Investigation into *Toxoplasma gondii* infection in relation to expression of neurodegenerative disease markers in natural mice populations

Ayed Alshammari

School of Science, Engineering and Environment

University of Salford, UK

Submitted in Fulfilment of the Requirements of the Degree of Doctor of Philosophy, 2020

Table of Contents

List of figures

List of tables

Acknowledgements

I will initially express my gratitude for God, owing to the fact that he provided me with this opportunity. Additionally, I will express my appreciation for the actions of King Salman bin Abdulaziz al-Saud, who decided to create the scholarship programme in order to help his subjects. Furthermore, I give many thanks to the Ministry of Higher Education, which has provided me both with trust and guidance. I also give thanks to both of my supervisors, Professor Geoff Hide and Dr Gemma Lace-Costigan, for their helpfulness, for their patience, and for their determination and trust to help me succeed. I will express my thanks for my entire family: my parents, all of whom have furnished me with good spirits and the support needed to succeed in this course. I will be eternally appreciative of their kindness and love. Finally, I give my thanks to all the staff in the Molecular Parasitology Laboratory, and, furthermore, to each of my friends; you are all responsible for creating the most enjoyable working conditions.

Declaration

Unless stated to the contrary, the work in this thesis is my own. I confirm that this work has not been submitted for a degree or any other qualification at the university of Salford or any other university.

Abbreviations

AIDS	Acquired immunodeficiency syndrome
AD	Alzheimer's diseases
Aβ	Amyloid beta
APP	Amyloid precursor protein
APCs	antigen-presenting cells
APOE	Apolipoprotein E
Arg	arginase
BBB	Blood brain barrier
Cre	Carbapenem-resistant Enterobacteriaceae
CNS	Central nervous system
CSF	Cerebrospinal fluid
CMA	Chaperone-mediated autophagy
CP	choroid plexus
CT	computed tomography
COX	Cyclooxygenases
DC	Dendritic cells
DNA	Deoxyribonucleic acid
DA	Dopamine
ELISA	Enzyme-linkedimmunosorbent assay
EC	Epithelial cell
EDTA	Ethylenediaminetetraacetic acid
GBP	guanylate-binding proteins
HRP	horseradish peroxidase
Hbmec	Human bone marrow endothelial cells
IgA	Immunoglobulin A
IgG	Immunoglobulin G
IgM	Immunoglobulin M
iNOS	inducible NO Synthase
IFN-γ	Interferon gamma
IL-10	Interleukin-10
IL-12	Interleukin-12
IL-2	Interleukin-2
IL-6	Interleukin-6
ITS-1	Internal transcribed spacer
LAMP2A	Lysosomal-associated membrane protein 2A
MA	Macroautophagy
MRI	Magnetic resonance imaging
mTOR	mammalian target of Rapamycin
LC3	microtubule-associated protein 1A/1B light chain 3
MAT	Modified agglutination test
MS	Multiple Sclerosis
NK	Natural Killer
NFT	Neurofibrillary tangles
NO	Nitric oxide
NMDA	N-methyl-D-aspartate
NSAIDs	non-steroidal anti-inflammatory drugs
SNPs	Nucleotide polymorphisms

PV	parasitophorous vacuole
PD	Parkinsons Disease
PGK1	Phosphoglycerate Kinase 1
PCR	Polymerase chain reaction
SNP	single nucleotide polymorphism
SDS	sodium dodecyl sulphate
SOCS1	Suppressor of cytokine signaling 1
SAG1	surface antigen gene-1
Th	T helper
HIV	the human immunodeficiency virus
TLR2	Toll-like receptor 2
TLR	Toll-like receptor 4
T.gondii	*Toxoplasma gondii*
TGF-β	Transforming growth factor beta
TBS	tris-buffered saline
TNF-a	Tumor necrosis factor alpha
VCAM-1	Vascular cell adhesion molecule 1

Abstract

Toxoplasma gondii is a highly prevalent opportunistic protozoan parasite that has a remarkable capacity to infect humans and a range of warm-blooded animals. This research had two broad aims. Firstly, to investigating whether detection of *T. gondii* in cadaveric brain tissue of patients, who had been previously diagnosed with Alzheimer's Disease (AD), could be used to investigate the role of this parasite in AD. Secondly to investigate *T. gondii* infection in naturally infected wood mice (*Apodemus sylvaticus)* populations and the parasite's role in the expression of a range of key autophagic and inflammatory genes associated with neurodegenerative diseases in humans.

Human brain tissue from the hippocampus and frontal lobe regions was obtained from 45 cadaveric specimens and analysed for the presence of *Toxoplasma gondii* cysts using visual microscopy. The human brain tissue did not show infection with *T. gondii* as judged by the absence of cysts found within the parenchyma of any of the subjects. Given the expected 10% prevalence in the UK and the small volume of brain tissue used and it was concluded that this was insufficient tissue material to investigate the association in this way. Due to the amount of brain tissue needed for scaling up and the unlikely capability of sourcing enough brain tissue, this approach was considered unfeasible.

A population of 21 *A. sylvaticus* (wood mice) were captured from a local peri-aquatic woodland ecosystem, euthanised followed by extraction and analysis of brain tissue. *Toxoplasma* infection was diagnosed using nested PCR with specific markers for B1, SAG1 and SAG2. Additionally, PCR and Western blotting were carried out to permit detection of the presence and expression of various autophagic and inflammatory genes/proteins.

As a result, this naturally infected murine population was used as model system for studying the interaction between infection and host brain gene expression. *T. gondii* was detected in five samples (5/21) of *A. sylvaticus* reflecting an infection rate of 23.8% (95% confidence interval: 10.2; 45.5%), which is a comparable prevalence to other recent studies in wood mice but considered to be a high for a species resident among a relatively feline-free habitat. Among the autophagy and inflammatory genes, PCR detection and DNA sequencing identified *Apodemus* homologues of human Beclin1, Lc3 and Lamp2, TLR2, Tau and APP in the murine brain tissue. Expression of various autophagy and inflammatory proteins were examined by western blotting. There were significantly increased levels of TLR2, LC3, Socs1, Arg1, in *T. gondii* infected mice compared to non-infected mice, but decreased levels of NMDAR1, Lamp2, P62, Beclin-1 and iNOS. No significant difference was observed in the levels of Tau and APP between infected and non-infected mice. The findings of this study are novel and insightful as they have revealed that the brain tissues of *T. gondii* infected mice, in the context of

a natural environment with natural infection, are undergoing changes in autophagic and inflammatory processes, often associated with neurodegenerative disease, in response to infection.. Overall, this study concludes that wild rodents could be used as a model natural population to explore gene expression relevant to human neurodegenerative diseases. This may pave the way to a greater understanding of the interactions of *T. gondii* with the brain and lead to new insights into its effect on neurological diseases.

Chapter 1: General Introduction

1.1 General Aims

Some parasites, like *Toxoplasma gondii*, pass through various stages in the brain. This can potentially interfere with the brain and behaviour. Links have been made between some disorders, like schizophrenia, and *Toxoplasma* infection. Currently, groups of researchers at Salford University are studying Alzheimer's disease and *Toxoplasma* infection, opening the opportunity to investigate the relationship between the two. It is possible to study this relationship directly in human brain tissue or to use proxy animal systems to investigate the parasite's effects on AD-related protein expressions in brain tissues, but, to the current researchers knowledge, no such study has ever been conducted among wild rodents. Therefore, the overall aim of this study was to investigate the relationship between *Toxoplasma* infection and Alzheimer's diseases .

1.1 General background on *Toxoplasma gondii*

Intracellular parasites are the microorganisms able to grow inside the cells of a host. There are two main categories where these parasites are divided into, facultative and obligate. *Toxoplasma gondii* (*T. gondii)* belongs to the second category, which means that it solely relies on intracellular resources (Dubey, 2009). It is a protozoan parasite (Apicomplexa phylum) that has a life cycle that involves a wide range of hosts, but the definitive-host specificity is restricted to felids. From then on it can infect humans, which act as an intermediate host, usually via the faecal-oral route, either by ingesting *T. gondii* cysts or oocysts. There have been reports that the vertical transmission is also susceptible to direct *T. gondii* infection (Figure 1.1).

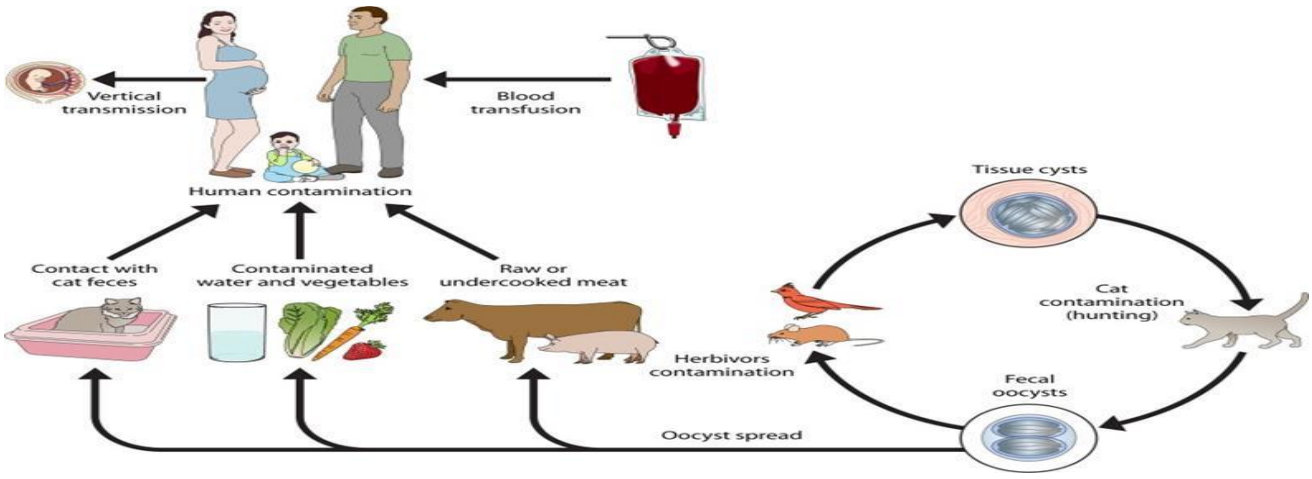


Figure 1.1: *Toxoplasma gondii* life cycle. Adapted from (Esch & Petersen, 2013).

Felines are the definitive hosts of *T. gondii*, where they are infected through consumption of raw meat from dead animals. Faecal oocysts are shed in large numbers by acutely infected cats for approximately 2 weeks. After shedding, parasite sporulation into infective oocysts takes place in 1 to 5 days. *T. gondii* rapidly proliferates within the intestine, developing into the highly invasive tachyzoite form. These in turn can infect at least three separate routes able to infect humans (intermediate hosts) via the oral and ocular route. Tissue cysts contain bradyzoites which can be found in undercooked meat or water, while oocysts contain sporozoites and usually found in feline faeces (Esch & Petersen, 2013).

Historically, *T. gondii* was considered to be grouped into predominant lineages; I, II and III, due to the lack of antigenic serotypes able to allow extensive screening (Sibley & Ajioka, 2008). It had been hypothesised that due to environmental factors, genetic bottlenecks occurred 10,000 to 20,000 years ago; it is now known that there is a higher heterogeneity for this parasite that these three main types. toxoplasmosis prevalence is well-spread, especially in developing countries, with the Control Disease Centre (CDC) underscoring it as a major etiopathogenetic factor of neurological disease such as schizophrenia and bipolar disorders (Jones-Brando, Torrey, & Yolken, 2003; Torrey & Yolken, 2003), increased risk taking and road traffic accidents (Flegr et al., 2009) and an increased risk of suicide (Lester, 2012). *T.gondii* infections affect populations all around the world. Depending upon location of the population being studied, the estimated seroprevalence ranges from 0% to 100% (Tenter et al., 2000). In 2009, global seroprevalence trends for *T. gondii* were published (Pappas et al., 2009). This considered the published data for seroprevalence in pregnant women and those of childbearing over the ten years covering January 1999 to December 2008. Figure 1.2 presents a global map of *T. gondii* seroprevalence; the map is based on the data provided by Pappas et al (2009).

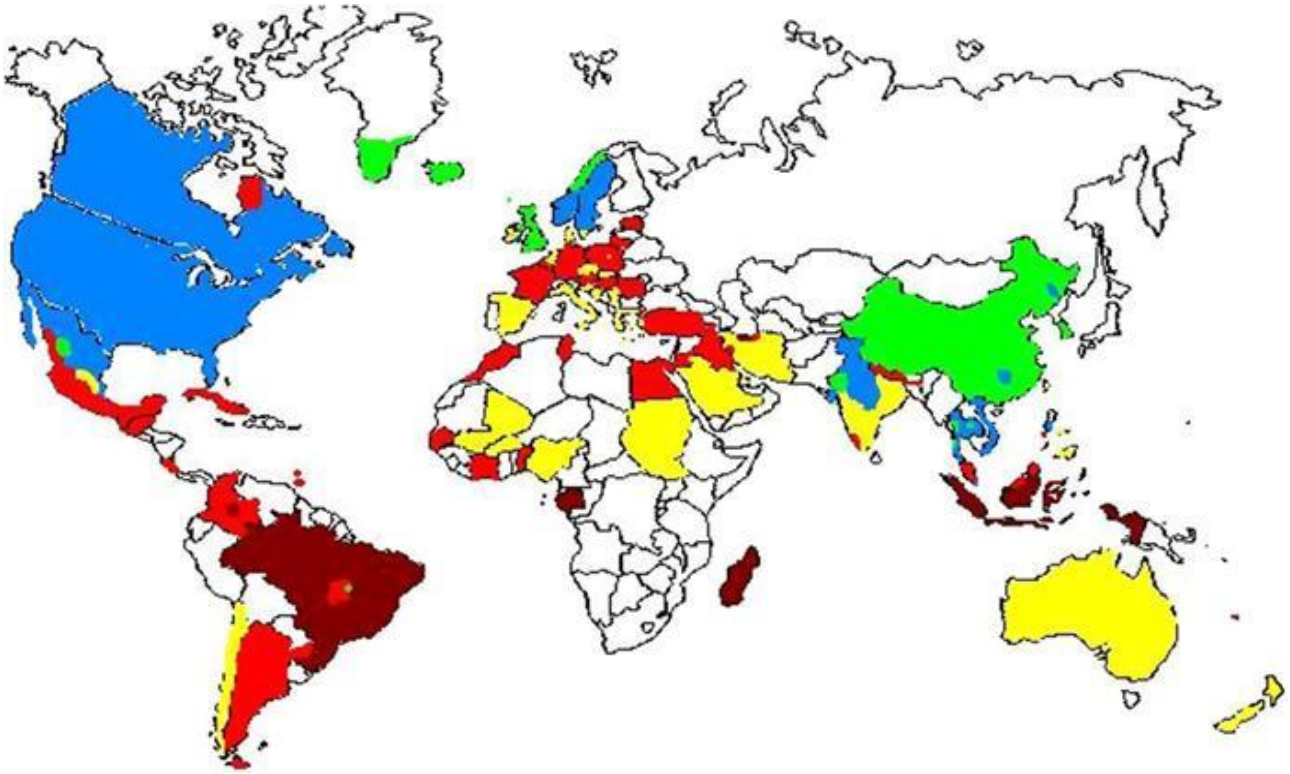


Figure 1.2: Global status of *T. gondii* prevalence. Dark red indicates high (>60%) prevalence, while light red is medium-high prevalence (40-60%). Yellow, blue and green indicate 20-40%, 10-20% and less than 10% prevalence. Adapted from (Pappas, Roussos, & Falagas, 2009).

South American countries, such as Brazil and Argentina, report high prevalence, with respective seroprevalences of 78% (n=503) and 53% (n=650) respectively (Porto et al., 2008; Rickard et al., 1999). Prevalence is lower in most European countries, as indicated by the Czech Republic (20% n=1,053) Denmark (28% n=89,873) and United Kingdom (9% n=1,897) (Kankova & Flegr, 2007; Lebech et al., 1999; Nash et al., 2005). However, some European countries report high levels of *T. gondii* infection. The seroprevalence data for Germany is 59% (n=4854) and France 54%, (n=13459) (Fiedler et al., 1999; Tenter et al., 2000). Even though France has a high seroprevalence, the incidence of *T. gondii* appears to be in decline. In 1980, the incidence in pregnant women was 7.5/1000; by 2010, this had dropped to 2.4/1000 (Nogareda et al., 2013). There may be multiple reasons for this improvement, but the most probable factors are changes in hygiene, meat production and dietary habits. The French Ministry of Agriculture reports that the consumption of sheep meat has fallen by 30%, which as a high-level source of contamination, could have a significant effect (Halos et al., 2010). Whilst there is data available that gives a general indication of the seroprevalence of *T. gondii* in humans, and the French studies described above give provide data about gravid women and those of childbearing age, more data is needed. To have a comprehensive understanding of the seroprevalence of *T. gondii* in humans, more research that considers males and females of all ages is required. The relationship to neurodegenerative disease such as AD is less well understood will be considered in the section 1.8.

1.2 *Toxoplasma gondii* infection in human brain

Once the parasite enters the body via immuno-privileged sites (i.e. via the oral route) or vertical transmission (Figure 1), it is then able to transmigrate the epithelial membranes of the placenta and intestine by direct binding interaction to intercellular adhesion molecule 1 (ICAM-1) (Barragan, Brossier, & Sibley, 2005; Barragan & Sibley, 2002; Da Gama, Ribeiro-Gomes, Guimaraes, & Arnholdt, 2004). There, after multiple replication rounds numerous tachyzoites (the rapidly dividing *T. gondii* parasites), they access the submucosal tissue, entering the circulation and able to infect cells of the innate immune system and professional antigen presenting cells, such as neutrophils, granulocytes, natural killer cells, dendritic cells and macrophages (Figure 1.3) (Courret et al., 2006; Denkers, Butcher, Del Rio, & Bennouna, 2004; Persson et al., 2009).

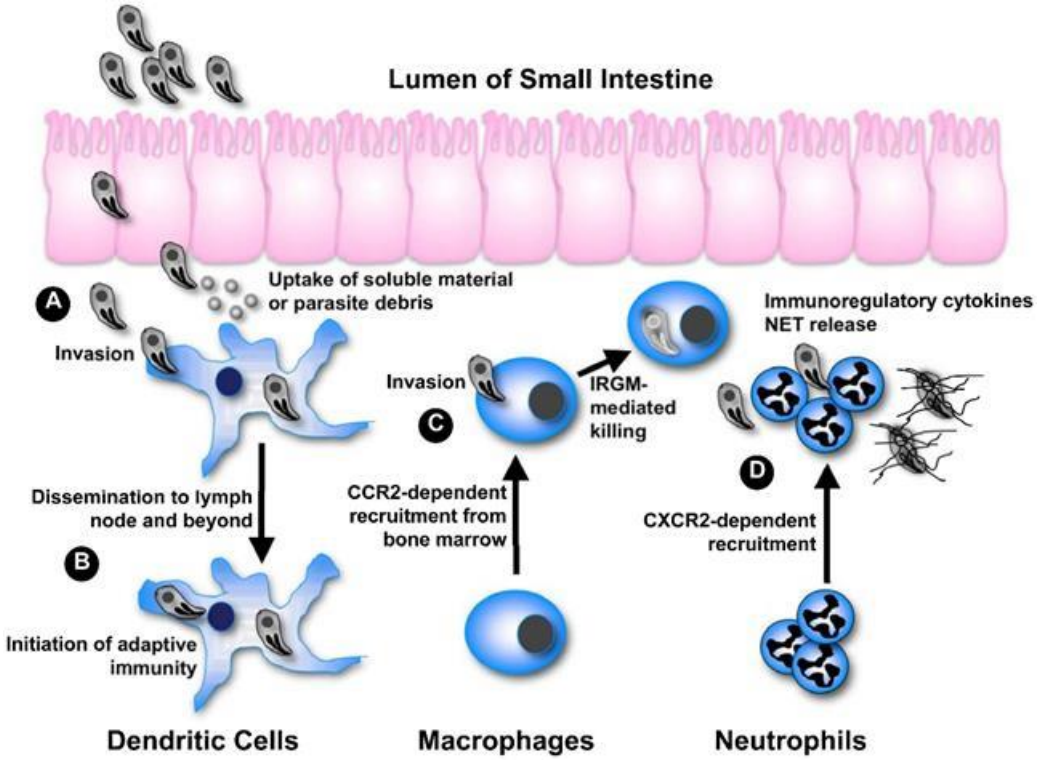


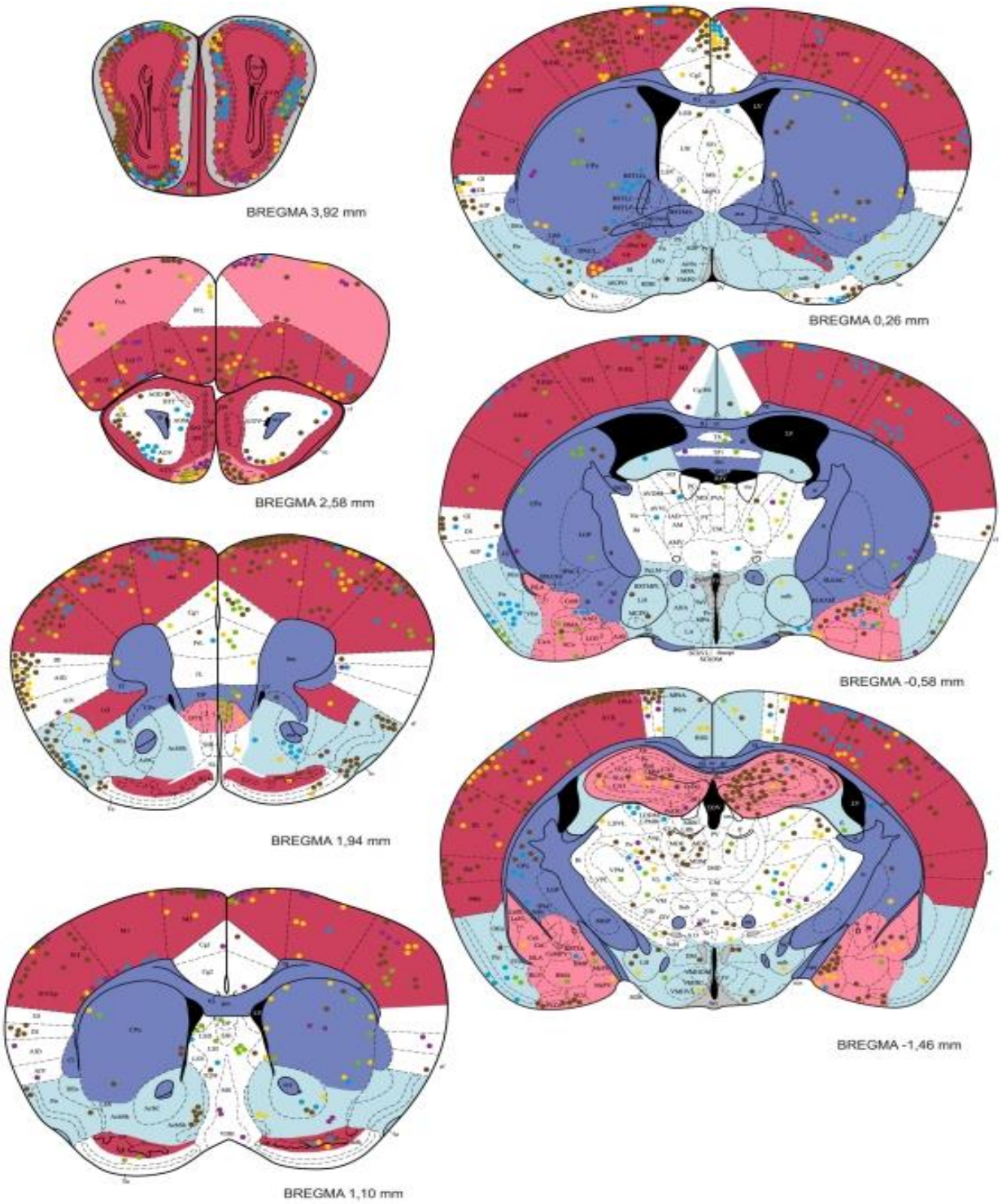
Figure 1. 3: *T. gondii* infection of innate immune cells. Adapted from (Denkers, Schneider, Cohen, & Butcher, 2012). (A) After crossing the epithelium of the small intestine, tachyzoites invade dendritic cells (DCs). (B) Infected DCs migrate to the draining lymph node and beyond, thereby disseminating infection. Infected as well as antigen-bearing DCs also initiate immunity in the lymph node. (C) Inflammatory macrophages are recruited to the site of inflammation/entry, able to destroy the majority of intracellular parasites. (D) Specific cytokines recruit CXCR2-dependent neutrophils.

This ability to infect cells of the immune system allows the protozoan access to the central nervous system (CNS) and developing foetus. The former contains three barriers, the arachnoid epithelium, the choroid plexus and the blood-brain barrier (BBB) (Feustel, Meissner, & Liesenfeld, 2012). These sites regulate the traffic of cells and substances and pathogens entering the brain, including *T. gondii* tachyzoites. The blood-brain barrier is very selective in terms of molecules (and cells) that penetrate into the brain matter. In healthy hosts, the presence of brain-specific professional antigen presenting cells, such as microglia and brain DC in that region is minimal, but upon *T. gondii* infection, the number is increased by 50- to 100-fold in mice (Fischer, Bonifas, & Reichmann, 2000). This localised asymptomatic inflammation causes increase in several endothelial molecules such as VCAM-1, ICAM-1, ICAM-2 and MadCam-1 and increases cytokine secretion, which alter the permeability of the blood-brain barrier, making it easier for tachyzoites or bradyzoites (within immune cells, possibly DC) to transmigrate directly into the brain. Also, it has been proven that altered blood brain barrier permeability has been implicated with AD (Yamazaki et al.,2017), which might influence the likelihood of Toxoplasma entering the brain of those with AD. Until today, the definitive entry site(s) of *T. gondii* to the brain are elusive, providing limited understanding on how to prevent CNS infections, although it has been shown recently to implicate endothelial cells in this mechanism (Konradt et al., 2016).

1.3 *Toxoplasma gondii brain* distribution

Experiments performed on model lab rodents indicated that upon oral infection, cysts were distributed throughout the brain with no apparent functional system being selectively more prone to infestation (Berenreiterova, Flegr, Kubena, & Nemec, 2011). Although it has been hypothesised that *T. gondii* interferes with dopamine metabolism (Prandovszky et al., 2011; Strobl, Goodwin, Rzigalinski, & Lindsay, 2012), and dopamine biosynthesis in dopaminergic murine cells (Martin et al., 2015), cyst prevalence was not associated with the hypothalamus and the dopaminergic system. In mice, it was reported that the olfactory bulb, the entorhinal sensory cortices and the hippocampus and amygdala were infected more than the rest of the brain. In their studies, all mice infected with *T. gondii* had tissue cysts in the brain, spread on average in over 92% of that organ (Figure 1.4). Most notably, the cerebral cortical areas exhibited higher cyst density in comparison to subcortical regions, where compact masses of myelinated axons reside. Evidence evaluating the distribution of *T. gondii* within the human brain taken from autopsy samples has shown that positivity on polymerase chain reaction was observed within the anterior horns of the lateral ventricles and the posterior horns of the ventricles and hippocampus (Samojłowicz et al., 2019). Other research has detected *T. gondii* within other and similar brain

regions, such as the cerebral cortex, diencephalon and hippocampus (Conejero-Goldberg, Torrey and Yolken, 2003).



