providing a possible mechanism for changes in catecholamine concentrations during infection. This change in DBH expression can cause norepinephrine concentration to be decreased, while dopamine in stockpiled in norepinephrinergic neurons; this is brain region specific *in vivo*. This down-regulation is *T. gondii* specific and not observed with other chronic neuronal infections. Further studies are required to delineate how catecholamine changes and DBH down-regulation contribute to various phenotypes associated with infection. Notably, the mechanism by which infection induces down-regulated DBH remains unknown. Elucidating this could provide insight into how *T. gondii* is able to alter expression of other genes, and possibly the mechanisms of gene regulation in the mammalian brain.

Chapter 4

Epigenetic changes during chronic CNS infection by *T. gondii*

4.1 Overview

Dynamic DNA Methylation in the CNS plays an important role in neuronal function and development. Many neurological disorders are associated with subtle changes in DNA methylation patterns. During mammalian methylation, a methyl group is added to the 5^{th} carbon of a cytosine creating 5-methylcytosine. Together with histone modifications this process facilitates the condensing of chromatin, inhibiting transcription factor binding; thus allowing gene down-regulation. Previous research has identified down-regulation of dopamine β-hydroxylase (DBH) mRNA during *T. gondii* infection (Chapter 2). Here, the mechanism governing this regulation was investigated. *T. gondii* infection was observed to induce DBH suppression and this was associated with elevated methylation upstream of the DBH gene. Previously, changes in catecholaminergic synthesis during chronic *T. gondii* infection have been identified. Furthermore, *T. gondii* infection is associated with down-regulation expression of the DBH gene, responsible for catalysing the conversion of dopamine to norepinephrine. Here it is reported that *T. gondii* bradyzoite-like infection increases methylation of the DBH promoter after 5 days of cell line infection as well as chronically infected rodent neuronal tissue. *T. gondii* infection did not alter global methylation during infection suggesting that increased methylation upstream of DBH gene is selective. Furthermore, using

whole-genome bisulphite sequencing other upstream sequences were identified as differentially methylated during infection. The observed effects on norepinephrine and catecholamine metabolism provides new insight into neuronal epigenetic regulation.

4.2 Introduction

Epigenetic changes regulate diverse gene expression profiles in mammalian cells and tissues throughout the body. A number of epigenetic mechanisms exist to alter gene expression, by remodelling chromatin structure. The architecture of chromatin determines how condensed genomic DNA is. This affects the binding of the regulatory transcription machinery proteins, and thereby controls gene expression. Within the nucleus of a cell, DNA is tightly organised into nucleosomes, these form the structural units of chromosomes. Each nucleosome contains a histone octamer at the centre, comprised of 2 copies of each of the 4 histone proteins H2A, H2B, H3 and H4, around which, DNA is tightly coiled[326,327].

DNA methylation and histone modifications are the major mechanisms of epigenetic regulation. During methylation a family of DNA methyltransferases (DNMTs) catalyse the transfer of a methyl group from S-Adenosyl methionine (SAM), a co-substrate synthesised from methionine, to the 5^{th} carbon of a cytosine residue, this creates 5' methyl-cytosine (Figure 4.1). Typically, these cytosine residues precede guanine nucleotides and are linked together by phosphate bonds (CpG). Areas of high CpG density are known as CpG islands, these are found in the promoter and first exon sequences of approximately 70% of mammalian genes[328–331].

There are 3 major classes of methyltransferase enzymes: DNMT1, DMNT3a and DNMT3b. The former, DNMT1, is implicated in DNA methylation maintenance. DNA methylation is heritable, and therefore, as cell replication occurs and DNA is copied, the methylation pattern must also be copied. This reaction is catalysed by DNMT1. DNMT3a and b induce nascent DNA methylation. DNMT3a and 3b play important roles in DNA methylation in the adult brain. DNMT3b expression is highest prenatally and subsequently declines; it is essential

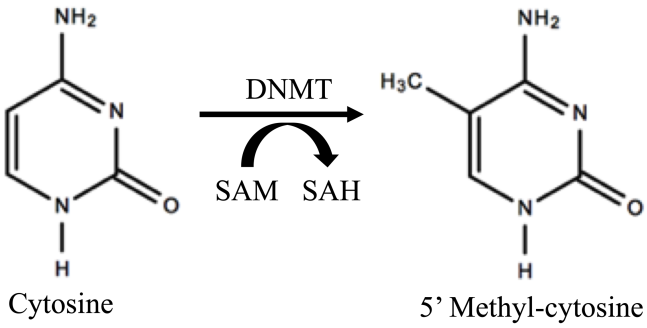


Figure 4.1: Scheme of chromatin structure and DNA methylation. Methyltransferase enzymes (DNMTs) catalyse the addition of a methyl group, donated from S-Adenosyl methionine (SAM) a co-substrate synthesised from methionine, to the 5th carbon of cytosine. This produces 5' methyl-cytosine and S-Adenosyl homocysteine (SAH)[326].

for embryonic development[332]. DNMT3a expression increases postnatally, peaking during infancy and declining with age[333]. Indeed, this decrease in DNMT3a expression with age is correlated with senescence associated hypomethylation which is linked to a deterioration in cognition[334]. DNMT3a and b dysfunction is associated with various neurological disorders and cancer development[335,336]. However, the molecular interactions underpinning this nascent methylation are poorly elucidated, particularly the mechanism of substrate recognition and CpG preference.

Although methylated cytosine residues were first reported in 1940s it was not until the 1980s that methylation was implicated in gene expression[337–340]. DNA methylation can directly inhibit the binding of transcription factors with their target sites[341–343]. Methylated cytosine residues can also act as a docking site for various methyl-binding proteins (MBP) such as MBD1, MBD2, MBD3, and methyl-CpG binding domain protein 2 (MeCP2). These MBPs contain a methyl-binding domain (MBD) which is able to recognise and bind to the methyl group on cytosine residues. MeCP2 was the first MBP characterised and contains 2 structural domains, an MBD and a transcriptional repressor domain[344]. MBPs are also recognised by various histone modifying enzymes, such as histone

deacetylases (HDACs)[345]. Indeed, MeCP2 can form a complex with HDACs and a co-repressor protein, Sin3a, to repress transcription in a methylation-dependent manner[346]. This process allows genomic DNA to condense and form heterochromatin which, in turn can bring about gene repression (Figure 4.2).

DNA methylation is closely linked to histone modifications (Figure 4.3). The N-terminal and C-terminal tails of core histones can be post-translationally modified at the lysine, arginine and serine residues. These residues can be methylated, acetylated, phosphorylated or ubiquitinated[348]. Modifications are catalysed by histone-modifying enzymes such as histone acetyltransferases and deacetylases, methyltransferases and demethylases, kinases and phosphatases, ubiquitin ligases and deubiquitinases, SUMO ligases and proteases. For example, Lys9, 14, 18 and 23 of H3 and Lys5, 8, 12 and 16 of H4, together with lysines on H2A and H2B, can be acetylated; whereas Lys4, 9 and 27, Arg2, 17 and 26 of H3, and Lys20 and Arg3 of H4 can be methylated[349]. Typically, methylation of Lys4 of H3 (H3K4) has been associated with active gene expression, whereas methylation of Lys9 of H3 (H3K9) has been associated with transcriptional silencing[350].

A combination of these modifications generate the histone code and determine the conformation of chromatin. These histone modification marks work in synergy to provide sufficient proof reading; this reduces the probability that a gene can be erroneously activated or deactivated[351]. Fundamentally, histone tail modifications alter the net charge between DNA and the histone proteins, this alters chromatin structure and thus facilitates gene regulation. Furthermore, histone tail modifications can also function as a binding surface for the recruitment of other proteins which can then directly or indirectly influence transcription[352]. These proteins contain domains which are able to recognise particular histone modifications. For example, acetylated histones are typically recognised by bromodomain and kinase domain molecules, whereas methylated histones are recognised by the chromodomain, PHD finger domain and others[353,354]. These proteins can act as transcriptional coactivators or repressors respectively.

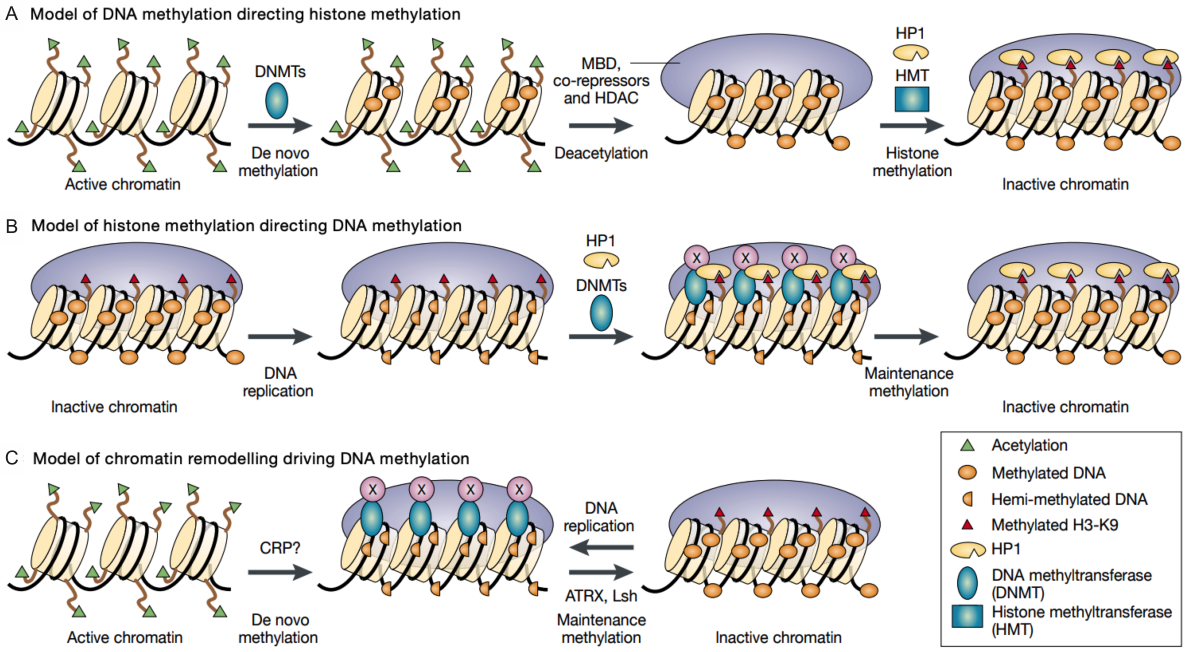


Figure 4.2: Scheme illustrating the interactions between DNA methylation, histone modifications and chromatin remodelling. A) A model of DNA directed histone methylation. DNA methyltransferases such as DNMT3a and DNMT3b induce DNA methylation patterns. Methyl binding proteins (MBPs) and histone deacetylase complexes (HDACs) are then recruited to the methylated CpG DNA in order to induce histone deacetylation, the formation of heterochromatin and gene silencing. B) A model of histone methylation directing DNA methylation. Methylated histone H3K9 acts as a signal for heterochromatin, by recruiting histone protein 1 to methylated histones. This may in turn recruit DNA methyltransferases directly or indirectly, in order to stabilise inactive chromatin. C) A model for chromatin remodelling driving DNA methylation. The ATP-dependent chromatin-remodelling and DNA helicase activities of proteins such as ATRX and Lsh, might facilitate DNA methylation and histone modification by unwinding nucleosomal DNA to increase its accessibility to DNMTs, HDACs and histone methyltransferases. Adapted from [347].

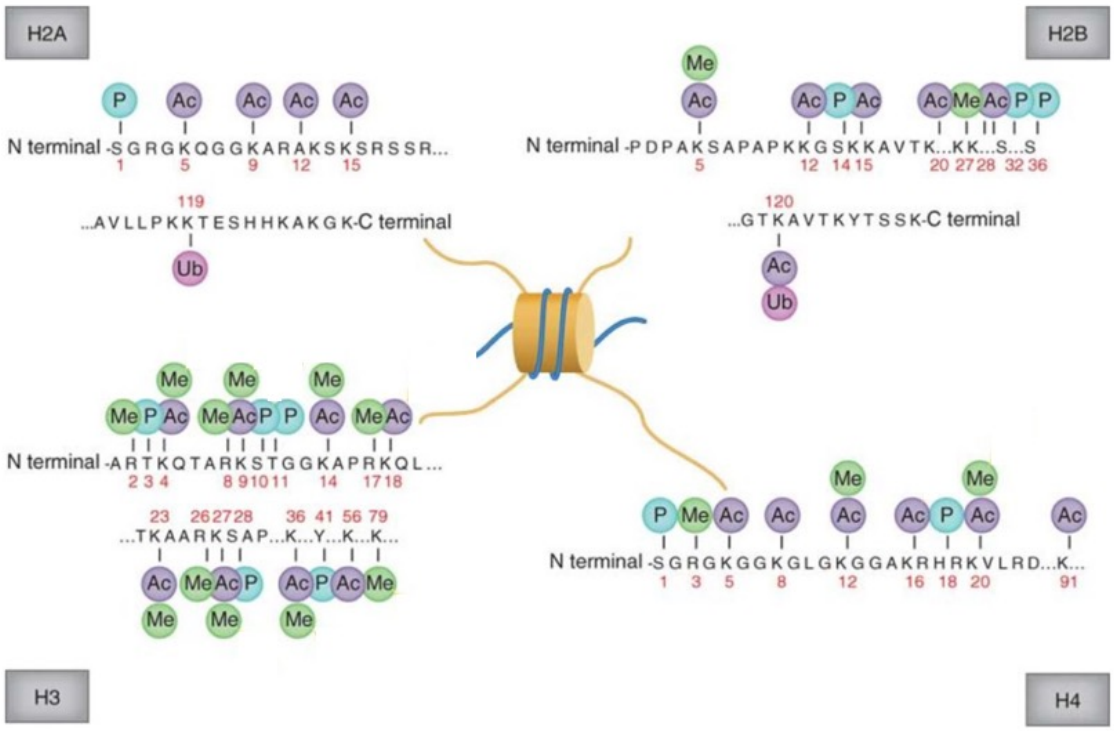


Figure 4.3: Scheme illustrating common post-translational modifications of histone tails. Genomic DNA is coiled around histone octamers in the nucleus of a cell. A nucleosome comprises 4 histone proteins and the 147bp of DNA wrapped around them. Histone tails protrude from the nucleosome core and can be modified by histone-modifying enzymes. Predominantly, these modifications occur on the protruding N-terminal tails, but also within their C-terminal regions, histones can undergo diverse post-translational modifications. In the right combination and translated by the appropriate effectors, these modifications contribute to establishing the global and local condensed or decondensed chromatin states that eventually determine gene expression. Ac, acetylation; Me, methylation; P, phosphorylation; Ub, ubiquitination. Reproduced from [355].

4.2.1 DNA Methylation in the brain

Historically, methylation was considered a stable epigenetic marker. Methylation was thought to occur during cellular differentiation only and maintained to preserve cellular function and identity[356,357]. However, there is emerging evidence of the importance of dynamic methylation particularly in the brain. Indeed, altered DNA Methylation patterns have been observed throughout the cell cycle[358]. Neurons are terminally differentiated. Despite this, dynamic DNA methylation is particularly important for adult CNS functions such as cognition, memory formation and synaptic plasticity[359–361].

Seminal work by Martinowich and colleagues first identified that neuronal depolarisation induced hypomethylation of brain-derived neurotrophic factor (BDNF) and a corresponding increase in BDNF mRNA expression *in vitro*[362]. Since then many publications have reported the role of DNA methylation in synaptic plasticity[363]. Furthermore, it was reported that DNMT1 knock out mice models exhibited synaptic alternations and impaired working memory[364]. The authors also observed that DNMT3a conditional knock-out mice exhibit impaired memory and learning. It has been observed that fear learning increases newly synthesised RNA expression of DNMTs[365,366]. This was accompanied by a decrease in transcript of protein phosphatase 1, catalytic subunit, beta (PPP1CB), a gene associated with suppression of synaptic plasticity and memory[364]. DNA methylation has also been identified as an important mechanism during the neuronal response to stress. Saunderson *et al* observed that traumatic events, such as forced swimming, induced altered DNA methylation in the dentate gyrus of a mouse model[367]. Increased methylation was observed in a region upstream of the of the c-Fos transcription start site. Additionally, stress specifically increased nascent mRNA expression of the DNA methyltransferase DNMT3a.

The structural and functional plasticity of neurons requires continual chromatin reorganisation and gene regulation. The dynamic DNA methylation landscape of an adult neuronal cell is able to adapt rapidly to external stimuli and alter neuronal gene expression accordingly[368]. Additionally, there is growing evidence that methylation also takes place at CpA, CpT and CpC sites[369]. This non-CpG methylation plays an important role in gene regulation particularly the

brain. It has been reported that non-CpG methylation is higher in the brain than in other tissues[370,371]. In the adult mouse dentate gyrus, neurons exhibit approximately 75% CpG methylation and 25% non-CpG methylation[372]. Non-CpG methylation is also enriched in neurons derived from embryonic stem (ES) cells and induced pluripotent stem (iPS) cells[373–376] (Figure 4.4).

Increasingly changes in neuronal DNA methylation are implicated in neurological diseases such as depression, Parkinson's disease, neurodevelopmental disorders and many more[378–380]. The MBP MeCP2 is expressed at high levels in the brain. Gabel *et al* demonstrated that MeCP2 has high affinity for non-CpG binding in transgenic mouse models and that mutations in MeCP2 lead to neurological diseases, such as Rett syndrome, in humans[381,382]. Throughout the last decade, as technology has advanced, our understanding of the role DNA methylation plays in disease and its potential as a biomarker for such has grown rapidly[383–386].

4.2.2 DNA Methylation and *T. gondii* infection

During histone methylation, methyl groups are transferred to the amino acids present on the N-terminal of histone tails. Histone lysine methylation has been widely reported as a key facet of epigenetic modification[387]. *T. gondii*-induced IFN-γ-stimulated gene (TgIST), a parasite-derived protein which localises to the host nucleus, is able to induce epigenetic regulation of STAT1. Repression of the TgIST was correlated with an increased recruitment of HDAC that resulted in hypoacetylated chromatin[388]. Interestingly, Gay and colleagues observed that TgIST is able to sequester host STAT1 and recruit host H3K4me3, an epigenetic marker usually associated with transcriptional activation[235].

It has also been demonstrated that chronic *T. gondii* infection is associated with decreased methylation of the promoter region of the vasopressin gene in the amygdala of male rats[190]. This provides new evidence that *T. gondii* can act to alter the epigenome of the host. Additionally, Vyas *et al* found that treating chronically infected rats with L-methionine, the precursor to SAM reversed the loss of fear phenotype[190]. This indicates that parasite induced epigenetic changes may play a role in host manipulation. However, globally altering methylation is

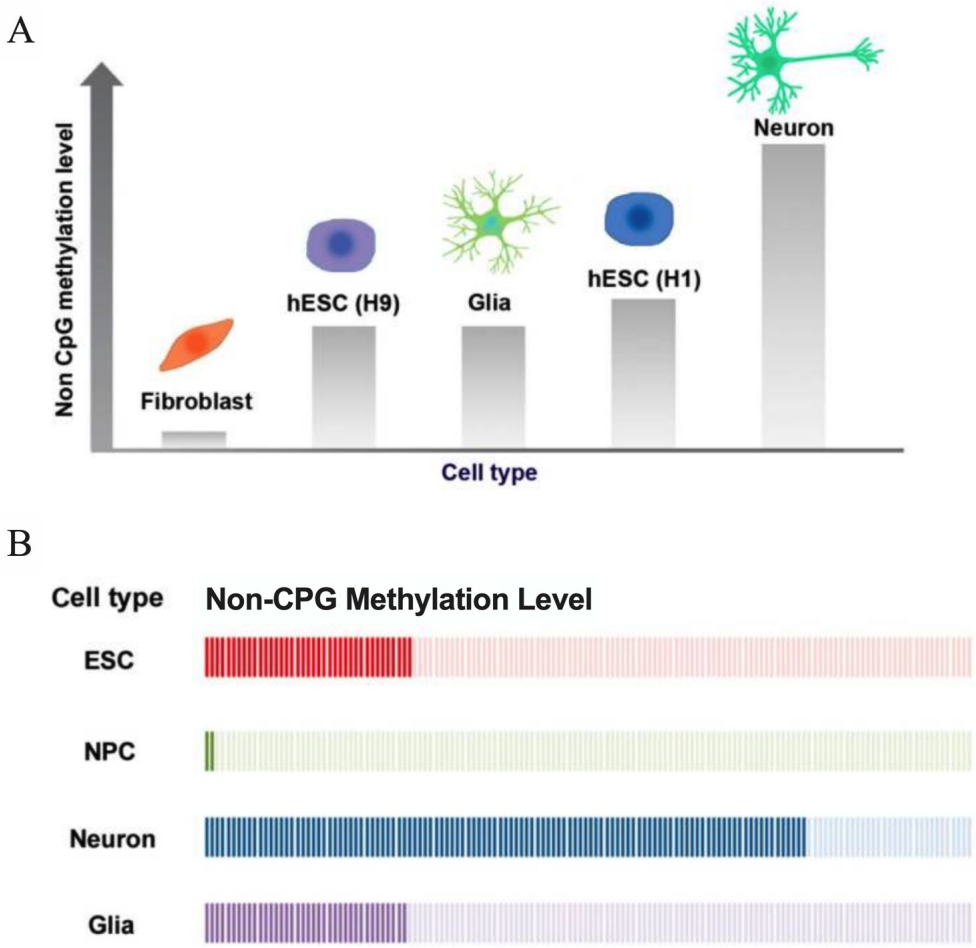


Figure 4.4: Non-CpG methylation levels in different cell types during differentiation of human ES cells. A) Cell-specific non-CpG methylation levels. Neurons exhibit the highest non-CpG methylation of any differentiated mammalian cell type examined. Non-CpG methylation is also enriched in embryonic stem (ES) cells. The male ES cell line H1 exhibits higher non-CpG methylation than the female ES cell line H9. B) Non-CpG methylation levels decrease when ES cells differentiate into neural progenitor cells (NPCs). When NPCs are further differentiated into neurons and glial cells, non-CpG methylation is increased again. Interestingly, non-CpG methylation levels are higher in neurons than in glial cells. Adapted from[377].

a blunt instrument that may affect many systems in the CNS. Additionally there is evidence that methionine treatment can induce cellular toxicity and infected neurons may be particularly vulnerable[104,389,390].