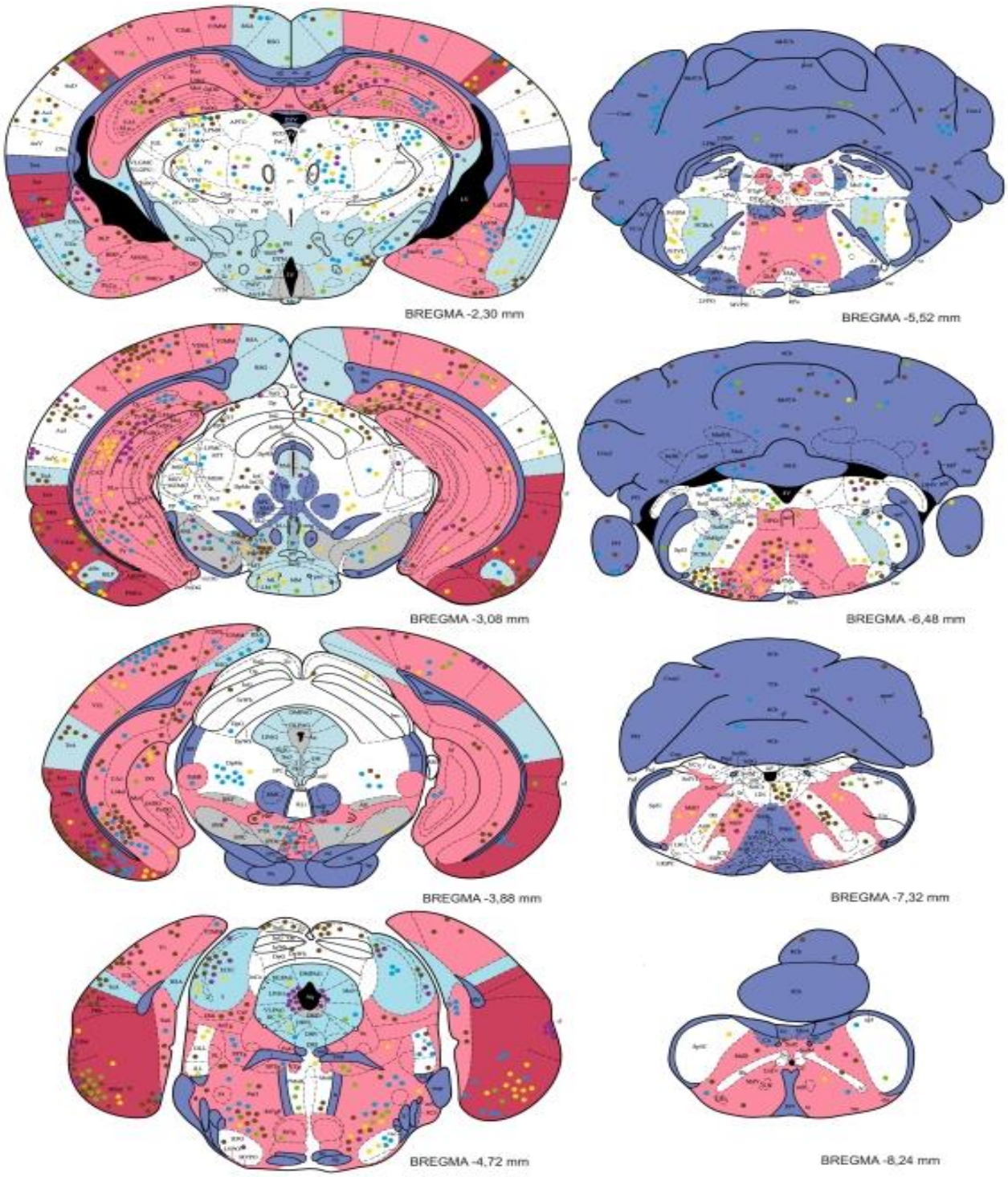


Figure 1. 4: The distribution of *T. gondii* cysts. Adapted from (Berenreiterova et al., 2011). in the forebrain, caudal forebrain, the cerebellum and the brainstem of a mouse with latent toxoplasmosis. Coronal-aspect diagrams of five CD1 mice infected with T. gondii. Different coloured circles indicate the locations of cysts in different mice and each circle denotes a single cyst. The density of the cysts in the different parts of brain is variable. To distinguish cyst density, areas are coloured differently– very high (red) high (pink), medium (white), low (blue),

and very low (dark blue). The dopaminergic system is coloured grey. Bregma anatomical points are used to determine the antero-posterior positions of the sections.

Hughes et al. (2008) carried out their research in wild rabbits and found the DNA of *T. gondii*, a close relative of *Neospora caninum*, to be more extensively distributed throughout the tissue. It was present in the brain, heart, liver tissue and tongue of rabbits and in the brain, cord tissue, heart and tongue of aborted lambs. To elucidate which brain cells are preferentially infected, several researchers employed mouse brain cells to show that tachyzoites are able to infect microglia, astrocytes, and neurons, where they readily form cysts (Fischer, Nitzgen, Reichmann, Gross, & Hadding, 1997). If *T.gondii* is found in a variety of CNS cells then it could have an impact on multiple systems and processes. When human fibroblasts cells were tested, the rate of infection was similar to that of mice (Schwartzman, 1987), although non-neural cells were also infected at the same rate in both species. Interestingly, a higher percentage of human astrocytes were infected in comparison to human neurons in cultured human brain tissues (Halonen, Lyman, & Chiu, 1996). Ultrastructural studies in aged mice brains indicated that the majority of cysts was found in the grey matter, predominantly within neurons (Ferguson & Hutchison, 1987a, 1987b). Recently, a study employing the Cre-recombinase lineage tracing system into *T. gondii* confirmed that neurons are the primary target of this parasite in vivo in mice (Cabral et al., 2016). When rats were orally inoculated with oocysts, their extensive brain distribution was confirmed (Dubey et al., 2016). The authors summarised data from previous researchers showing a random oocyst distribution in the brain or mice and rats, while their set of data correlated cyst number and distribution, size and location with *T. gondii* genotype. The number of cysts varied and irrespective of genotype or brain region. Therefore, cyst tropism could not be related to altered behaviour in rodents. The size of the brain cysts was dependent on the duration of infection, the strain of the parasite and the host cell parasitized. Most cysts were less than 40 μm in diameter and no greater than 58 μm. The density of cysts also confirmed a non-statistically significant spread, in both brain and eye. The hypothalamus was found with the least density of cysts, followed by the subpallium, and hippocampus, while the cerebellum, cerebral cortex, inferior and superior colliculus, mesencephalon, olfactory bulb, rhombencephalon, and thalamus had higher cyst density (Dubey et al., 2016). It is quite interesting that there was no difference in the presence of cysts in the olfactory bulb, since it has been reported that *T. gondii* manipulation appears to alter the rat's perception of cat predation risk, by making the infected rat not only be averted by cat odours, but also be attracted to feline urine (Berdoy, Webster, & Macdonald, 2000). Another theory supported that the parasite may affect pheromone perception centres in the brain (Vyas, Kim, Giacomini, Boothroyd, & Sapolsky, 2007). This is located in the hypothalamic zone and the amygdala, which did not show increase cyst density. It is still unknown how *T. gondii* is able to bypass this antipredator defensive behaviour, which gives the parasite a selective advantage is successfully infecting cats via rodents. There has been some evidence to show that the aberrant effects of *T. gondii* infection upon host brain function in AD may occur due to its injurious impact upon neurons and microglial cells, although the specific mechanism leading to cell dysfunction has not been elucidated (Jung et al., 2012; Li et al., 2019; Torres et al., 2018). *T.gondii* has been found in specific cell types or brain regions in AD and point out that there are in fact minimal studies using

human brain tissue.

1.4 *Apodemus sylvaticus*

Because felids naturally prey on rodents, the latter are effective intermediate hosts for *T. gondii*. The results of studies in which mice were infected with infective oocysts reveal that vertical transmission is common, passing from does to their offspring (Owen & Trees, 1998; Marshall et al., 2004). This implies that mice could serve as natural reservoirs of *T. gondii* in the wild. With the exception of Finland and northern Scandinavia, wood mice (*Apodemus sylvaticus)* are ubiquitous to Europe and the British Isles (Nowak, 1991). Typically, wood mice live in grassy fields, woodlands and urban areas; however, being highly adaptable, they are found in most environments. Wood mice nest in dense vegetation, inside hollow logs and spaces, and in tunnels or underground burrows. The territory of male mice normally extends to 109 m in diameter of, and the territory of female mice is generally 64m in diameter. Whilst most wood mice do not relocate, they can roam up to 400m in a single night (Nowak, 1991). They are skilled climbers and are typically nocturnal. As males have larger territories than females, breeding males may impregnate several females. The mice begin breeding in March, and, if it is mild and there is abundant food, breeding continues until early winter. This enables them to produce up to four litters a year, each litter containing four to seven young. The maximum lifespan of wood mice is considered to be between 18 and 20 months; however, many do not live for more than four months. Adult males are aggressive, negatively affecting juvenile survival (Bengston, 1989). Soon after weaning, young males are ejected from the nest. The overall population of mice varies over the year, with their numbers being lowest in spring and highest in autumn. Being primarily granivores, wood mice mainly eat grains, berries, seeds, nuts, roots and fruits, but their diet also includes insects and small snails (Nowak, 1991; Parker, 1990). Wood mice do not hibernate in winter but sleep together in groups. If the winter is particularly severe, the mice enter a kind of torpid state in which physiological activity is reduced. Wood mice are considered to be pests as they cause damage to crops and forest seedlings (Nowak, 1991). Thus far, few studies have explored *T. gondii* infections in *A. sylvaticus*. However, serological analyses have been conducted for *Apodemus agrarius*, a relative of the wood mouse. Those analyses found that, in Korea, the prevalence of *T. gondii* infection was 1.49% (Jeon et al., 2000), and, in the Czech Republic, it was 7.4% (Hejlicek et al., 1998). When using PCR to determine the prevalence of this infection in wood mice, different studies found the values to be variable: 14% (Kijlstra et al., 2008), 41% (Thomasson et al., 2011) and 35% (Bajnok et al., 2015). The purpose of the present study, therefore, was to explore the prevalence of *T. gondii* in natural populations of wood mice. It also aimed to identify patterns of brain protein expression in wild mice, which were naturally, rather than experimentally, infected with *T. gondii*. The research is described in full in Chapter 4.

1.5 The immune response to *Toxoplasma gondii*

The effects of *T. gondii* on brain cells is immediate. Microarray gene profiling of human foreskin fibroblasts showed that infection with *T. gondii* caused a pro-inflammatory cytokine increase, cytoskeletal rearrangements and protection from apoptosis within 1-2 hours after exposure to the parasite, a response that CNS cells may also have upon infection (Blader, Manger, & Boothroyd, 2001). A recent study using immature human neurons showed that *T. gondii* is able to infect these cells both from healthy and individuals with psychiatric disorders (Passeri et al., 2016). Twenty-four hours post-infection, after 2 to 4 cycles of parasitic replication, signs of metabolic alterations occur, presumably due to nutrient drainage. At that stage, the host's immune response will most likely determine the progression of the disease and whether it will establish a persistent (chronic) infection. This persistence in neurons, microglia and astrocytes is counteracted by a strong host response via an elevation in interferon -γ (IFN-γ), crucial for keeping the infection in check (Sa et al., 2015). Although astrocytes do increase their intracellular IFN-γ secretion via a GTPase activity to clear up to 90% of the parasite, neurons do not (Cabral et al., 2016; Halonen, Taylor, & Weiss, 2001), making the latter essentially a reservoir for parasites (bradyzoite-containing cysts). IFN-γ also plays a key role in recruiting immune cells to the site of infection during the next few days post-infection (Suzuki, 2002). In order for this parasite to be efficiently cleared from the host $CD4^+$ T-helper 1 effector cells must be activated. These cells will then orchestrate the adaptive immune response, by recruiting natural killer cells and macrophages that are able to release nitric oxide (which has microbicidal and microbiostatic effects), and priming cytotoxic $CD8^+$ T cells to kill infected host cells (neurons, microglia, astrocytes and other immune cells) in order to inhibit the spread of tachyzoites. Microglia also possess a defensive mechanism that involves the production of nitric oxide via the inducible nitric oxide enzyme upregulation. Again, this microglial activation is relied on IFN-γ expression along with another crucial cytokine tumour necrosis factor alpha (TNF-a). Other important cytokines that are secreted upon the acute stage of infection are interleukin (IL) IL-12, IL-6, IL-1 and IL-10 (Denkers & Gazzinelli, 1998), but are less important during chronic stages in relation to IFN-γ and TNF-a. Common features of neuroinflammation are activated astrocytes and microglia; these are considered to contribute to the advancement of AD neurodegeneration (Glass et al. 2010).

It is clear that *T. gondii* can infect healthy individuals, and usually there are no apparent clinical symptoms for the majority of the population, while 15-85% of the population is chronically infected, depending on demographics and lifestyle (Flegr, 2007). In those cases, the parasite benefits at the expense of the host, a condition called parasitism, in which an equilibrium is achieved between the parasite and the host's immune system. Therefore, in immunocompromised individuals with or without genetic polymorphisms that predispose them to be highly susceptible to this disease, brain

parasitism is dysregulated leading to pathogenesis and can be fatal or cause permanent brain damage if left untreated. There have been reports indicating that toxoplasmosis has been linked to schizophrenia (IgG antibody *T. gondii* titre in the schizophrenia patients was ~40% higher than in controls), depression, suicidal thoughts and attempts, personality disorders and decreased cognitive performance (Esshili et al., 2016; Hamidinejat et al., 2010; Hinze-Selch et al., 2007; Torrey & Yolken, 2003; T. Wang et al., 2013). These psychiatric illnesses were linked to aging, implying that the effect of a persistent *T. gondii* infection could account for these behavioural disorders, although only serological studies were performed (Alvarado-Esquivel et al., 2012; Beste, Getzmann, Gajewski, Golka, & Falkenstein, 2014; Perry et al., 2016). A recent study performed in a population-representative birth-cohort of individuals which were tested positive for *T. gondii* antibodies in their serum concluded that as far as human behaviour, this parasite was not responsible, contrasting previous reports derived from different patient groups of older age (Sugden et al., 2016). Still, this study did not take into account the correlation of older age (and chronic infection index), since most individuals in this study were between 30 and 40 years of age.

1.6 Diagnosis

When the parasite is able to have access to the host, it is able to transmigrate the epithelial membranes of the gut (and the placenta, gaining access to the unborn baby) (Barragan et al., 2005; Barragan & Sibley, 2002; Da Gama et al., 2004). This penetrance allows access the submucosal tissue, entering the bloodstream and lymphatic system, where it is then able to be detected by (and also infect) cells of the immune system such as neutrophils, granulocytes, natural killer cells, dendritic cells and macrophages (Courret et al., 2006; Denkers et al., 2004; Persson et al., 2009). Thus, the primary detection methods, at least for the stages of acute *T. gondii* detection initially relies on the humoral response to the parasite by the host's immune system. Remington et al. proposed in 1968 that the presence of IgM antibodies in the cord blood is a reliable indicator of toxoplasmosis since they cannot cross the placenta as the IgG subclass can (Remington, Miller, & Brownlee, 1968; Shaddel et al 2007). In fact, IgM antibody detection in the sera indicates an acute infection, while an IgG antibody detection can also mean a past or cleared infection. IgA antibodies can sometimes be elevated due to a chronic infection (Miller, Sunshine, & Remington, 1969). Remington also perfected *Toxoplasma* antibody detection by incorporating it to an enzyme-linked immunosorbent assay (ELISA) (Remington, 1969), while this method has also been evolved to detect polymorphic synthetic polypeptides (Kong, Grigg, Uyetake, Parmley, & Boothroyd, 2003). To that end, a number of serological methods have been developed, outlined below:

The most primitive one, now obsolete by several reference laboratories, is the dye test, developed in 1948 (reviewed in (Reiter-Owona et al., 1999)). The modified agglutination test (Fulton & Turk, 1959), the latex agglutination test (Mazumder, Chuang, Wentz, & Wiedbrauk, 1988) and the indirect hemagglutination test (Balfour, Bridges, & Harford, 1980). All of these tests rely on a reserve of *T. gondii* antigens (i.e. live cultures), which makes them unfavorable for routine detection.

More advanced methods of detection tried to depend on using platforms coated with specific human antibodies raised against *T. gondii*. These include the aforementioned ELISA assays, the indirect fluorescent antibody test (reviewed in (Rodrigues, Castro, Gomes, Amaral, & Avelino, 2009)), the immunosorbent agglutination and the piezoelectric immuno-agglutination assays (Desmonts, Naot, & Remington, 1981; H. Wang et al., 2004) and the immune-chromatographic test (Y. H. Wang et al., 2011). Finally, avidity tests can provide a snapshot of the timeline of *T. gondii* infection, even years after the first contact with the parasite (Abdel Hameed & Helmy, 2004; Pietkiewicz et al., 2007), (Gutierrez, Rodriguez, Piedrola, & del Carmen Maroto, 1997).

In addition to antibody-based detection techniques, molecular biology tools were employed. In 1989 Burg et al. managed to isolate *T. gondii* DNA from the blood by polymerase chain reaction (PCR) (Burg, Grover, Pouletty, & Boothroyd, 1989). For this method, a number of primers were raised to several genes' sequences, in order to create a highly sensitive method of detection. These genes are; the B1 gene, the internal transcribed spacer (ITS-1), the 529 base-pair repeat element, the 18S ribosomal DNA, as well as SAG1, SAG2 and GRA1. Real-time quantitative PCR has also been used, able to detect minuscule amounts of *T. gondii* DNA present in the bloodstream, amniotic fluid and cerebrospinal fluid (Kompalic-Cristo, Frotta, Suarez-Mutis, Fernandes, & Britto, 2007). The addition of molecular probes helped doctors to assess whether the parasite has been passed from an infected pregnant mother to the fetus.

Based on PCR, more expensive techniques, but of the same specificity, have been also introduced, such as the loop-mediated isothermal amplification, which targets four or five target genes on the same sample (Lau et al., 2010). Microsatellite analysis using tandem-short DNA motif repeats and microsatellite sequences are also in place for more accurate genotyping of the parasite, while multilocus sequence typing using single nucleotide polymorphisms (SNPs) has increased resolution by several-fold (Blackston et al., 2001). Other less used techniques include modified PCR protocols that involve restriction endonucleases, which then run on agarose gel electrophoresis, random amplified polymorphic DNA-PCR, and high resolution melting post-PCR.

Finally, a powerful, yet laborious, technique for post-natal (3-month old) diagnosis of congenital toxoplasmosis is Western Blotting (i.e. protein/antigen detection combined with antibody specificity) (Robert-Gangneux, Commerce, Tourte-Schaefer, & Dupouy-Camet, 1999). Of note, this method has a high sensitivity in detecting IgG antibodies in human saliva (Stroehle, Schmid, Heinzer, Naguleswaran, & Hemphill, 2005). To identify IgG interactions with *T. gondii* antigens when the titres of IgG are low, sensitive western blot (WB) assay could be useful. There are a number of antigens that IgG may detect, including the major tachyzoite surface protein, SAG-1. A commercially available kit (WB Toxo GII; LDBio) demonstrates 100% specificity and 99.2 % sensitivity for this type of analysis (Franck et al., 2008).

1.7 Histological changes associated with *Toxoplasma gondii* infection in the human brain

T. gondii is capable of infecting the central nervous system (CNS), by bypassing the highly-selectable blood-brain barrier using cells of the immune system as sentinels (Feustel et al., 2012; Fischer et al., 2000). Once in the brain, the parasite is able to form cysts throughout, with no apparent specificity (Berenreiterova et al., 2011). In chronic infections, serological screening may show no signs of infection, as IgM antibodies against *T. gondii* may not be present. But, this does not mean that the brain does not possess cysts of *T. gondii*. Although computed tomography (CT) and magnetic resonance imaging (MRI) are available for screening the human brain in a non-invasive manner (Collins & Cromwell, 1980), (Di Cristina et al., 2008), the definitive method for cyst identification and potential changes in brain morphology due to infection is histology.

There is a vast literature concerning histological findings of *T. gondii* cysts in the brain. An elegant study by Gatkowska et al. tried to assess cyst formation and behavioral changes in the mouse brain (Gatkowska, Wieczorek, Dziadek, Dzitko, & Dlugonska, 2012). Figure 1.5 depicts the findings of hematoxylin and eosin staining on brain slices from mice injected with the intermediate virulent *T. gondii* ME49 stain, in order to induce experimental toxoplasmosis. These cysts are distinct by their round morphology and darker in color than the surrounding tissue. Smaller, darker dots are found, which are bradyzoites. When cysts from the non-virulent HIF strain of *T. gondii* were injected into 6-month old mice, showed an uneven distribution of large cysts (>60 um) in the cerebral cortex (although not statistically different) (Berenreiterova et al., 2011) (Figure 1.6).

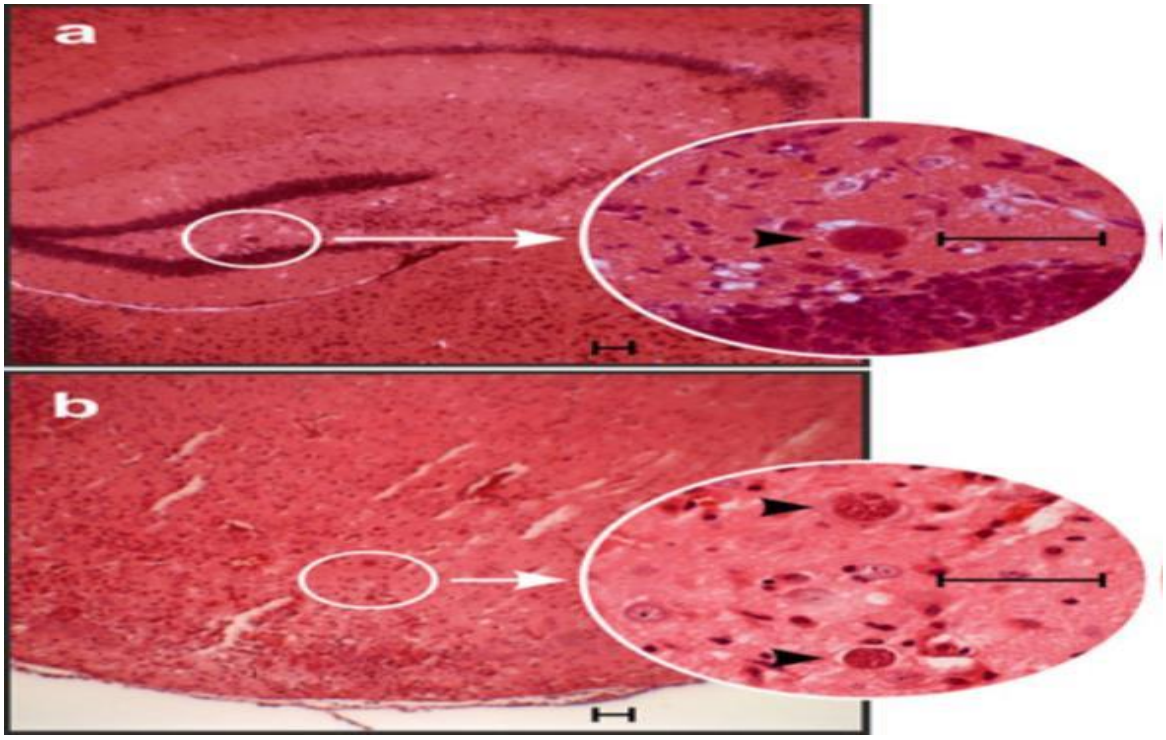


Figure 1.5: Light micrographs brain of sections in toxoplasmosis-induced mice. Exported from (Gatkowska et al., 2012). The characteristic demarcated cysts (black arrowheads) are present in the hippocampus (a) and amygdala (b), with an approximate diameter of 15 μm. Scale bar indicates 50 μm.

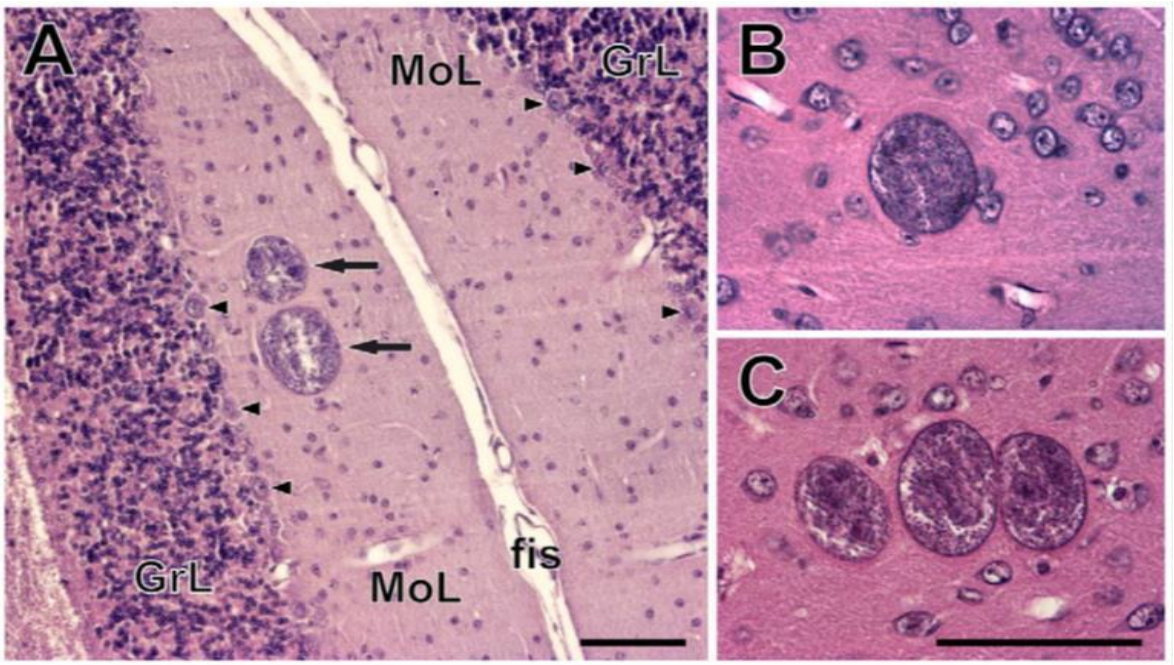


Figure 1. 6: Adapted from (Berenreiterova et al., 2011). Chronically infected CD1 mice were infected with cysts. (A) shows a pair of cysts (arrows) in the cerebellar cortex. Smaller round structures are Purkinje cells (arrowheads). GrL, granular layer; fis, cerebellar fissure; MoL, molecular layer. Higher magnification photos of a single cyst (B) and a cyst triad (C) in the isocortex. Scale bar, 100 μm.

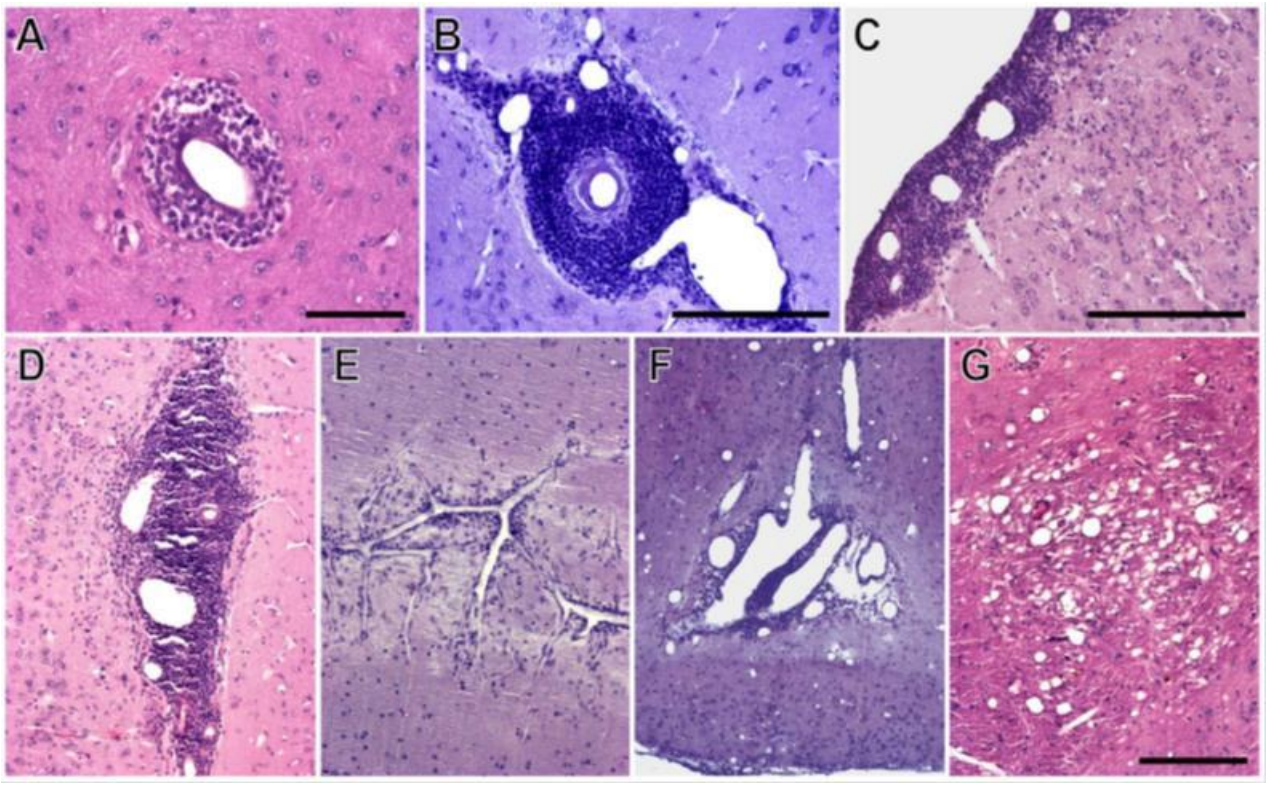


Figure1.7: Adapted from (Berenreiterova et al., 2011). Histopathological lesions due to latent toxoplasmosis. Inflammatory cells infiltrated the perivascular and leptomeningeal spaces (A–E), with on the surface of cerebral cortex (C), between the hemisphere region (D) and the cerebellum (E). (F) depicts potential necrosis occurring in the hypothalamus and (G) vacuolization of the thalamus. Scales, 100 um (A), 200 um (B, C, G, in G for D–G).

As such, the presence of a pathogen in the brain causes secondary lesions, primarily due to the increased presence of inflammatory cells (Figure 1.7). Although H&E staining has been informative, a better understanding of the spread of *T. gondii* infection in the brain has been achieved recently by the use of the Cre-LoxP system, able to trace the parasite in a spatiotemporal manner (Cabral et al., 2016). This powerful tool was able to detect with high specificity the CNS and immune cells that the parasite was able to infect, along with its distribution to the brain (Figure 1.8). Further, since there has been reported a link between reduction in AD and chronic toxoplasmosis, H&E and fluorescent immunolabeling have supported this notion by the reduction of Aβ plaque formation, were cysts were more prevalent (Figure 1.9) (Mohle et al., 2016).

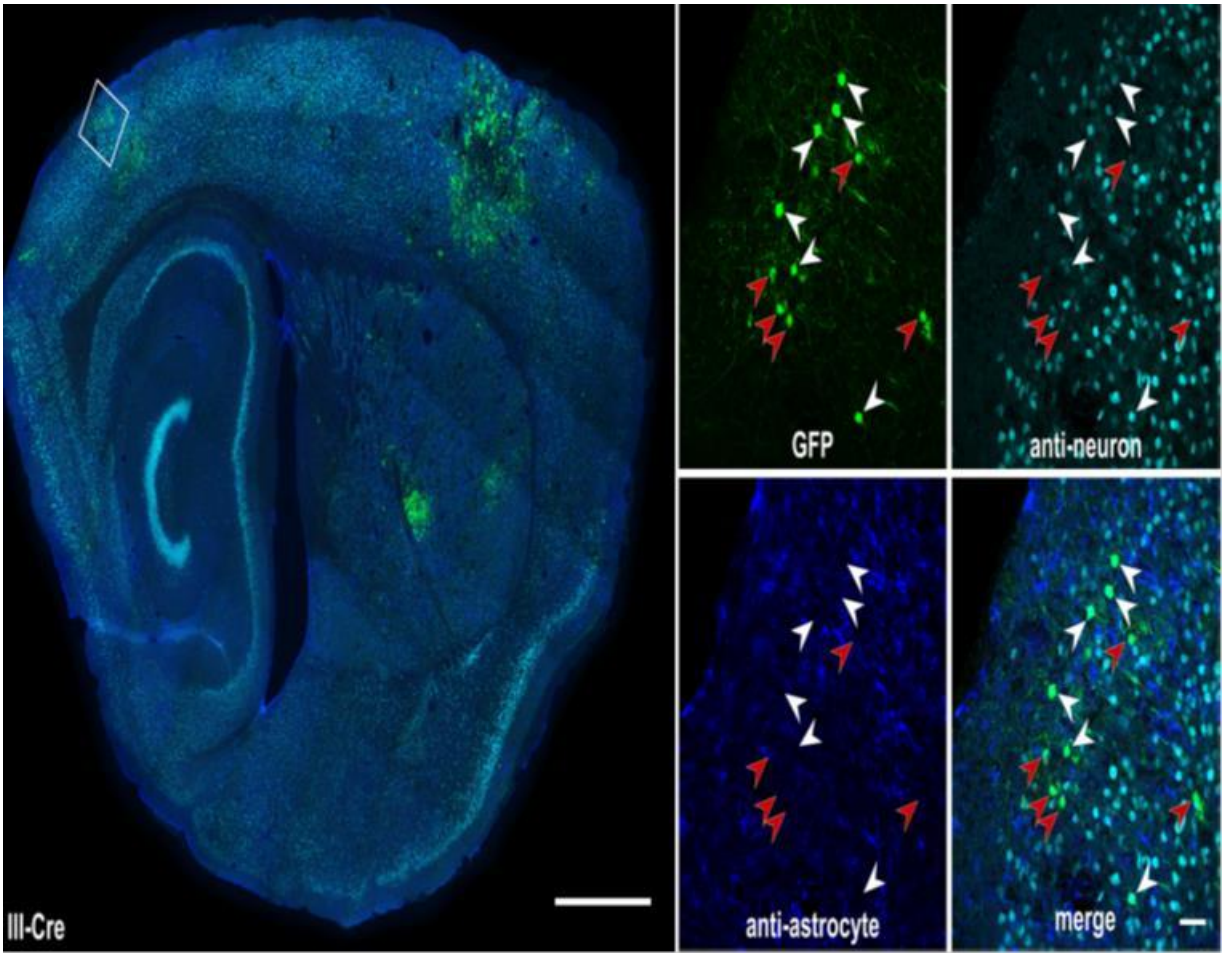


Figure 1.8: Adapted from (Berenreiterova et al., 2011; Cabral et al., 2016). A cre-mediated system for *Toxoplasma* infection tracing. Cre-mice were injected with III-Cre *T. gondii* and the infected brain was visualized 3 weeks post infection. Left image represents a whole brain slice. GFP indicate where *T. gondii* has been observed. Right, microphotographs show the presence of *T. gondii* (GFP) present in neurons (upper right), but not in astrocytes (lower left).

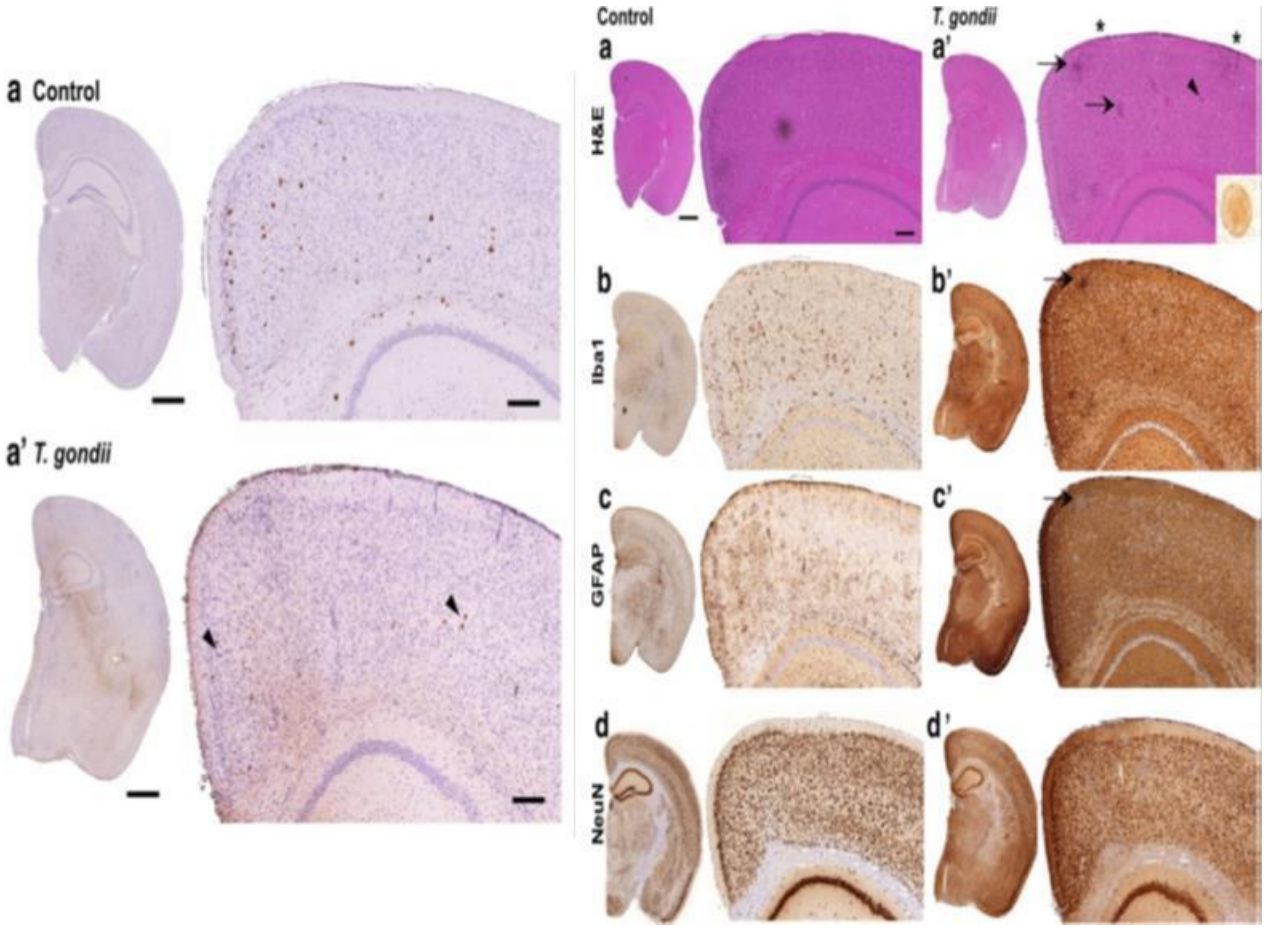


Figure 1.9: Adapted from (Mohle et al., 2016). Right; Uninfected and infected mouse brain showing the extent of A β labelling via immunohistochemical analysis along with H&E staining. Note the reduced plaque formation in the *T. gondii* cortical regions of the brain (black arrowheads). Scale bars 1000 μm (left) and 200 μm (right). Left; substantial histopathological changes occur in neurons (NeuN), astrocytes (Iba1) and microglia (GFAP). Scale bars represent 1000 μm (left column) and 200 μm (right column). Adapted from (Mohle et al., 2016).

1.8 Molecular inflammatory effects of *Toxoplasma gondii* infection in the human central nervous system

Txoplasmosis occurs when the *T. gondii* parasite has successfully invaded the host and established disease. A characteristic of the disease, in humans, is the presence of cysts in the central nervous system (CNS), causing cerebral toxoplasmosis, and in the muscular system (Nath and Sinai, 2003) (Figure 1.10). *T. gondii* parasites are able to establish a chronic infection, which in immunocompetent patients may be asymptomatic with no signs of infection and increased inflammation.

Yet, in immunocompromised patients, for example, those infected with the human immunodeficiency virus (HIV), active toxoplasmosis may cause severe encephalitis, which is fatal if left unchecked (Luft and Chua, 2000). Even in non-immunocompromised patients, *Toxoplasma* infection has shown to be involved in certain behavioural alterations such as cryptogenic epilepsy (Yazar et al., 2003) along with a modest yet significant increase of psychosis, major depression and/or schizophrenia in patients positive for anti-*Toxoplasma* IgG antibodies (Torrey et al., 2007; Al-Hussainy 2015). Therefore, a causal link between a functional immune system, *T. gondii* latent infection and inflammation in the CNS is evident and may be important for understanding CNS-related diseases.

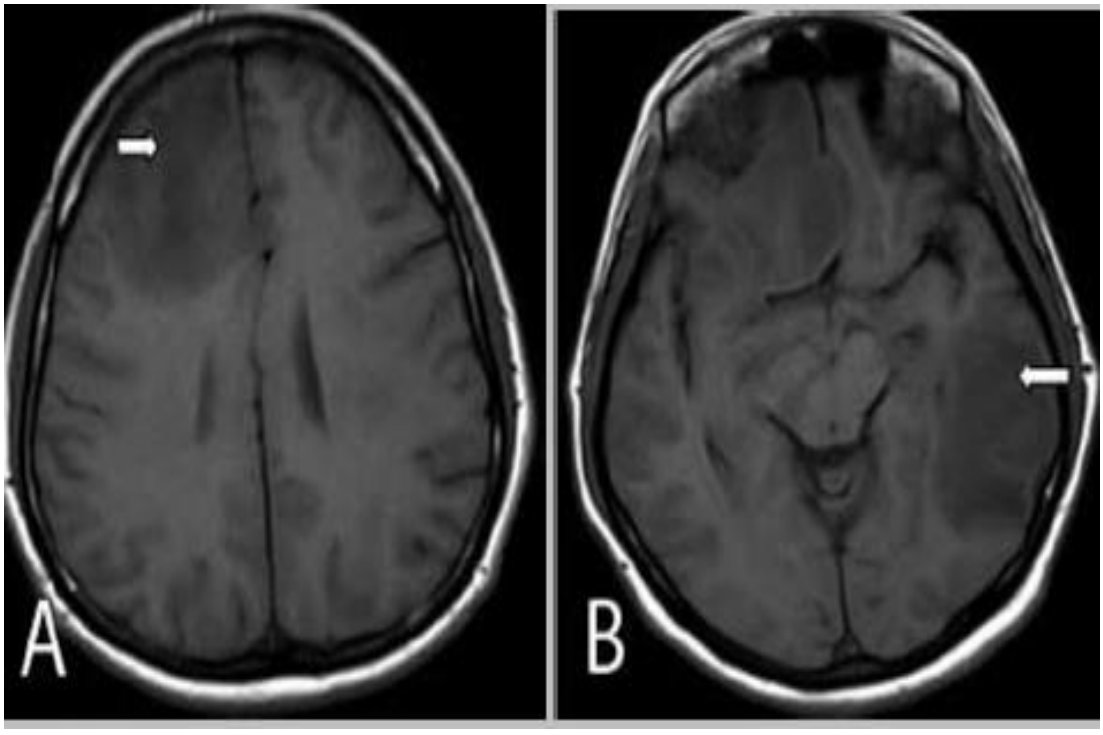


Figure 1.10. A case report of a patient admitted to hospital with a headache and neck pain for one week, along with persistent weight and appetite loss. Axial T1 images of the brain using Magnetic resonance imaging

(MRI) showed multiple hypointense lesions involving the right frontal (A, right arrow) and left temporoparietal lobes (B, left arrow) suspected to be caused from toxoplasmosis. Adapted from (Ramachandran et al., 2014).

When the cysts containing bradyzoites reach the CNS and switch to tachyzoites, the fast-replicating form of the parasite, a rapid immune response occurs with signs of increased inflammation of resident brain cells (reviewed in (Aliberti, 2005)). In humans, the correlation between brain cyst location and inflammation has not been shown, to date (Parlog et al., 2014) (Figure 1.11). The continuous secretion of inflammatory mediators are toxic to neurons and can cause neuroinflammation and neurodegeneration.

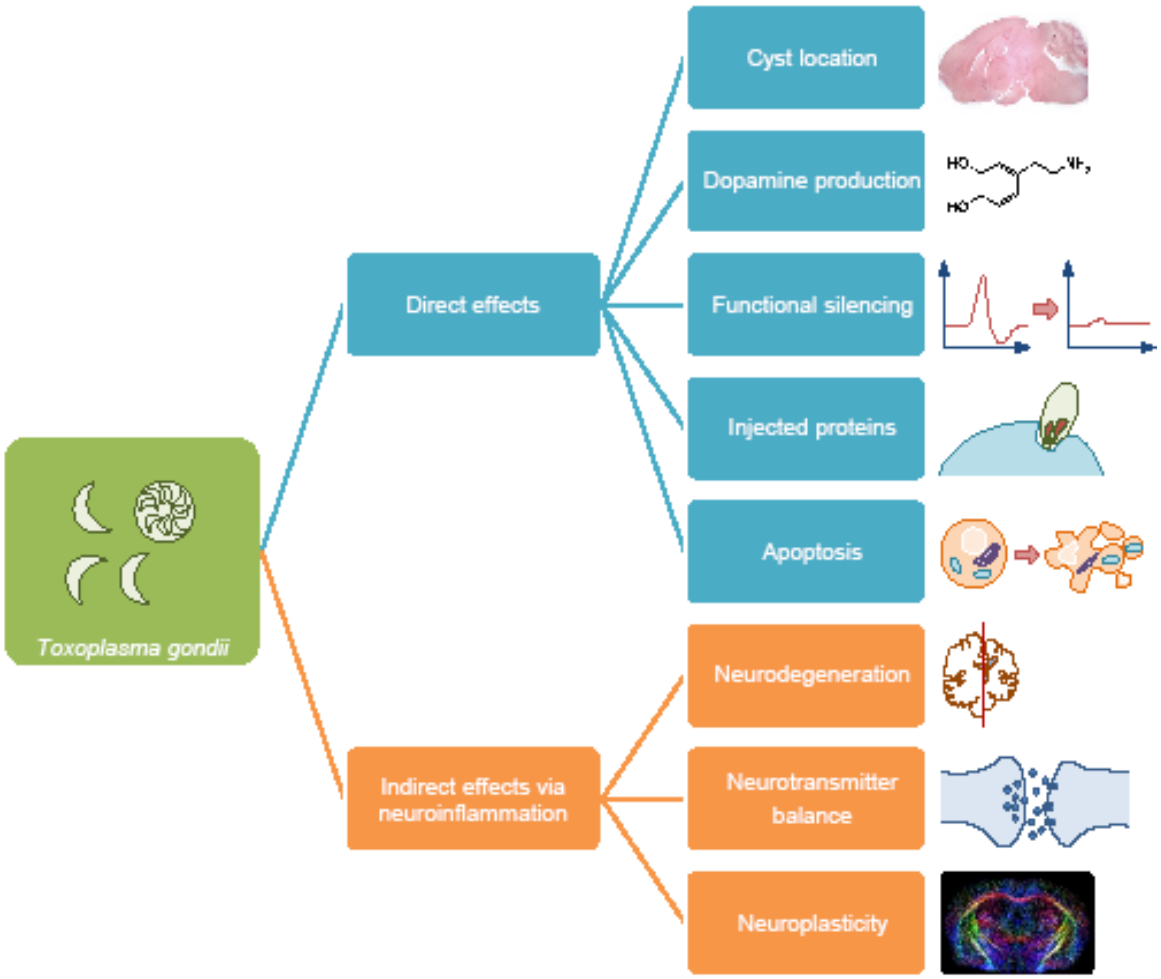


Figure 1. 11. *T. gondii* infection elicits systemic and localised effects on the CNS. Behavioral changes do occur in experimental murine models as well as in humans that have been challenged chronically with *T. gondii*. Specifically, subtle modifications in neuronal function and structure are evident. that may disrupt the physiological function of the CNS. Adapted from (Parlog et al., 2014).

From an evolutionary perspective, *T. gondii's* primary goal is to establish an infection inside the host, while keeping the host alive for as long as possible, is favourable for the parasite's survival and chance to infect another host. As such, although modest inflammation does occur at least in immunocompetent patients, it seems to be kept at bay. The classical inflammasome is initiated by innate immunity and involves the secretion of interleukin-12 (IL-12), interferon- γ (IFN- γ), nitric oxide (NO) and possibly tumor necrosis factor alpha (TNF-a) from Natural Killer (NK) cells, B lymphocytes and neutrophils as well as form local antigen-presenting cells (APCs) such macrophages and dendritic cells (DCs) causing systemic inflammation (Hunter et al., 1994, Sher et al., 1993). The parasite is able to skew this type-1 immune response, especially in late/chronic phases by inducing DCs to secrete anti-inflammatory cytokines such as transforming growth factor beta (TGF-β), IL-4, IL-27 and IL-10 (Gazzinelli et al., 1996) (Figure 1.12).

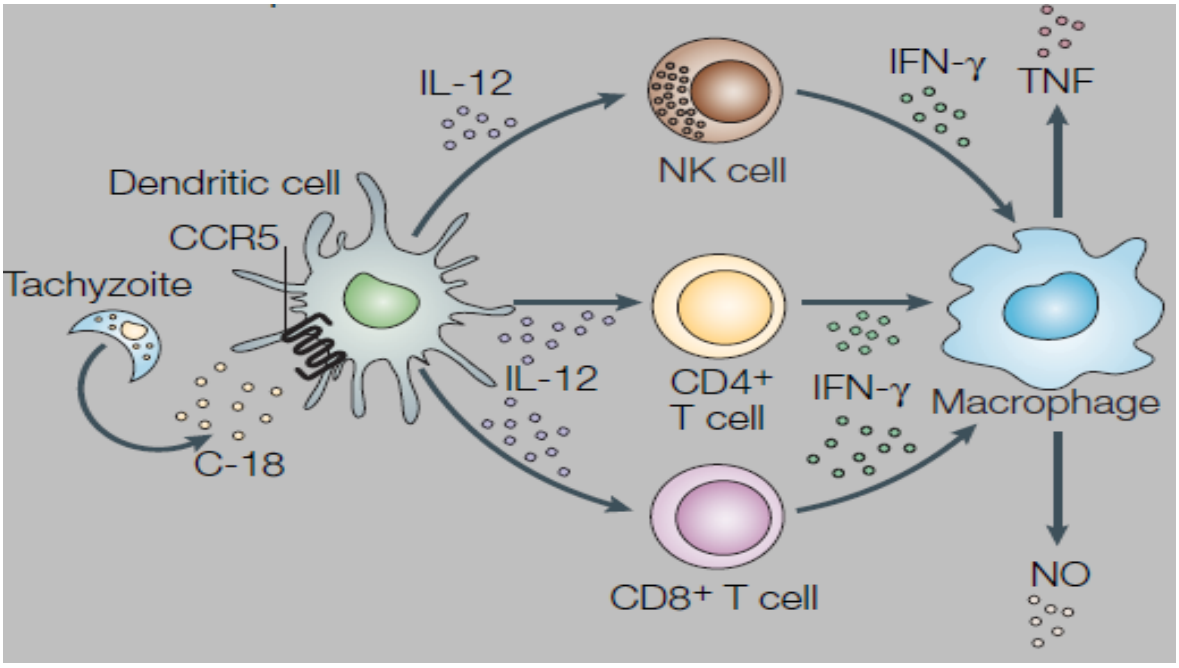


Figure 1.12. Upon the acute phase of infection, DCs secrete cytokines in response to products secreted by *T. gondii*. These cytokines activate (eventually) both CD4+ and CD8+ T lymphocytes and NK cells, along with eliciting macrophage activation for secreting TNF and NO. Adapted from (Aliberti, 2005).

In the brain, a neuroinflammatory milieu can occur from *T. gondii* infection by direct infection of resident macrophages, microglia, which secrete a similar array of the aforementioned cytokines, along with a centralised Th-1 type immune response. This alteration of the CNS microenvironment does alter neuronal function (Parlog et al., 2015). In addition, neurons and astrocytes have been

documented to be directly infected by the parasite both in tachyzoites and bradyzoites, which in turn may potentially cause localised inflammation (Halonen et al., 1996, Schluter et al., 2001). For a description of the effects the parasite induces to the immune system, in relation to CNS infection, please see Table 1, below.

Table 1.1 The immunologic response of CNS-resident cells to *T. gondii* infection. Adopted from (Kamerkar and Davis, 2012).

Brain cell type	*T. gondii* stage	Immune Activity in relation to CNS
Neuron	Tachyzoite	Parasites are able to infect neurons, directly
Neuron	Tachyzoite	Cytokine and chemokine production possibly favouring parasitic growth within neurons
Neuron	Bradyzoite	$CD8^+$ T cell detection evasion
Microglia	Tachyzoite, bradyzoite	Microglia cells are infected primarily, although parasitic growth is inhibited within the CNS
Microglia	Tachyzoite	IL-1 beta, IL-10 and TNF-a microglial-derived production is induced
Astrocyte, microglia	Tachyzoite	Infection reduces Major Histocompatibility Class (MHC) II expression
Endothelium	Tachyzoite	ICAM-1, IFN-γ R upregulation
Endothelium	Tachyzoite	Infection induces ICAM-1, IL-6, and MCP-1

Microglia, endothelium	Tachyzoite	CNS inflammation control via upregulation of CD200R and CD200 (OX-2 membrane glycoprotein)
Microglia	Tachyzoite	Major $CD8^+$ T cell-mediated activation with secretion of IL-12p40, iNOS, IL-1beta, TNF-a and MHC class I & II, ICAM-1 and LFA-1 upregulation
Astrocyte	Tachyzoite, bradyzoite	Tissue Inhibitor of Metalloproteinases-1 (TIMP-1) is induced by infection, invoking extracellular matrix alterations
Astrocyte	Tachyzoite	IFN-γ-activated indoleamine 2,3-dioxygenase (IDO) production inhibits parasitic growth. Autophagy may be involved

One important secreted substance, NO, has a toxic effect on neurons and has been implicated in a number of neurodegenerative and demyelinating diseases such as AD and Multiple Sclerosis (MS) (Giovannoni et al., 1998, Law et al., 2001). Yet, classical markers for neuronal death used in mice, such as anti-NeuN antibody and an anionic fluorescein derivative for detecting neuronal death (Schmued and Hopkins, 2000) did not show an increase in CNS-neuronal death (Jung et al., 2012). This implies that (i) *T. gondii* might be protective against neuronal death and this protection may be conferred via (ii) activated microglia (Rozenfeld et al., 2005). Rozenfield et al. showed that IFN-γ -activated microglia infected with the parasite, showed a proportional decrease in the production of inducible NO Synthase (iNOS), while TGF-β1 production was upregulated (Rozenfeld et al., 2005). In a study by Li et al. (2012) that compared *T. gondii* (virulent RH strain) infection in resistant and susceptible hosts, a correlation was detected between high iNOS and low arginase levels in resistant hosts (rats), whereas in susceptible hosts (mice) the correlation was with high arginase and low iNOS levels. Moreover, in the five strains of inbred rats, the study showed that

the ratios of iNOS/arginase and resistance to *T. gondii* (RH strain) were variable. To develop a systematic understanding of the situation with wood mice, the present study focused on their resistance and susceptibility to *T. gondii* infection. The results of this natural study may be useful to understanding aspects of toxoplasmosis in humans.