Previously published data has demonstrated that *T. gondii* increases dopamine levels in infected cells and in the brains of infected animals[213]. Concurrently, norepinephrine content in the brain is significantly reduced. As discussed in Chapter 2 infection induces down-regulation of DBH gene expression. Here the mechanism of this gene silencing is investigated. By understanding the mechanism of this gene change new insight into the host-parasite interaction can be obtained. Neurons are characterised by an adaptability; synaptic plasticity enables neurochemical responses to be rapidly altered according to external stimuli. Epigenetic mechanisms of gene expression are emerging as a driving force of neuronal function implicated in memory, cognition, movement and emotion. The neuronal epigenome is a rapidly developing area of research. By understanding how *T. gondii* can alter gene expression, further insight can be gained into the mechanisms governing epigenetic regulation of neuronal function.

4.3 Results

4.3.1 Nascent transcription is responsible for decreased DBH expression

The specifics of DBH mRNA changes during infection (as observed in Chapter 2) were further investigated. A nuclear run-on assay allows nascent transcription to be measured by incorporation of a tagged ribonucleotide into newly transcribed RNA in purified nuclei. In contrast to measuring steady state RNA in standard RT-qPCR assays, nuclear run-on assays examine newly transcribed RNA to distinguish transcriptional from post-transcriptional gene regulation. Five day infected rat dopaminergic and noradrenergic PC12 cells exhibited a 22.02-fold $\pm$4.8 ($p=0.0006$) down-regulation in nascent DBH transcription, as demonstrated by a nuclear run-on assay quantified by qPCR (Figure 4.6A). Similarly, human neuronal M17 cells infected with induced-parasites also exhibited significantly ($p=0.031$) down-regulated DBH (Figure 4.6B). During infection of M17 cells DBH

expression was 23.13-fold ±5.1 down-regulated. Taken together these data suggests that transcriptional regulation of the DBH gene occurs.

4.3.2 Epigenetic changes at the 5' region of the DBH promoter with infection

To assess epigenetic changes coincident with the transcriptional down-regulation of the DBH gene, methylation of the DBH promoter region was investigated using Methylation sensitive restriction enzyme (MSRE) qPCR. Methylation sensitive restriction enzymes are only able to cut unmethylated DNA. Hence, by comparing digested and undigested sample Cq values methylation status in the region of interest can be identified (Figure 2.4). Methylation of the 5' DBH promoter region was measured in rat PC12 and human M17 catecholaminergic cells after 0, 3 and 5 days of infection with *T. gondii*. At day 3, infection increased PC12 DBH promoter methylation from 20.05 ±4.6 in uninfected cells to 37.5% ±9.4 (p=0.19). At day 5, infected cells exhibited 66.0% ±3.7 methylation compared to 16.60% ±4.6 in uninfected cells at the DBH promoter ($p=4x10^{-5}$) (Figure 4.7A). M17 cells showed an increase from 25.14% ±6.2 in uninfected cells to 33.90% ±11.4 methylation in 3-day infected M17 cells (p=0.52). By day 5 infected cells had 68.96% ±9.1 methylation compared to 14.15% in ±6.0 in uninfected controls in the DBH promoter region (p=0.0011) (Figure 4.7B). Methylation of the promoter region in uninfected PC12 and M17 cells was not changed over 5 days (one-way ANOVA, p=0.9 and p=0.32 respectively).

In order to investigate the methylation effect of infection *in vivo*, triple brain slice co-cultures were initially used. These *ex vivo* cultures allow the relationship between methylation of the 5' DBH promoter and MOI to be assessed over a short period of time in the absence of systemic immune activation. Therefore, this provides insight into neuronal methylation changes resulting directly from infection. Slice cell numbers were estimated by DAPI staining, cell numbers were counted in 5 fields of view (data not shown). From each animal 3 coronal slices are taken and arranged on a single plate. Slices were infected with *T. gondii* and infection was monitored throughout the experiment. After 5 days of infection DNA was harvested and MSRE qPCR performed (Figure 4.8). Compared to uninfected

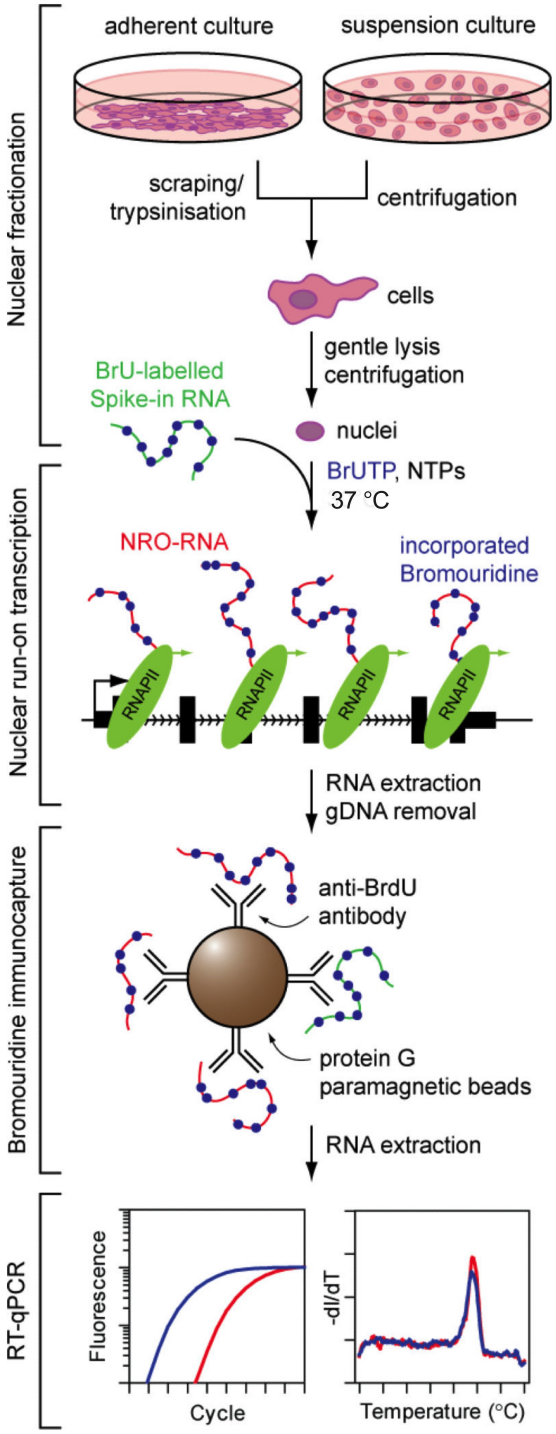


Figure 4.5: Scheme of the nuclear run on work-flow performed. Initially nuclei are isolated from uninfected or 5-day infected rat catecholaminergic cells. Nuclei are then incubated with BrUTP, and NTPs for 30 minutes at 37°C. RNA is then extracted, and nascent pre-mRNA is isolated using immunocapture. RNA is then reverse transcribed, and RT-qPCR performed. Adapted from [256]

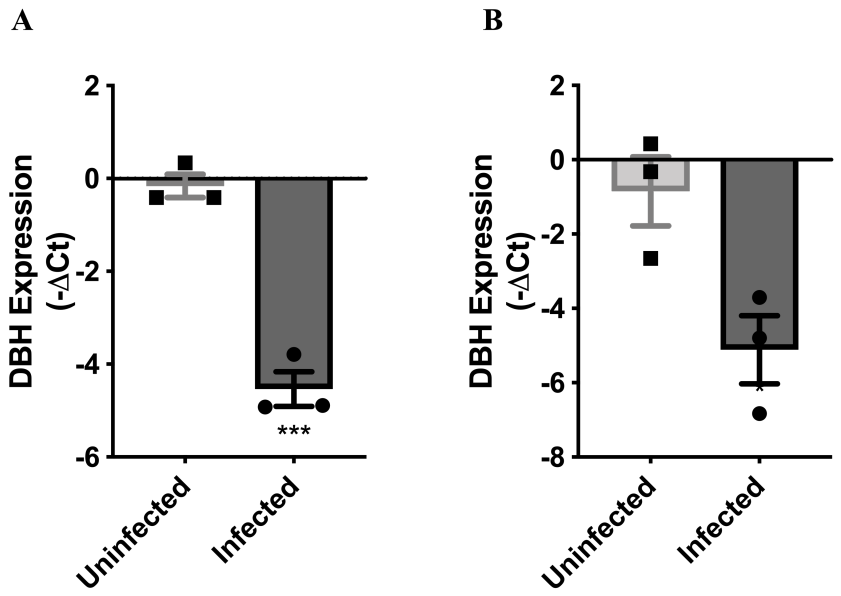


Figure 4.6: Nascent transcription of DBH is down-regulated during chronic infection. A) Rat catecholaminergic PC12 cells infected with bradyzoite induced parasites. After 5 days of infection nuclear run-on assays were performed. Nascent DBH pre-mRNA transcription is shown relative to GAPDH. ±SEM shown; MOI 1; n=5; ; unpaired student t test, ***$p<0.001$. B) Human neuronal M17 cells infected with induced-bradyzoites. After 5 days of infection nuclear run-on assays were performed. Nascent DBH expression relative to GAPDH shown. ±SEM shown; MOI 1; n=3; ; unpaired student t test, *$p<0.05$.

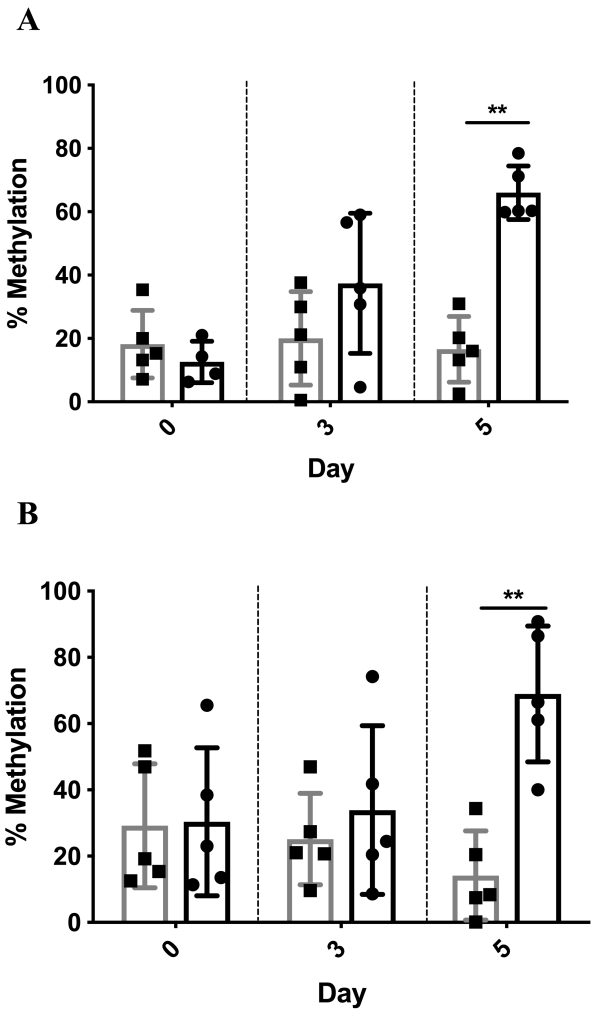


Figure 4.7: Methylation of the DBH promoter region is increased during chronic *T. gondii* infection. A) Percentage of methylation in the 5' promoter region of the DBH gene in PC12 cells was measured by MSRE qPCR. DNA was collected at 0, 3 and 5 days from uninfected (grey) or infected (black) rat catecholaminergic cells. MOI=1; ± SEM shown, n=5, unpaired student t test, **p<0.01. B) Percentage of methylation in the 5' promoter region of the DBH gene measured by MSRE qPCR. DNA was collected at 0; 3 and 5 days from uninfected (grey) or infected (black) human neuronal (M17) cells. MOI=1; ± SEM shown, n=5, unpaired student t test, **p<0.01.

controls methylation increased from 29.61% ±2.7 to 48.08% ±9.4 (p=0.04) when slices were infected at a MOI 5. Slices infected with MOI 7 exhibited 73.66% ±4.6 methylation (p<0.001).

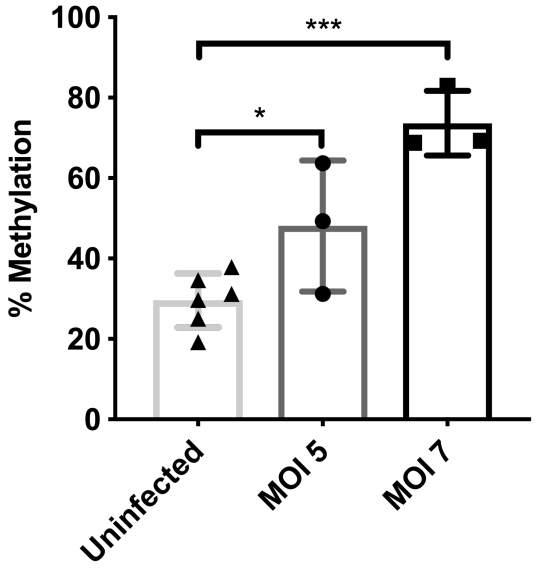


Figure 4.8: Triple slice co-culture DBH promoter methylation was measured with MSRE qPCR. Triple slice co-cultures were uninfected (light grey, n=6), infected with wild-type PRU at MOI 5 (dark grey, n=3) and MOI 7 (black, n=3). Infection was monitored for 5 days of infection by light microscopy; ± SEM shown, n=3 biological repeats containing 3 slices from the same rat per well, Student t test * p<0.05, *** p<0.001.

Altered methylation of the DBH promoter region was also observed *in vivo*. Brains were harvested from mice chronically infected with wild-type Pru (after 5 weeks of infection mice were sacrificed). To increase sensitivity of the assay so that changes in noradrenergic neurons could be detected, neuronal nuclei were isolated from brain tissue. Typically, during rodent *T. gondii* infection, only a small number of neurons are infected, therefore epigenetic changes may be nullified when analysing whole brain homogenate. Unlike RNA, changes in the neuronal DNA status induced by infection can be difficult to detect because change is only in

a small number of cells *in vivo*. Therefore, this method increases the likelihood that methylation changes in DBH will be measurable. A neuronal nuclei isolation was first performed which may allow more nuanced changes in DBH promoter methylation to be detected. Nuclei isolated from chronically-infected and control mice were purified and labelled with Alexa 488 conjugated anti-NeuN antibody. Based on this, fluorescent signal neuronal nuclei are separated to isolate neurons from other brain tissue (Figure 4.9A). MSRE qPCR revealed that methylation was increased at the DBH promoter region during chronic *T. gondii* infection (Figure 4.9B). Methylation at the DBH promoter region was 6.63% ±2.0 in uninfected male mouse neurons, whereas chronically infected mice neurons exhibited 53.12% ±7.7 methylation ($p<0.0001$) (Figure 4.9C).

It is possible that methylation in the 5' promoter region of DBH is a consequence of DBH down-regulation rather than the cause. To begin to characterise the type of host chromatin reorganisation in neural cells changes during *T. gondii* infection, cell cultures were treated with methyltransferase inhibitor compounds (Figure 4.11). RG108 is a DNMT inhibitor, 5-Azacytidine (5-AC) is a potent DNMT1 inhibitor and Trichostatin A (TSA) is a histone deacetylase (HDAC) inhibitor. As a control to assess whether drug treatment would affect parasite viability or differentiation a Pru strain expressing GFP linked to a BAG1 promoter (and possessing a KU80 mutation, making parasite deficient in nonhomologous end joining) and Type I RH strain tachyzoites with fluorescent tag via a golgi marker were grown at various drug concentrations and no change to parasite growth was observed[391] (Figure S2).

Initially, uninfected and *T. gondii* infected rat catecholaminergic PC12 cells were treated with TSA, 5-AC and RG108 at 10μM, 1μM and 1μM respectively (Figure 4.10A). After 5 days of infection RNA was harvested and RT-qPCR performed. Infected cells exhibited 4.9-fold ± 2.0 down-regulation of DBH mRNA, relative to the housekeeping gene GAPDH, only during TSA treatment. 5-AC and RG108 treated cells exhibited no down-regulation in DBH expression ($p=0.072$ and $p=0.83$) respectively. Uninfected cells did not exhibit significantly altered DBH expression under and drug treatment condition (one-way ANOVA, $p=0.093$). To asses if this observation was cell-type dependent uninfected and *T. gondii* infected human neuronal M17 cells were also subjected to

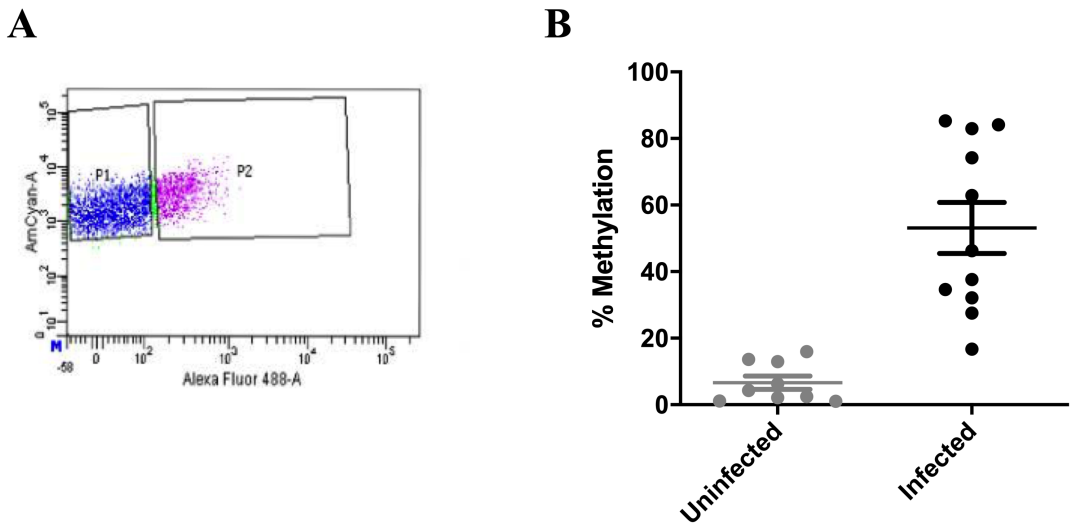


Figure 4.9: Chronic *T. gondii* infection induces methylation of the DBH promoter *in vivo*. A) Representative FACS report of chronically infected and uninfected mouse brain samples. Plot shows Hoechst 33342 (y axis) and NeuN AlexaFluor-488 (x axis) signal. Sort gates are illustrated as P1 representing NeuN negative non-neuronal cells and P2 represents NeuN positive neuronal cells; P2 represents isolated neuronal nuclei. P2 samples were processed for MSRE qPCR . B) Plot shows methylation at the DBH promoter region in neurons from uninfected (grey, n=9) and chronically infected (black, n=11) mouse brain samples measured by MSRE qPCR. ±SEM shown, student t test p=0.0004

treatment with TSA, 5-AC and RG108 at 10μM, 1μM and 1μM respectively (Figure 4.10B). Infected M17 cells exhibited 7-fold ±1.7 DBH down-regulation, relative to the housekeeping gene GAPDH, during TSA treatment only. 5-AC and RG108 treated cells exhibited no down-regulation in DBH expression (p=0.66 and p=0.61) respectively. Uninfected cells did not exhibit significantly altered DBH expression under and drug treatment condition (one-way ANOVA, p=0.72).

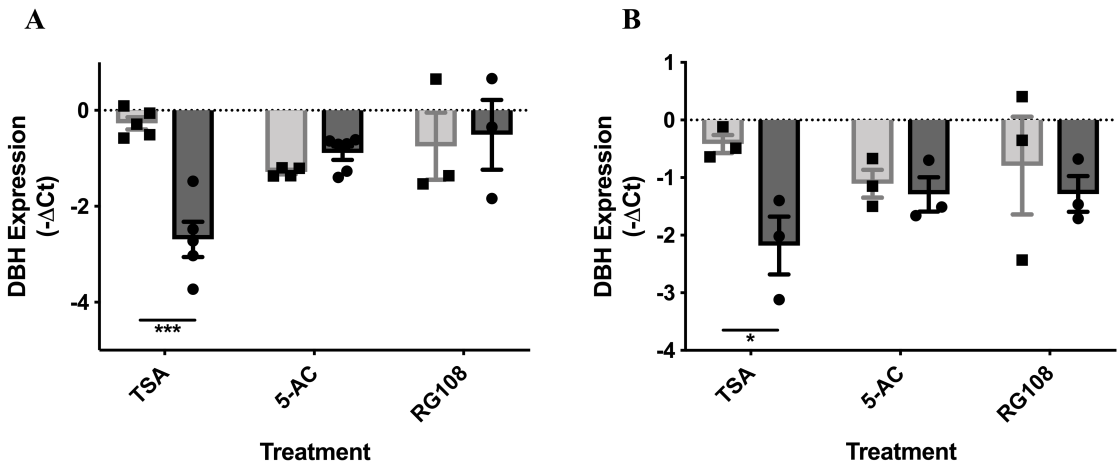


Figure 4.10: DBH expression during treatment with histone deacetylase and methyltransferase inhibitors. A) Plot shows expression of DBH relative to the housekeeping gene GAPDH. Uninfected (grey) and infected (black) rat catecholaminergic PC12 cells treated with TSA, 5-AC and RG108; ±SEM shown, n=5, student t test ** p<0.01. B) Plot shows expression of DBH relative to the housekeeping gene GAPDH. Uninfected (grey) and infected (black) human neuronal M17 cells treated with TSA, 5-AC and RG108; ±SEM shown, n=5, student t test * p<0.05.