The link between AD severity/onset and neuroinflammation is well-established (Bouvier and Murai, 2015, Prokop et al., 2013, Combs, 2009), with many similarities and substantial differences when compared to autoimmune disorders such as MS (reviewed extensively by (Schwartz and Deczkowska, 2016)) (Figure 1.13). In the latter, exacerbation of systemic inflammation (increased IFN- γ and localised immune activation, and invasion, is evident with a breach of the Blood-Brain-Barrier (BBB) and the choroid plexus (CP) (Fabis et al., 2007, Dendrou et al., 2015). Its pathology is characterised by demyelinated areas both in the brain and spinal cord, with astrocytic aberrant proliferation. As the disease progresses, $CD4^+$ T, $CD8^+$ T and plasma B cell infiltration is evident with establishing an inflammatory niche within the CNS (Dendrou et al., 2015). In a similar fashion, Parkinsons Disease (PD) is characterised by increased inflammation since TNF-a attenuation prevents disease onset in experimental animals (McCoy et al., 2006).

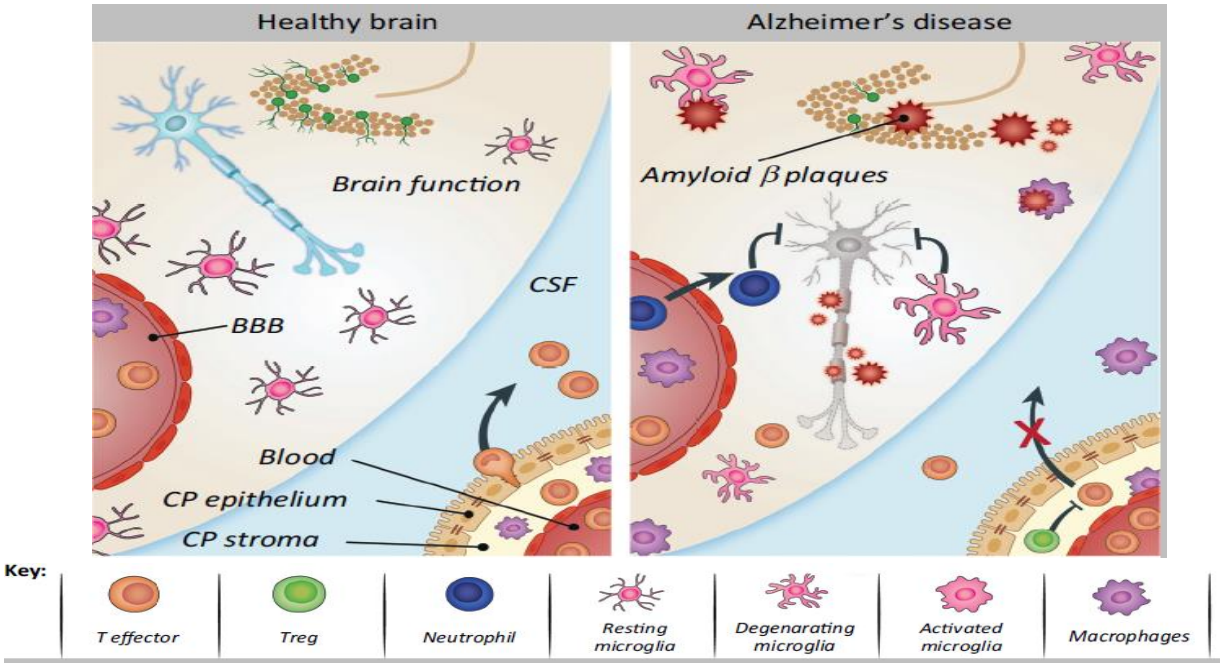


Figure 1.13. The immune system indirectly supports optimal CNS function and low-level regeneration from the choroid plexus (CP) and the cerebrospinal fluid (CSF), with the blood-brain barrier (BBB) preventing a direct access to the CNS. During AD, amyloid beta aggregates cause aberrant microglial activation, which involves neuronal dysfunction and BBB breaching primarily by neutrophils. Peripheral macrophages are unable to clear the plaques due to the inability of the CP epithelium to allow access to those cells. Adapted from (Schwartz and

Deczkowska, 2016).

In the former, neuroinflammation is somewhat suppressed with an altered regulation of the adaptive immune response, as observed by the increased presence of $Foxp3^+$ T regulatory (T_{regs}) (in mice) and $CD4^+$ T-helper 1 (T_{h1}) lymphocytes (Baruch et al., 2015) as well as decreased phagocytic capability (Hickman et al., 2008). Specifically, activation of microglia in AD is caused by soluble amyloid beta (A β) aggregates as well as hyperphosphorylated Tau proteins. In this manner, *T. gondii* chronic infection of the brain may be a primer for AD's onset since the pathophysiology and immunological footprint between the two conditions is similar. It is therefore clear that there is a cross-talk between AD and immune system homeostasis, although it has not been established if inflammation drives the disease or is a secondary phenomenon (Wyss-Coray and Rogers, 2012). In respect to other age-related CNS diseases, such as dementia onset, and its subsequent progression, there is no consensus on the role of inflammation in epidemiological studies (Peila and Launer, 2006), while a causal link between chronic depression (that encompasses chronic inflammation) and dementia has been shown (Leonard, 2007, Leonard and Myint, 2006). In addition, it has not been established whether neuroinflammation is neuroprotective or neurodegenerative when it comes to cognitive disorders. This discrepancy has pushed towards a re-evaluation of treating CNS-related diseases with anti-inflammatory agents, by introducing a spatiotemporal factor to it. To this end, non-steroidal anti-inflammatory drugs (NSAIDs) have been introduced in preventing CNS-related disease by targeting Cyclooxygenases (COX) (Warner and Mitchell, 2004). Concerning AD, COX-2 inhibitors have shown very promising results in reducing disease risk, but this success was not shown in subsequent clinical trials underscoring the importance of when, for how long, and which preventive agent needs to be administered for an optimal outcome (Koch and Szecsey, 2000, Reines et al., 2004).

As the search for preventing or ameliorating age-related CNS disease continues, it is important to assess whether inflammation is a friend or foe. Evidently, low-level inflammation is important to be maintained for immuno-privileged sites such as the CNS, ensuring that there is no abnormal immune activation (Elahy et al., 2015). Yet, heightened immune cell infiltration is a key factor in disease exacerbation. Following this notion, *T. gondii* infection may provide an advantage towards preventing, or significantly delaying, the onset of AD or other neurodegenerative diseases. It has yet to be shown that an attenuated non-pathogenic strain of this parasite may be used as a preventive agent against experimentally-induced CNS disease.

Another important immune effector mechanism is the activation of the complement system (Tenner, 1999). This system is able to recognise pathogenic molecular patterns including apoptotic or

necrotic cells. This is achieved via C1q or C3 proteins or through mannose-binding receptors (Sahu and Lambris, 2001). Once activated, the complement pathway can attract immune cells, promote phagocytosis and/or cytolysis (via the formation of the membrane attack complex). In AD, with the presence of A β and Tau proteins, and perhaps in MS, with the presence of necrotic cells, the complement system is overactivated in the CNS, initiating a vicious circle of white and grey matter degeneration. Additionally, activation of innate immune pattern recognition receptors, such as Toll-like receptors (TLR), may also confer to CNS diseases, including AD (Janeway and Medzhitov, 2002, Su et al., 2016, Gambuzza et al., 2014, Landreth and Reed-Geaghan, 2009). This is turn activates local cells such as microglia and astrocytes and oligodendrocytes inducing neuronal stress, but this is yet to be shown to cause protection or degeneration across all patients examined, due to a vast array of different conditions used in the clinical trials [for a review (Wyss-Coray and Rogers, 2012)].

The vast difference in the response to NSAIDs and other drugs to CNS-related disease may also be due to genetic variability between individuals i.e. genetic polymorphisms. Indeed, two genome-wide association (GWA) studies have indicated two genes named Clusterin and Complement C3b/C4b Receptor 1 (CR1) as important for sporadic AD onset (Harold et al., 2009, Lambert et al., 2009). Additionally, age-related transcriptional changes play a pivotal role in disease progression (Blalock et al., 2005) as shown by gene expression arrays pinpointing differences in lymphocytic, inflammatory and immune pathways from young vs. old and age-matched patients (Fiala et al., 2007, Kalman et al., 2005, Maes et al., 2007). It is therefore evident that the genetic makeup of each patient needs to be explored when assessing for any CNS-related disease in order to decide which drugs are more appropriate to be administered.

1.9 *Toxoplasma gondii* infection in Alzheimer's disease

Based on the knowledge that It is, therefore, plausible to examine whether a chronic *T. gondii* infection increases the risk for age-related diseases that affect brain function. In 2011, AD was a devastating neurodegenerative disorder affecting about 26 million worldwide (Reitz, Brayne, & Mayeux, 2011) , whereas, in 2017, dementia affected around 50 million people who suffer globally (ARUK, dementia statistics, 2017) and the effect of *T. gondii* on this multifactorial disease is under debate (Kusbeci, Miman, Yaman, Aktepe, & Yazar, 2011). One factor in AD pathology that has been identified as being

of significance is inflammation in the central nervous system (CNS); this is known as neuroinflammation (Heppner et al., 2015). AD is categorised as impaired cognition that in its advanced stage, leads to functional impairment. Early stage symptoms of AD include apathy, depression, short-term memory loss, and planning and problem-solving difficulties (Bäckman, 2004). As the disease progresses to moderate AD, sufferers experience aphasia, disorientation, irritability and long-term memory loss, (DeFina, 2013). Patients with advanced AD become unable to communicate and move independently. For over one hundred years, AD neuropathology has been characterised by the presence of diverse microscopic lesions. These include amyloid/senile plaques, cerebral amyloid angiopathy and glial responses, granulovacuoles and eosinophilic rod-like structures (Hirano bodies), neurofibrillary tangles, Serrano-Pozo, 2011). However, amyloid plaques and neurofibrillary tangles are the primary physical traits of AD; it is the presence of these features that are used to confirm a diagnosis of AD (Perl, 2010; Tanghe, 2010).

A paper published in 2011, reported serologic rate comparison of anti-*T. gondii* antibodies between 34 AD patients of 53-84 years of age and 37 healthy individuals of 56-69 years of age, and it was found that antibody levels were higher in the AD group (Kusbeci et al., 2011). Although this result was significant, the sample size was too small and acute *T. gondii* infection (IgM anti-*T.gondii* serology) was not performed. This could have been assessed by investigating IgG and IgM anti-*T. gondii* levels. In 2016, Perry et al. performed a similar study on 105 subjects diagnosed with AD against 114 age-matched controls (up to 98 years of age and as young as 62 years of age) without signs of dementia (Perry et al., 2016). The authors did find a 10% higher prevalence of latent toxoplasmosis in AD patients (40% in AD against 30% in healthy controls), but this finding was not statistically significant. Based on these studies, it is not conclusive on whether *T. gondii* is involved in AD pathophysiology, but given the impact of *T. gondii* infection on the inflammatory process, the ability of the parasite to cross the BBB and the understanding that multiple cells types of the CNS can become infected, one might predict that *T.gondii* infection could influence AD disease pathogenesis. Of note, toxoplasmosis has only recently been reported to be involved in other neurodegenerative diseases such as anosmia and Parkinson's disease, yet the nature of this involvement remains unclear (Alvarado-Esquivel et al., 2017; Celik et al., 2010; Mahami Oskouei et al., 2016; Miman, Kusbeci, Aktepe, & Cetinkaya, 2010; Prandota, 2014).There is currently no cure for AD and no treatment for slowing down the progression or reversing the disease. Recently, two large clinical trials with drugs that interfere with Aβ production or its clearance that hypothetically could slow down the progression of the disease were unsuccessful (Prins, Visser, & Scheltens, 2010; Salloway et al., 2014). Although the negative results of these clinical trials could be related to the study design or the choice of dosage, it is increasingly

recognized that the understanding of the mechanisms of AD pathogenesis is paramount for efficient drug designing.

The ApoE gene is highly expressed in the liver and brain (Zannis, Cole, Jackson, Kurnit, & Karathanasis, 1985). A very large number of studies has shown that ApoE gene mutations are a major risk factor for AD (Mahley, Weisgraber, & Huang, 2006), with ApoE being a main protein of the lipoprotein transport system that plays a critical role in atherosclerosis, dyslipidaemia and various neuropathological processes (Mahley et al., 2006). Studies in ApoE-deficient mice infected with *T. gondii* showed an exacerbation of atherosclerosis, with a concomitant reduction in serum lipoproteins and cholesterol (Portugal et al., 2004). The authors stipulated that the parasite was involved in the pro-inflammatory response in the aortae and modulation of the lipoprotein transport system, common to AD and atherosclerosis. Growing evidence suggests that neuroinflammation may also play an important role in the initiation or progression of AD (Glass, Saijo, Winner, Marchetto, & Gage, 2010; Wyss-Coray, 2006) and as described previously, *T. gondii* infection is known to induce neuroinflammatory responses (Jung et al 2012; Lang 2018). It has been hypothesized that chronic activation of inflammatory pathways may be either the consequence of or the driving force for AD pathogenesis and *T.gondii* might have a role in changing the inflammatory state of the brain thus influencing AD risk and/or disease progression. It is also possible that some aspects of inflammation may be protective against AD or that the disease process inhibits beneficial inflammatory responses (Glass et al., 2010; Wyss-Coray, 2006). The next section will explore the hypothesis that chronic toxoplasmosis may be protective against the onset of AD.

1.10 Chronic Toxoplasmosis may protect against the onset of Alzheimer's disease

It has been suggested that the production, oligomerisation and deposition of A β play central roles in AD (Haass & Selkoe, 2007). Aβ is a ~4 kiloDalton (kDa)) peptide fragment generated by sequential proteolytic cleavage of the transmembrane amyloid precursor protein (APP). Intraneuronal accumulation of Aβ is considered an early event in AD pathogenesis, leading to brain plaque formation (LaFerla, Green, & Oddo, 2007; Oddo et al., 2003; Welikovitch 2018). This involves a number of cells of the immune and CNS, while pathogens could be causative factors in the pathophysiology of this disease (Harris & Harris, 2015; Krstic et al., 2012). Interestingly, chronic *Toxoplasma* infection with Type II *T. gondii* cysts, ameliorated age-dependent Aβ accumulation in the brain, possibly by

indirectly promoting myeloid-derived monocytes to differentiate into macrophages which were able to phagocytose and degrade soluble A β (Mohle et al., 2016).

When the humanised mouse model of AD was employed (Tg2576 mice expressing the mutated human APP695 gene (Hsiao et al., 1996)) for investigating the effect of latent *T. gondii* infection and AD progression, it was shown that not only A β deposition was inhibited, rather a more extensive suppression of AD pathogenesis takes place (Jung et al., 2012). Specifically, latent toxoplasmosis inhibited spontaneous neuronal degeneration that usually occurs in this mouse model from 6 months onwards. This effect was inhibited possibly due to the increase of the immune-supressing cytokines IL-10 and Transforming Growth Factor beta (TGF -β) while having no effect on IFN- γ levels. As aforementioned, microglia are able to produce an inducible form of nitric oxide, which although protective against intracellular pathogens, it may cause damage to nearby cells. Upon co-inoculation of *T. gondii* lysates and IL-10 or TGF -β microglia reduced their nitric oxide synthetic capability, which is known to be important for AD pathogenesis. In order for the authors to confirm that there were behavioural changes to this model, Morris water maze and the Y-maze behavioural tests were used, indicating a marked decrease in learning and memory impairments, in comparison to controls (Jung et al., 2012).

This implies the potential of regulatory T cells (T_{reg}) to be employed by latent toxoplasmosis to the sites of infection. It has been documented that these cells do inhibit AD progression in mice (Dansokho et al., 2016). These immune cells are $CD4^{+}CD25^{hi}Foxp3^{+}$ (both in human and mice) and they can be readily detected by triple immunofluorescent labeling in brain tissue slices. Additionally, IL-17-producing T cells ($T_{reg}17$) have also been shown to confer to CNS age-related disease in humans (Singh, Schwartz, Sandrock, Bellemore, & Nikoopour, 2013). It is plausible that *T. gondii* primes the recruitment and proliferation of specific T_{reg} types to the sites of infection and as a secondary effect, it offers protection against age-related A β plaque formation and AD onset and/or progression.

1.11 Amyloid precursor protein in Alzheimer's diseases

One important factor related to AD's aetiology is amyloid precursor protein (APP) deposits (Beyreuther & Masters, 1991). Found in various tissues, the integral membrane protein, amyloid precursor protein (APP), is also present at the synapses of neurons. Whilst APP's function in non-disease states has yet to be fully determined, it is considered to play a role in the plasticity of neurons, regulating the formation of synapses and cell survival (O'Brien and Wong, 2011). It has been shown that point mutations in the APP695 gene on chromosome 21 that encodes a transmembrane precursor of alpha beta amyloid, A β is the cause for familial AD (Okamoto et al., 1996), setting the pace for identifying signalling pathways that are distorted in both hereditary

and sporadic AD. Follow-up studies on APP revealed that some neuronal subtypes seem to show an intrinsic sensitivity to APP fluctuations that may contribute to AD (Saitoh & Mook-Jung, 1999; Small, 1998). Umeda et al., 2017 showed recently that mutations in APP in vivo, that promoted A β accumulation induced memory impairment, neuropathology, aberrant synaptic activity and GABAergic neuron loss (Umeda et al., 2017). In the posttranslational level, APP metabolism and degradation may also be responsible for AD onset. It has been shown recently that nerve growth factor (NGF) dysfunction conferred to sporadic AD neuropathology, partially by Aβ deposition in humans (Canu et al., 2017). These findings indicate that regulation of APP is critical in the onset of neurodegenerative disease, including AD. In the study undertaken by Jung et al., (2012), APP was implicated in the formation of β-amyloid plaques. The researchers found an association between *T. gondii* infection and reduced APP, which suggests the spread of AD may be slowed by *Toxoplasma* brain infections.

1.12 Toxoplasmosis and N-methyl-D-aspartate receptor in Alzheimer's diseases

AD pathophysiology has not been fully elucidated (Folch et al., 2018). It has been suggested that AD is established through a chronic and aberrant inflammatory microenvironment (Kusbeci, Miman, Yaman, Aktepe, & Yazar, 2011). Here, genes that encode proteins that have been found to be important in toxoplasmosis and inflammation, with a potential link to AD onset/progression, will be discussed, to assess their importance in human pathophysiology. One of the major characteristics of AD pathophysiology is synaptic loss in the entorhinal cortex that eventually leads to dementia (Yang et al., 2018). The glutamate receptor N-methyl-D-aspartate (NMDAR) has been shown to regulate synaptic transmission intensity and mediate amyloid-β peptide (Aβ) accumulation in neurons through glutamate release regulation (Lacor et al., 2007; Manabe, 2017). The aforementioned NMDA receptors are important in controlling synaptic activity involved in learning and short-term memory function (Foster, Kyritsopoulos, & Kumar, 2017).

Overactivation of NMDAR activity causes synaptic excitotoxicity (Paula-Lima, Brito-Moreira, & Ferreira, 2013), and therefore its blockade, or regulation in general, may be beneficial for treating early AD symptoms (Lipton, 2006). When mice were infected with *T. gondii* it was shown that the NMDAR signal was attenuated in CNS nerve cells (Torres et al., 2018). Specifically, the authors claimed that Aβ aggregates significantly reduced NMDAR in neurons in the cortex and hippocampus of infected mice 15 days post-infection, reaching a peak at 60 days post-infection. Of note, the link between Aβ accumulation and NMDAR intensity changes was not shown directly (Torres et al., 2018); this was noted indirectly in AD-like mice (Tg2576-APPswe) where $A\beta_{1-42}$ peptides were able to bind and disrupt the entorhinal–CA1 synapses (where NMDAR site of action is) (Yang et al., 2018). In

addition, NMDARs were found within *T. gondii* cysts indicating that cognitive impairment may be due to this ectopic concentration of this neurotransmitter. As such, the working hypothesis for *T. gondii* infection in the AD context and NMDAR, is that Aβ aggregates are formed due to IFN- γ mediated innate immune response (presumably localised in the brain) that bind to the NMDAR, that in turn primes astrocytes to release glutamate promoting NMDAR-driven excitotoxicity in adjacent neurons, leading to mitochondrial dysfunction and neuronal death over time (Torres et al., 2018). Finally, *T. gondii* -infected mice displayed elevated anxiety levels (yet normal motor activity), in comparison to controls, although these findings may not be exclusively related to the decrease in NDMAR levels (Torres et al., 2018). *Toxoplasma* infection has been found to impair NMDAR signalling – a receptor critical to neuronal homeostasis, that in turn, has only been inferred to lead to synaptic dysfunction with impairments in learning and memory in AD (Foster, Kyritsopoulos and Kumar, 2017; Torres et al., 2018)

A recent study has also shown that cholinergic [α7 nicotinic acetylcholine receptors (nAChRs)] and glutamatergic systems are engaged in cross-talks in order to mediate cholinergic and glutamatergic neurotransmission, respectively (Jaso et al., 2017). These complexes have shown to affect murine and brain function, while in AD, these complexes are being disrupted by Aβ complexes (Elnagar et al., 2017). Therefore, NMDAR regulation has the potential to mitigate AD symptoms and inhibit CNS neurodegeneration in both chronic *T. gondii*-infected and non-infected AD patients. Whilst it is clear *T.gondii* has the ability to influence inflammatory and immune pathways, which are known to also be involved in AD pathogenesis, other AD associated pathways, such as autophagy, are not as well studied in *T.gondii* infected patients and this will be explored further in next section

1.13 Autophagy in the brain

Mammalian cells are micro-factories that continuously produce a vast amount of proteins and energy. Autophagy, meaning self-eating, is a physiological and regulated process that clears the cytosol and various organelles via delivery to the lysosomes (Stavoe & Holzbaur, 2018). Under changing cellular conditions, or stress, autophagy plays a critical role in removing damaged organelles and toxic proteins restoring homeostasis. Recently, it has been also shown that autophagy participates in innate and adaptive immunity for the elimination of pathogens (Boya, Reggiori, & Codogno, 2013). Autophagy can be described by three interlinked mechanisms called microautophagy, chaperone-mediated autophagy and macroautophagy (Mizushima & Komatsu, 2011). Macroautophagy is the main

mechanism of action, while all three classes will transfer degraded organelles and peptides to the lysosome, for digestion and recycling.

The main mechanism of autophagy is macroautophagy with Beclin 1 (Bcn1) and Microtubule-associated proteins 1A/1B light chain 3B (LC3 protein and MAP1LC3B gene) playing important roles in its regulation. In the CNS, Bcn1 was discovered to play an important role in Parkinson's Disease (PD), where silencing mutations in PINK1, the main driver of PD, promoted a dysregulation in autophagy via BCN1 (Michiorri et al., 2010). The authors also found that PINK1 physically interacts with BCN1, paving the way for other degenerative diseases to also alter autophagy this way, including AD. Knocking down BCN1 also promotes tumorigenesis via increased proliferation and decreased apoptosis of epithelial cells (Wang et al., 2013), including brain tumours (J. Huang et al., 2017; Lapierre, Rodriguez, Ojha, & El-Hage, 2018; Lin & Tsai, 2017; Xuan et al., 2016). Under hypoxic or glutamine-deprived conditions, Bcn1 is activated via the mammalian target of rapamycin (mTOR)-mediated phosphorylation inhibition of Arrest-defective protein 1 (ARD1), leading to Phosphoglycerate Kinase 1 (PGK1) acetylation that phosphorylates Bcn1 (and activates it) (Qian et al., 2017).

Another protein of interest LC3 that has played a major role in solid tumour expansion in mammals (Holt et al., 2011). It is a ubiquitin-like protein that resides both in the cytoplasm and nucleus of eukaryotic cells (Kouno et al., 2005). Under starvation conditions, nuclear LC3 is acetylated by Sirtuin 1 and activated in order to promote autophagy (Huang et al., 2015). This is achieved by the direct binding of the acetylated LC3 that exits the nucleus after has formed a complex with the diabetes- and obesity-regulated gene (DOR) (Mauvezin et al., 2010), which in turn binds to Autophagy Related 7 (Atg7) that primes macroautophagy.

Finally, another mechanism of cellular organelle turnover is achieved by chaperone-mediated autophagy, where Lysosome-associated membrane protein 2 alpha (LAMP2A, CD107b) plays a central role (Saha, 2012). Two mechanisms of action have been recently elucidated into how LAMP2A is activated. In the first, the unfolded protein response (UPR) and autophagy are the processes linked to the endoplasmic reticulum (ER) and lysosomal function. When a stress signal is present, the ER activates lysosomes via the activation of p38 Mitogen-activated protein kinase (MAPK) (Li et al., 2017). Proteostasis is also achieved through oxidative stress. The nuclear factor, erythroid derived 2, like 2 (NFE2L2) has shown to directly bind to the LAMP2 gene, with NFE2L2-deficient mice to demonstrate a profound decrease in LAMP2A levels and therefore in chaperone-mediated autophagy

(Pajares et al., 2018). In conclusion, it seems that autophagy is regulated on a DNA and protein-protein interaction levels.

In the central nervous system (CNS), autophagy has just started to be elucidated. Neurons are postmitotic cells, which need a high energy input and therefore misfolded proteins and aggregates, as well as damaged organelles, such as mitochondria (mitophagy), need to be cleared for optimal performance. Recent studies using transgenic mouse models have showed that autophagy is not detectable (or is very low) in healthy neurons (Mizushima, Yamamoto, Matsui, Yoshimori, & Ohsumi, 2004), while this increases in pathological conditions such as in AD (Nixon, Cataldo, & Mathews, 2000). One explanation for this lack of an autophagy machinery was its high efficiency. To test this, researchers induced the inhibition of lysosomal degradation which caused autophagosomal accumulation in the CNS, confirming its action in physiological conditions and more importantly, its deregulation during AD (Boland et al., 2008). In order to address the genetic pathways involved in autophagy in the CNS, conditional knock-out (KO) mice have been developed, such as the Atg5 KO transgene (Hara et al., 2006). The authors of this seminal study showed that autophagy-deficient neurons had an abnormal turnover of cytoplasmic contents, with abnormal protein accumulation and inclusion bodies that eventually disrupted neuronal function. Similar results were reported when Atg7 was specifically ablated from CNS cells (Komatsu et al., 2006; Komatsu et al., 2007).

It is now evident that autophagy plays a major protective role in CNS neurons, astrocytes and glial cells (Osellame et al., 2013). It is essentially a homeostatic mechanism, which is altered under stressful conditions such as systemic or local inflammation and in nutrient-deprived conditions. Thus, a relationship between autophagy (including lysosomal degradation) and the onset of neurodegenerative disease is highly likely. As such, autophagy is being investigated as a mechanism that is affected in age-related diseases such as AD (Nixon & Yang, 2011; Wolfe et al., 2013). A defective autophagy-lysosomal pathway is a prime candidate for the characteristic accumulation of extracellular amyloid plaques and intraneuronal tau protein aggregates, both hallmarks of sporadic and hereditary AD onset (Querfurth & LaFerla, 2010). It is, therefore, important to investigate whether CNS inflammation and/or infection can trigger a cascade of events that hinder the autophagy-lysosomal machinery. One potential candidate that will be examined is the effect of chronic *T. gondii* infection and AD's onset in relation to autophagy regulation. The relationship between autophagy and *Toxoplasma* in the brain will be considered in the next section 1.12

1.14 *Toxoplasmosis* and autophagy

Toxoplasmosis most frequently affects the function of the central nervous system (CNS) (Vidadala et al., 2016). One of the mechanisms that seem to play a role in whether the parasite will induce a chronic infection, at least in mice, is autophagy (Gao et al., 2014; Ghosh, Walton, Roepe, & Sinai, 2012; Halonen, 2009; Lee, Song, Lee, Ryu, & Ahn, 2013).

Upon invasion by the *T. gondii* parasite, the host's immune system will employ and augment, several defence mechanisms. Naturally, autophagy is one of those prime mechanisms, especially for intracellular parasitic infections, in order to clear the pathogen (Subauste, 2009). Fusion of microbes with lysosomes is an important host defence mechanism and in toxoplasmosis, this mechanism deviates from its normal cascade providing a window for the parasite to survive and perhaps establish a chronic infection, especially in the CNS and in macrophages (Halonen, 2009). It has been reported that the parasite has the ability to hack the macroautophagy mechanism, promoting increased autophagy, and yet benefiting from that increase (Wang, Weiss, & Orlofsky, 2009). Specifically, the authors observed an increase in parasitic growth in line with increase expression of Atg5, independent of host mammalian target of rapamycin (mTOR) suppression upregulation/activation. This resulted in nutrient consumption to shift from host cell mechanisms towards parasitic growth.

The activation of autophagy by *T. gondii* may ensure host's cell survival by inhibiting necrotic or apoptosis as part of the innate immune response (Krishnamurthy, Konstantinou, Young, Gold, & Saeij, 2017). Also, autophagy may also be a survival mechanism for the parasite. Ablation of either Atg3, Atg4 and Atg8 in mice, indicated a dramatic growth inhibition in tachyzoite formation in infected cells [for a review see (Besteiro, 2012)]. These findings are indications that the parasite needs to manipulate the autophagosome machinery in order to thrive; tachyzoites once inside the host cell are engulfed in a parasitophorous vacuole (PV) that shields them from host's surveillance (Wang et al., 2009). Researchers indicated that for transmission between host's cells, the autophagosome may help the parasite's spread in the body (Li et al., 2016). In the case of the CNS, the astrocytic and macrophagic autophagosomes have been investigated, in order to elucidate the mechanism of establishing chronic *toxoplasmosis* (Halonen, 2009). In macrophages, autophagy's most prominent activator is interferon gamma (IFN-γ) that was seen to eliminate the PV and clear the parasite via p47 GTPases (Ling et al.,

2006), or direct fusion to host's lysosomes via CD40 induction (Andrade, Wessendarp, Gubbels, Striepen, & Subauste, 2006). The mechanism of PV elimination was not observed in astrocytes, where parasites were directly lysed in the cytoplasm (Melzer, Duffy, Weiss, & Halonen, 2008). In that case, it was suggested that the PV was directly fused to the endoplasmic reticulum (RE) in order for a calcium influx leading to PV degradation.

Currently, autophagy is an active field of research since it has been shown to be activated upon intracellular infections such as parasites, viruses and some bacteria. In the case of toxoplasmosis, disruption of the PV is important for avoiding chronic infection (Saeij & Frickel, 2017), while several new studies are revealing new signalling pathways which orchestrate lysosome and the ER against *T. gondii* (Nguyen, Berry, Sullivan, & Besteiro, 2017; Portillo et al., 2017). In addition, it is now clear that autophagy plays a crucial role in orchestrating the IFN-γ -dependent innate immune response to this parasite and several new lines of evidence have emerged on how to enhance that response (Ohshima et al., 2014). Build-up of abnormal protein has been linked to autophagy deficits in AD, but also in Parkinson's disease and Huntington's disease (Meng et al., 2019). If *Toxoplasma* upregulates autophagy by causing a small amount of cellular stress, this could underlie some of the 'protectivity' in AD through increased degradation of damaged proteins.

The gene Sequestosome 1 (SQSTM1, p62) encodes a protein that is involved in autophagy capable for binding to ubiquitin (Cohen-Kaplan, Ciechanover, & Livneh, 2016; Lippai & Low, 2014; Peng et al., 2017). P62 is a multifactorial protein that acts as a scaffold protein able to activate the nuclear factor kappa-B (NF-kB) signalling pathway through Tumour Necrosis Factor (TNF) receptor 6 and interferon gamma (IFN-γ) (Su & Wang, 2011). During parasitic infections, including *T. gondii*, IFN-γ -mediated immune responses prime the assembly of p62 and ubiquitin complexes, directing them to the intracellular parasite-entrapped vacuoles destined for digestion, in order to provide antigens for loading to major histocompatibility (MHC) I and sufficient presentation to the host's infected cell surface. These complexes will be then recognised from professional antigen-presenting cells (APC) that in turn will activate cytotoxic $CD8^+$ T cells (Lee et al., 2015). The disruption of this mechanism may be involved in *T. gondii* prevalence in humans (Clough et al., 2016), and could contribute to AD.

Experiments performed in p62-deficient mice indicated a diminished antigen-presenting capability for APC that led to a global reduction in cytotoxic $CD8^+$ T cell activation and reduced capability in clearing *T. gondii* in murine embryonic fibroblasts (Lee et al., 2015) and Human Umbilical Vein Endothelial Cells (HUVEC) (Clough et al., 2016). Specifically, T. gondii-infected HUVEC cell cultures recruit and activate their intracellular ubiquitin system, in the infected vacuoles, through IFN-

γ (Perrin, Jiang, Birmingham, So, & Brumell, 2004). Clough et al. indicated that the binding proteins p62 and NDP52 were being recruited to the parasitophorous vacuoles (PV), but only on half of those PVs the ubiquitin-p62-NDP52 complex was activated possibly because they were bearing specific *T. gondii* Type II strains (Clough et al., 2016). Thus, the restriction of p62 recruitment in PV may be a key survival mechanism for specific *T. gondii* strains.

The absence of p62 (by blocking the p62 promoter region) has been shown to induce an AD-like phenotype in mouse brains, while AD patients showed a decreased expression of p62, in comparison to aged-matched subjects (Du, Wooten, Gearing, & Wooten, 2009). This dysregulation was caused by oxidative stress that has been linked to age-related neurodegenerative diseases, including AD (Filipcik, Cente, Ferencik, Hulin, & Novak, 2006). Du et al. primarily focused on whether the absence of p62 causes oxidative stress and vice versa, in mice up to 12 months of age (Du et al., 2009). Their findings indicated that the p62 promoter region accumulates damage from oxidative stress that in turn significantly reduces P62 expression in mice and humans. Finally, a large cohort study in European subjects showed that p62 polymorphisms may be indicative for late-, but not early-onset, AD (Cuyvers et al., 2015). Thus, *T. gondii* CNS infection may be a precursor for AD onset in predisposed individuals by hacking the host's innate immune defence pathways. These findings indicate that p62 is important in clearing host-infected cells in the CNS and *T. gondii* may inhibit its function, indirectly contributing to AD onset and/or progression.

1.15 *Toxoplasmosis* and innate immunity in Alzheimer's diseases

Autophagy has also been implicated in regulating innate immunity (Deretic & Levine, 2018). Its role has been instrumental in pathogen recognition by pattern-recognition receptors called Toll-like receptors (TLRs) (Into, Inomata, Takayama, & Takigawa, 2012). Of interest, TLR-2 has shown to be also implicated in neuronal function during infection with *T. gondii* (Schmitz, Mages, Heit, Lang, & Wagner, 2004; Umeda et al., 2017). Experiments performed in murine brain cells (astrocytes, microglia, neurons and peritoneal macrophages) deficient in TLR2 showed that infection with *T. gondii* tachyzoites significantly altered the gene expression profile of the TLR2-deficient murine cells, in comparison to infected and uninfected wild-type cell cultures (Umeda et al., 2017). The authors analysed the transcriptome of brain cells during *Toxoplasma* infection and identified gene expression level differences in relation to the infected cell-type, with 19 common genes being upregulated in all neuronal types and 5 downregulated when compared to control cell cultures. In earlier, similar yet in

vivo, experiments, TLR2-, TLR4- and myeloid differentiation primary response 88 (MyD88)-deficient mice were infected intraperitoneally with a high dose of *T. gondii* (Mun et al., 2003). The authors did not find alterations in nitric oxide (NO) levels in mice infected with the parasite, with increased levels of pro-inflammatory cytokines being produced by immune cells, especially in TLR-4 deficient mice. Of note, TLR-2 deficient mice had an increased infiltration of innate immune cells in the lungs resulting in severe interstitial pneumonia. When Chinese hamster ovary (CHO) cells were examined for their TLR expression upon infection with glycosylphosphatidylinositols derived from *T. gondii*, it was shown that only MyD88 was indispensable for TNF-alpha production in macrophages, not TLR2 or TLR4 (Debierre-Grockiego et al., 2007). Finally, a large study that employed 138 patients with bipolar disorder and 167 healthy individuals showed that *T. gondii* infection status and bipolar disorder had a strong correlation, which implicates a single nucleotide polymorphism (SNP) in the TLR2 locus, resulting in increased risk for this disease (Oliveira et al., 2016). These reports indicate that the TLR signalling pathway may play an important role in chronic toxoplasmosis and perhaps is key in AD progression as well. Another potential target for therapeutic intervention is the suppressor of cytokine signalling protein 1 (SOCS1) (Liau et al., 2018). The gene encodes a STAT inhibitor that negatively regulates cytokine signalling on its downstream targets on their cascade pathways. Most prominent targets are pathways that are activated by IL-2, IL-3, GM-CSF, EPO and IFN-γ. As such, SOCS1 gene expression may be a prominent target for viral and parasitic infections in order to circumvent the host's immune system (Charoenthongtrakul, Zhou, Shembade, Harhaj, & Harhaj, 2011). Indeed, in mice that have been induced to be chronically infected with *T. gondii*, the overall inflammasome in the CNS was upregulated contributing to increased neuronal and glial cell death (Hwang et al., 2018). Specifically, C57BL/6 mice were injected intraperitoneally with 10 *T. gondii* cysts isolated from brains of infected mice. Infected mice were sacrificed at 3-month intervals up to 12 months for assessment. Among important immune regulators, SOCS1 mRNA and protein levels were increased by 2.63-fold by week 3 post-infection (PI) and 12-weeks PI respectively, when compared to controls. The authors claimed that an increase in SOCS1 affected nitric oxide (NO) production, a crucial component against clearing intracellular parasitic infections; when NO production is inhibited by *T. gondii*, it may cause a delay in AD-related symptoms, as judged by the significantly low Aβ plaque accumulation and increased cognitive abilities of infected mice, in relation to uninfected mice (Jung et al., 2012). In addition, SOCS1 downregulation both in gene and protein expression levels may induce a systemic dampening of the innate and adaptive immune response by reducing IFN-γ and T-cell activation, respectively (Whyte et al., 2011).

Until today, there has been no definitive link between SOCS1 expression levels and AD. *T. gondii* infection has shown to inhibit neuronal degeneration in AD-specific transgenic mouse strains (Tg2576) by limiting learning and memory impairments in those mice (Jung et al., 2012). As such it is plausible to assume that SOCS1 regulation, either at the mRNA or protein levels may ameliorate AD symptoms. In conclusion, the genes described above play pivotal roles in inducing innate immune responses against intracellular pathogens and are important in the progression of chronic toxoplasmosis-related AD, both in humans and mice, yet their spatiotemporal significance has not been fully elucidated.

1.16 The importance of establishing human cell culture systems for studying *Toxoplasma* infection

In order to study the effect of *T. gondii* infection in human tissues and cell populations, it is important to set up a reliable and reproducible cell culture systems. It is well documented that toxoplasmosis is a condition where the parasite is able to infect multiple organs in humans, using multiple routes of infection (Cowen and Wolf, 1951, Cowen and Wolf, 1950a, Cowen and Wolf, 1950b). There are human immortalised cell lines which are used for studying the effects of tachyzoites and bradyzoites in humans. The most recent ones used are going to be described along with other laboratory techniques that can be applied to assess the cycle of the parasite intracellularly and the changes that occur in human cells.

A prominent human cell line is the SH-SY5Y human cell line derived from a four-year-old female child's bone marrow biopsy-diagnosed with neuroblastoma (Biedler et al., 1973). The original cell line was called SK-N-SH and a neuroblast-like subclone of this cell line has been used extensively in studying *Toxoplasma* infection. To study the disease in relation to age-related diseases and the brain, other human cell lines have been employed such as the microglial CMH5 cell line (Mammari et al., 2015, Mammari et al., 2014). An important feature of this parasitic infection is the formation of cysts in the brain from bradyzoites, as soon as some tachyzoites have managed to evade the host's immune response, especially in immunocompromised patients (Skariah et al., 2010). Of note, brain cyst location has been thoroughly examined in mouse-infected brains but not in humans (Gonzalez et al., 2007; Vyas et al., 2007a; Di Cristina et al., 2008;Berenreiterova et al., 2011) (Figure 1.14).

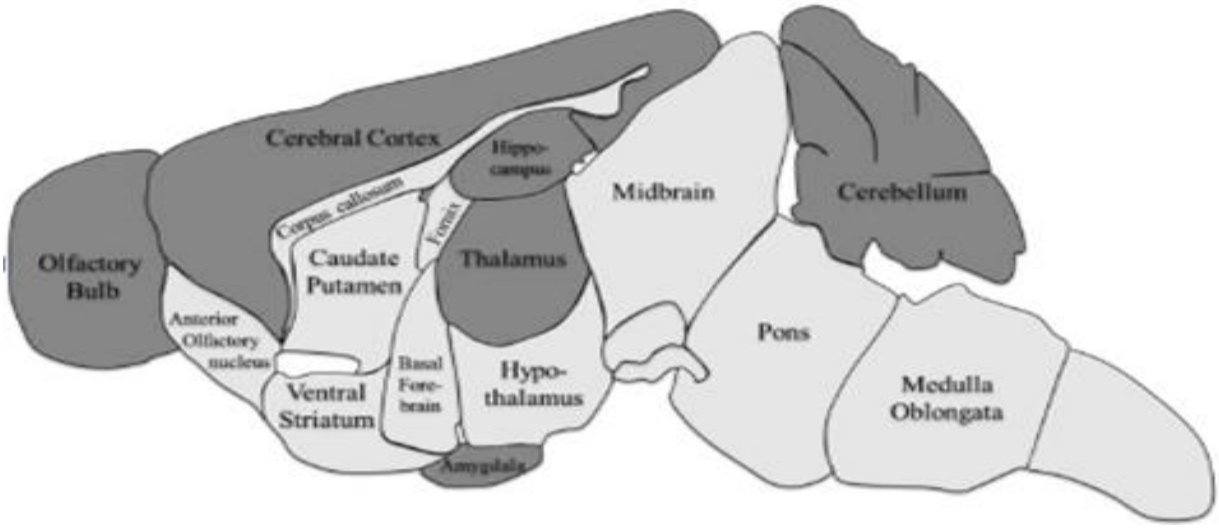


Figure 1. 14: *T. gondii* cyst locations in the brain of a rodent. A graphic of the cross section of a murine brain along the median plane, highlighting the locations of *T. gondii* cysts within the brain of a subject suffering chronic toxoplasmosis. Larger amounts of cysts, shown in a darker grey, were identified in the amygdala, cerebellum, cerebral cortex, hippocampus, thalamus and olfactory bulb (Gonzales et al., 2007; Vyas et al., 2007a; Di Cristina at al., 2008' Berenreiterova et al., 2011).

The human cell line SH-SY5Y has shed light on many intracellular molecular mechanisms that *T. gondii* affects. Work performed by Mammari et al. used both CMH5 and SH-SY5Y as well as a bone-marrow-derived endothelial cell lines (Mammari et al., 2014). In that study, murine-derived tachyzoites were used to infect the aforementioned human cell lines in order to assess immune mediators in a time-dependent manner. Results from co-cultures of *T. gondii* cysts and neuroblastoma cells indicated peak expression levels in interleukins 6 (IL-6) and 8 (IL-8), in the first 8 hours post-infection, and dropped in proportion to parasite burden. The same research group investigated the effects of interferon gamma (IFN- γ) on *T. gondii*-infected human nerve cells (Mammari et al., 2015). Results indicated changes in growth factors as well as SICAM and Serpin E1.

In another set of experiments, the same human cell line was transfected with the virulent factor *Toxoplasma* rhoptry protein 16 (ROP16) (Fan et al., 2016). Transfected cells' RNA was used for microarray analysis and compared to human cells transfected with empty vector. Differentially expressed genes (DEGs) underscored biological processes affected that included immune response, apoptosis, neural development and metabolism. In addition, an array of transcription factors were up- and down-regulated in the ROP16-transfected cells, in comparison to non-transfected cells.

Gene ontology showed that cellular metabolism is affected by the presence of ROP16. Of interest, the expression levels of a gene involved in AD onset (Liu et al., 2013), Apolipoprotein E (APOE), were decreased significantly. Although the authors did not comment on which allele showed a decrease, it is still important to assess the link between parasitic infection and APOE gene expression regulation. Finally, Topgene-derived results indicated a subcluster of immune genes affected by ROP16 transfection that have been reported to be important in AD and toxoplasmosis, such as Toll-like receptor signalling, lymphocytic migration and adhesion, and NK and T lymphocyte cell activation (Landreth and Reed-Geaghan, 2009, Lee and Lee, 2002, Su et al., 2016, Kokkinopoulos et al., 2005).

From the above results, ROP16 transfection of human neuron-like cells indicated a vast change in the transcriptional landscape of these cells. In order to create effective therapeutic and preventive strategies, its molecular mechanism of action should be elucidated. Suang Chang et al. showed that ROP16 is able to induce partial apoptosis of human SH-SY5Y, according to flow cytometric analysis and Annexin V membrane staining (Chang et al., 2015). Further, agarose gel electrophoresis revealed extensive DNA fragmentation after ROP16 transfection. Cell cycle analysis of the transfected SH-SY5Y human cells in comparison to controls showed a marginal and transient increase in G0/G1 phase. Reciprocal co-immunoprecipitation using FLAG-ROP16 in human SH-SY5Y cells revealed direct colocalisation with the P53 protein, in the nucleus. This was also visualised under fluorescence microscopy in the nucleus of the same cells. These results underscore the notion the ROP16 induces cell cycle arrest and potentially apoptosis. To confirm this, further Western Blot analysis revealed an increased expression of BAX and P21 proteins in transfected human cells. In vivo and in vitro experiments using an array of different P53-phosphorylated constructs indicated that ROP16 destabilises the phosphorylation at Serines 15 and 37 on P53.

Albeit the identification of a single target for one *T. gondii* protein, a more powerful tool is needed for mapping susceptibility genes that affect the human brain. Huân M. Ngô and colleagues performed genetic and proteomic analyses on human genes affected by toxoplasmosis (Ngo et al., 2017). Two reconstructions revealed important upstream regulators including inflammation, innate immunity and oncogenes such as IFN- γ, TLR4, TLR9, ALOX12, NALP1, TAP1, JUN, MYC, TGFB1, HLA genes and TNFR, along with 112 upstream genes of these regulators. For the second

reconstruction, two primary human temporal-lobe neural stem cell lines were employed showing vast similarity in genetic transcriptional alterations. MicroRNA analyses underpinned several epigenetics "switches" that may also be used as biomarkers in patients with AD (mir-139, mir-29a and mir-107). Proteomics analysis indicated that ATXN2L, NSC2, FXR1, WDFY1, UBE3A, USP8 and PPP4C proteins were differentially expressed, affecting iron ion homeostasis, RNA splicing (marginally), protein transport and localisation and TOR-signalling (marginally) as well as oxidative phosphorylation that is affected in AD. Finally, deconvolution analysis indicated that *T. gondii* induces activation of the NFκB pathway in human neuronal cells. Ingenuity pathway analysis showed that AD-related pathways are indeed affected by *T. gondii* infected human brains, for example via APOA1 regulation, while 12 olfactory human receptors are modulated by the parasite.

1.17 Immunohistochemical and immunocytochemical analysis tools for *Toxoplasma gondii* detection

In order to assess host infectivity and potential disease progression, cell culture systems (as the ones aforementioned) have been established. Fluorescent-tagged antibodies are readily employed in diagnostic and research fields for assessing pathogen-related infections and disease progression (Cabral et al., 2016). In *T. gondii* infection, not only the parasite's presence must be confirmed, but it is important to detect changes that occur in CNS cells and amyloid beta deposition, which has been related to AD.

There is a limited number of antibodies that have been raised against *T. gondii* specific antigens. Most diagnostic tools detect the antibody responses to this parasite or the presence of specific *T. gondii* antigens using ELISA. A major target protein is *T. gondii's* surface antigen 1 (SAG1) which its antigens have been used as target. Anti-*Toxoplasma* gondii p30 antibody (especially the N-terminus) has been used to detect the parasite in vitro (Figure 1.15). This antibody is fluorescently tagger with Fluorescein IsoThioCyanate (FITC) with an emission spectrum at 495-520 nm (green-yellow light). An unconjugated monoclonal IgG2a antibody against SAG-1 native protein has also been raised in mice (Biswas et al., 2017). SAG1 is the prototypic protein that belongs to the superfamily of glycosylphosphatidylinositol (GPI)-linked proteins (SRSs) that coat the surface of

T. gondii (He et al., 2002). This family of proteins are involved in host cell attachment and directly linked to this parasite's virulence (Figure 1.16).

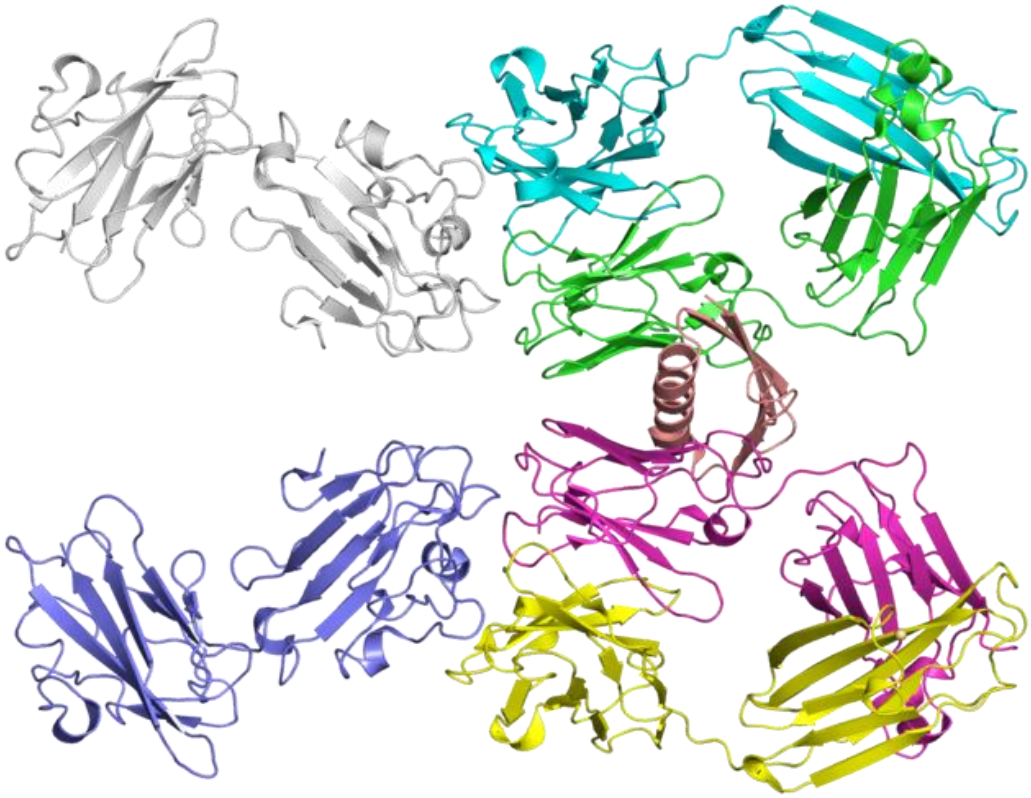


Figure 1.15. Graphical representation of the crystal structure of the immunodominant epitope displayed by the Surface Antigen 1 (SAG1) of *Toxoplasma* gondii bound to a human antigen-binding (Fab) fragment. The structure represents a 1.7 ångström units (Å) and contains 2 copies of 4F11E12 Fab variable light chain region and 2 copies of heavy chain region, one copy of protein L, 2 copies of major surface antigen p30 and two copies of the cadmiun ion. Adapted from (Graille et al., 2005).

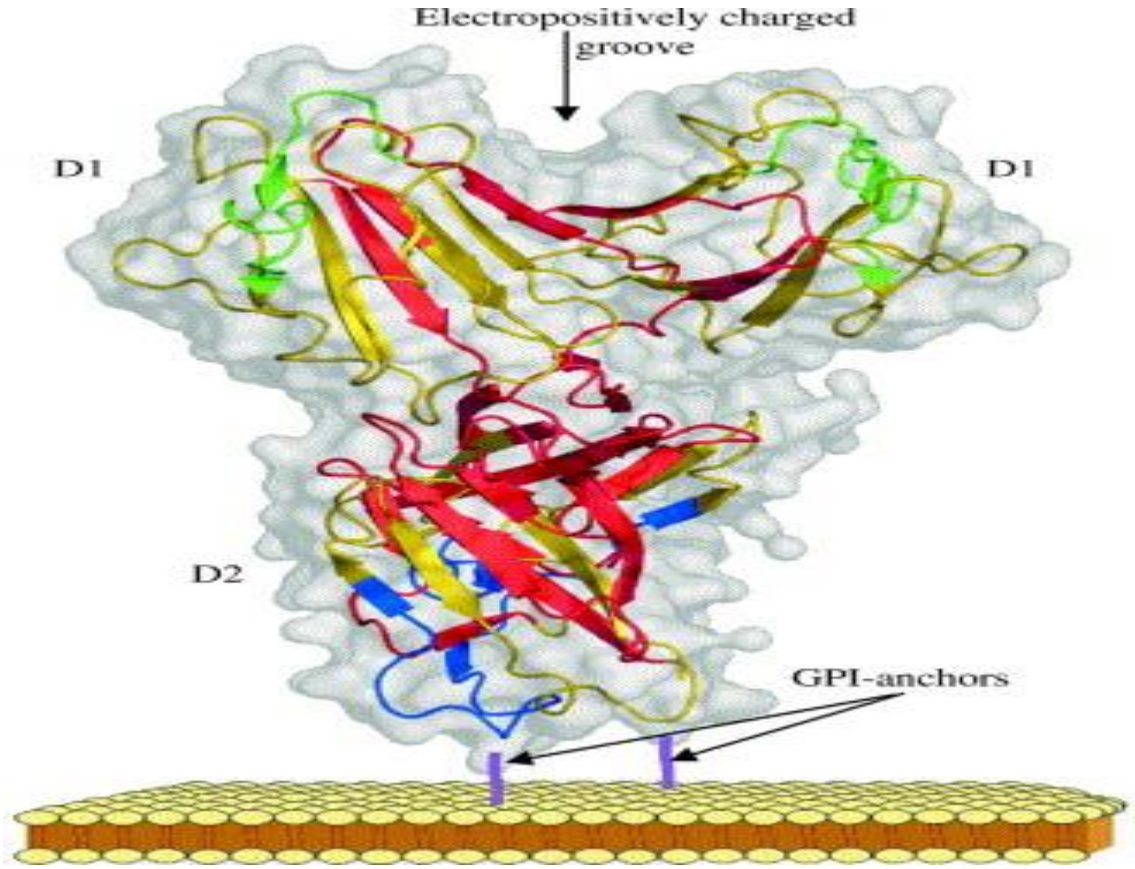


Figure 1. 16. Graphical representation of SAG1 function of adherence to the host's cell surface., adapted from (Graille et al., 2005). Crystal structure of the immunodominant epitope. Upon homodimerisation of SAG1 (yellow), structural epitopes are revealed allowing the protein to reveal its GPI anchors reorientating the parasite's scaffold proteins (red, green and blue).