Next, the effect of drug treatment on methylation of the 5' promoter region of DBH was investigated. Uninfected and *T. gondii* infected rat catecholaminergic PC12 cells were treated with TSA, 5-AC and RG108 at 10μM, 1μM and 1μM respectively (Figure 4.11A). After 5 days DNA was harvested and MSRE qPCR performed. After TSA treatment methylation of the 5' promoter region of DBH was increased from 6.60% ±1.8 in uninfected cells to 45.21% ±4.3 during infec-

tion ($p<0.001$). No difference was found between infected and uninfected PC12 cells when treated with 5-AC and RG108 ($p=0.53$ and $p=0.54$ respectively). To investigate if this can be observed during human neuronal cellular infection M17 cells were also treated with TSA, 5-AC and RG108 (Figure 4.11B). Similarly, only TSA treatment resulted in a difference between infected and uninfected samples: methylation of the DBH promoter was increased from 21.63% ±11.0 in uninfected cell to 62.09% ±11.8 during infection ($p=0.045$). No difference in methylation of the DBH promoter was observed between infected and uninfected M17 cells when treated with 5-AC and RG108 ($p=0.69$ and $p=0.66$ respectively). There was no difference in uninfected DBH 5' promoter region methylation across the various drug treatments in PC12 or M17 cells (one-way ANOVA $p=0.666$ and $p=0.701$ respectively). Taken together this data indicates DNA methyltransferase plays an important role during parasite induced suppression of DBH expression and methylation of the 5' DBH promoter region.

4.3.3 Preliminary whole-genome bisulphite sequencing

MSRE qPCR is limited by the presence of restriction sites in the DBH promoter region. Whole-genome sequencing of bisulphite treated DNA was performed to investigate genome wide-alterations in methylation of promoter regions induced by *T. gondii* infection. Whole-genome bisulphite sequencing combines bisulphite treatment, which converts cytosine residues to uracil, but leaves 5-methylcytosine residues unaffected, with next-generation sequencing technologies. It is currently considered to be the gold standard for a comprehensive and quantitative analysis of DNA methylation throughout the genome[392].

Rat catecholaminergic cells were uninfected or 5 day infected with *T. gondii*. DNA was collected from 5 biological repeats and pooled. Samples were pooled to minimise cost while providing preliminary data. After sequencing only 2kb regions upstream of a gene were analysed as these provided the highest sequencing reads. Of these, 250 genes exhibited statistically significant differential methylation. Due to CpG island bias there was insufficient read depth of the DBH promoter region to confirm MSRE qPCR. Analysis of methylation within promoter sequences revealed differential methylation was primarily found in the promoters

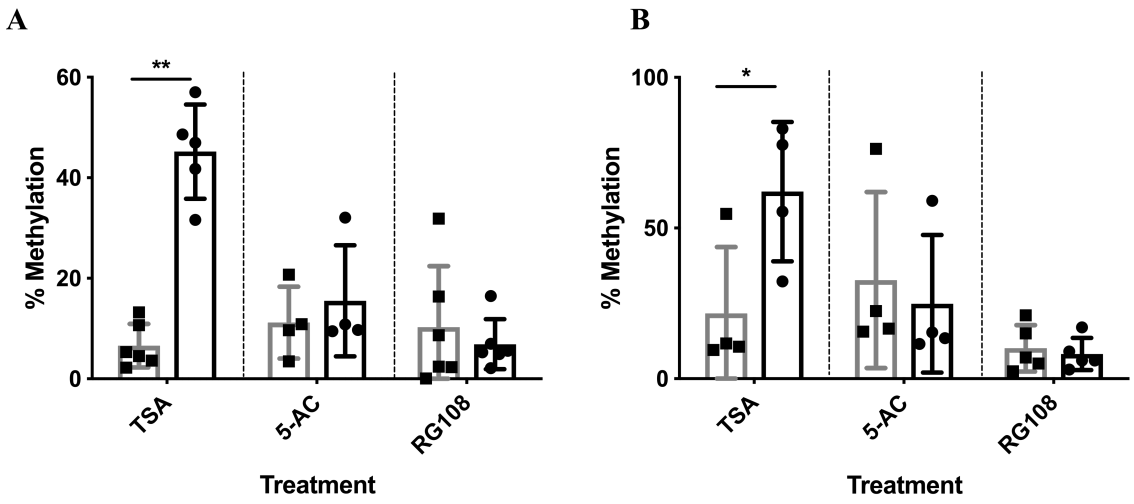


Figure 4.11: Cell cultures treated with histone deacetylase and methyltransferase inhibitors. Methylation of the 5' DBH promoter region was then measured via MSRE qPCR and expressed as a percentage. A) Uninfected (grey) and infected (black) rat catecholaminergic PC12 cells treated with TSA, 5-AC and RG108; ± SEM shown, n=5, student t test ** $p<0.01$. B) Uninfected (grey) and infected (black) human neuronal M17 cells treated with TSA, 5-AC and RG108; ± SEM shown, n=5, student t test * $p<0.05$.

of genes associated with the immune response, neuronal signalling and intracellular signalling (Figure 4.12). Pie charts represent GO term analysis of promoter sequences exhibiting more than a 20% decrease in methylation during infection. Infection induced decreased methylation in the 5' promoters of genes associated with neuronal signalling and the immune response. Increased methylation was primarily observed in the 5' promoter sequences of gene associated with neuronal signalling, metabolism of immune signalling (Figure S6). However, as pooled samples were analysed individual sample variation cannot be estimated.

4.3.4 Validation of whole-genome bisulphite sequencing: Ascl1 represents another example of altered methylation in the 5' promoter of a gene

To validate whole-genome sequencing and identify other genes that may exhibit transcriptional regulation during *T. gondii* infection, whole-genome bisulphite data was compared to RNAseq[274]. Of these genes, Achaete-scute homolog 1 (Ascl1, also known as Mash1) was identified as a gene exhibiting mRNA down-regulation coupled with increased methylation at the 5' promoter region. Ascl1 expression and promoter methylation was then investigated using MSRE qPCR (Figure 4.13). Initially, total RNA was harvested from uninfected and 5-day infected PC12 cells; Ascl1 expression was 1.78-fold ±0.23 down-regulated relative to GAPDH after 5 days of *T. gondii* infection (p=0.029) (Figure 4.13A). To assess the effect of infection on nascent RNA transcription a nuclear run on assay was performed, revealing newly synthesised Ascl1 RNA was 36.2-fold ±9.0 down-regulated (p=0.0006) (Figure 4.13B) relative to the housekeeping gene GAPDH.

Methylation at the promoter of Ascl1 was measured via MSRE qPCR (Figure 4.14). A time course of DNA harvested from uninfected and infected PC12 cells was made. Similar to DBH promoter methylation, a change with infection was only observed after 5 days of infection. Methylation was increased from 27.86% ±8.5 in uninfected cells to 65.58% ±9.0 in infected samples (p=0.0378). Uninfected PC12s did not exhibit a significant change in methylation between 0 and 3 or 5 days (one-way ANOVA, p=0.842).

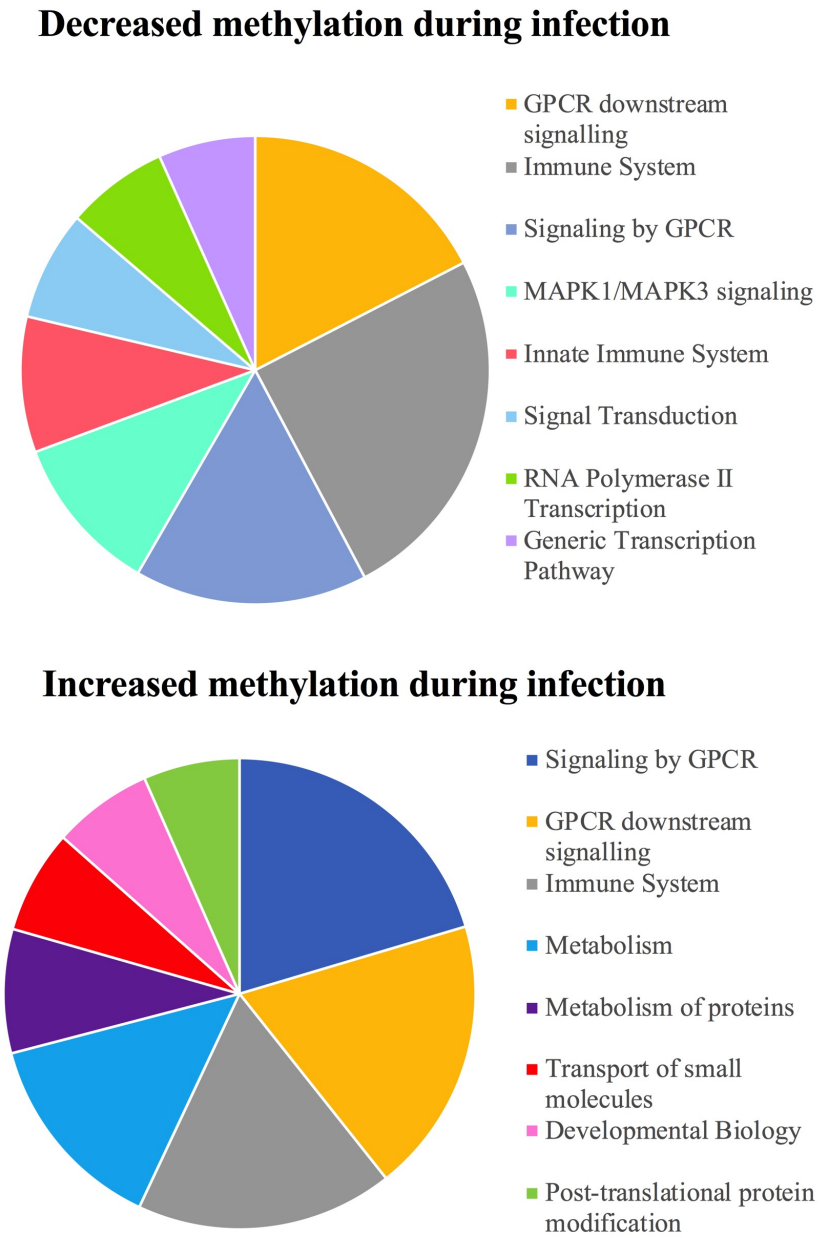


Figure 4.12: Cellular pathways associated with the 200 most differentially methylated genes as measured by whole-genome bisulphite sequencing. Methylation was measured in the 2kb region upstream of a gene was analysed with whole-genome bisulphite sequencing. Promoter regions exhibiting decreased (top panel) and increased (bottom panel) methylation were plotted. DNA was extracted from uninfected and 5 day *T.gondii* infected rat catecholaminergic cells; 5 biological repeats where pooled prior to whole-genome bisulphite sequencing.

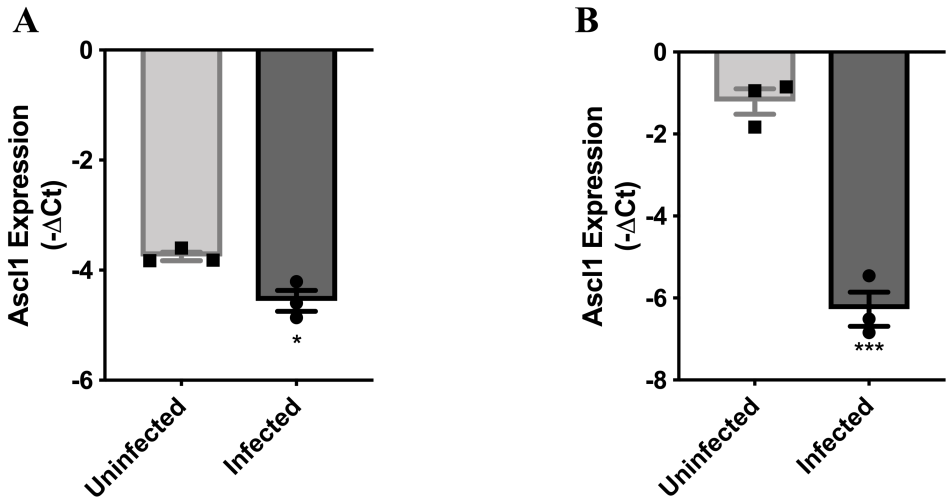


Figure 4.13: Ascl1 expression is altered via methylation of the promoter during *T. gondii* infection. A) Plot showing expression of Ascl1, RT-qPCR shown with respect to the housekeeping gene GAPDH. PC12 cells were uninfected (light grey) or 5-day infected with *T. gondii* (dark grey). ±SEM shown, n=3; student t test * $p<0.05$. B) M17 cells were either uninfected or infected with induced-bradyzoites. After 5 days of infection nuclear run-on performed. Newly transcribed Ascl1 expression is shown relative to GAPDH. ±SEM shown; MOI 1; n=5; ; unpaired student t test, *** $p<0.001$.

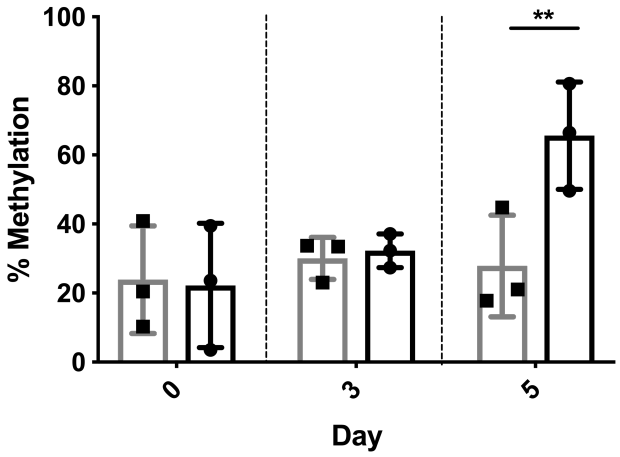


Figure 4.14: Methylation of the 5' Ascl1 promoter region is altered during *T. gondii* infection. Plot shows percentage methylation of the Ascl1 5' promoter region as measured by MSRE qPCR. Rat catecholaminergic cells were infected with *T. gondii* over a time course of 5 days. DNA was collected at 0; 3 and 5 days from uninfected (grey) or infected (black) PC12 cells; MOI=1, ± SEM shown, n=3, unpaired student t test, **$p<0.01$.

4.3.5 Altered methylation of the 5' DBH promoter is not due to global methylation changes during *T. gondii* infection

It remains possible that the observed change in DBH promoter methylation is due to a universal increase in host cell methylation with infection. Hence, global methylation changes during chronic *T. gondii* infection were investigated. An ELISA was performed to assess whether global methylation was changed in cell lines and rat neuronal tissue during infection (Figure 4.15). *T. gondii* infection did not alter global methylation in rat catecholaminergic PC12 cells (p=0.56); with average methylation of uninfected cells 3.3% ±3.0 and chronically infected cells exhibited 6.1% ±2.8 (Figure 4.15A). Similarly, infection did not alter global methylation of human neuronal M17 cells (p=0.588). Average methylation of uninfected M17 was 13.7% ±5.7 and infected cells exhibited 9.3% ±1.9 (Figure 4.15B). DNA isolated from neuronal nuclei purified from mouse brain tissue did not show any changes in global methylation (p=0.15), uninfected methylation was observed to be 23.3% ±6.5 and infected neuronal DNA was 14.8% ±0.8 methylated (Figure 4.15C).

4.4 Discussion

Jähner *et al* first described altered host DNA methylation during infection. It was observed that retrovirus integration into the host genome correlated with increased DNA methylation, resulting in transcriptional silencing of target genes, which facilitated viral survival[393]. *T. gondii* has previously been described as an epigenator altering host gene expression via hypomethylation of host DNA[190]. Here, for the first time specific increased promoter methylation is described during chronic *T. gondii* infection. This provides, a possible mechanism for altered catecholaminergic signalling that have been widely observed. DBH down-regulation observed during *T. gondii* infection, described in Chapter 2, was investigated. Down-regulation of nascent DBH gene transcription during *T. gondii* infection is reported (Figure 4.6). Traditionally, transcription is monitored by measuring incorporated radiolabelled nucleotides in isolated nuclei. This has been adapted

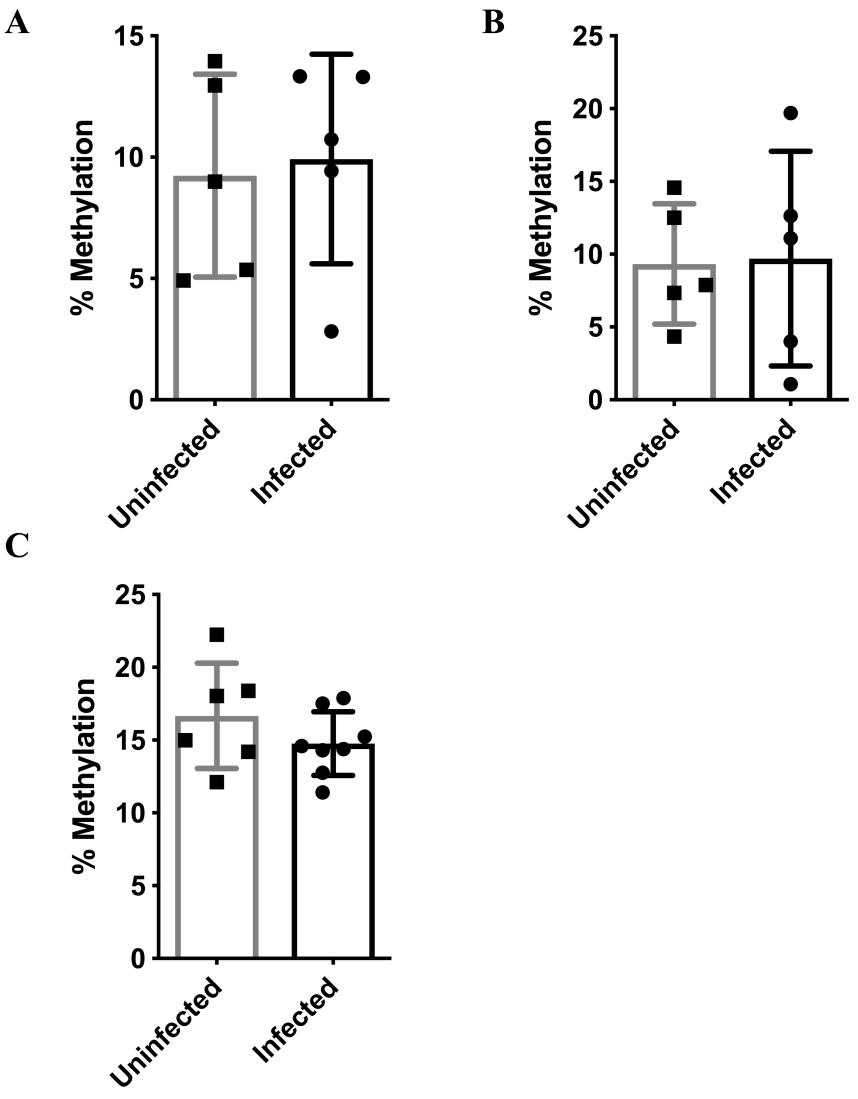


Figure 4.15: Global methylation during chronic *T. gondii* infection. A) Plot shows total methylation measured by ELISA of both uninfected and infected rat catecholaminergic PC12 cells. No significant change in methylation was identified (p=0.515); ± SEM shown, n=5. B) Plot shows total methylation measured by ELISA of both uninfected and infected human neuronal M17 cells. ± SEM shown, n=5, student t test not significant, p=0.588. C) Global methylation of uninfected (n=6) and chronically infected (n=8) neuronal nuclei isolated from mouse brain tissue; ± SEM shown, student t test, no significance, p=0.153

to avoid radioactivity with the incorporation of bromodeoxyuridine during transcription. Instead isolation of the bromo containing RNA by immunocapture is used. During a nuclear run-on assay, radiolabelled nucleotides can be incorporated into RNA transcripts being synthesised at the time of sample collection. DBH expression was significantly down-regulated in infected cells compared to controls showing regulation of DBH gene expression prior to RNA transcription.

Methylation in a 5' upstream region of the DBH gene was observed in rodent neural cells, human neuronal cells, and neurons isolated from mouse brain as shown by MSRE-qPCR. Hence universal change in noradrenergic cells can be observed during with *T. gondii* infection. This may explain the observed decrease in transcription of DBH as described in Chapter 2. However, only one 250bp region 2.6kb upstream of the DBH gene was investigated. Primarily, this is due to limitations of MSRE qPCR as MSRE sites were not present in sufficient number closer to the transcription start site. Furthermore, the upstream region of the DBH gene contains relatively few CpG sites and the whole genome sequencing method used favoured CpG islands. Therefore, comprehensive methylation data across the whole 5' promoter region is lacking and MSRE qPCR data urgently requires confirmation. Given the high frequency of non-CpG site methylation in the brain it is possible that the DBH promoter methylation observed is due to CpT, CpA or CpC methylation. Techniques such as Sanger sequencing or pyrosequencing may provide insight into base pair methylation across the whole upstream region[394].