Virreira Winter et al., 2011 .reported that fluorescently-labelled antibodies against purified tachyzoites were able to detect their presence in IFN- γ -activated vacuoles within host cells (Virreira Winter et al., 2011). Yet, the direct detection of parasites using only this antibody was not evident as it had to be colocalised with IFN-γ induced FLAGed p65 guanylate-binding proteins (GBP) 1 and the mCherry-expressing type II *T. gondii* construct (Figure 1.17).

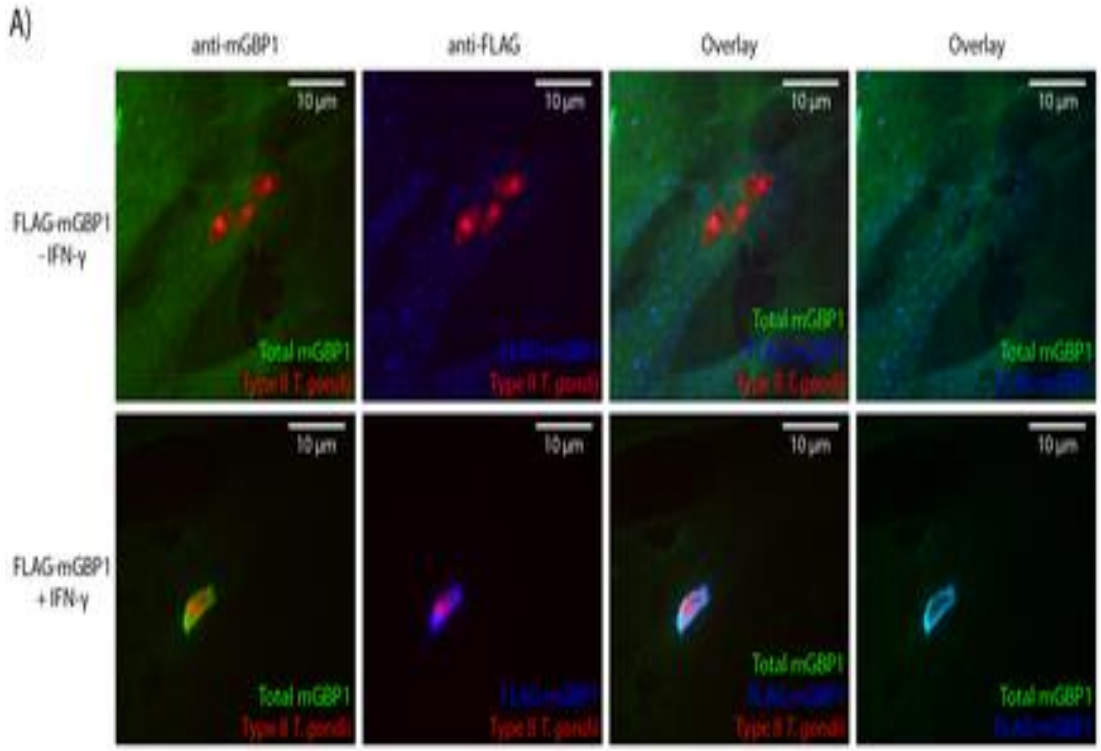


Figure 1.17. Spinning disk confocal microscopy photomicrographs of mGBP1 accumulation on vacuoles. Mouse embryonic fibroblasts (MEFs) expressing FLAG-mGBP1 were stimulated with 200U/ml IFN- γ and were then infected with type II *T. gondii* for 1 hour. A rabbit polyclonal anti-mGBP1 antiserum (green) and a mouse monoclonal anti-FLAG antibody (blue) and Type II *T. gondii* construct (red) were used for stainings. Adapted from (Virreira Winter et al., 2011).

Finally, Koshy et al. created a *Toxoplasma* strain able to inject Carbapenem-resistant Enterobacteriaceae (Cre) fusion recombinase into host cells, making possible the detection and interaction of *T. gondii* proteins and infected cells (Koshy et al., 2010). The fusion protein chosen was Toxofilin, an Actin-binding *T. gondii* protein strongly coupling to G-actin (Poupel et al., 2000). The fused product was able to infect 95% of the host cells, based on the high expression of the reporter constructs (Figure 1.18).

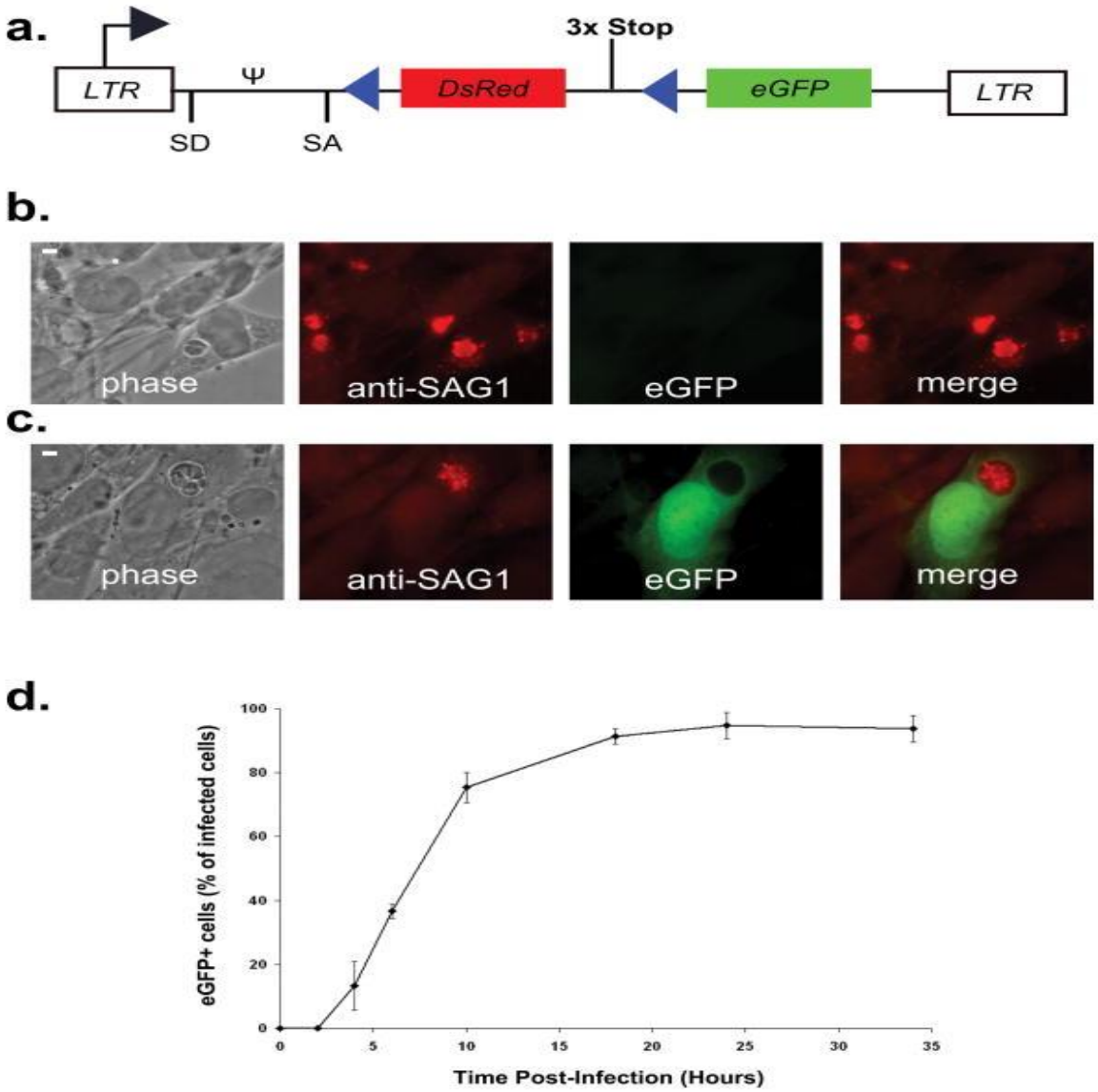


Figure 1.18. Activation of fluorescent-tagged with enhanced Green-Fluorescent Protein (GFP) in human cell lines. Adapted from (Koshy et al., 2010). (a) Schematic representation of the retroviral construct for the creation of the Cre-reporter cell line. A promoter in the long terminal repeat (LTR) drives the expression of Discosoma sp. red fluorescent protein (DsRed) and eGFP. There are 3 stop codons between them, which allow only eGFP to be expressed (in the direction the black arrow indicates transcriptional activation). The blue triangles indicate the loxP sites and their orientation. Upon excision of the LoxP sites by Cre-recombinase the stop codons are removed discontinuing DsRed expression. SD is splice donor, SA is splice acceptor, and Psi (ψ) is a retroviral packaging signal. (b,c) Cre-reporter cells 24 hours after infection with two *T. gondii* strains. Panels indicate DsRed expression and staining with *Toxoplasma*-specific anti-SAG1 antibody (both red), while green shows eGFP expression. (d) Percentage of genetically modified parasite-infected Cre-reporter cells that showed eGFP expression is plotted against specific time points. Scale bars, 5 μm.

T. gondii has the ability to infect the central nervous system (Feustel et al., 2012). Upon entering the brain, cysts are formed which are retained for extended periods of time and are one of the prime signs of latent infection (Berenreiterova et al., 2011). In infected patients, the majority of the brain tissue can be found to have cysts (Abdel Razek, Watcharakorn, & Castillo, 2011; Pedersen, Mortensen, Norgaard-Pedersen, & Postolache, 2012) (Figure 1.19). In experimental animals it has been noted that these cysts could have behaviour-altering effects, in mice (Gatkowska et al., 2012) and cats (Prandota, 2014). As such, it has been noted that latent toxoplasmosis (Figure 1. 20) may confer to AD progression or alleviation of symptoms (Mahami-Oskouei et al., 2016; Mohle et al., 2016).

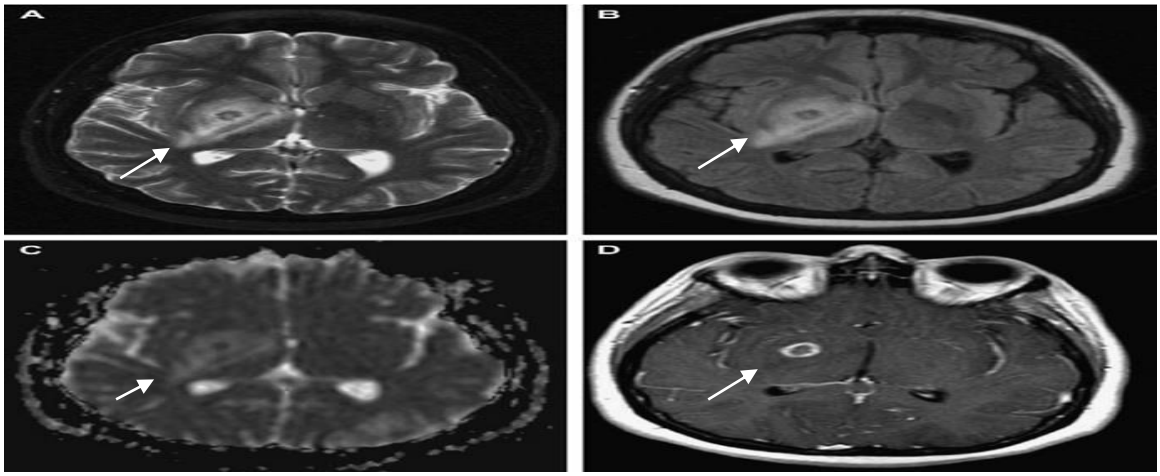


Figure 1.19: showing potential toxoplasmosis lesion or cyst in human brain (white arrow), using Magnetic resonance imaging (MRI). Adapted from (Abdel Razek et al., 2011).

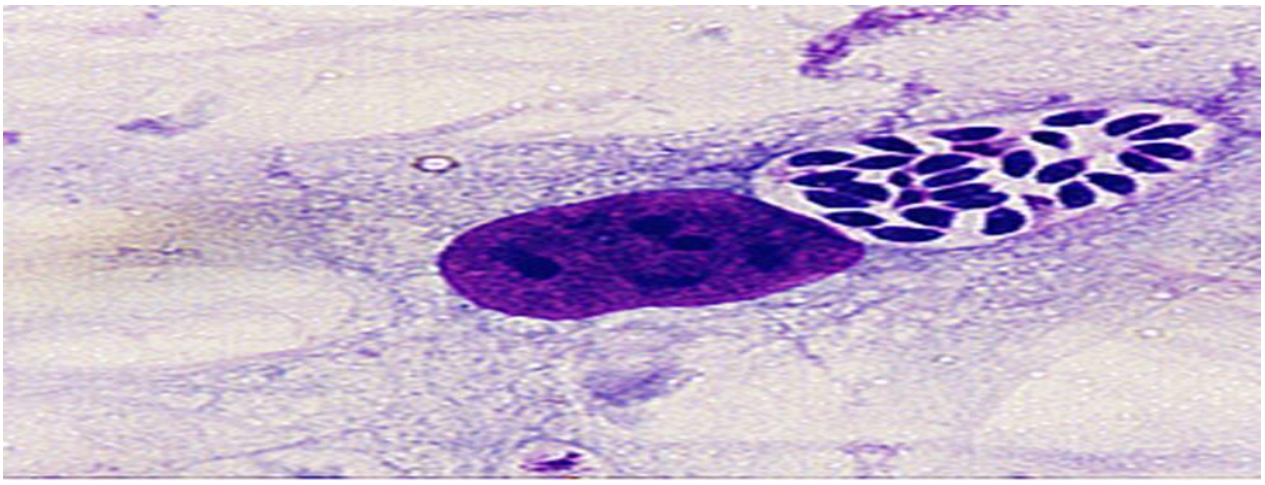


Figure 1. 20: bradyzoite cyst with the dense cyst wall surrounding the bradyzoites, Adapted from (Dubey, 1998).The left shows a bradyzoite cyst with the dense cyst wall surrounding the bradyzoites (a possible sign

of latent infection). The right shows tachyzoites inside the parasitophorous vacuole (present during an active infection stage).

Toxoplasmosis is a disease that affects approximately 30% of the population, with the vast majority of cases being asymptomatic. In the elderly, dementia including AD affects around 12% of people above the age of 60 and the percentage increase dramatically above 80 years of age. Although *T. gondii* infects and persists primarily the brain (and muscle) in humans, this parasitic infection could be translated into a symbiotic relationship, one which both organisms benefit from; the parasite prolongs its presence to its intermediate host, while the non-immunocompromised host is partially protected from age-related CNS diseases.

Generic approaches, including animal models, must be developed for studying humans. To explore possible relationships between *T. gondii* and AD, this study examined post-mortem tissue samples from the brains of AD patients. These were obtained from the Manchester Brain Bank for the purpose of detecting *Toxoplasma* cysts in frontal and hippocampal tissues. The prevalence of *T. gondii* was also investigated, as well as the expression of various relevant proteins in the sampled natural population of wood mice. It is unclear whether there is increased *Toxoplasma* infection in AD or if markers of inflammation and autophagy vary between infected and non-infected mice. This research has, thus, aimed to address some of the gaps in current knowledge about *T. gondii's* relationship to AD.

1.18 Aims and objectives

This research has aimed to explore the relationship between toxoplasmosis and Alzheimer's disease by examining brain samples from human cadaver brain tissues. It has, secondly, sought to determine the prevalence of *T. gondii* in a natural rodent population and to compare the expression of important inflammatory genes associated with brain disorders (e.g. Alzheimer's) in response to infection with the parasite *Toxoplasma gondii*. The study has, specifically, pursued the following objectives:

1- Conduct a pilot study to investigate the possibility of using samples from the Manchester Brain Bank as a material source for investigating possible interactions between *Toxoplasma gondii* infection and Alzheimer's disease.
2- Developing and using molecular diagnostic tools to detect *Toxoplasma* in the brain tissues of a natural population of wood mice.
3- Investigating the presence of autophagy and inflammation genes in the brain tissues of a natural population of wood mice.
4- Investigating the expression of autophagy and inflammation proteins in the *Toxoplasma* infected and uninfected brain tissues of a natural population of wood mice.

Chapter 2: Materials and methods

2.1 Immunohistochemistry on human brain tissue

In order to investigate the presence of *Toxoplasma* cysts and their relationship with AD, 90 stained slides were examined for the expression of specific proteins via immunohistochemistry. Human brain tissue from 45 individuals from two different brain regions Hippocampus and frontal cortex was examined for the presence of cysts. These tissues had been stained with antibodies for pathological proteins tau of AD (AT8) and anti-Beclin-1 (Abcam) (Jaeger & Wyss-Coray, 2010; Pickford et al., 2008). The counterstain was Haematoxylin) as part of a different study investigating autophagy impairments in AD. *Toxoplasma* cysts have previously been shown to be identifiable using haematoxylin (Mohle et al., 2016).

The staging of AD-related neurofibrillary pathology was performed on thick sections (50 um) using a modern silver technique and reflected the progress of the disease process based chiefly on the topographic expansion of the lesions (Braak & Braak, 1991). According to the standard classification stages of AD progression, there are six stages of disease progression, able to be distinguished with respect to the location of the tangle-bearing neurons and the severity of changes as well as the presence of extracellular amyloid deposits; (transentorhinal stages I-II: clinically silent/asymptomatic cases; limbic stages Ill-IV: incipient AD; neocortical stages V-VI: fully developed AD (Braak & Braak, 1995; Braak et al., 1995).

These two brain regions were specifically examined as it has been reported that AD primarily affects those two regions (Reitz & Mayeux, 2014) and thus it was hypothesised that the presence or absence of cysts in these areas may be relevant to human pathophysiology. The age of both groups was between 77-94 of mixed sex (median age 85.5). The 45 cases comprise of 15 cases of low Braak (No AD), 15 cases of mid Braak (Possibly AD), 15 cases of high Braak (Highly likely AD).

Human tissue samples were obtained from Manchester Brain Bank. Tissue collection and consent forms were followed according to the Bank's regulations. Samples had already been immunostained from previous PhD students at Salford University and we examined the pre-stained sections for the presence of cysts. Also, the methods carried out were done by the previous students.

In order to determine the presence of tau, each slide of the hippocampus was stained using the AT8 antibody, while the frontal cortex tissue samples were labelled with Beclin-1. Tissue sections were dewaxed by being immersed in Histoclear for 5 min before being rehydrated by soaking them in100% ethanol for 5 min. The latter step was repeated and followed by two further 5 min immersions in 95% and 75% ethanol. Tissue sections were then washed in three changes of dH_2O for 5 min. To prevent

endogenous peroxidase activity, tissue samples were immersed for 20 min in a solution of 3% H_2O_2 in methanol then incubated with AT8 and Beclin-1 for 1 h.

Tri-sodium citrate buffer (0.01M; pH 6.5) was made to retrieve antigens. Retrieval was achieved using a Lab Vision™ Module (Thermo Fisher) that was heated to 98° C for 20 min. Slides were quickly moved to TBS-T. Slides were washed in dH_2O five times. Immunodetection was achieved by using a VECTASTAIN Elite ABC (mouse IgG) kit (Vector), made up with 1.5% horse normal blocking serum in accordance with the manufacturer's instructions. Samples were incubated at room temperature for 30 min followed by 60 min with AT8 antibody (1:500). Incubation with Beclin-1 antibody (1:2000) was at 4° C for 16 h (overnight).

Sections were then rinsed in TBS-T (Tris-buffered saline + 0.1% Tween 20) before being incubated for 30 min at room temperature with ABC (avidin-biotin complex) (Vector). This was made to a 1:25 ratio. Samples were again rinsed in TBS-T five times then incubated with 1.5% normal blocking serum and biotinylated anti-mouse/rabbit antibody (1:200) (Vector). After 30 min incubation, samples were washed with TBS-T then incubated with ABC. To develop peroxidase activity, samples were exposed to diaminobenzidine/H_2O_2 for 5 min; the reaction was interrupted with tap water. Slides were counterstained with haematoxylin (Vector Lab); a few drops were added to each slide for 3 seconds then rinsed with tap water. Slides were transferred to dH_2O then dehydrated in serial increments of ethanol (75%, 95%, 100%, 100%) for 5 min each time. A 5 min incubation in Histoclear was used then coverslips applied to the slide using DPX. The investigation for the presence of potential *toxoplasmosis* was investigated using light microscopy, with a full scanning of the entire brain section, looking at different subregions of the hippocampus and the frontal cortex at different optical magnifications (x10, x40 and x100) inclusive of both grey and white matter.

2.2 Collection of *Apodemus sylvaticus* tissues

Before commencing this work, all relevant permissions were obtained in accordance with Boyce et al. (2012, 2013) and the ethical approval of the University of Salford Research Ethics and Governance Committee (STR1819-12) was obtained. Longworth traps were employed over three-night periods in three different sites on woodland belonging to the Malham Tarn field centre, Yorkshire Dales (Figure 2.1) for the collection of live wood mice (*Apodemus sylvaticus*, n=21). Four lines of 15 traps were set up with 3-m intervals between successive traps. When possible, traps containing food and straw were strategically positioned next to or on top of natural features such as undergrowth (Figure 2.2), grass tussocks, fallen tree trunks, or the branches of shrubs. The traps were set in the evening and collected early the following morning. During sample collection, the use

of aseptic techniques was central to all protocols used by myself. Between sample collections, all the instruments were wiped, thoroughly washed and sterilized by immersing in alcohol and then by flaming. Significant pains were taken to avoid contamination between successive samples and between each sample and the environment. The collected mice were evaluated for various characteristics including sex, body length and weight. The mice were then euthanised before aseptically dissecting the brains, transferring the tissue to sterile tubes containing Allprotect Tissue Reagent (400 µl) and storing in the frozen state for later use in DNA extraction. The dissections, DNA extractions, and PCR *T.gondii* B1, SAG1, SAG2 tests were all performed by myself

Figure 2.1: Locationof Malham Tarn field centre, Yorkshire Dales. (from www.ciaofamiglia.com/frfletcher/fletchlinks.htm, accessed 20/01/2020)

Figure 2.2: A Longworth small animal trap as typically positioned in woodland belonging to the Malham Tarn field centre. Adapted from Thesis (Hughes, 2009).

2.2.1 Extraction of DNA from tissue samples

The DNeasy Blood & Tissue kit (Qiagen) was employed in keeping with the manufacturer's guidelines for the preparation of DNA from samples of mouse organ. The procedure involved cutting tissue samples into pieces of small size and introducing them in 1.5 ml microcentrifuge tubes, followed by the addition of 180 µl tissue lysis buffer (Qiagen) and 20 µl proteinase K (600 mAU/ml) (Qiagen). Vortexing was performed for proper mixing of the samples, which were subsequently subjected to centrifugation, followed by overnight incubation in a thermomixer at 56°C. After they were taken out of the incubator, the samples were vortexed again, followed by addition of 200 µl buffer AL and renewed vortexing. They were then introduced in a heat block for 10 minutes, at 70°C, followed by addition of 200 µl 100% ethanol. The whole volume was afterwards moved to a spin column. The next step was 60-second centrifugation at 8000 rpm, with the flow-through being subsequently eliminated. Afterwards, 500 µl buffer AW1 was added, followed by renewed 60-second centrifugation at 8000 rpm, with the flow-through being subsequently eliminated. This was followed by addition of 500 µl buffer AW2 and three-minute centrifugation at 13000 rpm, with the flow-through being subsequently eliminated. The spin column was then introduced in UV-crosslinked 1.5 ml tube, followed by addition of 200 µl buffer AE. After a settling period of one minute, 60-second centrifugation at 8000 rpm was performed again.

2.2.2 Synthesis of Primers

A search was carried out for *Apodemus sylvaticus* MAP1LC3,LAMP2a, Beclin1, TLR2, APP and Tau DNA sequences within the National Centre for Biotechnology Information (NCBI) database and as there were no recovered sequences for this genome (the first mouse species of choice), gene sequences from *Mus musculus* and *Rattus norvegicus* were identified for alternative use.

The Gene bank database at NCBI (http://www.ncbi.nlm.nih.gov/) and Ensembl (http://www.ensembl.org/index.html) provided the gene sequences for variants of autophagy and inflammation genes. The DNA variant sequences were subsequently subjected to ClustalW alignment by employing the alignment instrument supplied by EBI (http://www.ebi.ac.uk/Tools/msa/clustalo/). This was followed by creation of potential primers and addition of ambiguity codes to enable identification of multiple bases. Primer 3 (http://primer3.ut.ee/) was online software that enabled confirmation of primers and verification of every criterion that had to be satisfied to achieve best performance. Eurofins Genomics was used for primer synthesis (Table 2.1).

Table 2.1. Primer sequences, annealing temperature and anticipated fragment size for the autophagy and inflammation primers.

Name of gene	Primer (forward)	Primer (reverse)	Annealing temperatur	Size (bp)	Exon
MAP1LC3	5' GGACCTGCTGCCTTCTCTAA 3'	5' GCTGTTGTCTTCACAGCTGACA 3'	59°C	559	Exon 4
Beclin1	5' GCAGTGGCGGCTCCTATTCC 3'	5' TGTGTGCCACAAGCATCTCA 3'	57°C	317	Exon12
Lamp2a	5' TGGTCTCAAGCGCCATCATA 3'	5' GGCATCTTAGGTTAGGATCCCA 3	60°C	516	Exon 9
TLR2	5' TGACAGCTGTGCTGTAACAC 3'	5' TGGAGACCAGCAGAACACTC 3'	59°C	373	Exon 18-16
Tau	5' CCCCTCGGAGTCAGACATAG 3'	5' ATGTCAAGGGCTGTCAGGTT 3'	58°C	210	Exon 4-2
APP	5' CCTTCAAAAGCAGGCCAGC 3'	5' GCAGAAAGTGACCACCATGAG 3'	58°C	670	Exon 12-10

2.2.3 PCR methods

A Robocycler 96 (Stratagene, UK) was used to carry out all PCR and flat-capped PCR tubes with 0.2 ml thick walls (Advanced Biotechnologies, UK) were employed for every PCR reaction. Eurofins mwg/operon (Germany), previously known as MWG-Biotech, was used for the synthesis of every primer. Delivery of primers was done in lyophilised form and after they were received, they were dissolved in 100 pM/ul ultrapure DNase free water and placed in storage frozen for dilution as necessary.

2.2.4 Tubulin-specific DNA amplification using *Apodemus sylvaticus* DNA.

Alpha-tubulin-specific PCR, using primers designed to amplify 21 wood mice (*Apodemus sylvaticus*). Human genomic DNA (Promega), Catalog number G304A , genomic alpha-tubulin DNA, was performed as the control for the research project and in order to confirm adequate DNA recovery from suitable samples for further investigation (Draberova et al., 2013). PCR amplification carried out with a total volume of 25 µl which contained 2.5µl Bioline Bioline NH4 PCR buffer, 1µl MgCl2 (50 mM), 0.25 µl of dNTP mix (25 mM each), 0.5 µl of each forward 5'CGTGAGTGCATCTCCATCCAT 3' and reverse 5'GCCCTCACCCACATACCAGTG 3' 25 pM/µl, 18.75 µl of water, 0.5 µl of Taq DNA

polymerase (5 units/µl) and 1 µl of DNA. Positive control DNA was included in PCR run and consisted of human DNA, negative control was also included and consisted of PCR water, apart from using DNA, to monitor contamination in PCR reaction. It is possible to calculate the baseline PCR cycling condition by using DNA denaturation. First, this was conducted for ten minutes, at a temperature of 94°C. It lasted for a single cycle. Then, the process was repeated for forty cycle throughout, over forty seconds, with the primer galvanising at 60°C for 40 seconds. This was expanded to 72°C for a period of 90 seconds. Finally, the process ended with an extended rotation at 72°C for ten minutes**.**

2.2.5 PCR product gel electrophoresis.

The techniques described here have been used by researchers for some time (Kern and Brody, 2004). They are a popular way to conduct PCR and form an important part of many scientific studies. All data and visuals are recorded. The next step, after denaturation, is to carry out electrophoresis by adding agarose gel. It is useful for preparing the PCR DNA end-result. To make the gel, 0.3g of agarose is directed into a conical container (250ml). Then, 30ml of tri-borate-EDTA (TBE) is combined with this, because it acts as a buffer. The mixture is put in a microwave and warmed for a period of half a minute. After which, it requires vigorous agitation (usually by hand) and a further thirty seconds in the microwave. When it is melted, the container gets put on an agitator (artificial movement) until the temperature drops to 50°C. The substance is heated at full power, so it shouldn't take long for the agarose to break down. Following this, 30 µl of GEL RED (1,000X) is incorporated. It too needs to be gently agitated and swirled into the rest of the mixture (by hand).

The casting comb and dams must be prepared in place before you start pouring. To set the mixture, direct into a gel casting device. Then, it is left to rest and set. When it is ready, 200ml of TBE buffer gets added, directly on top of the original mixture. Eventually, the comb and dams are extracted. They can be cleaned, dried, and put back in their normal area now, because they're no longer needed. The mixture is added to the electrophoresis machine and so is the DNA analysis targeted for testing and an additional marker. The machine is left to operate at 70V (5V/cm) and 100mA for sixty minutes. When the bromophenol creates a distinct blue patch and it rests somewhere around the halfway point of the mixture, it can be removed. The UV trans-illuminator is then used to map out the DNA profile.

2.2.6 SAG1 polymerase chain reaction (PCR)

A wood mouse (Apodemus sylvaticus) was collected from an area close to Malham Tarn, North Yorkshire. Having been euthanased and dissected, as described section 2.2, was used to extract DNA from brain tissue. T. gondii was determined through the use of nested PCR amplification of the surface antigen gene-1 (SAG-1) (Savva et al., 1990, Duncanson et al., 2001, Terry et al. 2001). *T. gondii* was determined through the use of nested PCR amplification of the surface antigen gene-1 (SAG-1) (Savva et al., 1990, Duncanson et al., 2001, Terry et al. 2001).

The first round of PCR reaction was achieved in a total volume of 25 µl which contained 25 µl of PCR consisted of 2.5 µl Bioline (excluding $MgCl_2$), 2.5 µl β-mercaptoethanol (50 mM), 1µl MgCl2 (50 mM), 0.25 µl of dNTP mix (25 mM each), 2.5 µl of each external forward 5' TTGCCGCGCCCACACTGATG 3' and reverse primers 5' CGCGACACAAGCTGCGATAG 3' (10 pM/µl), 12.25 µl of water, 0.5 µl of Taq DNA polymerase (5 units/µl) and 1 µl of DNA. This reaction mixture was heated at 95°C for 5 minutes for 1 cycle, followed by 40 cycles of denaturation at 95°C for 40 seconds, annealing at 63°C for 40 seconds and extension at 72°C for 1 minutes 10 seconds, and finally cycle of extension at 72°C for 10 minutes.

Nested PCR was performed in a total volume of 25 µl containing 25 µl of PCR master mix consisted of 2.5 µl Bioline (excluding $MgCl_2$), 2.5 µl β-mercaptoethanol (50 mM), 1µl MgCl2 (50 mM), 0.25 µl of dNTP mix (25 mM each), 2.5 µl of each internal forward 5' CGACAGCCGCGGTCATTCTC and reverse Primers 5' GCAACCAGTCAGCGTCGTCC 3' 10 pM/µl, 11.25 µl of water, 0.5 µl of Taq DNA polymerase (5 units/µl) and 2µl of first around product.

2.2.7 SAG2 polymerase chain reaction (PCR)

According to Howe et al. (1997), the locus of type II SAG2 displays polymorphic sites at the 3 end. Amplification of this locus was therefore performed separately. Following the optimization protocol of Fuentes et al. (2001), two successive PCR reactions were performed with two sets of primers (nested PCR), one of which targeted the 3 end of the SAG2 gene. The nucleotide sequences of the outer and inner set of primers are detailed below.

The amplification was performed in a solution containing a mixture of 25mM KCl Bioline buffer (2.7 µl) and 25mM dNTP (0.32 µl) along with 10 pM forward and reverse primer (1 µl) and 5 U/µl Taq DNA polymerase (0.4 µl, Bioline) to a total volume of 20 µl. As a template, 2 µl of DNA was used. The first amplification cycle was initiated by 4 mins denaturation at 95 °C and was followed by 20 cycles consisting of successive incubations

at 94 °C (30 s), 55 °C (60 s) and 72 °C (120 s). Finally, the reactions were incubated for 10 min at 72 °C. After diluting the amplification products in water (1/10), the diluted product (2 µl) was used as a template in a second round of 35 amplification cycles at an annealing temperature of 60 °C with the inner primer sets designated below. The nPCR targeting the 3 end of SAG2 gene was regarded as positive when products with 222 base pairs were identified at the end of the second amplification round.

2.2.8 B1 Gene Amplification

External primers for the initial amplification round, comprising the forward primer 5 GGAACTGCATCCGTT CATGAG-3' and the reverse primer 5'TCTTTAAAGCGTTCGTGGTC-3', which yields a 193 bp product (nucleotides 694-887), and internal primers for the second round of amplification, comprising the forward primer 5' TGCATAGGTTGCAGTCACTG-3' and the reverse primer 5'GGCGACCAATCTGCGAATACACC-3', which yields a 96 bp product post-nPCR amplification, were the nested primers that targeted the B1 gene (Jones et al. 2000).

In the initial round of amplification, the composition of PCRs included 10 mM TRIS-HCl, pH 8.3, 50 mM KCl buffer, 2 mM $MgCl_2$ (Bioline KCL buffer with 15 mM $MgCl_2$ was used), 0.1 µM each primer, 0.1 mM each dNTP, 1.25 U Taq DNA polymerase and 2 µl parasite DNA. After cycling 40 times and 10-second denaturation at 93°C, reactions were annealed at 57°C for 10 seconds and extended at 72°C for half a minute. Diverging from the initial protocol, further 10-minute extension at 72°C was conducted.

In the second round of amplification, the PCR composition included 1µ of the first-round product, 10 mM Tris-HCl, pH 8.3, 50 mM KCL, 3 mM MgCl2, 0.5 µl each primer, 0.1 mM each dNTP and 1 U Taq DNA polymerase. After cycling 40 times and 10-second denaturation at 93°C, nested PCRs were annealed at 62°C for 10 seconds and extended at 72°C for 15 seconds. The nested reactions also comprised the negative control samples from the previous amplification. Further 10-minute extension at 72°C was conducted. Following electrophoresis on 2.5% TBE gel with Hyperladder V (Bioline) serving as marker, visualisation of 10 µl amplification products was achieved under UV illumination. In the two rounds of N-PCR, PCR-water and *T. gondii* RH strain DNA were respectively employed as negative and positive control. Manipulation of these was done in a different space than the PCR setup so that cross-contamination with PCR products did not occur. Furthermore, repeated pipetting in the same tube was prevented by storing PCR reagents and DNA in small aliquots. Visualisation of PCR products was enabled by GelRed™ (biotium) staining on a 1% TBE (Trisborate-EDTA) with 2.5% for B1 gene.

2.2.9 *Apodemus sylvaticus* LC3, Beclin1, LAMP2a, TLR2, Tau and APP-specific PCR

The content of the prepared master mix included 2.5 µl 10x Bioline NH4 PCR buffer, 1 µl 50 mM $MgCl_2$ (Bioline), 0.25 µl each of 25 mM dNTPs, 0.5 25pm/µl forward primer, 0.25 of 25pm/l of the created reverse primer, 18.75 µl distilled water and 0.5µl of 5unit/l Taq DNA polymerase. The conventional procedures of PCR cycling were applied, with 10-minute DNA denaturation at 94°C for one cycle, 40-second DNA denaturation at 94°C for 40 cycles. Furthermore, primer annealing was performed for 40 seconds at various temperature depending on primer type, followed by 90-second extension at 72°C. Further 10-minute extension at 72°C was performed as well. For certain PCRs, a Bioline MyTaq Mix was employed at particular times. This mix consisted of MyTaq DNA Polymerase buffer, dNTPs and MgCl2, with addition of 1 l of 25 pm/µl forward primer, 1 µl of 25 pm/l reverse primer, 1 µl DNA templet, 12.5 µl MyTaq Mix and 9.5 µl water.

2.2.10 PCR Optimisation

Annealing temperature and $MgCl_2$ concentration were the two parameters of PCR optimisation. The employed procedure of optimisation was intended to increase the number of target segments and make them more specific. Furthermore, a temperature gradient was established over a thermocycler plate to establish the ideal annealing temperature. For instance, temperature will rise by 1°C for every column in a 12-column PCR plate. In addition, to make it easier to recover PCR product, a MgCl2 dilution series was employed in certain cases.

2.2.11 Clean-up of PCR Products

This procedure was undertaken with a Bioline Isolated II PCR Kit. Sample preparation involved addition of water to obtain a 50 µl overall volume, followed by mixing of one sample volume with two CB binding buffer volumes. The introduction of the isolated II PCR column in a 2 ml collection tube was followed by sample loading and half-minute centrifugation at 11000 rpm. After removal of the flow-through, the isolated II PCR column was enriched with 700 µl CW washing buffer, followed by additional half-minute centrifugation at 11000 rpm. For elimination and attenuation of chaotropic salt carry-over, the preceding step was performed again. After removal of the flow-through, centrifugation was conducted again to eliminate ethanol from the washing buffer. Following introduction in a 1.5 ml

tube with around 25 µl elution buffer, the isolated II PCR column was incubated at room temperature for 60 seconds. Subsequently, final 60-second centrifugation at 11000 rpm was conducted.

2.2.12 Measurement of DNA concentration

The measurement of the concentration of the extracted PCR product was undertaken with a nanodrop spectrophotometer (ThermoFisher Scientific). This involved placing a 2–3 µl deionised water aliquot on the inferior optical surface, with closure of the superior optical arm to allow cleaning of both optical surfaces. The water was subsequently wiped off, followed by addition of 2-3 buffer to the optical surface and closure of the arm to blank the measurement. Closed, double-stranded DNA was the sample type used, with loading of 1 µl of the sample on the inferior optical surface and closure of the superior arm for concentration measurement.

2.2.13 PCR product for sequences

Preparation of PCR products was undertaken for delivery to Source Bioscience Lifesciences Company for sequencing. This company imposed certain conditions to ensure sequence data of high quality, such as 5 µl volume and 46 of 3.2 pmol/µl concentration for the forward and reverse primers, and 5 µl volume and 1ng/µl per 100bp concentration of samples for every PCR product. Furthermore, primers were18-23 base pairs long, the range of primer annealing temperature was 55-60°C, and primer GC content was 40-60%. FinchTV(http://officialsite.pp.ua/?p=2958497), NCBI blast search (https://blast.ncbi.nlm.nih.gov/Blast.cgi?PAGE_TYPE=BlastSearch) and clustal alignment (http://www.ebi.ac.uk/Tools/msa/clustalo/) were the software programs employed in data analysis. More specifically, sequencing was visualised and analysed via FinchTV, comparability to mouse references was assessed vi NCBI blast search, and sequences were arranged and discrepancies between them were searched via clustal alignment.

2.3 Western blotting

2.3.1 Reagents and materials

The conventional reagents and chemicals for this study were obtained from UK suppliers. Reagents for molecular

biology (e.g. protein ladders), rabbit monoclonal and goat anti-rabbit IgG (H+L) conjugated to HRP secondary antibodies, rabbit monoclonal LC3, Lamp2a, Anti-Beclin1, Anti-SQSTM1/p62, Anti-TLR2, Anti-NMDAR1, Anti-SOCS1, and GAPDH loading control were supplied by Abcam; Immun-Blot® PVDF membrane, Mini Protean Tetra cell®, Mini-PROTEAN® TGX™ precast protein gels, tris-glycine buffer and electrophoresis mini buffer tank were supplied by Bio-Rad; N-PER neuronal tissue protein extraction reagent and SuperSignal™ West Femto HRP substrate for the Western blot analysis were supplied by Thermo Fisher. The 10x re-blot solution (catalogue number 2502) was supplied by Merck Millipore the antibodies were incubated using dried milk powder (Marvel). The 3x SDS sample buffer (1M Tris pH 6.95), glycerol, 2-Mercaptoethanol, and sodium dodecyl sulphate (SDS) were supplied by Sigma. Dri-block® DB-2P heaters from Techne Digital were used to incubate the samples before loading for electrophoresis.

2.3.2 Protein extraction from mice brain tissue

The N-PER neuronal tissue protein extraction reagent was used in accordance with the manufacturer's protocol for total protein isolation. The neuronal tissue was harvested, washed with phosphate-buffered saline and stored on ice for later use. The sample was weighed and homogenised with N-PER Reagent in a tissue-to-reagent ratio of 0.1g to 0.1 ml. To obtain the optimum yield of protein, a Dounce homogeniser was employed with 10 to 20 passes on ice. The homogenate was then transferred to a suitable microcentrifuge tube and incubated for 10 min on ice before pelletising the cell debris via centrifugation at 4°C and 10,000 g for 10 min. The supernatant was then collected and stored at −80 °C for further purification or subsequent analysis.

2.3.3 Determination of protein concentration

The total protein concentration in the tissue samples was determined using the Bradford colorimetric (dye-binding) protein assay, which is based on changes in the colour of Coomassie Brilliant Blue G-250 (CBBG) dye upon binding to various concentrations of protein. The protein concentration is then determined by the absorbance measurement at 595 nm. To prepare the active Bio-Rad solution, distilled water (800 µl) was added to the Bio-Rad reagent (200µl) in a 1.6 ml, 1 cm-path UV-vis cuvette (giving a water-to-reagent ratio of 1:4). Calibration was performed using the N-PER neuronal tissue protein extraction reagent (2 µl) as a blank, with the spectrophotometer set to 595 nm. The absorbance of protein sample (2 µl) mixed with diluted dye was measured for normalisation. Equal quantities of protein were mixed with 3x SDS sample buffer (1M Tris pH 6.95), 10% glycerol, 5% 2-Mercaptoethanol, and SDS (0.6g) and used for sodium dodecyl sulphate-polyacrylamide gel electrophoresis (PAGE) on the same day or else stored at −20 °C.

2.3.4 Protein separation and preparation by SDS-PAGE

Electrophoresis is primarily used to separate macromolecules by molecular weight. In this technique, a polyacrylamide gel is used as a supporting medium and the proteins are denatured by SDS. The application of a voltage causes the denatured proteins, which are negatively charged, to move toward the positive electrode. In the present work, the Mini Protean Tetra cell® was used with 12% Mini-PROTEAN® TGX™ precast protein gels and tris-glycine as the running buffer for SDS-PAGE.

2.3.5 Sample loading and running

The SDS-Gel was loaded into the electrophoresis mini buffer tank containing 1x SDS-PAGE running buffer (0.25M Tris, 1.9M glycine, and 30mM SDS). The samples were incubated for 5 min at 95 °C prior to loading. The well combs were then removed and fine pipette tips (Prot/Elec™) were used to load the total protein samples (25 µg) and the pre-stained protein ladder molecular weight marker (5 µl). Electrophoresis was run for 20 min at 80 V to allow the samples to reach the resolving gel, then at 110 V for 95 min.

1.3.6 Western blotting and detection of proteins

In Western blotting, the individual proteins present in a mixture are identified by the addition of antibodies specific to each protein. The target protein is bound by the primary antibody, while a secondary antibody carrying a fluorescent label is bound to the primary antibody and is detected by its fluorescent light. The polyvinylidene difluoride (PVDF) membrane was first soaked for 30 s in methanol, while some filter paper was soaked in 1x SDS-PAGE transfer buffer. The gel and PVDF membrane were then sandwiched between two pre-soaked filter papers in a cassette in preparation for transferring the protein from the gel to the membrane. The cassette was positioned between electrodes immersed in 1x SDS transfer buffer. Cassettes and icebox were then placed in the tank with 1x SDS-PAGE transfer buffer and a stir-bar was used to keep the membrane and buffer cool. The transfer was performed at 0.4 amps for 2 h, with the ice being changed after 1 h. To avoid non-specific binding, the membrane was then blocked for 1 h with 5% skimmed milk in 1x TBS Tween. The nitro-cellulose membrane was then incubated at 4°C over-night on a rocking platform in the presence of a primary antibody at dilutions of 1:1,000 – 1:2,000 in 2.5% skimmed milk, 1x TBS and 0.1% Tween. On the following day, the membrane was subjected to three washes, each of 10 min duration, with 1x TBS and 0.1%Tween buffer prior to incubation with Horse Radish Peroxidase (HRP) conjugated secondary antibody in a 1:3,000 dilution dissolved in 2.5% skimmed milk/1x PBS/0.1%Tween (10 ml). This was followed by another three washes (10 min each) with 1x PBS/0.1%Tween. The PVDF membrane was then incubated with SuperSignalTM West Femto

Chemiluminescent substrates for visualisation of the protein bands. The chemiluminescent signal was detected using the G: Box Chemi XX6 (Syngene).

2.3.7 Western blot quantification

The results of western blotting were quantified using the Image J software version1.48. Each protein band reading was normalised to its matching GAPDH band, expressed with respect to the control lane, and the results presented on bar charts. The software allowed a square to be drawn around the protein bands, after which a tool could be used to select specific bands for representation as distinct peaks. The density indicated by the peaks was then calculated in a separate window.

2.3.8 Membrane stripping

After detection of the first protein, the membranes were incubated at room temperature for 15 min in stripping buffer consisting of 2-Mercaptoethanol (100 mM), 2% SDS, Tris-HCl (62.5 mM, pH 6.7) and washed three times in 0.1% TBS Tween as and when needed in order to strip the antibody from the membranes. The membranes were then blocked using the selected primary antibody and could be re-probed a number of times before stripping and subjecting it to a different selected antibody. The stripping procedure unbinds whichever primary antibody is present, thus enabling the new primary antibody to bind to its specific protein on the membrane.

The blot was stripped by addition of 10% SDS solution (10 ml) and 1M Tris (3.2 ml, pH 6.95) to the universal tube, after which distilled water was added to make the volume up to 50 ml. This was followed by addition of 2-mercaptoethanol in a fume hood, inverting the tube to achieve mixing, then heating the tube for 30 min at 50 °C in a water bath. After discarding the solution under the fume hood, the membrane was washed three times with PBS/0.1% Tween and blocked according to the usual procedure.

Re-blotting and the use of a mild stripping buffer allows gentle removal of primary and secondary antibodies from the PVDF membrane. The stripping solution was diluted by addition of distilled water (18 ml) to 10x re-blot solution (2 ml) before placing the membrane in a plastic tray containing the appropriate quantity of 1x stripping solution. The membrane was then stripped with 15 to 20 min of incubation time on a rotor machine, then the standard blocking procedure was performed for 1 h using 5% skimmed milk in 1x PBS. The blot was then ready for re-probing with the selected antibodies.

Chapter 3: Investigating the presence of *Toxoplasma gondii* in the tissue of human brains

3.1 Introduction

This research aims to conduct a pilot study, determining if brain samples from the Manchester Brain Bank may be used to investigate whether there is an association between *T. gondii* infection and AD. Pre-stained haematoxylin slides will be used to detect *Toxoplasma* cysts in human brain tissue samples – the same samples previously examined by Stan et al. (2019).

Cysts formed in the brains of hosts due to *T. gondii* infection primarily occur when the parasite is in its bradyzoite stage. The bradyzoites produce various proteins, which act as integral structures of the cyst walls; however, the precise mechanism of cyst formation remains unclear (Peng, Tan & Lindsay, 2015; Dubey, Lindsay & Speer, 1998). Whilst cyst formation is recognised as a virulence factor of *T. gondii*, helping the parasite escape from its hosts' immune systems, it has become increasingly evident that latent infection in these cysts is associated with brain dysfunction. In this regard, studies have shown that cysts may induce neuronal dysfunction, given their clinical correlates with headaches, seizures and psychiatric and behavioural disturbances (Sugden et al., 2016; Haroon et al., 2012; Samojłowicz et al., 2019).

Haroon et al. (2012) found that chronic *T. gondii* infection and cyst formation resulted in neuronal dysfunction by inducing aberrations in calcium signalling as a response to glutamate stimulation. In effect, this either lowered or heightened the neuro-responsiveness of infected neurons and, in turn, contributed to aberrant murine behaviour. Other murine studies have shown that *T. gondii* infection can also lead to hyperactivity, as well as to the impairment of other cognitive functions, such as working memory (Kannan et al., 2010; Webster, 2001).

In humans, one of the most significant associations related to *T. gondii* infection has been schizophrenia, with observational studies and case reports noting a high infection positivity rate in persons living with the psychiatric disorder (Torrey & Yolken, 2003). Indeed, high quality meta-analytical evidence has shown that the likelihood of schizophrenia in persons with chronic *T. gondii* infections is 2.7-fold greater than that in non-infected persons (Torrey, Bartko & Yolken, 2012).

Several studies have demonstrated positive associations between *T. gondii* infection, as determined by the detection of anti-*Toxoplasma* antibodies, and AD, with the likelihood ratios, among studies of reasonable sample sizes, ranging between 1.55 and 2.46 (Kusbeci et al., 2011; Mahami-Oskouei et al., 2016; Menati Rashno et al., 2016). Moreover, Nayeri Chegeni et al. (2019) conducted a recent systematic review and meta-analysis – including a new examination of their former meta-analysis, alongside other key studies – showed the association between Ad and *T. gondii* immunoglobulin G antibodies was significant (OR 1.53; 95% CI 1.07, 2.18).

However, the evidence, either way, is currently inconclusive, and this raises the important question of how the issue can be definitively addressed. To find essential evidence of *Toxoplasma* cysts in Alzheimer's patients, it will be necessary to take brain samples from human Alzheimer's patients and use techniques, such as immunohistochemistry, to measure the density of *T. gondii* cysts in their brains. Concerning the present study, in determining whether the sampled slides contained enough tissue to allow for the detection of *Toxoplasma* infections, the researcher concluded that the Manchester Brain Bank could supply enough tissue for the desired work.

The Manchester Brain Bank was founded by David Mann in 1986. Over 30 years, it has conducted fundamental, clinical research into the spectrum of frontotemporal dementia. Brain samples are recruited for the Brain Bank via its support of the Brains for Dementia Research initiative, as well as the assistance of tissue donation programmes (jointly funded by the Alzheimer's Society, Alzheimer's Research UK, the Manchester and Newcastle Longitudinal Ageing Cohort and the Cerebral Function Unit at Salford Royal Hospital).

The human brain tissue samples used in this research were obtained, post-mortem, from the Manchester Brain Bank after having been processed by the Brain Bank staff. Half of the tissue is snap frozen, and the other half is formalin fixed. These samples, all of which are pre-stained with haematoxylin, were taken from 45 cases presenting with no AD to late AD pathology and varied between Braak group (Stan et al., 2019). These samples were also used in a previous study (Stan et al., 2019), and the immunohistochemical staining present in them is designed for another protein. However, the background haematoxylin staining should enable the identification of *T. gondii* cysts. This method lends itself very well to uncovering the pathological processes that lead to non-standard neurological operations. In a previous study by Berenreiterova et al. (2011), the hippocampus and frontal lobe were found to have links to *Toxoplasma* infection, and so the conclusion was drawn that *T. gondii* cysts will most likely be identified in grey matter, which contains glial cells, neurons and blood vessels. Thus, the research seeks to determine the possibility of using these pre-existing human brain samples, obtained from the Manchester Brain Bank, to investigate the occurrence and distribution of *Toxoplasma* infections in Alzheimer's patients.

The objectives are:

- Use inspection to investigate the presence of *Toxoplasma* cysts in frontal and hippocampal derived brain human tissue and explore if this varied between Braak group.

3.2 Materials and Methods

Tissue samples from 45 subjects were taken from both the frontal cortex and hippocampus and then examined for the presence of *T. gondii* cysts. The 45 cases used in this study were had been pre-stained with Beclin 1 antibody an autophagy associated protein which would have allowed morphological identification of *Toxoplasma*. The 45 cases used in the study were selected based on degree of AD type pathology, using a system

called Braak staging (Braak *et al*, 1991; Braak *et al*, 1997) which looks at increasing deposition of tau pathology. The 15 cases were Braak stage 0-II (Unlikely AD or prodromal disease), 15 Braak III-IV (Likely AD, early/mid disease) and 15 Braak V-VI (Probable AD, late disease). Immunostained slides were evaluated using quantitative criteria (ImageJ) to provide a percentage score that represented the degree of tissue coverage. Three randomly selected x40 magnification microscope fields were taken for each patient (slide). Slides of the tissues were examined, using light microscopy, to pick out *T.gondii* cysts by eye in the brain tissue. Figure 2 shows an example of the size and structure of the trophozoite stage and a *T. gondii* cyst (Ionita et al., 2004). Typically, the trophozoites (Tachyzoites) are around 1 µM in length and typically cysts can be between 10 – 50 µM in diameter. Cysts are generally spherical or ovoid but can be very long and thin in some tissues such as muscle.

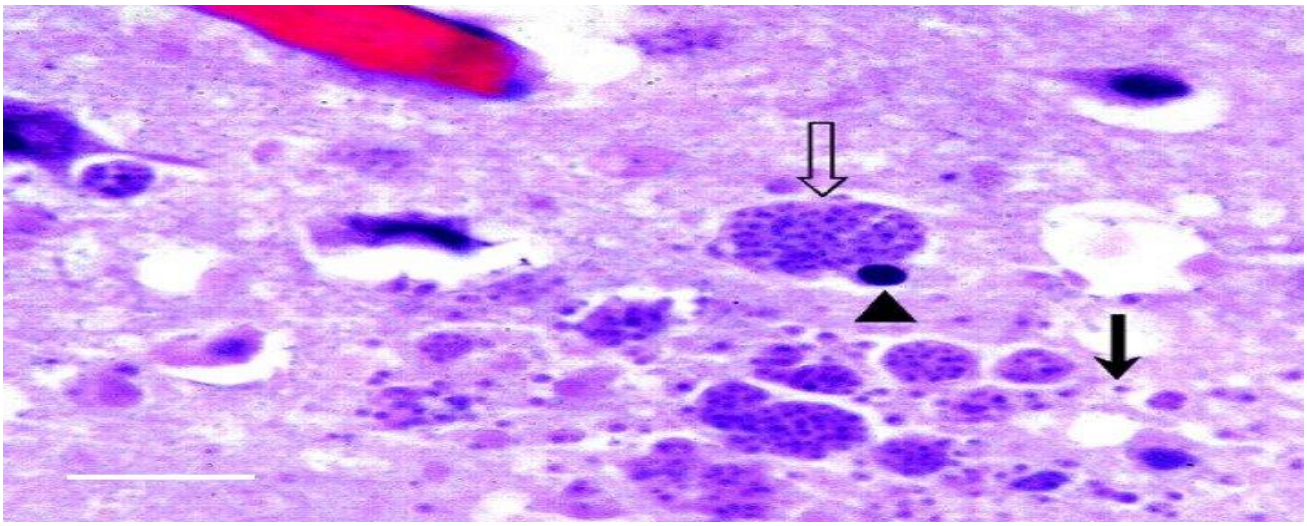


Figure 3.1: Microphotograph of *T. gondii* cysts in an H&E stained photomicrograph of a Toxoplasma infected patient. The image presented (magnification ×600) reveals multiple *Toxoplasma* cysts (open arrow) Scale bar = 50um, probable neuronal cells with round, dark nuclei (arrowhead), as well as trophozoites (Tachyzoites) in the extracellular space after the rupture of cysts (straight arrow). Adapted from (Ionita, Wasay, Balos, & Bakshi, 2004).