In order to identify the mechanism of epigenetic regulation, inhibitors of methylation and histone deacetylation were used. The use of DNMT inhibitors rather than DNMT knock out cell lines enables normal cell and parasite growth prior to the assay and specific time points to be analysed. However, 5-AC and RG108 incorporate into DNA and disrupt the binding of DNA methyltransferase (DNMT) enzymes[395,396]. The exact timepoint DNMTs become inhibited was unknown because nucleoside inhibitors must first be incorporated into DNA in order to inhibit DNMT activity. Therefore, RNA and DNA were harvested at 5-days only[397]. Nucleoside inhibitors can also be toxic to cells, although normal cell and parasite growth was observed over 5 days (Figure S2). 5-AC and RG108 treatment abrogated the DBH down-regulation observed with infection

(Figure 4.11). Furthermore, drug treatments also inhibited the hypermethylation of the DBH promoter region. TSA is an inhibitor of mammalian histone deacetylases (HDAC). During the formation of inactive chromatin, these enzymes remove acetyl groups from amino acids in the N-terminus of histone tails, this allows chromatin to condense and, can induce a down-regulation of gene expression[398,399]. TSA treatment did not alter expression, nor the DNA methylation pattern observed with infection. These findings suggest that the mechanism of DBH regulation observed during *T. gondii* infection is dependent upon the action of DNMTs and may not, at least during the 5-days of the experiment, involve HDAC. This may be because DNA methylation is able to directly inhibit transcription factor binding, decreasing DBH expression and chromatin has not yet condensed[400,401]. Additionally, it is possible that in this system histone methylation drives DNA methylation causing transcript inactivation[402] (Figure 4.2B). However, qPCR of control genes known to be regulation via HDAC should be used to confirm TSA is effective at these concentrations.

A global change in host DNA methylation did not accompany *T. gondii* infection. This was observed in rodent and human neuronal cell lines and within the neurons of adult mouse brain (Figure 4.15). Several publications have reported methylation is increased globally during cellular stress and injury[403–405]. The absence of a change to global methylation suggests that DBH down-regulation is a targeted effect of *T. gondii* infection, rather than a cellular response to stress. This was further supported by whole-genome bisulphite sequencing of uninfected and chronically infect rat catecholaminergic cells (Figure S6). No global change in methylation across the genome was identified. Reduced methylation of the 5' promoter regions of some neuroimmune associated genes were observed during infection (Figure 4.12). This supports previously published transcriptomic analysis identifying increased immune associated gene expression during infection[223]. Importantly, genes associated with apoptosis or necrosis were not differentially methylated during infection, suggesting that, observations were not due to cell death. Interestingly, the promoter regions of neuronal signalling genes exhibited increased and reduced methylation. However, analysis of whole-genome sequencing data is incomplete and further analysis is required. Additionally, MSRE

qPCR of other differentially methylation genes is required to validate these findings. Future experiments should focus on repeating the whole-genome sequencing with un-pooled neurons isolated from chronically infected rodents.

Whole-genome bisulphite sequencing revealed increased Ascl1, also known as Mash1, promoter methylation during infection. The observed Ascl1 gene down-regulation and corresponding DNA methylation provides further validation of MSRE qPCR and whole-genome bisulphite sequencing to identify epigenetic changes during infection (Figure 4.13). Infection had a larger effect on the abundance of nascent Ascl1 RNA expression compared to mRNA. This may be because of large amounts of steady state RNA within the cell, HOWEVER, further investigation is required. Ascl1 is a transcription factor important during neuronal differentiation[406–408]. GABAergic and serotonergic neuronal differentiation is dependent upon Ascl1 expression[409,410]. However, it's relevance during chronic *T. gondii* infection has not yet been characterised and requires further study.

Given that global DNA methylation is not altered, increased methylation of the DBH promoter region may be a specific phenotype of *T. gondii* infection. Interestingly, within the *T. gondii* genome there are three genes with methyltransferase homology, TGME49027660, TGME49310070 and TGME4926061021[411]. Indeed, two functional DNMTs have been identified in *T. gondii* able to induce CpG methylation[412]. It is possible that these are secreted into the host cell, and are able to induce methylation of host DNA. It has also been demonstrated that hepatitis B virus is able to recruit host DNMT3A2 inducing hypermethylation of a target promoter sequence[413,414]. Furthermore, Negri *et al* have found evidence that *Drosophila* infection with alpha-proteobacterium *Wolbachia pipientis* is able to induce host feminisation via epigenetic modifications[415]. Interestingly, there is evidence that this is catalysed by *Wolbachia pipientis* methytransferase enzymes[416]. During chronic *T. gondii* infection, host DNMT1, DNMT3b and DNMT3L ($p=0.0017$, $p=0.0072$ and $p=0.0036$ respectively) are 2-fold up-regulated in transcriptome data of chronically infected mouse brain samples[276].

Using MSRE pPCR, increased methylation of the promoter region of DBH was identified during infection with bradyzoite-induced *T. gondii* (Figure 4.7). Although DBH down-regulation was observed at 3 days of infection, methylation

of the promoter was only identified after 5 days (Figure 3.4). Interestingly, non-coding RNA associated methylation is correlated with a delay in DNA methylation. Gene silencing may down-regulate target gene expression and induce methylation concordantly[417,418]. Typically, miRNA mediated chromatin remodelling occurs after 72-96 hours[419]. The delayed DBH transcriptional silencing reported here may be an indication that methylation in this region is a by-product of RNA mediated DNA methylation. It is also possible that methylation in another region of the promoter may induce down regulation of DBH. As heterochromatin is formed other CpGs are methylated to facilitate DNA condensing, including those within the region examined using MSRE qPCR. MSRE restriction sites could not be identified throughout the promoter limiting further investigation. Although whole-genome bisulphite sequencing revealed methylation patterns observed at the promoter, only 1 time point, 5 days of infection, was investigated. Other techniques such as methylated DNA immunoprecipitation (MeDIP) would facilitate further investigation of the DNA methylation timescale[420].

Unexpectedly, there is a significant difference between control and infected neuronal tissue samples *in vivo* (Figure 4.9). Given that tissue cysts are relatively low in number, few neurons are expected to be directly affected by infection. Global change has been observed in DBH gene expression (Figure 3.5). Additionally publications have also reported global changes in gene expression and protein localisation during infection[213,221,222]. This may be due to length of infection, cysts are able to develop over a longer duration, up to 4 months resulting in larger cysts containing more parasites. There is also evidence that *T. gondii* can inject rhoptry proteins into neurons that are not infected[243]. Using this mechanism, it is possible that these proteins directly affect cellular function in such a way as to induce DBH down-regulation. It is also possible that host derived mechanisms such as the immune response may induce these changes.

Epigenetic changes during infection represent a paradigm shift in the study of the host-parasite interaction[421]. There is growing evidence that parasites are able to alter host immune response, facilitating parasite survival. *T. gondii* infected macrophages exhibit impaired acetylation of core histones preventing chromatin remodelling, this prohibits IFN-γ signalling during infection[388]. Whereas, Marr *et al* identified altered CpG methylation at promoter regions associated

with JAK/STAT signalling, with *Leishmania donovani* infected macrophages[422]. Pennini *et al* identified a *Mycobacterium tuberculosis* lipoprotein able to inhibit IFN-γ induced chromatin remodelling[423]. Although evidence of infection induced epigenetic changes are increasing the mechanisms governing these remain poorly delineated.

DNA methylation patterns can provide new insight into the mechanisms that may predispose infected individuals to a range of mental health, sensorimotor and coordination disorders. The correlation between schizophrenia and *T. gondii* infection has been reported in more than 60 publications[127,128]. *T. gondii* seroprevalence is 2.5 times higher in the schizophrenia population compared to healthy controls. In a recent meta-analysis of CpG methylation changes in peripheral blood and saliva from schizophrenia patients, hypermethylation of DAT1 and COMT was observed[424]. Although this data does not directly investigate changes in neuronal DNA methylation, it suggests that dysregulation of the dopamine signalling pathway can be identified even outside the CNS. Recently published data has identified epigenetic dysregulation of dopaminergic signalling during tachyzoite *T. gondii* infection[425].

Methylation induced by *T. gondii* may play a significant role in the association between mental health disorders and *T. gondii* infection. Several publications have found *T. gondii* seroprevalence is correlated with suicide attempts[129,130,426]. Suicide attempts are a result of complex environmental and genetic factors, often associated with depression. Recently published data has found that suicide completers exhibit hypermethylation of the DBH promoter region within the brain[427]. Indeed, depleted LC neurons and reduced norepinephrine are associated with suicide[428,429]. These findings provide new insight into epigenetic factors that may cause *T. gondii* infected individuals to become predisposed to mental health disorders and suicide.

4.5 Conclusion

The data presented here provides the first evidence that chronic *T. gondii* infection is able to alter the dopaminergic metabolic pathway via epigenetic mechanisms. Epigenetic changes may provide an explanation for observed behavioural

changes of infected rodents even after parasite clearance[100]. For the first time evidence is presented that targeted hypermethylation of a promoter region is induced by chronic *T. gondii* infection. This study provides an insight into the changes occurring at one significant gene, however, *T. gondii* induced methylation could affect many aspects of the host-parasite interaction. These epigenetic changes could hold to the key to understanding what predisposes *T. gondii* infected individuals to certain behavioural, motor and cognitive disorders.

Chapter 5

The role of extracellular vesicles during chronic *T. gondii* infection

5.1 Overview

This, the final results chapter, describes changes in cell-cell communication during chronic *T. gondii* infection. Extracellular vesicles are excreted by almost all cells. They may contain DNA, RNA and protein and function to facilitate intercellular communication. Here, DBH transcriptional regulation is reported in cells that are uninfected but exposed to *T. gondii* infected cells. The extracellular vesicles excreted by neuronal cells infected with *T. gondii* are characterised. Extracellular vesicles were isolated by ultracentrifugation and then characterised using electron microscopy and western blotting. Extracellular vesicles purified from infected cell lines are able to induce down-regulation of the DBH gene, as well as DNA methylation of the DBH promoter sequence. Cell cultures were treated with purified extracellular vesicles and changes in gene expression and DNA methylation were observed. The mechanism of DBH promoter methylation was then investigated. UV-ablation of extracellular vesicles inhibited the observed change in methylation suggesting that RNA or DNA may induce the observed epigenetic changes. This work addresses extracellular vesicle communication between neuronal cells; for the first time, extracellular vesicle induced epigenetic change is described in neuronal cells.

5.2 Introduction

Multicellular organisms require coordinated cellular function; for this to occur, robust intercellular communication is essential. Intercellular communication pathways, such as via direct contact with cell surface receptor binding or soluble factors, are relatively well characterized. Less well described are extracellular vesicles that over the last 20 years have emerged as a third mechanism of cell-cell communication. Three major heterogeneous classes of extracellular vesicle demarcated based on size and biogenesis are: 1) exosomes 50-150nm in size, considered to be formed by endocytosis; 2) microvesicles 100-2000nm in size formed by budding of the plasma membrane; and 3) apoptotic bodies 1000-4000nm in size and released by cells undergoing programmed cell death[430] (Figure 5.1). Of these, the latter play only a limited role in cell-cell communication and are rapidly phagocytosed after release.

Extracellular vesicles were first described by Wolf as platelet dust 50 years ago[431]. Trams *et al* first used microvesicles to describe extracellular vesicles with 5'-nucleotidase activity[432]. Exosomes purified from cell culture were first described in 1987 by Johnstone and colleagues who proposed that they were mainly involved in the removal of cellular waste[433]. It is now known that extracellular vesicles are able to alter the intercellular milieu affecting function of both proximal and distal cells. As well as normal cell-cell communication, extracellular vesicles have been implicated in a plethora of human diseases, notably cancer and neurodegenerative disorders[434–437].

5.2.1 A note on nomenclature

Similarity of morphology, size and common cargo of exosomes and microvesicles has hampered the identification and functional characterisation of these extracellular vesicles. Much of the literature investigating the biological roles of extracellular vesicles primarily focuses on function rather than vesicle biogenesis. This conflates the roles of exosomes and microvesicles. Indeed, as the extracellular vesicle field has expanded in recent years controversy has surrounded inconsistent nomenclature and isolation methods[438]. Throughout this chapter the term

extracellular vesicles will be used because extensive characterisation of the studied extracellular vesicles has not yet been undertaken. Therefore either exosomes, microvesicles - or a combination of both may be producing the observed biological effect.

5.2.2 Biogenesis, release and uptake

Microvesicles

Microvesicle biogenesis occurs via blebbing and pinching of the plasma membrane; this process both forms and releases the microvesicle into the intercellular space (Figure 5.1). The mechanisms governing this process remain incompletely understood, however it has been proposed that this process may be similar to viral budding[440]. It was observed that formation of microvesicles was driven by tumour susceptibility gene 101 (TSG101) interacting with arrestin domain-containing protein 1 found throughout the plasma membrane. Indeed, TSG101 has been widely identified within the cargo of microvesicles. Plasma membrane protein localisation can alter the membrane curvature[441]. It has also been observed that changes in plasma membrane composition, such as recruitment of membrane protein and lipid components able to modulate changes in membrane rigidity and morphology, precede microvesicle formation[442]. Additionally, this process is associated with vertical redistribution of the microvesicle cargo along the plasma membrane[443].

Unlike apoptotic bodies, the microvesicle cargo is not indiscriminately selected. It was observed that the GTP-binding protein ARF6 facilitates selective protein cargo sorting into microvesicles[444]. The endosomal sorting complex required for transport (ESCRT) also plays an essential role in cargo selection and vesicle budding. Proteomic analysis of tumour derived microvesicles has revealed that they contain metabolism, signalling, mRNA processing, angiogenesis, and cell growth associated proteins, as well as proteins required for budding[445,446]. Transcriptome analysis of microvesicles has shown that they contain protein coding RNAs, pseudogene transcripts, long noncoding RNAs (lncRNA) and short noncoding RNAs (miRNA). The proportion of each of these subpopulations is dependent upon the cell type analysed[447].

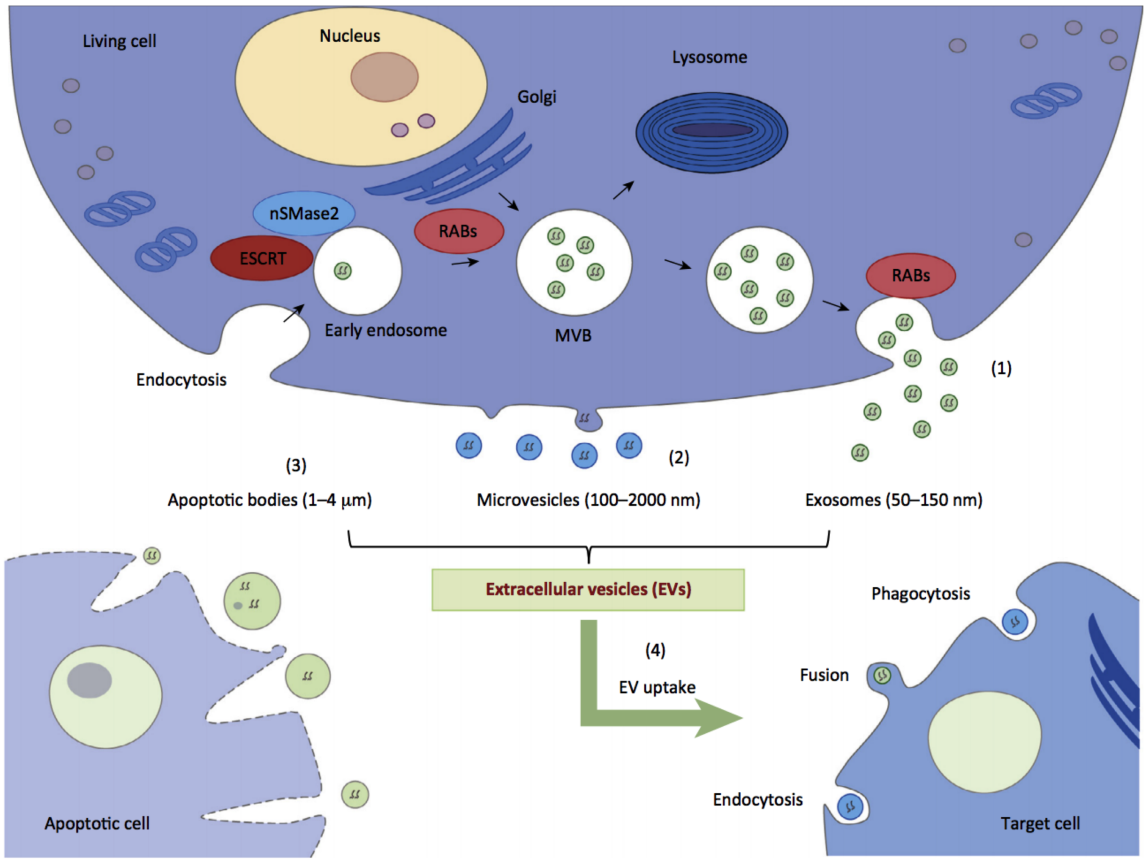


Figure 5.1: Scheme of mammalian extracellular vesicle biogenesis. Biogenesis of 1) Exosomes, 2) Microvesicles and 3) Apoptotic bodies. Exosomes are formed by endocytosis; initially the early endosome matures as cargo is selected, this forms the multi-vesicular body (MVB). After maturation this fuses with the cell membrane releasing exosomes. Microvesicles and apoptotic bodies are formed via plasma membrane blebbing and pinching. During microvesicle biogenesis, cargo is sorted at the plasma membrane. Uptake occurs at the target cell via endocytosis, fusion and phagocytosis. Reproduced from [439]

Exosomes

Exosome formation occurs by the formation of interluminal vesicles during endocytosis (Figure 5.1). Endocytosis is an umbrella term describing various cellular uptake pathways of compounds, particles, fluids or macromolecules which are ingested by invagination of the plasma membrane[448]. The molecular interactions that determine early endosome formation are poorly delineated. The early endosome membrane is likely to be lipid rich, containing sphingolipids, ESCRT complexes and membrane protein aggregates[449]. Late endosome, or multi-vesicular body (MVB), formation is facilitated by Rab7, a regulatory guanosine triphosphatase associated with endosomal maturation[450].

Initially, lipids and membrane associated proteins cluster at the endosomal membrane. This membrane then invaginates, generating interluminal vesicles; which when released will become exosomes[451]. This process can occur via several mechanisms in an ESCRT protein dependent or independent manner[430]. Delineation of the ESCRT pathway was the first breakthrough, characterising one mechanism of exosomal biogenesis. Sequentially, ESCRT-0 to ESCRT-III localise and complex driving membrane shaping and scission[452]. Stuffers *et al* observed that even when all key protein domains for ESCRT machinery were depleted in *Saccharomyces cerevisae*, exosomal production was still possible[453]. An RNAi screening conducted by Colombo *et al* observed TGS101 and STAM1 depletion decreased exosomal shedding but did not inhibit it completely, whereas when ESCRT associated CHMP4C, VPS4B, VTA1 and ALIX proteins were knocked down exosomal production increased. However, silencing of ALIX alone did not alter the volume of exosomes produced but altered exosomal cargo, suggestive of a role in cargo selection[454]. Taken together, this suggests redundancy in the system of exosomal biogenesis.

Exosome cargo is transported to the endosome primarily via the Golgi apparatus[455]. This cargo is similar to that of microvesicles, however, each extracellular vesicle subtype is associated with a specific RNA and protein enrichment signature. The heterogeneity of EV purification methods impacts proteomic and transcriptomic studies making it difficult to characterise common exosomal cargo[456]. In general, the RNA cargo of extracellular vesicles mirrors that of the cells own

cytoplasmic RNA profile. It was observed that cellular stress conditions, and miRNAs associated with this, are reflected in extracellular vesicle cargo[457]. Furthermore, Squadrito and colleagues observed that extracellular vesicles, purified from cells induced to over-express target miRNAs, were also enriched with these miRNAs[458].

Different mechanisms of extracellular vesicle cargo selection have been proposed[459]. Guduric-Fuchs *et al* have observed argonaut-2 (AGO2), a protein associated with RNA mediated gene silencing, may contribute to miRNA cargo selection[460]. AGO2 knock-out cells exhibited dysregulation in exosomal miRNA content. Additionally, AGO proteins are enriched in extracellular vesicles[461]. Sequence motifs have been identified on many miRNAs, enriched in exosomes, which may determine cellular localisation[462,463]. Finally, post-translational modifications, such as 3' end uridylation, have been observed on many miRNAs found to be enriched in exosomes[464].

Experimental evidence suggests that, once secreted, extracellular vesicles can travel via the blood throughout the body to distant organs[465]. Current data suggests that extracellular vesicles are taken up via endocytosis[466]. Extracellular vesicle uptake has been observed via caveolin-dependent endocytosis, clathrin-mediated endocytosis, phagocytosis and macropinocytosis. However, the molecular interactions which define the process of microvesicle uptake remain incompletely characterised. This process can be rapid, with extracellular vesicles identified within cells just 15 minutes after treatment[467,468]. Many publications report that extracellular vesicle uptake is reduced at 4°C[469–473]. Additionally, paraformaldehyde fixed cells are unable to endocytose vesicles[474,475]. Taken together, this may indicate vesicle uptake is an active rather than passive process requiring energy expenditure.

5.2.3 The role of extracellular vesicles in neuronal function

Many cells of the CNS can release extracellular vesicles including astrocytes, glia, oligodendrocytes, Schwann cells and neurons[476–478]. Extracellular vesicle secretion is emerging as an important mechanism of cellular communication within the CNS. Cultured neurons can release exosomes; it was observed this process

can be modulated by glutamatergic synaptic activity[479]. Indeed, it has also been observed that neuronal extracellular vesicle release can be regulated by depolarisation suggesting that extracellular vesicle release is part of normal synaptic neuronal communication[480]. Goldie *et al* observed that depolarisation of the neuron was associated with depletion of specific miRNA extracellular vesicle cargo[481].

There is evidence that neuron derived exosomes are taken up by other neurons and glial cells[482]. It has been observed both in culture and the mouse brain, extracellular vesicles released by oligodendroglial cells can enter neurons altering gene expression[483,484]. Interestingly, it was observed that serotonin treatment increases extracellular vesicle secretion by microglia[485]. This glial-neuron communication via extracellular vesicles is an important feature of normal maintenance of the CNS, and may be coupled to synaptic activity[486]. There is mounting evidence that extracellular vesicles play an important role in synaptic plasticity[487].

Extracellular vesicles also play an important role during CNS injury. Several studies have observed that when treated with ATP, an indicator of neuronal injury, microglia and astrocyte derived extracellular vesicles contain the pro-inflammatory cytokine IL-β1[488,489]. Recently published data has demonstrated that extracellular vesicles, purified from cultured microglia treated with ATP, exhibit enriched miR-146a-5p. Delivery of these extracellular vesicles to the hippocampus of mice resulted in neuronal down-regulation of neuroligin 1 and synaptotagmin 1, as well as concordant reduction in synaptic strength[490]. As well as facilitating inflammation and neurodegeneration, they play an active role in the pathology of some diseases of the CNS, such as prion disease[491–493]. Extracellular vesicles likely also play an important role in Alzheimer's disease, facilitating the spread of β-amyloid plaques, a hallmark of the disease[494].

5.2.4 Extracellular vesicles and the host-parasite interaction

There is growing evidence that extracellular vesicles play an important role in the host-parasite interaction, particularly by modulating the host immune response. Work by Hassani and colleagues found extracellular vesicles purified

from macrophages infected with *Leishmania mexicana* were able to induce phosphorylation of signalling proteins and up-regulate immune-associated genes in treated macrophages[495]. Indeed, it was observed that extracellular vesicles purified from *Leishmania amazonensis* infected macrophages can prime naive macrophages, initiating an anti-parasitic Th1 response and inducing the release of pro-inflammatory cytokines such as IL-1β, TNF and IL-12[496]. CMV infected endothelial cells release extracellular vesicles able to induce recruitment and activation of $CD4^+$ cytokines[497]. Mantel *et al* observed *Plasmodium falciparum*-infected erythrocytes produce extracellular vesicles that can induce the monocyte secretion of pro-inflammatory cytokines such as IL-12, IL-6 and IL-1β[498]. Additionally, *Trypanosoma cruzi* can also induce the release of extracellular vesicles from infected cells. These extracellular vesicles contain transforming growth factor beta (TGF-β). Interestingly, TGF-β treatment can facilitate host-cell invasion by propagating *T. cruzi* parasitic spread[499].

T. gondii can alter the cargo of extracellular vesicles produced by infected fibroblasts. Extracellular vesicles were enriched with proteins associated with neuronal morphogenesis and development such as Rab-13; eukaryotic translation elongation factor 1 alpha 1; and thymosin beta 4[500]. Additionally, infected fibroblasts produced extracellular vesicles containing up-regulated miR-23b, a well characterised regulator of IL-17. These shifts in extracellular vesicle cargo hint at the neurophysiological changes that *T. gondii* may be able to exert.

As discussed in Section 1.5.3, during chronic *T. gondii* infection cysts can be found throughout the brain and during human or rat infection less than 10 neurons may be infected. However, global changes in gene expression can be observed[501]. In previous chapters, global changes in DBH mRNA expression and methylation of the 5' promoter region have been described throughout neuronal tissue of chronically infected animals. Currently, global changes in gene expression are explained by *T. gondii*'s 'kiss and spit' mechanism observed by Koshy *et al*; wherein the parasite is able to inject rhoptry proteins into cells that it does not invade. However, this mechanism is predominately observed during the tachyzoite life cycle, whereas global DBH transcriptional regulation is observed during chronic infection. Additionally, the 'kiss and spit' was detected at a frequency of 20 times the number of cysts found *in vivo*, however, given the small

number of cysts this is still a relatively small number of cells in the brain [243,502]. Therefore, we sought to investigate if *T. gondii* is able to co-opt host cell-cell communication, manipulating the host neurophysiology environment to facilitate parasite survival.

5.3 Results

5.3.1 DBH mRNA expression and 5' promoter methylation is altered by exposure to *T. gondii* infected cells

As described in Chapters 2 and 3, changes in transcriptional regulation and mRNA expression of DBH during chronic *T. gondii* infection cannot be accounted for with the small number of cysts found in the brain. Only a small number of neuronal cells are infected by the parasite during *in vivo* infection, yet silencing of DBH mRNA appears to be global. To characterise the mechanisms governing this global change, cell-cell communication during infection was investigated. DBH mRNA down-regulation and 5' promoter methylation were used as markers of infection-induced cellular change throughout this chapter.

To investigate whether epigenetic changes associated with *T. gondii* can be observed in neuronal cells that are uninfected but have been exposed to infected cells in cultures, fluorescence-activated cell sorting (FACS) was used to enrich for cells exposed to infected cells in a growing culture (Figure 5.2). A wild-type Prugniard-GFP strain (kind gift from Boothroyd) allowed a cell culture to be sorted isolating GFP-positive infected cells and GFP-negative uninfected, but exposed cells (Figure 5.2A). After sorting, DNA was harvested and MSRE qPCR performed. Methylation at 5' promoter region was increased from 12.60% ±3.0 in uninfected cells to 55.19% ±14.0 in 5 day infected human neuronal cells. This is similar to values obtained in earlier experiments using infected cultures (Figure 4.7). The enriched uninfected human neuronal M17 cells that were exposed to infected cells exhibited 79.60% ±19.9 (p=0.0052) methylation in the promoter region. Promoter methylation was not significantly altered between infected and exposed cells (p=0.226). This increased methylation of the DBH promoter ob-

served in exposed bystander cells suggests that transcriptional regulation may be conferred via cell-cell communication.

To determine whether cell-cell contact is necessary for the reported epigenetic change observed in bystander cells, rat catecholaminergic PC12 cells were grown in a transwell system. This two-layer plate allows two cell cultures to be grown in proximity, separated by a 0.4μm membrane through which small vesicles, proteins and nucleic acid can be exchanged (see Figure 2.1). Cells grown on the lower layer are then exposed to either uninfected or *T. gondii* infected cells in the upper well. Rat catecholaminergic PC12 cells were exposed to the same cells, infected or mock infected cells above. After 5 days RNA was harvested from the exposed cells and RT-qPCR performed (Figure 5.3A). DBH was 4.87-fold ±2.3 down-regulated in PC12 cells exposed to infected cells and 6.97-fold ±3.2 down-regulated compared to mock-infected (p=0.45). Human neuronal M17 cells were also grown in a transwell system and exposed to 5 day infected or uninfected M17 cells on the upper layer. Neuronal cells exposed to infected cells exhibited 42.57-fold ±24.6 (p=0.0038) down-regulation of DBH expression (Figure 5.3B). Hence, both human and rat neuronal cells exhibit DBH mRNA down-regulation in response to exposure to cells infected with live parasites suggesting that some factor or vesicle excreted by infected cells regulates DBH expression .

The cell-cell communication of DBH down-regulation was investigated to establish whether hypermethylation of the 5' promoter region is associated, as previously observed in infected cultures and mice (Figure 4.7). The transwell experiment was repeated as above with PC12 cells exposed to uninfected or *T. gondii* infected cells and methylation of the DBH promoter measured by MSRE qPCR (Figure 5.4). After 5 days DNA was harvested from the exposed cells and MSRE qPCR performed. Methylation of the DBH 5' promoter region was 44.44% ±5.5 higher (p=0.0013) in rat catecholaminergic PC12 cells exposed to infected cells compared to those exposed to uninfected control cells (Figure 5.4A). This was also examined with human neuronal M17 cells to assess the generality of this mechanism. Human neuronal cells exposed to infected cells exhibited 48.85% ±18.7 (p=0.0003) increased DBH promoter methylation compared to those only exposed to uninfected control cells (Figure 5.4B). The level of hypermethylation

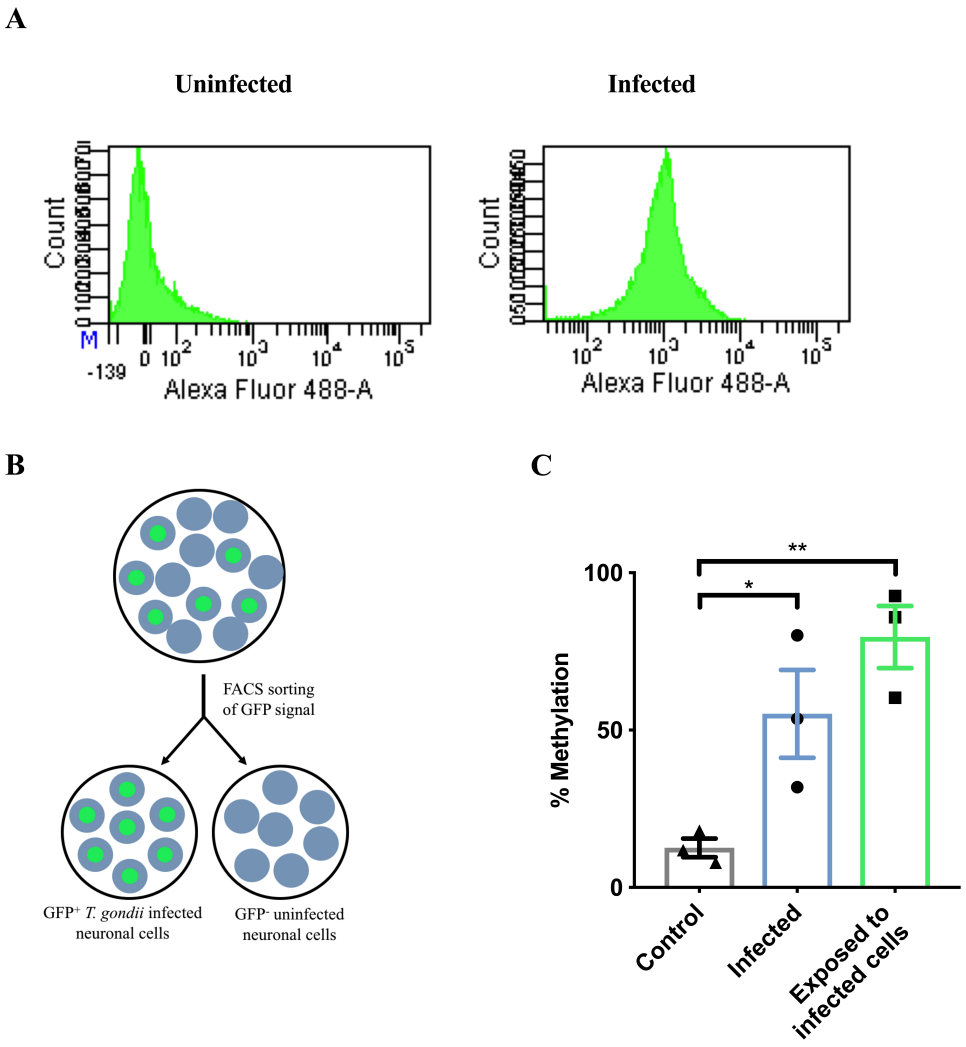


Figure 5.2: Fluorescence-activated cell sorting (FACS) to enrich for infected vs uninfected neighbour cells ("exposed") human neuronal M17 cells grown in an infected culture. A) Representative plot showing cell count against GFP signal for GFP-negative uninfected exposed cells and infected GFP positive cells. B) A schematic representation of experimental design. Human neuronal M17 cells were infected with bradyzoite-induced *T. gondii*, after 5 days of infection FACS was performed and DNA was then harvested. MSRE qPCR was utilised to measure DBH promoter methylation. C) Plot showing percentage methylation at the 5' prime DBH promoter region measured using MSRE qPCR; ±SEM shown, n=3, Student t test, * $p<0.05$, ** $p<0.01$.

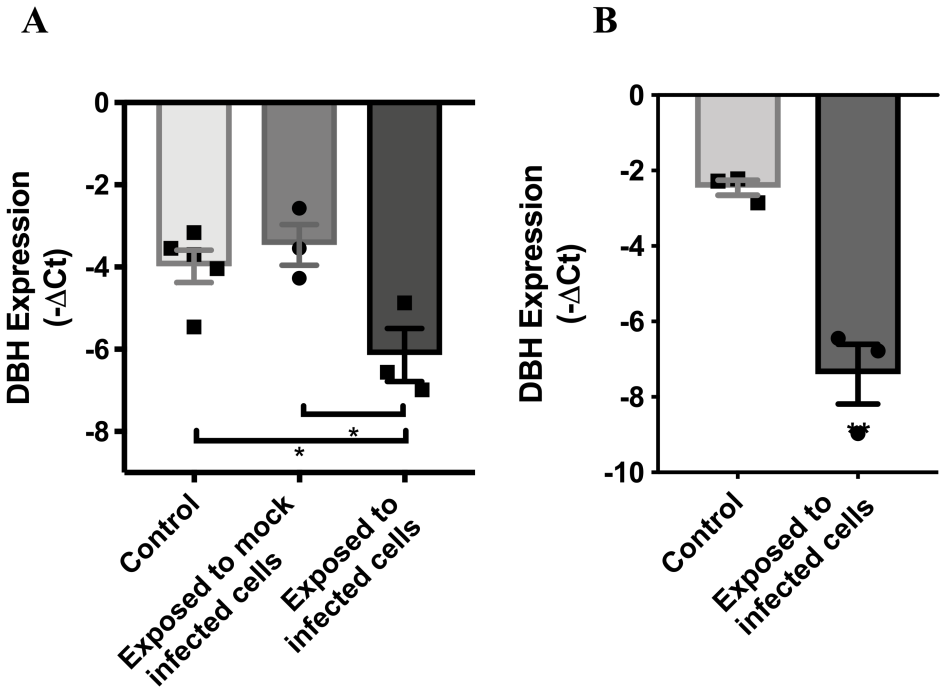


Figure 5.3: Neuronal cells exposed to infected cells exhibit DBH down-regulation in a transwell cell culture system. A) Plot showing rat catecholaminergic PC12 cell DBH expression measured using RT-qPCR and expressed with respect to the housekeeping gene GAPDH. PC12 cells were cultured in a transwell with either uninfected control, *T. gondii* infected or mock-infected cells on the upper transwell layer; RNA was harvested after 5 days. Mock infected were a mixture of PC12 cells and heat-killed *T. gondii* tachyzoites. B) Plot showing human neuronal M17 cells expression of DBH mRNA measured using RT-qPCR, expressed with respect to the housekeeping gene GAPDH. M17 cells were cultured in a transwell with either uninfected control or *T. gondii* infected cells on the upper transwell layer; RNA was then harvested after 5 days; ±SEM shown; n=5; Student t test, * $p<0.05$, ** $p<0.01$.

mirrors that observed in *T. gondii* infected cultures. The corresponding hypermethylation with mRNA levels may suggest that DBH expression is transcriptionally regulated.

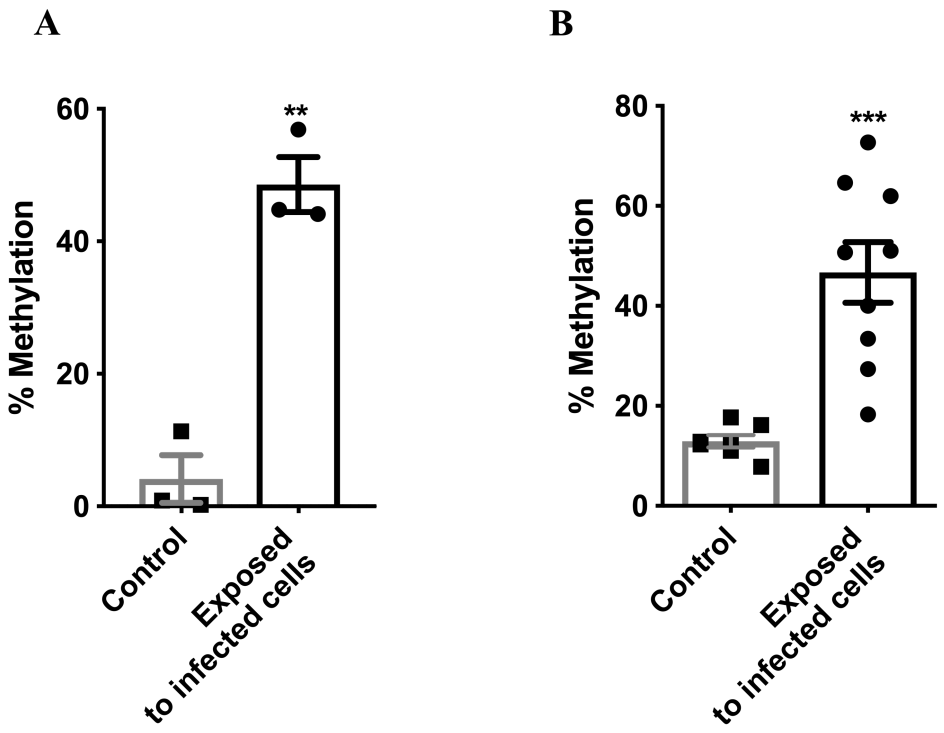


Figure 5.4: Cells exposed to *T. gondii* infected cells exhibit increased methylation of the 5' DBH promoter region measured by MSRE qPCR. A) Percentage 5' DBH promoter region methylation plotted of PC12 cells cultured with uninfected or 5 day bradyzoite-induced *T. gondii* infected cells on the upper transwell layer. ± SEM shown, n=3, Student t test, ** $p<0.01$. B) M17 cell DBH promoter methylation measured via MSRE qPCR. M17 cells were cultured with either uninfected (n=4) or 5 day *T. gondii* infected M17 cells (n=9). ± SEM shown, Student t test, *** $p<0.001$.

The observed changes in DBH mRNA expression and 5' promoter methylation could be induced by a number of factors excreted by cells including extracellular vesicles. In order to elucidate this mechanism a transwell culture was treated

with a neutral sphingomyelinase (N-SMase) inhibitor GW4869, preventing endocytosis, a key stage in exosomal biogenesis. PC12 cells were either exposed to *T. gondii* infected or uninfected cells on the upper transwell layer of a transwell system and supplemented with 0-100μM of GW4869. Methylation of the 5' DBH promoter region in purified DNA of the exposed cells by MSRE qPCR showed that cells exposed to *T. gondii* infected or uninfected cells on the upper transwell layer. After 5 days of infection DNA was harvested and methylation of the 5' promoter region examined using MSRE qPCR (Figure 5.5). PC12 cells exposed to *T. gondii* infected cells exhibited 63.45% ±11.6 compared to 6.67% ±3.4 methylation in cells exposed to uninfected cells without inhibitor (p=0.0093). In striking contrast, cells exposed to infected cells treated with 10μM and 100μM N-SMase inhibitor did not exhibit any increase in 5' prime DBH promoter methylation (p=0.81 and p=0.46 respectively) compared to controls. Control cells, did not exhibit significantly altered DBH methylation at any concentration of N-SMase inhibitor (one-way ANOVA, p=0.23). Inhibition of N-SMase prevents the formation of a multivesicular body, an essential stage of exosomal biogenesis, as discussed in Section 5.2.2. Taken together this data suggests extracellular vesicles, particularly exosomes secreted from infected cells, may be able to induce epigenetic changes in cells exposed to *T. gondii* infected catecholaminergic and neuronal cells.

5.3.2 Extracellular vesicle characterisation

In order to characterise and compare extracellular vesicles secreted by infected and uninfected cells isolation via ultracentrifugation and purification on a sucrose gradient was undertaken (Figure 2.2). Electron microscopy remains the gold standard of extracellular vesicle identification[254]. Extracellular vesicles were purified from 5 day infected rat catecholaminergic cultures. Transmission electron microscopy was performed and using ImageJ analysis software infected and uninfected extracellular vesicle size quantified and morphology were compared. All extracellular vesicles appeared to be a regular spherical shape, with no obvious morphological differences between infected and uninfected vesicles observed (Figure 5.6A). Furthermore, extracellular vesicles isolated from infected

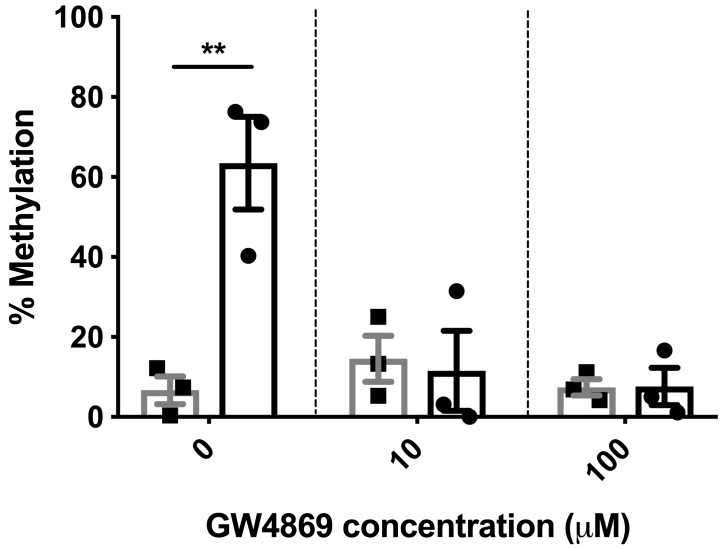


Figure 5.5: Treatment with an inhibitor of endocytosis and exosome biogenesis disrupted hypermethylation by diffusible factors from infected cells. PC12 cells were exposed in a transwell system to uninfected (grey) or *T. gondii* infected (black) PC12 cells on the upper layer. Media was supplemented with 0μM, 10μM or 100μM of GW4869 a neutral sphingomyelinase (N-SMase) inhibitor. $\pm$ SEM shown, n=3, Student t test, ** $p<0.01$.

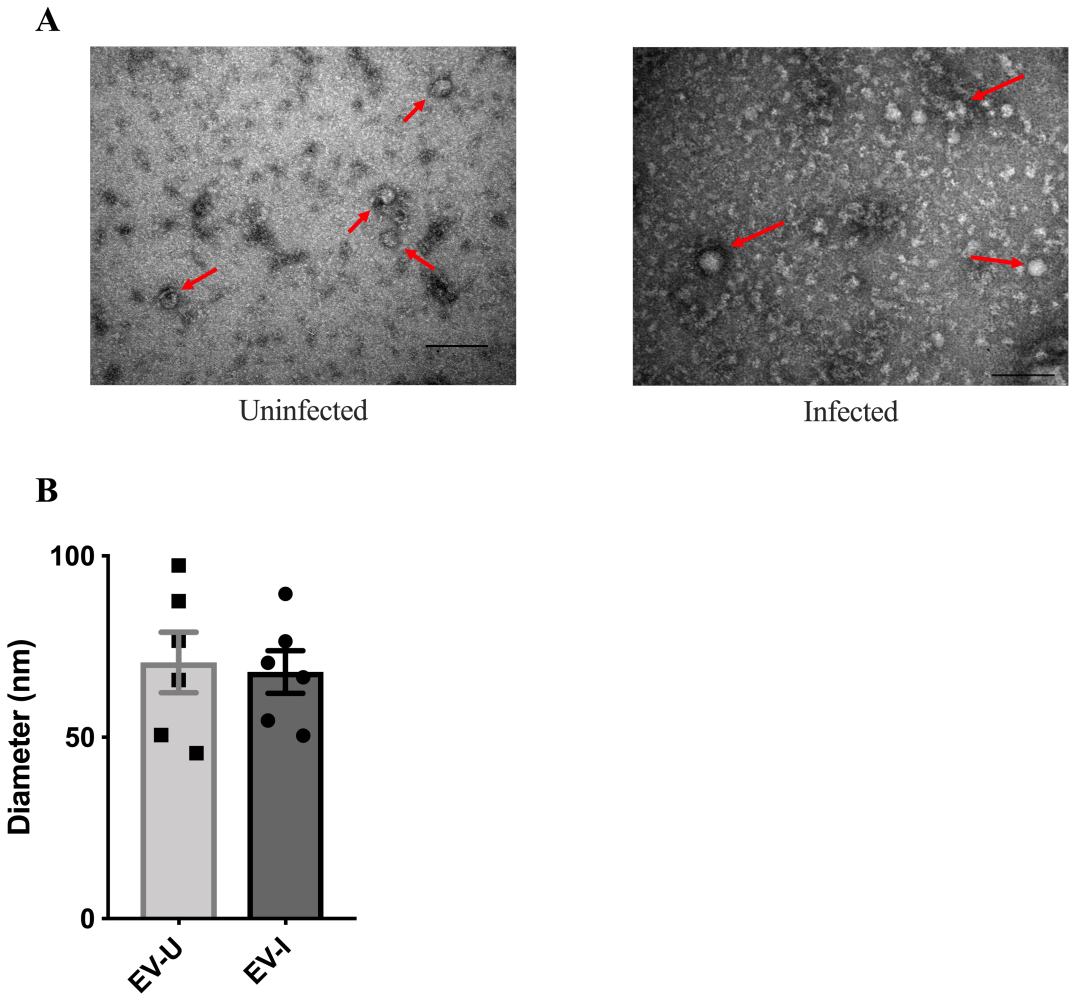


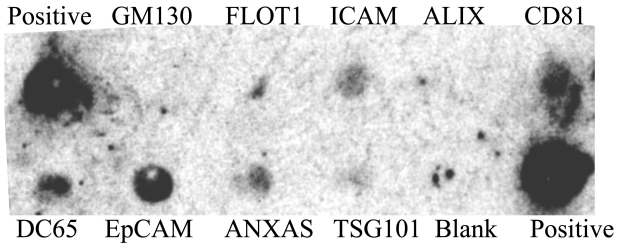
Figure 5.6: Characterisation of extracellular vesicles secreted from rat catecholaminergic cells. A) Transmission electron microscopy of extracellular vesicles isolated from uninfected (left) and infected (right) rat catecholaminergic cells. Extracellular vesicles are indicated by arrows, scale bar represents 100nm. B) Plot showing extracellular vesicle diameter of uninfected and 5 day *T. gondii* infected PC12 cells; as measured by ImageJ. ±SEM, n=10, p=0.81.

and uninfected rat catecholaminergic PC12 cells did not differ significantly in size (p=0.806) with mean diameters of 70.59nm ±8.3 and 68.02nm ±5.9 and respectively (Figure 5.6B).

Next, extracellular vesicle protein content was assessed. An array of eight antibodies targeting well characterised exosomal markers, CD63, CD81, ALIX, FLOT1, ICAM1, EpCam, ANXA5 and TSG101 from Cambridge bioscience was probed (Figure 5.7). Additionally, a GM130 antibody was used to indicate cellular contamination. The array was performed using 300μg of extracellular vesicles isolated by ultracentrifugation and sucrose purification from 5 day *T. gondii* infected rat catecholaminergic cells. Extracellular vesicles were lysed and bound to a horseradish peroxidase (HRP) reporter; the mixture was then incubated with the dot blot and light signal measured using X-ray film (Figure 5.7A). Detection was semi-quantified using imageJ software to analyse average pixel saturation at each dot point (Figure 5.7B and C). The presence of FLOT-1, ICAM, CD81, CD63, EpCAM, ANXA5 and TSG101 proteins were detected. Possibly FLOT-1 is present but this is not clear on the dot blot. Importantly, GM130, a cis-golgi marker and indicator of cellular contamination, was not detected. This taken together with electron microscopy images, suggests the ultracentrifugation and purification method used produce purified extracellular vesicles. *T. gondii* infection does not appear to significantly alter the morphology or size of exosomes secreted by cells.

The importance of cell type in DBH mRNA down-regulation by a diffusible factor from infected cells was investigated using the transwell system. Human fibroblast cells, *T. gondii* infected or uninfected, were grown on the upper layer and human neuronal M17 cells grown on the lower layer. The M17 cells express DBH whereas DBH is not expressed in fibroblasts. DBH mRNA expression in the M17 cells was not significantly altered by exposure to infected human fibroblasts (p=0.84) (Figure 5.8). Hence infected fibroblasts do not release factor(s) that down-regulate DBH suggesting that the observed change in DBH expression is cell dependent and may be restricted to neural cells. In this system, parasite-derived factors should remain constant suggesting that the observed down-regulation of DBH mRNA observed is induced by endogenous cellular effectors induced by infection.

A



B

Protein	Signal (Relative Pixel saturation)	Protein Function
Positive	24957.2	NA
GM130	0	Golgi
FLOT-1	2319.3	Vesicle trafficking and cell morphology
ICAM	3443.7	Integrin binding
ALIX	0	Anatomical structure morphogenesis
CD81	6347.3	Integrin binding
CD63	6183.8	Cell surface receptor for TIMP1 and ITGB1
EpCAM	7862.1	Cadherin binding involved in cell-cell adhesion
ANXA5	1402.1	Calcium-dependent phospholipid binding
TSG101	2194.0	Regulator of vesicular trafficking process
Blank	0	NA
Positive	22612.4	NA

C

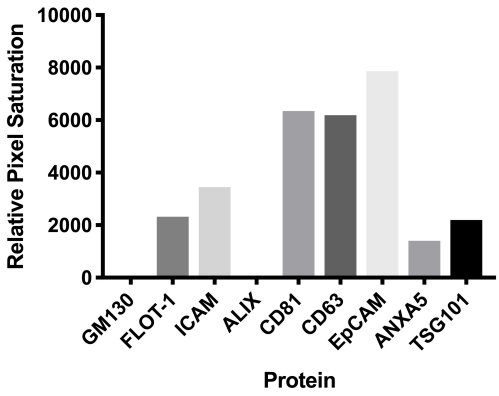


Figure 5.7: Extracellular vesicles isolated from infected cells exhibit classical exosomal markers. Protein from *T. gondii* infected rat PC12 cells was labelled and used to probe a commercial blot (Cambridge bioscience, UK) containing common exosomal antibody markers. A) Image dot blot on X-ray film with markers from right to left: HRP-conjugated antibody (positive control), D63, CD81, ALIX, FLOT1, ICAM1, EpCam, ANXA5 TSG101, blank, HRP-conjugated antibody (positive control). B) Table summarising results of quantitation of blot. C) A plot representing average relative light signal, as stated in B) for each target protein.

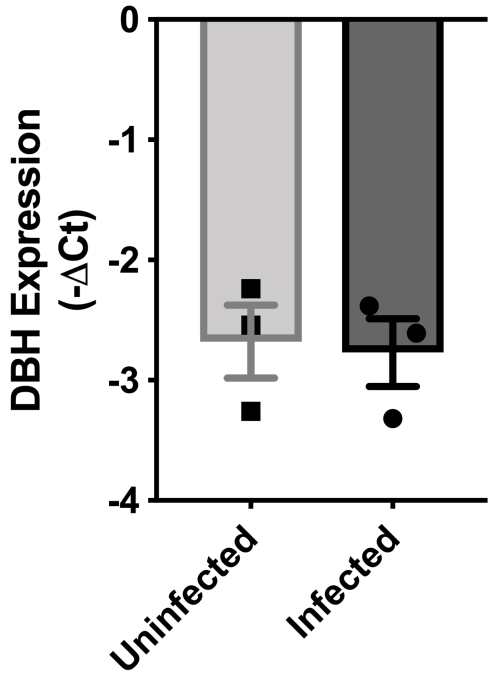


Figure 5.8: The DBH down-regulation observed with exposure to infected cells is neuronal cell dependent. Human neuronal M17 cells were exposed to *T. gondii* infected or uninfected human fibroblasts. After 5 days RNA was harvested and RT-qPCR performed. Plot showing expression of DBH with respect to the housekeeping gene GAPDH. DBH gene expression was not significantly altered by exposure to infected fibroblasts (p=0.84); ±SEM shown, n=3, Student t test.

5.3.3 Extracellular vesicles purified from *T. gondii* infected cells induce DBH transcriptional regulation.

To investigate whether purified extracellular vesicles induce DBH transcriptional regulation, PC12 cells were treated with extracellular vesicles purified from 5 day *T. gondii* infected cells. Treated cells exhibited 191.6-fold ±67.0 (p=0.0062) DBH down-regulation compared to cells treated with extracellular vesicles purified from uninfected PC12 cells (Figure 5.9A). To characterise this mRNA down regulation, methylation of the 5' promoter region of DBH was analysed. Rat catecholaminergic PC12 cells were treated over three days with fresh exosomes. DNA was harvested at 24, 48 and 96 hours. Methylation of the 5' promoter region was measured via MSRE qPCR (Figure 5.9B). No difference was observed after 24 or 48 hours (p=0.44 and p=0.43 respectively). After 96 hours of treatment a difference of 64.22% ±9.1 methylation (p=0.0021) was observed. Taken together the observed changes in DBH mRNA expression and promoter methylation suggest that purified extracellular vesicles secreted by infected rat catecholaminergic cells are able to induce these changes. As observed during rat catecholaminergic cellular *T. gondii* infection methylation of the promoter occurs over a longer timescale than that of DBH mRNA down regulation. This may indicate the same mechanism of transcriptional regulation as observed during infection.

Global changes in gene expression can be observed during *in vivo* chronic *T. gondii* infection. Indeed, the loss of fear phenotype can be observed in rodents with only a small number of neuronal cells infected seemingly anywhere in the brain. The observation that extracellular vesicles secreted by infected cells can alter transcriptional regulation represents a possible new mechanism of neurophysiological change induced by *T. gondii* infection. To investigate this mechanism *in vivo*, extracellular vesicles were delivered directly to the locus coeruleus of rats. Vesicles purified from uninfected and 5 day infected PC12 cells were used (Figure 5.10A). DBH mRNA expression relative to Microtubule-associated protein 2 (MAP2) expression (Figure 5.10B). DBH mRNA expression was 61.84-fold ±43.2 down-regulated in the rear brain containing the locus coeruleus of rats treated with extracellular vesicles purified from *T. gondii* infected rat catecholaminergic cells compared to those treated with extracellular vesicles purified from uninfected

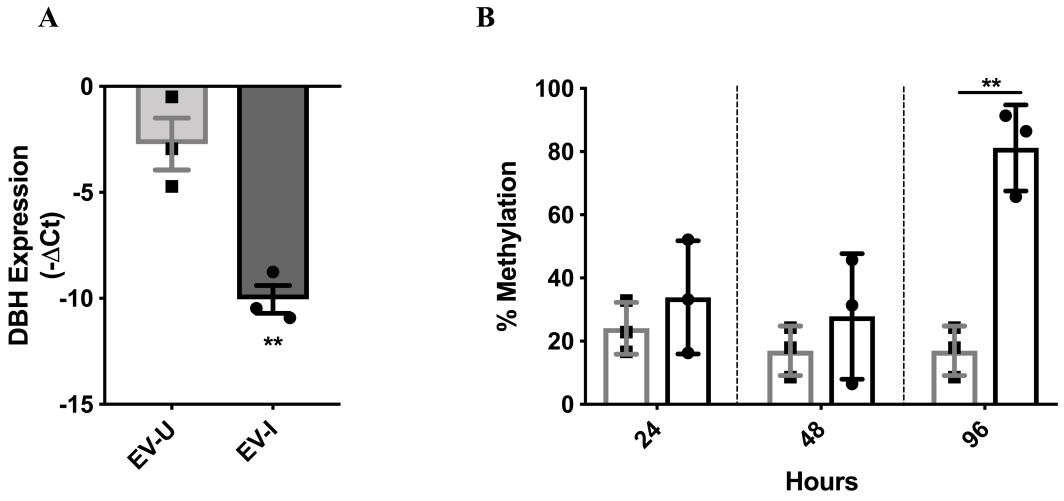


Figure 5.9: Treatment with extracellular vesicles purified from *T. gondii* infected cells can induce transcriptional regulation of DBH. A) Plot of DBH mRNA expression shown with respect to GAPDH. PC12 cells were treated with extracellular vesicles from uninfected (grey) or 5 day *T. gondii* infected (black) PC12 cells and RNA isolated after 24 hours for RT-qPCR analysis. ±SEM shown, n=3, Student t test ** $p<0.01$. B) Percentage methylation of the 5' DBH promoter region shown. Rat catecholaminergic PC12 cells were treated every 24 hours with extracellular vesicles purified from uninfected (grey) or 5 day *T. gondii* infected (black) PC12 cells. DNA was harvested after 24, 48 and 96 hours of treatment. ± SEM shown, n=3, **$p<0.01$.

cells (p=0.016). DBH mRNA expression was not altered by extracellular vesicle purified from infected PC12 cells in the mid-brain (p=0.26) or the prefrontal cortex regions (p=0.13). The locus coeruleus is the centre of norepinephrine signalling within the CNS with efferents for noradrenergic signalling throughout the brain and hence DBH transcriptional regulation will have widespread effects.

5.3.4 DBH transcriptional regulation is dependent upon the RNA cargo of extracellular vesicles

To begin elucidating the mechanism of transcriptional regulation induced by infection-derived extracellular vesicles UV ablation was used. Short UV light exposure can damage nucleic acids whilst leaving protein intact[503,504]. Extracellular vesicles purified from infected PC12 cells were exposed to short wave 302nm to 254nm UV prior to addition to PC12 cell cultures. PC12 were treated with UV-treated or mock-treated extracellular vesicles for 72 hours and DNA was then harvested. Methylation of the 5' promoter region of DBH was increased from 23.93% ±7.6 in cells treated with EV-U to 80.79% ±6.6 in cells treated with EV-I (p=0002). UV treated vesicles had no effect (Figure 5.11A). Indeed, 5' DBH promoter methylation was not significantly different between cells treated with either extracellular vesicles from uninfected PC12, UV ablated extracellular vesicles of uninfected cells or untreated control PC12 cells (one-way ANOVA, p=0.76). The observed inhibition of infection-associated DBH transcriptional regulation suggests that this process may be dependent upon an extracellular vesicle-derived RNA. In sum, these results highlight the role of extracellular vesicles during *T. gondii* infection. Disruption of the process of extracellular vesicle biogenesis or RNA cargo inhibits the observed DBH transcriptional regulation.

5.4 Discussion

Current evidence suggests that bradyzoite cysts are distributed randomly throughout the brain (Table 1.3)[107]. Despite this, several publications have observed global changes in gene expression throughout the brains of chronically infected rodents[104,221,223]. Furthermore, in Chapters 2 and 3 transcriptional regulation

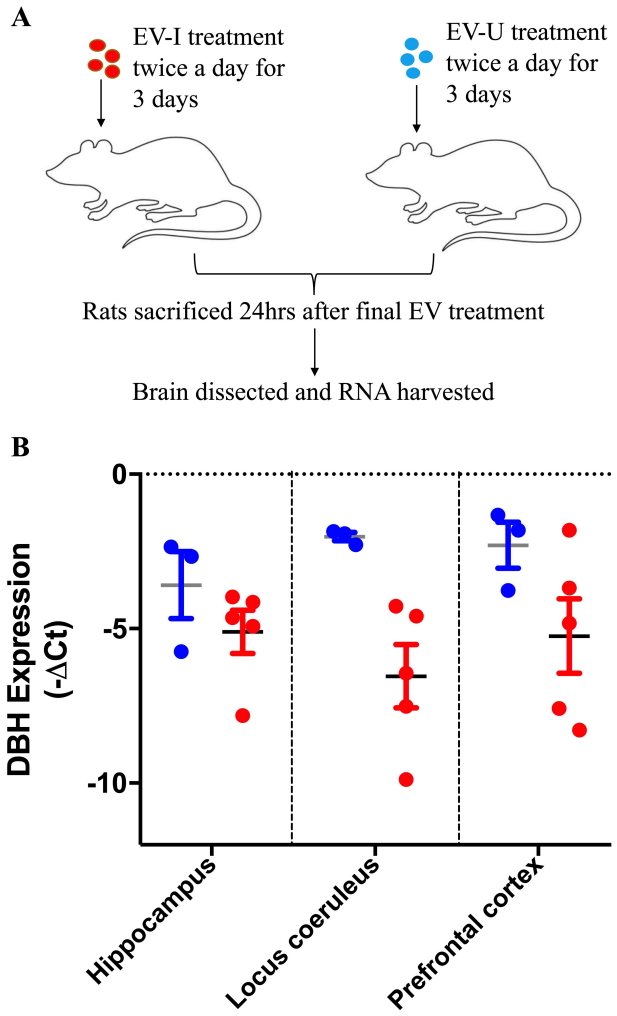


Figure 5.10: Extracellular vesicles purified from infected rat catecholaminergic cells induced DBH mRNA down-regulation in rats. A) A schematic representation of experimental design. Extracellular vesicles purified from uninfected or 5 day *T. gondii* infected rat catecholaminergic PC12 cells were delivered via injection to the locus coeruleus of Sprague-Dawley rats twice a day for 3 days. 24 hours after the final injection animals were sacrificed. The CNS was harvested and the brain dissected to isolate the prefrontal cortex, mid-brain, and rear brain regions. RNA was then harvested and RT-qPCR performed. B) Plot of DBH mRNA expression with respect to Microtubule-associated protein 2 (MAP2) expression. Rats were injected with extracellular vesicles purified from uninfected (grey, n=3, EV-U) or *T. gondii* infected (black, n=5, EV-I) PC12 cells. DBH expression in the region containing the locus coeruleus only was altered by treatment (p=0.016); expression in the mid-brain (p=0.26) and the prefrontal cortex (p=0.13) were not significant. ±SEM shown; horizontal line indicates the mean; each dot represents 1 animal, n=8.

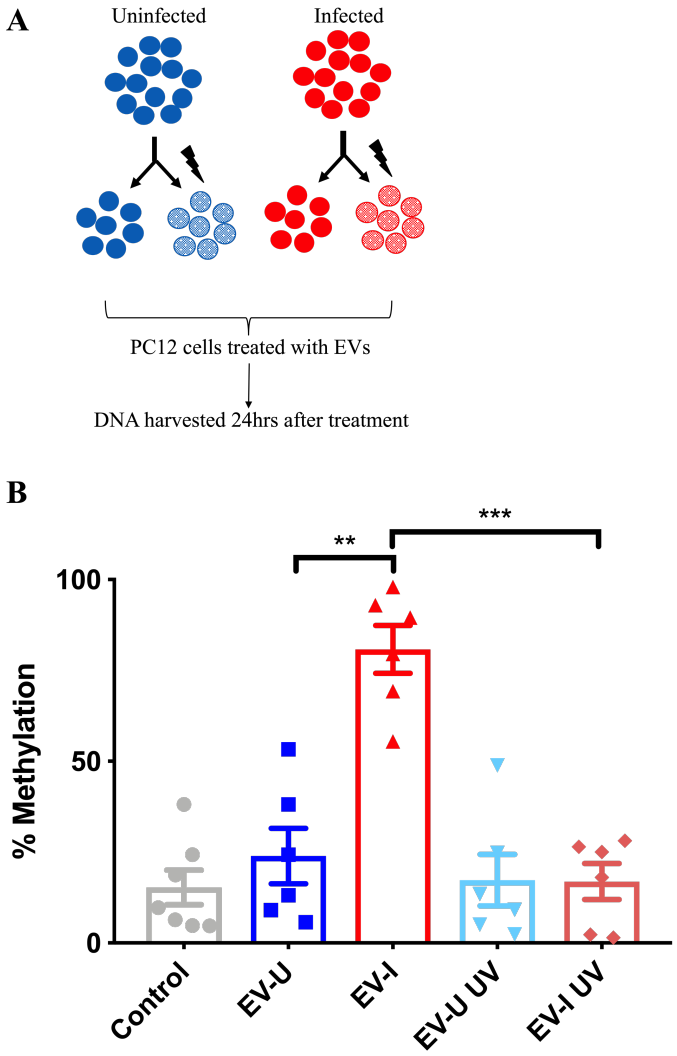


Figure 5.11: UV ablation reverses the effect of infection-derived extracellular vesicles on 5' promoter methylation of DBH. A) A schematic representation of experimental design. Extracellular vesicles were purified from uninfected or *T. gondii* infected rat catecholaminergic PC12 cells. Extracellular vesicles were then mock or UV ablated for 10 minutes at 4°C. PC12 cells were then treated with extracellular vesicles for 3 days and DNA harvested 24hrs after final treatment. B) Percentage methylation of the 5' promoter region of DBH measured by MSRE qPCR. PC12 cells were untreated (grey) or treated with extracellular vesicles purified from uninfected mock-UV ablated (dark blue) or UV-ablated (light blue), infected mock-UV ablated (dark red) or UV-ablated (light red) PC12 cells. Boxes represent mean and interquartile range, n=3, *$p<0.05$, **$p<0.01$.

of the DBH gene is observed throughout the neuronal tissue of chronically infected mice and rats. Indeed, the loss of innate fear phenotype associated with chronic *T. gondii* infection can be observed independent of cyst density or distribution[96,100]. Although several papers suggest that the immune response may be involved, extracellular vesicles provide a compelling mechanism to facilitate these global changes during chronic infection.

There is growing evidence that *T. gondii* is able to alter the extracellular vesicles secreted by the host cell. A recent publication observed pro-inflammatory extracellular vesicles from *T. gondii* infected cells able to recruit and activate immune cells[505]. There is also evidence that parasite-exposed dendritic cells produce extracellular vesicles able to induce a *T. gondii* specific Th1 response[506]. However, targeted down-regulation of a gene has not previously been reported. Here, down-regulation of DBH mRNA in rat catecholaminergic and human neuronal cells exposed to infected cells is described (Figure 5.3). Furthermore, this down-regulation of DBH is associated with increased methylation within the 5' promoter region of the DBH gene, this can be observed in rat catecholaminergic and human neuronal cells exposed to *T. gondii* infected cells (Figure 5.4). Increased methylation of the 5' promoter region can be observed in uninfected human neuronal cells co-cultured with *T. gondii* infected cells (Figure 5.2).

Neurons can use a variety of mechanisms to induce gene regulation in neighbouring cells through synaptic junctions, secreted effector molecules or extracellular vesicles. Extracellular vesicles purified from infected rat catecholaminergic cells were able induce down-regulation of DBH mRNA 24 hrs after treatment (Figure 5.9). Additionally, treatment every 24 hours, for 48 hours induced methylation in the 5' upstream promoter region of DBH. This suggests that purified extracellular vesicles alone are able to induce transcriptional regulation of the DBH gene. This was also observed *in vivo*. Rats treated with purified extracellular vesicles from infected rat catecholaminergic cells twice a day for 3 days exhibited DBH mRNA down-regulation in the locus coeruleus (Figure 5.10). Injecting extracellular vesicles into the brain has been associated with an increased immune response[507]. The associated immune response causes up-regulation of GAPDH in the brain, this may nullify changes in neuronal DBH expression if GAPDH is used as a housekeeping gene. Therefore, DBH down-regulation was measured

by RT-qPCR relative to MAP2, a neuronal cellular marker that does not change during chronic *T. gondii* infection (Figure 3.5 and Figure 3.6). Injection with extracellular vesicles purified from rat catecholaminergic PC12 cells represents an easily reproducible model to study extracellular vesicles, however, PC12 cells have some genetic abnormalities which may not accurately represent the *in vivo* environment[508]. Extracellular vesicles isolated from *T. gondii* infected primary rat neuronal cultures may advance future study.

It was observed that extracellular vesicles were between 45.6 and 97.4 nm in diameter (Figure 5.6). Exosomes typically have a diameter of 30-150nm, whereas, microvesicles can be between 100-1000nm in diameter[509,510]. However, size is not an absolute measure of extracellular vesicle type as it can be altered depending on cell type and extracellular vesicle cargo, as well as analysis method used[511]. It was observed that increased methylation within the 5' promoter sequence of DBH was absent if cells were treated with an N-SMase inhibitor (Figure 5.5). This observation suggests that methylation of the promoter sequence of DBH is dependent upon N-SMase, which, plays an essential role in exosomal biogenesis[430]. This provides evidence that methylation at the 5' promoter region of DBH and corresponding mRNA down-regulation may be mediated by exosomes. Western blotting revealed that extracellular vesicles contained protein markers typical of exosomal cargo; extracellular vesicles were enriched with CD81, CD63 and EpCAM (Figure 5.7). Predominantly, these proteins facilitate extracellular vesicle cargo selection, they act as scaffold proteins facilitating cellular sorting and thus are essential for cargo selection[512]. However, future investigation is required to fully characterise the extracellular vesicle subtypes involved. In addition to protein cargo extracellular vesicles carry DNA, miRNA and lncRNA.We observed that hypermethylation of 5' promoter region was reversed when extracellular vesicles were treated with UV. This damages the RNA, leaving the protein intact (Figure 5.11). This is suggestive of RNA mediated transcriptional silencing.

Targeted down-regulation, mediated by extracellular vesicles, has been described in the tumour micro-environment. It has been observed that tumour derived extracellular vesicles are able to induce targeted down-regulation of PTEN

in surrounding cells, this promotes metastases[513]. Zhang *et al* identified miRNA-146a was able to induce down-regulation, however the mechanism regulating the process was not investigated. Recently published data by Kimura and colleagues identified that the induction of regulatory $CD4^+$ cells is inhibited via extracellular vesicles during multiple sclerosis[514]. It was observed that let-7i, a miRNA precursor, is enriched in extracellular vesicles purified from multiple sclerosis patients. This is able to induce down-regulation of IGF1R and TGFBR1, proteins found on the surface of naive $CD4^+$ cells and essential for T cell recruitment. Currently, there are few examples of circulating miRNAs inducing methylation, however, there is growing bioinformatic evidence that, as in plants and worms, mammalian miRNAs in extracellular vesicles can induce epigenetic inheritance[515–517]. Studies of the cancer microenvironment have revealed that tumour derived extracellular vesicles increase methyltransferase expression and induce global hypermethylation in target cells[518].

The intracellular pathways by which *T. gondii* is able to alter host extracellular vesicle cargo are poorly delineated. Recently published data has demonstrated that during host cell invasion rhoptry neck proteins (RON2, RON4 and RON5) actively recruit host ESCRT components, ALIX and TSG101[519]. RON proteins mimic the conserved binding interfaces of these extracellular vesicle biogenesis associated proteins, with high specificity. This process facilitates host cell entry during acute infection, however, it may also facilitate altered extracellular vesicle communication during chronic infection. Interestingly, extracellular vesicles derived from dendritic cells exposed to *T. gondii* infection carried parasite antigens and were able to induce a Th1 response[506]. This suggests that the parasite can interact with cargo selection. Cannella and colleagues observed that during chronic neuronal infection expression of the miR-146a was down-regulated by the parasite protein ROP16[520]. It has been observed that miR-146a plays an important role in the extracellular vesicle mediated neuroimmune response, and low expression is associated with a higher instance of host, and therefore, parasite survival[521].

This work provides the first evidence of the down-regulation of a gene by extracellular vesicles derived from neurons. Furthermore, it is the first example of extracellular vesicle communication affecting catecholaminergic signalling.

Extracellular vesicles secreted from *T. gondii* infected human fibroblasts did not induce DBH mRNA down-regulation in human neuronal cells (Figure 5.8). Given that parasite derived factors are likely to be the same regardless of cell type infected, this suggests that DBH regulation is mediated by neuron specific factors. Extracellular vesicles can be released by neurons upon synaptic excitation, this has been observed in rat catecholaminergic PC12 cells and *in vivo*[479,522]. Extracellular vesicles secreted after glutamatergic activation are selectively taken up by neurons only, not other cells of the CNS such as glia[482]. Morel *et al* observed that extracellular vesicles excreted from neurons were enriched with miR-124a and able to induce down-regulation of GLT1 expression in target astrocytes[523]. Down-regulation of astrocyte GLT1 expression is associated with reduced neuronal synaptic activity[524]. Furthermore, it has been observed that depolarised neurons release extracellular vesicles enriched with Map1b a regulator of synaptic pruning[481]. Interestingly, neuron derived extracellular vesicles containing miR-193a facilitated neurogenesis in neighbouring neurons by blocking proliferation-associated gene *in vivo*[525]. Taken together this suggests that extracellular vesicle secretion is an important facet of neuronal signalling regulation, however, the mechanisms underlying these observations remain unclear.

5.5 Conclusion

Chronic *T. gondii* infection is associated with many nuanced neurophysiology changes within the CNS. Generally, these changes are independent of bradyzoite cyst density or distribution. Here, using DBH transcriptional regulation as a marker of *T. gondii* influence, altered cell-cell communication via extracellular vesicles is described. Extracellular vesicles purified from *T. gondii* infected rat catecholaminergic and human neuronal cells are able to induce transcriptional regulation both *in vitro* and *in vivo*. DBH regulation may be mediated by the RNA cargo of the extracellular vesicles. This is the first example of transcriptional regulation by extracellular vesicles in neuronal cells. Extracellular vesicles are emerging as important regulators of neuronal signalling, plasticity, and neurogenesis. *T. gondii* has evolved to co-opt many mechanisms of mammalian neu-

ronal function, this manipulation of cell-cell signalling provides a unique model to further investigate the role of extracellular vesicles within the CNS.

Chapter 6

Conclusions and Future work

6.1 Elucidating the mechanism of catecholaminergic change observed during chronic *T. gondii* infection in the CNS

Since 1985, when Stibbs and colleagues published their seminal experiments demonstrating that neurotransmitter concentrations are altered during chronic *T. gondii* infection, many groups have found altered catecholamine concentrations[10,159,212,213,223]. Characteristically for the CNS, changes in dopamine and norepinephrine concentration during chronic infection can be subtle. Although there is a lack of consensus in the literature regarding the mechanisms governing dopamine change, the validity of altered catecholaminergic signalling is evident. This thesis sought to investigate altered catecholaminergic signalling during chronic infection.

Here, an increase in dopamine and concordant decrease in norepinephrine concentration during chronic infection is reported. Interestingly, a common finding throughout many publications is a reduction in norepinephrine content during chronic *T. gondii* infection. Transcriptomic analysis undertaken by I. Alsaady identified down-regulation of the DBH gene[245]. This gene encodes the DBH enzyme that is able to metabolise dopamine to norepinephrine. Rat catecholaminergic cells were infected with *T. gondii* induced-bradyzoites and after 5 days of infection down-regulation of DBH was identified. This was also observed in

human neuronal cells, further verifying that DBH down-regulation can occur in other neuronal cells types. Down-regulation of DBH was also observed in the homogenised brain tissue of chronically infected rats and mice. In Chapter 2 the mechanism governing DBH down-regulation was sought. Nascent RNA transcription was analysed to investigate the mechanism of DBH change. It was observed that nascent DBH pre-mRNA was down-regulated, suggesting that DBH is subject to transcriptional regulation. Furthermore, it was observed that methylation in the 5' promoter region of DBH is increased during *T. gondii* infection of rat catecholaminergic and human neuronal cells. Indeed, methylation of the 5' promoter region was also observed in neurons isolated from chronically infected male mice. These findings provide the first evidence of a possible mechanism to explain altered catecholamine concentration during infection.

Methylation of the DBH promoter and the corresponding mRNA down-regulation will likely reduce DBH concentration in the brain. This will limit the amount of norepinephrine metabolised from dopamine, thus, reducing norepinephrine while concordantly increasing dopamine content. Future experiments should seek to verify this using western blotting or immunohistochemical staining to visualise DBH protein content *in situ* during chronic *T. gondii* infection. It was observed that DBH down-regulation was most significant in the locus coeruleus and prefrontal cortex of chronically infected male mice, therefore, is it possible that down-regulation occurs selectively in norepinephrine producing neurons. There are approximately 400,000 dopaminergic neurons throughout the brain, whereas, there are only approximately 35,000 noradrenergic neurons[526]. Therefore, changes in gene expression in this small number of cells can be nullified when whole brain homogenate or selected areas only are analysed.

6.2 Reduced noradrenergic signalling during chronic infection

Evidence presented here suggests that chronic *T. gondii* infection may target noradrenergic signalling. Norepinephrine signalling in the CNS is implicated in

many cognitive functions such as attention, memory and learning[45]. In addition, noradrenergic neurons are part of complex cellular networks determining dopaminergic, glutamatergic and neuroimmune function[60,325]. Small changes in norepinephrine can affect many mammalian systems throughout the body. Altered norepinephrine signalling is associated with a broad spectrum of neurophysiological changes, many of which share commonality with *T. gondii* associated phenotypes. Movement disorders and seizures have been widely reported in patients with low norepinephrine and *T. gondii* seropositivity[527,528]. Interestingly, hypermethylation of the DBH promoter has been associated with bipolar disorder[424]. Several publications have found *T. gondii* seropositivity is correlated with bipolar disorder[128,529]. Poor working memory is associated with both *T. gondii* infection and reduced noradrenergic signalling. Compared to healthy controls, *DBH-/-* mice perform poorly in an elevated plus maize[530]. Many publications have reported poor working memory and low concentration during *T. gondii* infection[103,104]. Methylation is essential during memory formation. DNMT3a knock out mice models exhibit synaptic alterations as well as learning deficits[531]. Here, hypermethylation of DBH during infection is reported, the resulting changes to noradrenergic signalling may affect memory formation.

These neurophysiological changes may also provide insights into the mechanisms responsible for the loss of fear phenotype observed during chronic *T. gondii* infection[96]. The effect of reduced norepinephrine on novelty seeking and fear associated behaviours has yet to be fully assessed. Future investigation of the behavioural changes exhibited by *DBH-/-* mice models, described by Schank *et al*, may provide insight into how altered DBH expression contributes to *T gondii* associated behaviour phenotypes[532]. These changes may facilitate *T. gondii* survival and proliferation. However, this thesis has not investigated what evolutionary benefit the parasite may gain from global down-regulation of DBH expression. Future investigation could utilise mouse models with altered DBH expression such as a DBH over-expressing mouse model to assess if this effects parasite growth, cyst density or distribution in any way.

6.3 Sex-differences during chronic *T. gondii* infection of the CNS

Down-regulation of DBH mRNA and a concordant increase in norepinephrine was observed in male but not female mice. This may explain the reported sex-differences observed during chronic *T. gondii* infection. It has previously been noted that neurotransmitter changes during chronic infection are sex-dependent [215]. Furthermore, several publications have found that males but not females exhibit an attraction towards cat odour [99,175]. This suggests that altered catecholamine synthesis may contribute to behaviour changes during infection. Regulation of DBH by oestrogen has been demonstrated in PC12 cells, with two oestrogen response element (ERE) sites being identified [318]. The hypermethylation in the promoter region of DBH with infection may alter ERE binding although DBH expression correlated with ESR expression regardless of infection status. This suggests that the DBH suppression induced by infection is less powerful than the oestrogen regulation and oestrogen binding to the DBH promoter may prevent *T. gondii* induced down-regulation. Some transcription factors such as SP1 are capable of binding DNA and initiating transcription even when the binding site is CpG methylated [533]. Ung *et al* demonstrated that ESR1 may be one such transcription factor, able to exert its effect regardless of DNA methylation within its binding regions altering gene expression during breast cancer [534].

6.4 Methylation of the DBH promoter - cause or consequence of mRNA down-regulation?

Methylation was observed at 5 days of infection in rat catecholaminergic and human neuronal cells, whereas, down-regulation of DBH mRNA was evident after only 3 days of infection. Down-regulation of nascent RNA transcription clearly indicates that the reduced mRNA is not due to post-translational modification/ degradation such as Post-Transcriptional Gene Silencing (PTGS). As extracellular vesicles carry miRNAs and many miRNAs are involved in PTGS it is reasonable to assume that the decrease in DBH mRNA observed was due

to miRNA-mediated degradation of mRNA in the cytosol yet the run-on experiments clearly support regulation at the transcriptional level. Methylation of the DBH promoter was measured using MSRE qPCR, however, this technique is limited by restriction site selection allowing a small region of 251bp that is 2.6kb upstream of the DBH gene to be analysed. Therefore, methylation changes closer to the transcription start site will be missed. Pyrosequencing or bisulphite sequencing of the DBH promoter over a time scale of 2 to 5 days may reveal the sequential methylation pattern that occurs

Interestingly, there is also increasing evidence that intergenic CpG islands, known as CpG shores, not within 1kb of a gene, are highly conserved and may regulate gene expression[535]. Of these, several were identified in the extended promoter of DBH[536]. Furthermore, during colon rectal cancers it has been observed that the majority of differential methylation occurs in a region 2kb upstream of the transcription start site[537]. These regions are associated with gene expression as well as tissue specific DNA methylation patterns[538].

6.5 The mechanism of methylation

The identification of DNA methylation only after 5-days of infection may be due to the mechanism of transcriptional regulation. This may be due to the mechanism of 5' methylation of the DBH promoter sequence. If RNA in the extracellular vesicles is required, as suggested by the UV-ablation, then this suggests that RNA may mediate DBH transcriptional regulation. In plants this is a well characterised mechanism of gene down-regulation by dsRNA targeted DNA methylation[539]. Groundbreaking work by Morris *et al* reported the first example of RNA mediated DNA methylation in mammalian cells. It was demonstrated that a promoter-directed siRNA inhibited elongation factor 1α expression[540,541]. Using a nuclear run-on assay, it was demonstrated that this was transcriptionally regulated. Furthermore, DBH down-regulation was inhibited after treatment with 5-AC and TSA demonstrating that this expression was regulated via methylation. These findings confirmed that miRNAs can induce epigenetic modification of mammalian DNA, altering gene expression. RNA directed transcriptional si-

lencing has since been demonstrated to affect expression of many mammalian genes including brain-derived neurotrophic factor (BDNF) in the CNS[542].

It remains possible that DBH hypermethylation follows a miRNA mediated sequence of events as found for other genes. Sequential studies have determined that siRNAs targeted to promoter sequences interact first with the Argonautes 1 and 2 (AGO1 and AGO2)[543]. The AGO protein family play an important role in RNA mediated gene silencing, their role in post-translational silencing via the RNA-induced silencing complex (RISC) is well characterised[544]. However, AGO1 and 2 play an increasingly important role in RNA mediated DNA methylation[545,546]. Kim *et al* observed that siRNA-AGO interactions at the target promoter site are identified within the first 24 hours, this is rapidly followed by the recruitment of H3K9me2 and H3K27me3[543]. DNA methyltransferase is then recruited, and DNA methylation can be observed after 72-96 hours[419]. 5' promoter methylation was only observed after 96 hours of extracellular vesicle treatment, in my studies suggestive of this mechanism of RNA mediated DNA methylation. Interestingly, it was reported that AGO2 is enriched in many extracellular vesicles[460] further supporting the model of extracellular vesicle miRNA mediated transcriptional silencing.

Identifying the miRNA able to induce DBH transcriptional regulation is an essential next step to understanding this mechanism. Future experiments should focus on transcriptomic analysis of all miRNA and lncRNA cargo of extracellular vesicles purified from infected cells[547]. It may also be possible to construct an expression library. High through-put screening could then be performed, wherein, cell cultures are transfected with plasmids containing miRNAs and DBH down-regulation investigated using RT-qPCR[548]. Moreover, once a miRNA candidate is identified it may be possible, using microRNA-expressing lentiviruses, to deliver miRNA directly to the CNS and measure DBH down-regulation[549].

6.6 Extracellular vesicles during *T. gondii* infection

T. gondii has previously been identified as an epigenator. Dass and colleagues reported hypomethylation of the arginine vasopressin promoter in the medial amygdala of male rats during chronic infection[190]. During this study *T. gondii* bradyzoite cyst distribution is not reported, however, it is possible this hypomethylation is also mediated by extracellular vesicles. Findings reported here suggest that *T. gondii* is able to co-opt cell-cell communication, inducing transcriptional regulation throughout the CNS. It is demonstrated that DBH transcriptional regulation was mediated by extracellular vesicles resulting in increased methylation of the DBH promoter. Future experiments should focus on inhibiting exosomal biogenesis in vivo. The *Cre-loxP* system allows site-specific recombination to occur in targeted genes. Koshy and colleagues have described an engineered *T. gondii* strain able to induce site-specific recombination of infected cells only[550]. In addition, *Cre-loxP* mice models are now widely available[551]. Using this technique, it may be possible to inhibit a gene, such as N-Smase, essential for exosome biogenesis in infected cells only.

Many publications report global neurophysiological changes during chronic *T. gondii* infection. Historically, these observations have been attributed to global changes induced by the inflammatory response. However, many neurophysiological changes are observed only during chronic infection when the immune response is lowest. Furthermore, there are many alterations in neuronal signalling, motor function and cognition not observed during other chronic infections of the CNS. Altered cell-cell communication via extracellular vesicles may account for many neurophysiological changes observed during chronic infection. Altered cell-cell communication may play a role in other *T. gondii* associated phenotypes such as glutamate-1 (GLT1), a glutamate transporter, during chronic *T. gondii* infection of mice[221]. Particularly as extracellular vesicles containing miR-124, secreted by neurons, are able to induce translational regulation of astroglial GLT1[523]. Global changes observed in glutamate decarboxylase (GAD) localization may be due to alterations in a protein for localization in neurons that may involve extracellular vesicles throughout the CNS[222].

How *T. gondii* is able to alter host extracellular vesicle cargo remains unknown. This mechanism may be via parasite-derived secretory proteins. Some *T. gondii* rhoptry proteins are able to alter host cell signalling such as ROP16 which is able to induce host cell down-regulation of proinflammatory cytokine signalling[238]. Additionally, *T. gondii* infection induces Myc which has been found to regulate a set of miRNAs that were induced with infection of fibroblasts[49,552]. Whether this explains the neuronal specific response described herein or another mechanism is involved is the subject of future studies. Pathways regulating miRNA expression and extracellular vesicle packaging should be the subject of future investigations, particularly as little is known about the mechanisms governing extracellular vesicle cargo selection.

6.7 The role of extracellular vesicles in the CNS

The CNS is the centre of cognition, motor function, memory and learning. Many of these functions are governed by complex neuronal networks communicating via neurotransmitters and cellular effectors. Neurons are uniquely capable of adapting to changes in their environment; altering their morphology, synaptic plasticity and neurotransmitter release depending upon environmental signals. Dynamic methylation is essential for many of these processes. Altered methylation of BDNF resulted in chromatin remodelling and was associated with increased synaptic plasticity and memory consolidation[362,553]. Interestingly, it has been observed that extracellular vesicles containing miR-132 can regulate neuronal BDNF expression[554,555]. Findings reported here demonstrate that environmental changes in a small number of neurons can alter methylation in disparate neurons throughout the CNS. This mechanism enables neurons to regulate signalling via synaptic plasticity throughout the CNS system.

There is increasing evidence that extracellular vesicles propagate neurodegenerative disease pathology. Exosomes isolated from Alzheimer's disease patients contain misfolded tau and amyloid-β[556]. Similarly, during Parkinson's disease exosomes were found to contain α-synuclein[557]. Little is known about why extracellular vesicles contain these protein aggregates during neurodegenerative diseases. Trojan extracellular vesicles may play an important role in the mechanisms

underlying neuronal death. Furthermore, extracellular vesicle cargo may act as biomarkers for disease aiding early diagnosis[558]. They may even become vectors for disease treatment in the future[559]. Thus, the study of extracellular vesicle biogenesis, cargo selection and uptake is of great therapeutic interest and has implications for a huge range of diseases.

The results reported here represent the first example of neuron-neuron transcriptional regulation mediated by extracellular vesicles. In addition to DBH, extracellular vesicles may regulate many other neurotransmitter pathways. This thesis demonstrates that *T. gondii* infection may be a model system by which to study neurophysiological mechanisms. The neurophysiological role of cell-cell communication can be delineated by investigating how it is altered during *T. gondii* infection. Findings reported here shed new light on neuronal epigenetic mechanisms.

6.8 Final remarks

Here it is demonstrated that *T. gondii* alters host catecholaminergic signalling by transcriptional regulation of DBH. Furthermore, bradyzoite tissue cysts are able to alter extracellular vesicles communication to facilitate DBH transcriptional regulation throughout the brain, altering the epigenome of uninfected but exposed neurons. This pathway may provide an explanation for neurophysiological changes observed during *T. gondii* infection. In addition, *T. gondii* is a unique model to study mammalian neurological function, by examining the influence the parasite is able to exert over cell-cell communication, we may be able to further understand the mechanisms governing CNS function and dysfunction.

Appendix A

Appendix A

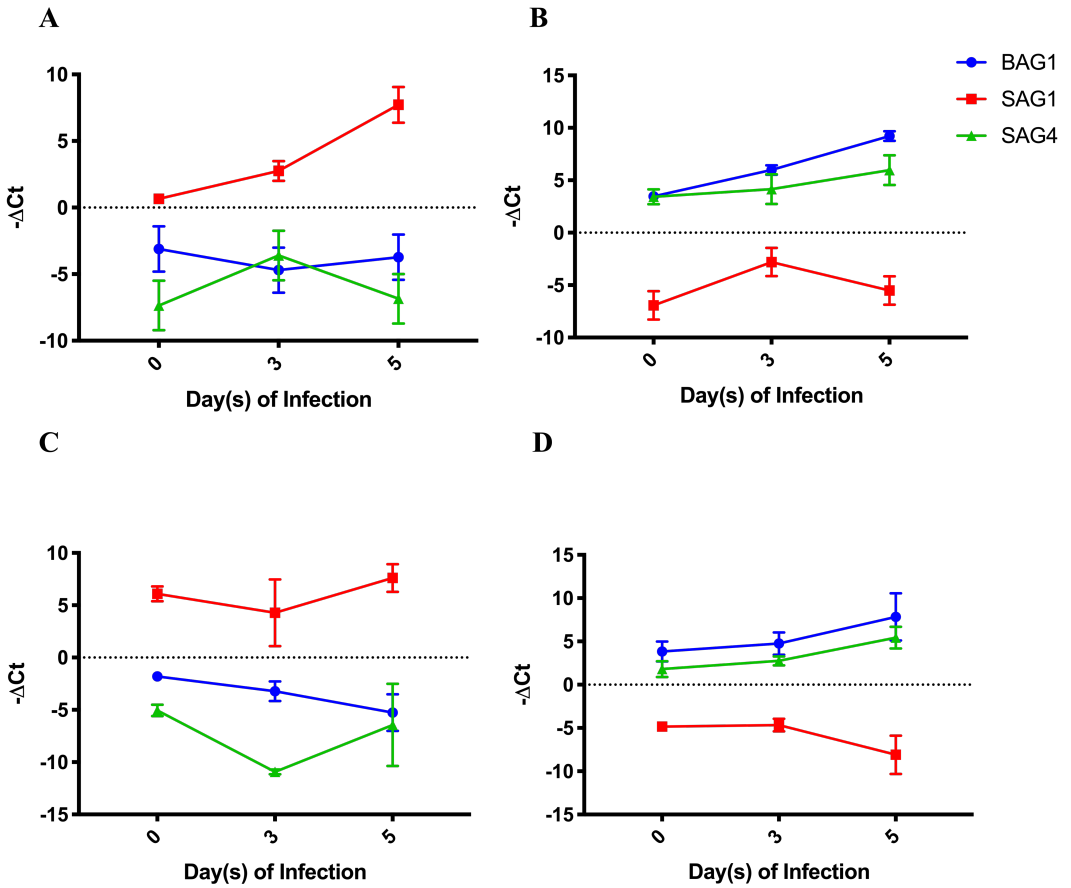


Figure S1: pH shocking of liberated *T. gondii* induces a bradyzoite-like phenotype. All plots show expression of the bradyzoite markers BAG1 (blue) and SAG4 (green), as well as the tachyzoite marker SAG1 (red) expressed in relation to the housekeeping gene GAPDH over a time course of 5 days of infection. A) RNA was collected over a time course of 5 days from tachyzoite infected rat catecholaminergic PC12 cells. ±SEM shown, n=2 biological repeats. B) Rat catecholaminergic PC12 cells were infected with pH shocked *T. gondii* and cultured for 5 days. RNA was collected on day(s) 0, 3 and 5; RT-qPCR was then performed. ±SEM shown, n=2 biological repeats. C) Human neuronal M17 cells were infected with *T. gondii* tachyzoites. RNA was collected over a time course of 5 days from uninfected and tachyzoite infected human neuronal M17 cells. ±SEM shown, n=2 biological repeats. D) Human neuronal M17 cells were infected with pH shocked *T. gondii* and cultured for 5 days. RNA was collected on day(s) 0, 3 and 5; RT-qPCR was then performed ±SEM shown, n=2 biological repeats.

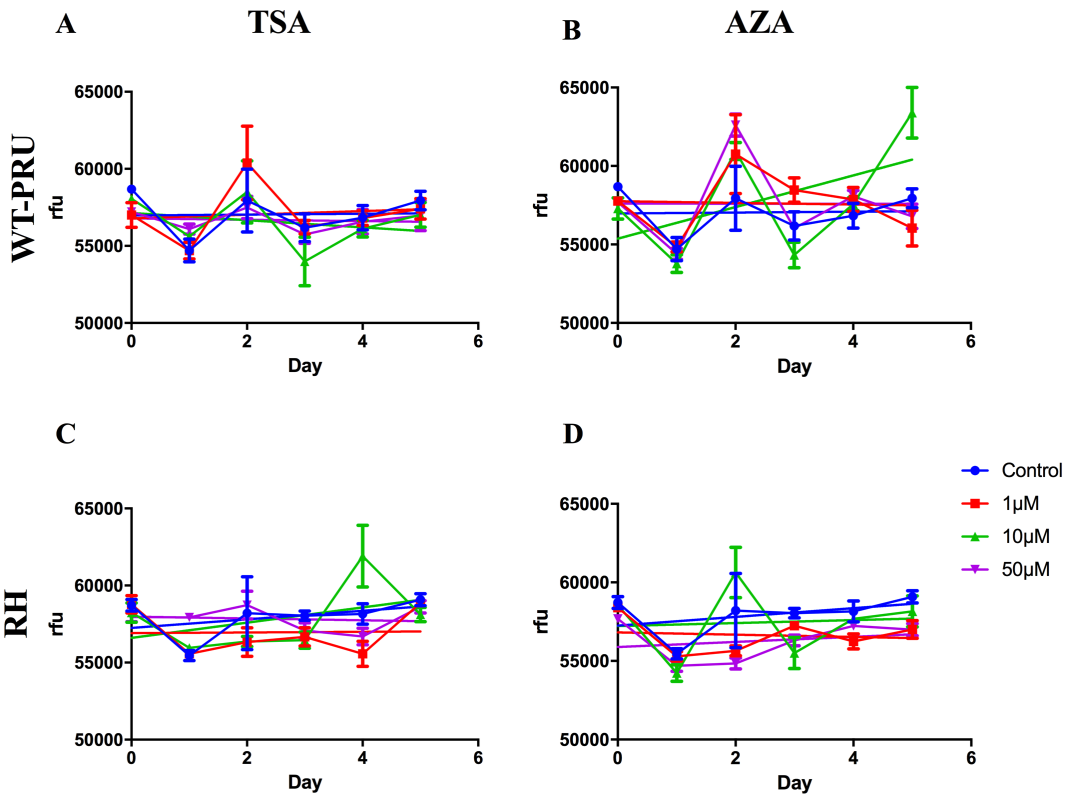


Figure S2: Parasite growth during treatment with methylation inhibitors. Growth of KU80-GFP and RH-YFP *T. gondii* parasites cultured on human fibroblasts and subjected to treatment with TSA and 5AZA for a time course of 6 days. Fluorescence was measured each day using a plate reader. ±SEM shown, no significant difference observed.

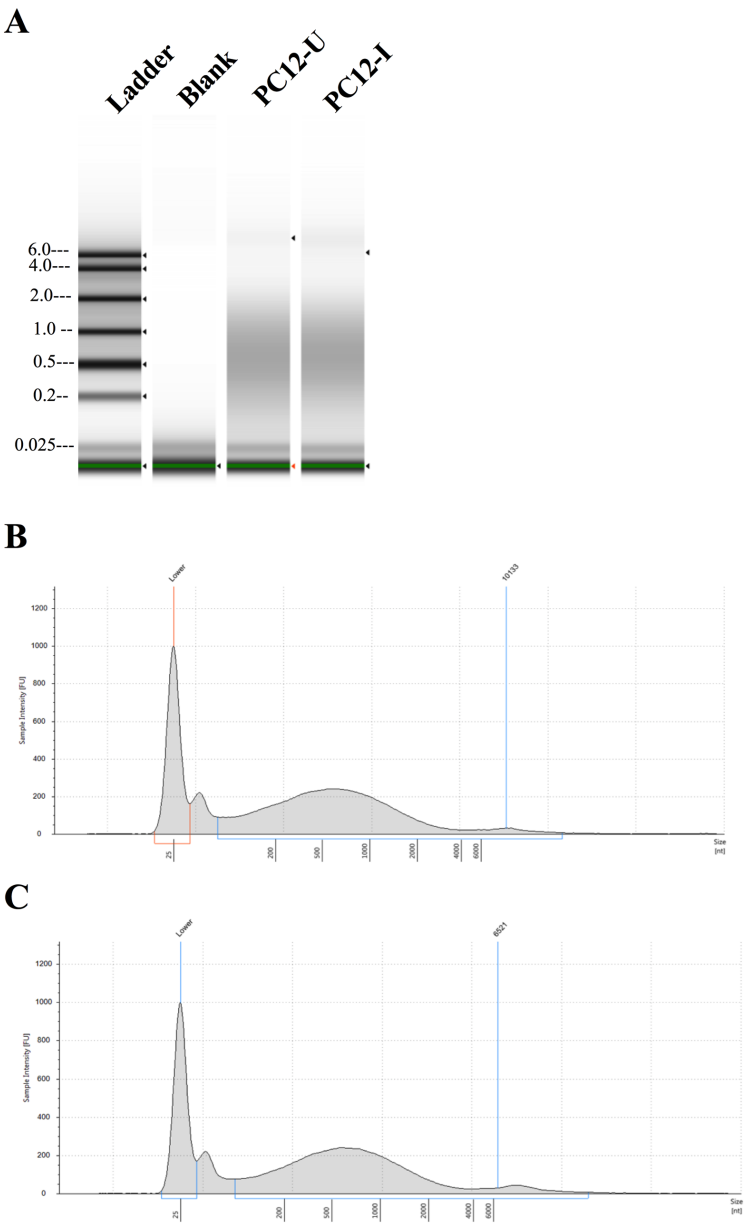


Figure S3: QC Analysis of Truseq Library Prep. A) Tape station analysis of library prep samples. The Illumina protocol states that you should expect to see a peak of between 260-380 bp. Gel shows ladder, blank PC12 uninfected, PC12 infected samples. B) In-depth report of uninfected PC12 sample. C) In-depth report of 5-day infected with induced bradyzoite infected PC12 cells. Samples are pools of 5 biological repeats, n=10.

Figure S4: DBH promoter sequence analysed with MSRE qPCR. CpG sites are highlighted in yellow, MSRE sites are indicated in red and primer sequences are underlined.

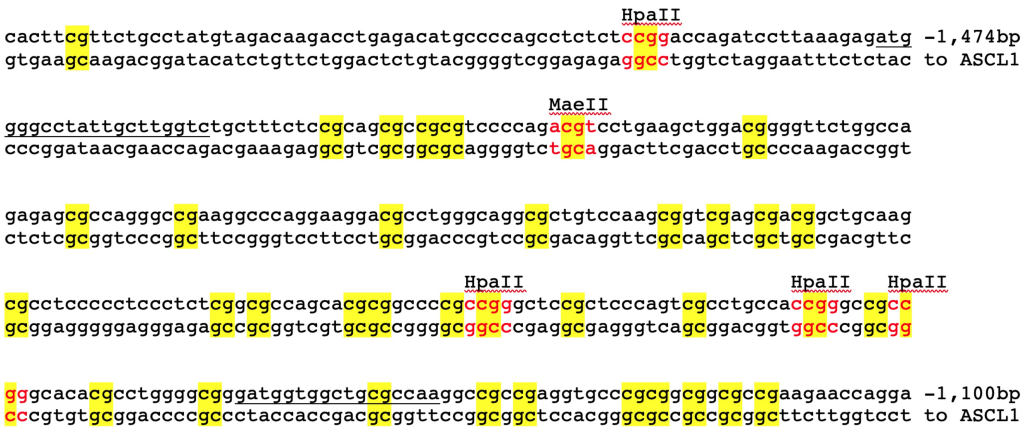


Figure S5: Ascl1 promoter sequence analysed with MSRE qPCR. CpG sites are highlighted in yellow, MSRE sites are indicated in red and primer sequences are underlined.

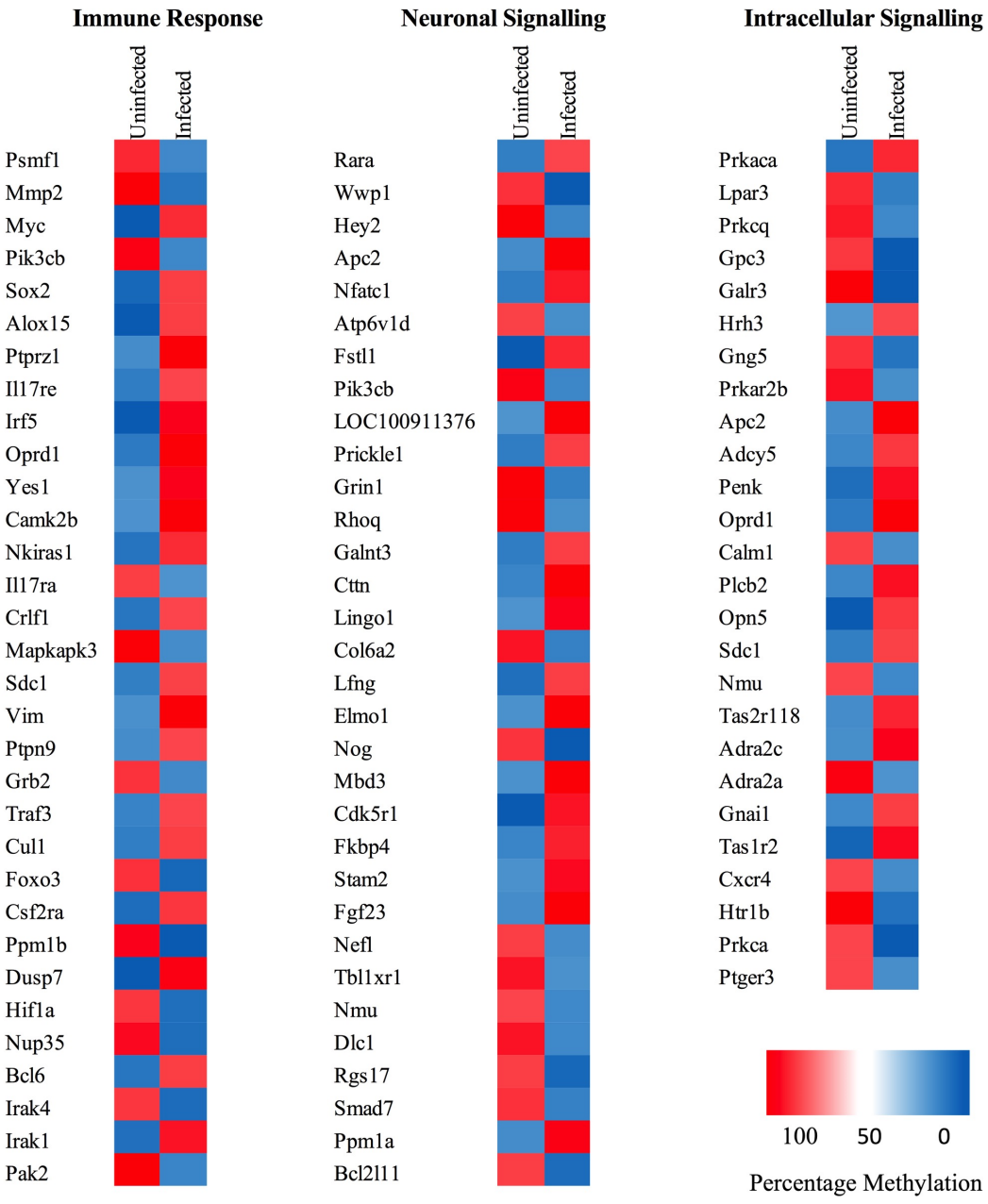


Figure S6: Heat map of promoter methylation of genes grouped into cellular pathways measured by whole-genome bisulphite sequencing. DNA was extracted from uninfected and *T.gondii* infected rat catecholaminergic cells, 5 biological repeats were pooled prior to whole-genome bisulphite sequencing.

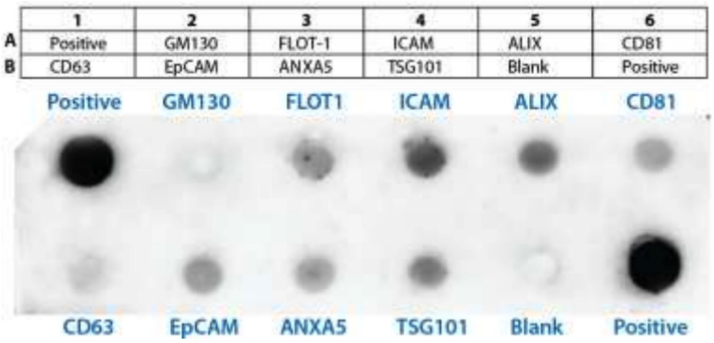


Figure S7: An example Exocheck blot provided by Cambridge Bioscience (UK). Exosomes were isolated from human HT1080 lung sarcoma cell line media. Table indicates each target protein.

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