3.3 Results

3.3.1 Investigation into the distribution of *Toxoplasma gondii* in frontal brain tissue from Alzheimer's patients

It was found 45 slides containing human frontal brain tissue from Alzheimer's patients (Stan et al., 2019) were used to test for the presence of *Toxoplasma* cysts. Stan et al. (2019) had previously stained and counterstained the slides with haematoxylin to visualise their cell structures and immunostained them with Beclin-1. This haematoxylin staining was also useful for visualising *T. gondii*, but the immunostaining was not. Because these

were pre-existing, pre-stained tissues, they could not be further stained with anti-*Toxoplasma* antibodies. It was also not possible for the current researcher to stain new slides, since no such slides were available, and there was no ethical clearance for conducting new staining of this sort.

A visual investigation was carried out, using light microscopy, and the results for the 45 samples are shown in Table 3.1. Figure 3.2 provides examples of brain section photomicrographs. The tissue samples were investigated and visualised under three magnification conditions (×10, ×40 and ×100). As the purpose of this study was to detect *T. gondii* cysts, it was necessary to study example cysts to be able to recognise them. The brain is first infected by *T. gondii* in its tachyzoite stage, and, after entering a neuron cell, it transforms into its bradyzoite stage, as *T. gondii* cell division slows down until it has reached the dormant cyst stage. *Toxoplasma* cysts can appear in any part of the frontal cortex, particularly in the grey matter and surrounding blood vessels due to the proximity of the bloodstream. Hence, in this research, cysts were expected to be found in tissue from any part of the brain, including the hippocampus and the frontal area (an area associated with AD damage). The slides in Figure 3.2 reveal the grey matter and white matter of the outer cerebral cortex, which incorporates the neuron cell bodies and glial cells. Also visible are sections containing oligodendrocytes. These images show that no *Toxoplasma* cysts were found in the sampled frontal brain tissues. Figure 3.2A illustrates a haematoxylin positive spherical feature and, but their structures and sizes do not conform to a classical *T. gondii* cyst. Notably, Figure 3.2C highlights a Beclin-1 positive neuronal cell body, showing a potential cyst; however, this potential cyst does not present the typical bradyzoite clustering within a defined cyst wall (at x400 magnification) commonly associated with *Toxoplasma* cysts (Figure 3.1). After careful analysis of all 45 samples (Table 3.1), the researchers could find no evidence of *Toxoplasma* infection in any of the frontal brain tissue slides. Since it may be the case that the frontal brain area is not the preferred area for cyst formation, tissue from another region in the brains of the same patients – the hippocampus – was also considered.

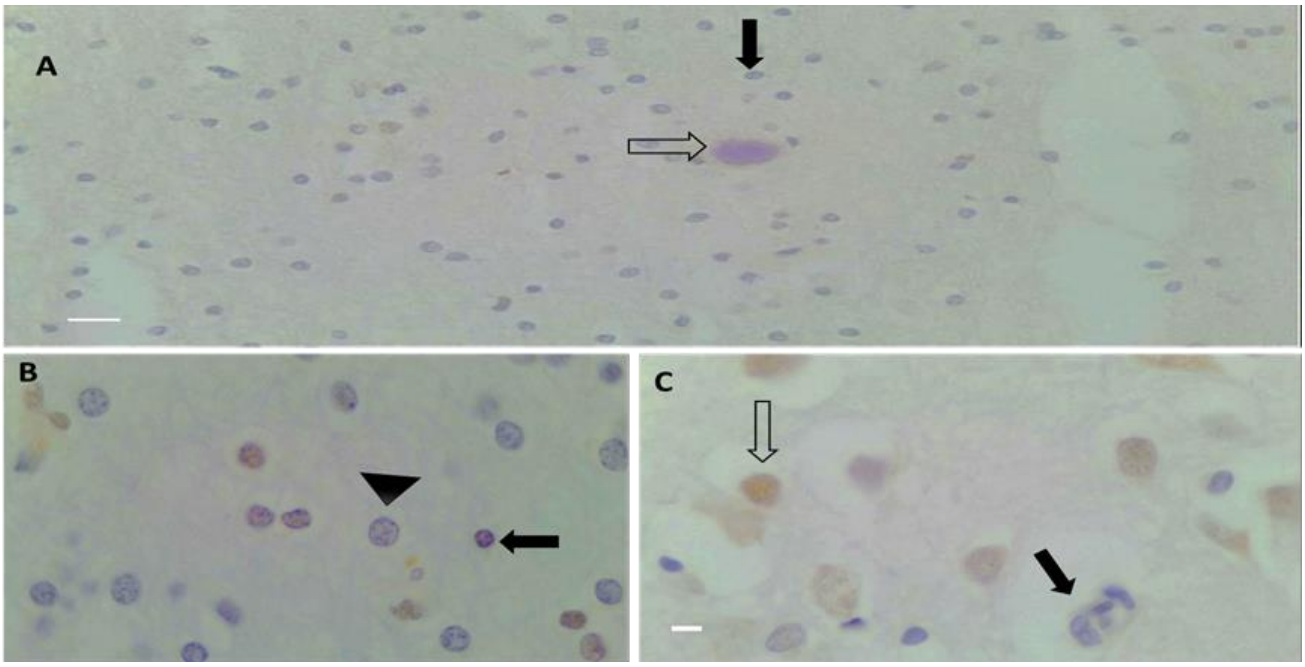


Figure 3.2: Example of photomicrograph (magnification ×40) from human frontal brain sections, which were stained, for a previous study (Stan et al., 2019), by immunohistochemistry with antibodies against Beclin-1. (A) Braak stage IV, showing neuronal cell bodies (straight arrow) and a haematoxylin positive spherical feature (open arrow); scale bar = 8um. (B) Brakk stage V-VI, showing oligodendrocytes (open arrow) and glial cells (straight arrow). (C) Brakk stage II, showing glial cells (straight arrow) and a Beclin-1 positive neuronal cell body (open arrow); scale bar = 5um.

3.3.2 Investigation into the distribution of *Toxoplasma gondii* in hippocampal brain tissue from Alzheimers patients

The presence of *Toxoplasma* cysts in human hippocampus tissue was investigated through 45 slides obtained from AD patients (Table 3.1). As described in Section 3.3.1, the slides had already been stained by Stan et al. (2019), and, therefore, they could not be stained with *Toxoplasma* antibodies. Moreover, no new slides were available to be stained for *T. gondii*, and ethical approval had not been obtained for such a procedure. All samples are shown in Table 3.2. A variety of tissue samples from Alzheimer's patients were examined under the magnifications of ×10, ×40 and ×100, as shown in Figure 3.3. Grey matter and white matter were visualised in the outer cortical layer, which incorporates the neuron cell bodies. Parts of the hippocampus, such as the glial cells, were also visualised, as was the accumulation of neurofibrillary tangles within the neuronal cell bodies. The microphotographs of AD cases revealed that no cysts were found in the hippocampal region. Figures 3.3A and B indicate Tau-positive neuronal cell bodies, and, as with the frontal brain tissue samples, structures resembling cysts could be confirmed as not being cysts due to the lack of classical cyst morphological features, such as size and parasite (bradyzoites) presence (see Figure 3.1). At first glance, a possible cyst was evident, but it was determined not to be a *Toxoplasma* cyst because it differed from the common *Toxoplasma* cyst expression; they usually appear (at ×400 magnification) as bradyzoites. Similar to the negative results for the frontal brain slides, *Toxoplasma* infection was not found in any of the 45 hippocampal brain tissue slides.

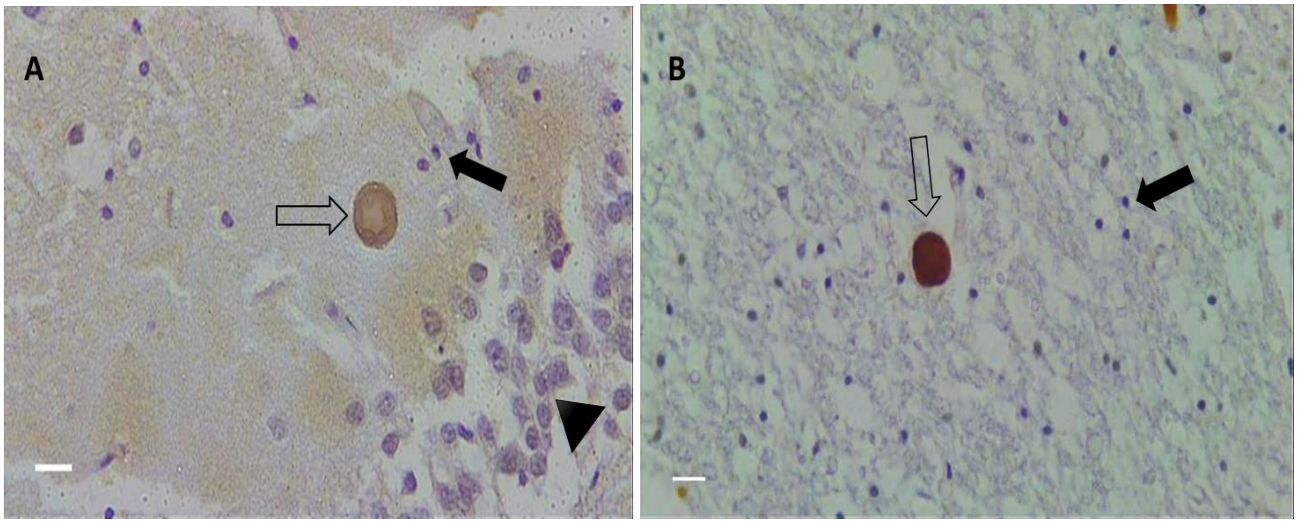


Figure 3. 3: Photomicrograph (×40 magnification) of the human hippocampus brain tissue., which was stained with Tau antibodies via immunohistochemistry for a previous study (Stan et al., 2019). (A) Braak stage I-II, showing glial cells (straight arrow), neural cells (arrowhead) and a Tau-positive neuronal cell body (open arrow); scale bar = 8um. (B) Braak stage V-VI, showing glial cells (straight arrow) and a Tau-positive neuronal cell body (open arrow); scale bar = 9um.

Summary of prevalence

In this study we have detected 0% prevalence of *T. gondii* in Alzheimer's patients using pre-stained Haematoxylin for immune-histological techniques. The 45 cases consist of 15 cases of Braak stage 0-II (Unlikely AD or prodromal disease), 15 Braak III-IV (Likely AD, early/mid disease) and 15 Braak V-VI (Probable AD, late disease). Several different tissue samples were investigated and visualised

under three different magnification conditions (x10, x40 and x100) and took a Photograph of them (Table 3.1). Overall the IHC showed the following results that the sections had been macroscopically assessed at autopsy and showed given that none of cases exhibited any microscopic evidence of *Toxoplasma*.

Table 3.1. Human frontal and hippocampus tissue samples obtained, from AD cadavers, from the Manchester Brain Bank and investigated for the presence of *Toxoplasma* cysts.

Braak groups	**magnification X10**	**magnification X40**	**magnification x100**	**Photograph**	***T.gondii* cysts**
0-II	✓	✓	✓	✓	0%
III-IV	✓	✓	✓	✓	0%
V-VI	✓	✓	✓	✓	0%

3.5 Discussion

This study was to investigate the presence of *T. gondii* cysts in the brain of AD patients, compared to healthy subjects, and specifically focussing on the frontal lobe and hippocampal regions. The aim of this limited study was to act as a pilot study to see if sufficient justification could be put together to develop a full study with the Manchester Brain Bank. The assessment was performed on fixed cadaver brain slices obtained from the Manchester Brain Bank. Latent toxoplasmosis is a condition that has been reported to be involved with AD (Mahami-Oskouei et al., 2016), while in felines it has been shown to induce anosmia (Prandota, 2014). In this study, it was hypothesised that *Toxoplasma* cyst location in specific regions of the human brain may contribute to our understanding of how this parasite is involved in AD. Of interest, chronic *Toxoplasma* infection has been shown to ameliorate AD symptoms such as amyloid-beta accumulation in the brain, a prime prognostic marker of AD (Mohle et al., 2016). Thus, the presence, or absence, of *T. gondii* cysts could be linked with AD severity, a prospect that to our knowledge has not been thoroughly examined, to date. With the help of samples from the Manchester Brain Bank, it was feasible to access precious human cadaver brain tissue from the frontal lobe and hippocampus, which was used in this study to assess whether cysts were present in AD/dementia patients and control patients. No confirmation of toxoplasmosis was possible on these samples and this can be justified by the sample sizes we used. The lack of evidence for infection can be completely explained by the small volume of brain tissue and relatively small quantity of samples examined.

In the United Kingdom, symptomatic toxoplasmosis is present in around 10-20% of the 350,000 people that are being infected annually (Advisory Committee on the Microbiological Safety of Food, 2012). As such, if one of the characteristics of latent toxoplasmosis would be cyst presence in the brain, in the elderly either with confirmed dementia or not, it would not also have been shown in our samples if they were too small as we argued.

This was not the case, thus making data interpretation difficult. There were several disadvantages for relying on this method. Our immunohistochemical investigation of the two brain regions indicated that *Toxoplasma* cysts were absent on both control and AD/dementia patients, irrespective of disease severity (Grade levels 0-IV). From this, it can be assumed that (a) patients from both groups did not have chronic toxoplasmosis and thus no cysts could be found, or (b) that some patients had been infected with *T. gondii,* but with those regions, relevant to AD, not being affected by the disease.

Finally, (c) it is possible for cysts for this parasitic infection not to be present in the human brain because there was not enough tissue to enable detection of the parasite. For a better understanding of these results, it would be important to confirm the infection prevalence via serology or molecular tools and also investigate, if possible, other brain regions to ensure that cysts were indeed absent from the majority of the brain tissue. Another possibility is that the brain tissue slides obtained were not of adequate quality in order to perform a valid immunohistochemical analysis for *Toxoplasma*. As mentioned in the results, there was an indication of some structures *resembling* cysts, but could not be verified due to low resolution. Yet, more samples will be needed to be investigated and compared with patients with and without AD and latent toxoplasmosis in order to obtain a more definitive answer on whether latent toxoplasmosis affects AD progression.

The technical limitations with respect to the area and quantity of brain tissues examined in the present study explain the contrasts between the outcomes of this study and those obtained Burrells et al. (2016). In Scotland, molecular detection of the parasite was performed using 3 g of brain tissue, which represents 0.2% of the human brain volume. This allowed an estimate of 17.9% for *Toxoplasma* prevalence. The average human male brain weighs 1,370 g and the average female brain weighs 1,200 g, giving an overall average of 1,285 g (Cosgrove et al., 2007; Harrison et al., 2003). However, the present investigation used 0.126 ml of brain tissue, with just 1 μL being seen under the light microscope in each field of view. Thus, only 0.0001% of the human brain volume was accurately examined for the presence of *Toxoplasma* cysts under low and high magnification. Images of the area of interest were obtained but no cysts were detected. Hence, the sample sizes may have been too small to allow the parasite to be detected. The above discussion suggests that a very large quantity of human brain tissue samples may have to be obtained and subsequently stained with *Toxoplasma* antibody. It is critical to identify the appropriate *T.gondii* immunohistochemistry stain, e.g. bradyzoite antigen1 (anti-bag1) and tachyzoite surface antigen1 (antigen sag1) (Passeri et al., 2016; Tomita et al., 2017). In addition, the samples may need to involve different parts of the brain. Consequently, the original proposal for acquiring samples from the Manchester Brain Bank has proved unfeasible – since we would not be able to get access to the volumes of tissue required for accurate analysis. A possible resolution to this problem might be to use animals such as rodents, which have smaller brains, to investigate the interactions of *T. gondii* infection with proteins that have demonstrated involvement in human Alzheimer's. Hence, given that we are unable to proceed with human brain tissue, as initially proposed, we have decided to work with rodent tissue.

Chapter 4: Investigation of *Toxoplasma gondii* prevalence in *Apodemus sylvaticus* mouse brain tissue

4.1. Introduction

This study aims to collect brain tissues from a wild population of wood mice (*Apodemus sylvaticus*), which can be used to investigate the relationship between *Toxoplasma gondii* infection and the expression of genes associated with AD (and other neurological diseases) in humans.

The pilot study, detailed in Chapter 3, illustrates that there are challenges to obtaining enough human brain material to conduct successful studies on natural *T. gondii* infections and AD. So, this research has been adapted to consider natural infections in wild mice populations.

Many studies on diseases such as Alzheimer's are conducted in model systems, either via laboratory animals or cell cultures. In natural populations, such as humans, considerable variability is observed between individuals; infectious diseases are delivered by natural routes; and many of these variabilities are not reflected in model laboratory systems. Thus, the novelty of the approach presented in this proposal is the planned investigation of the relationship between *T. gondii* infections and gene expressions related to AD in a natural population of wood mice (*A. sylvaticus)*.

This study intends to investigate the distribution of *T. gondii* infections in brain tissue, collected in an ordered manner, from a series of localized. *A. sylvaticus* populations (Boyce et al., 2012). Concentrations of these creatures are predominantly found in areas with relatively few cats. For instance, Hughes et al. (2008) quote a figure of fewer than 2.5 cats per square kilometre. Additionally, according to Thomasson et al. (2011) and Bajnok et al. (2015), previous findings in the area chosen for the present research showed a high commonness of *T. gondii* infections. To amplify the molecular diagnostic tools used for screening *Toxoplasma* brain tissue and to determine the prevalence of *T. gondii* in wood mice, this research plans to use the nested polymerase chain reaction (PCR) as designed by Su et al. (2006). Several PCR techniques are available to test for *Toxoplasma*. For instance, Duncanson et al. (2001) and Terry et al. (2001) use *T. gondii* marker SAG1; Bajnok et al. (2015) use *T. gondii* markers B1, SAG1 and SAG2 in wood mice; and Bajnok et al. (2019) use *T. gondii* markers B1, SAG1 and SAG2 in humans. The proposed study will examine

the brain tissue from a population of wood mice (*A. sylvaticus*) to extract DNA and test for the *T. gondii* parasite in the tissue, using common *T. gondii* markers B1, SAG1 and SAG2 determining the parasite's importance as a potential candidate for Alzheimer's screening. This study will also use protein extraction to examine Alzheimer's-related gene expression.

Objectives:

- Collection of wild *Apodemus sylvaticus* for brain samples
- Extraction of DNA from mouse brain samples.
- To confirm the quality of DNA extraction using mammalian tubulin PCR.
- To detect the presence of *T. gondii* using nested PCR amplification of *T. gondii* specific genes (B1, SAG1, SAG2).

4.2. Materials and Methods

The study involved collection of 21 a wild sample of *A. sylvaticus* mice taken from the Malham Tarn Nature Reserve, England, which were tested for detection of *Toxoplasma* in brains. Longworth Traps were used to capture *A. sylvaticus* (commonly known as wood mice), in the immediate area around Malham Tarn Field Centre (see Chapter 2 for details). Using sterile methods so as to not contaminate the subjects, the brains were removed and placed in tubes under 400 µl of Allprotect Tissue Reagent and then stored at a temperature of -20 °C. Extracted DNA was tested for mammalian tubulin, to ensure the DNA was viable for the use in diagnostic PCR (Terry et al. 2001; Chapter 2). This would remove the possibility of false negatives due to PCR inhibition All PCR reactions were performed using published primer sequences as described in Chapter 2. All samples were tested a minimum of five times with each marker. Development of methods of *Toxoplasma* detection in rodents to enable us to have a population of rodents to investigate in later chapters using markers for inflammation and other neurological events. Several methods of PCR amplification were used to ensure robust results: SAG1, a method credited to Savva et al., 1990 (later modified by Morley et al., in 2005) used nested PCR amplification of the Surface Antigen Gene 1; Positive amplification was confirmed by nested PCR amplification with other sets of *T. gondii* specific primers (SAG2) as described by Su et al. (2006) and Shwab et al. (2013). a third method, credited to Jones et al., 1999, involved nested PCR amplification of the repetitive and conserved gene B1; As a positive control, *T. gondii* RH strain DNA was used, with sterile water acting at the negative control. The Stratagene ROBOCYCLER™ was used for all PCR operations. The PCR products ran on 1.5% agarose TBE gel containing GELRED, which was then graphically represented on Alpha Imager™ 1220. For each phase of the operation, different locations were used to reduce the risk of contamination. To make sure that partial digestion did not skew the results, all the band sizes from all markers were carefully examined and compared to previously published DNA sequences and other studies. For achieving optimal amplification outcome devoid of non-specific amplicons, nested PCR was employed for amplification of genomic DNA extracted from the *Apodemus sylvaticus* specimen using freshly thawed aliquots of the same primer pair set and an internal primer pair. It has been

reported that nested PCR is efficient in effectively detecting *T. gondii* infections both in water and mammalian tissues (Kourenti & Karanis, 2006; Mahittikorn, Wickert, & Sukthana, 2005). In brief, nested PCR allows for minimal carryover of non-specific binding by introducing nested primers, which will be able to bind only to the designated target sequence, after a specific number of thermal cycles. This technique offers an intrinsic protection over by-products, providing a greater amount of target product in a number of human diseases (Eiamprapai et al., 2013; Skotnikova et al., 2000; Takahashi & Nakayama, 2006).

4.3 Results

A programme of polymerase chain reactions (PCRs) was carried out, employing *T. gondii* specific PCR primers, to scrutinise the extent of *T. gondii* in the *A. sylvaticus* population. Tests were conducted on DNA successfully removed from the brains of 21 mice. Amplification of the α-tubulin gene was used to establish the absence of PCR inhibition.

In all cases, the animal's body weight and length were measured at the time of collection, and the sex was determined. Body length was measured from the animal's nose to its anus. The mouse morphometrics, as collected during the fieldwork, are summarised below (Table 4.1).

Table 4.1. Summary of the captured details and morphometric data from the 21 sampled *A. sylvaticus* wood mice.

Samples No	Sex	Length (CM)	Weight (g)
475	male	7.9	14
476	female	7.7	19
477	female	8.3	20
478	female	7.6	15
479	male	8	21
480	male	8	18
481	female	7.5	16
482	male	7.3	19
483	male	7	13
484	female	7.6	19
485	male	8.6	18
486	male	7.3	17
487	female	7.6	16

488	male	8.1	18
489	female	8.4	17
490	male	7.2	16
491	male	9.1	20
492	female	7.9	13
MW1	male	7	13
MW2	male	8.8	16
MW3	female	8.2	17

4.3.1 Mammalian α- tubulin PCR

The aim of this objective was to develop the α-tubulin gene PCR technique to be used as a positive control for amplifying DNA samples. PCR products using a set of α-tubulin primer pairs were run in a 1% agarose gel to detect any amplified genomic products. The anticipated band size for α-tubulin was ~1300bp. As a negative control, distilled water was used instead of DNA during the PCR amplification steps. For the same primer pair sets (old and new aliquots) used in the PCR, five gel electrophoresis attempts are shown in Figure 4.1.

In Figure 4.1A, the primary experiment for the human genomic DNA sample (Promega), which was used as a positive control, shows two bands of approximately 1300bp, indicating that PCR amplification of the α-tubulin gene was successful. There were no by-products, even after 40 amplification cycles, although the smear found in all bands, including the negative control, may be indicative of non-specific primer binding. The bands visualised were faint – implying that either the starting DNA template was low upon PCR amplification or that fresh PCR reagents should have been used. As such, new primer pairs for α-tubulin were employed, and Figure 4.1B indicates that the new aliquots of primer pairs and the fresh reagents exhibited a better outcome, confirming that this sample collection method could provide adequate DNA. To rate the quality of the *A. sylvaticus*

DNA – 475, 476, 477 and 478 mammalian-specific – ß-tubulin PCR was used. 1300bp was the expected band size for the PCR product. To identify the expected band, as shown in Figure 4.1B, 1% agarose gel was used. Human DNA was used as a positive control, and sterile water was used as a negative control.

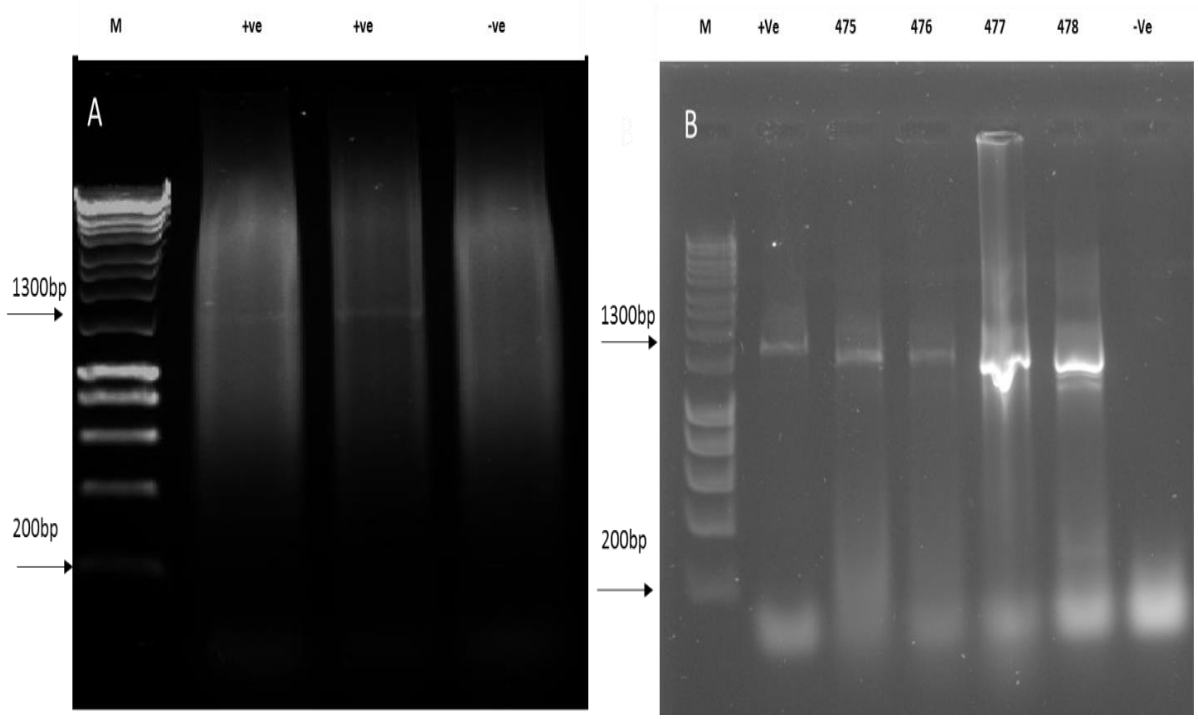


Figure 4 1: PCR amplification of the α-tubulin gene to check for PCR inhibition. A) Lanes 2–3: experimental PCR sample (human DNA). Lane 4: negative control (water). M: Hyperladder 1Kb (Invitrogen, UK). B) PCR amplification of the α-tubulin gene to check for PCR inhibition. Lane 2: positive PCR control (human DNA). Lanes 3–6: test samples *A. sylvaticus* targeted fragment (1300bp). Lane 7: negative control (water). M: Hyperladder 1Kb (Invitrogen, UK).

4.3.2 B1 PCR Amplification

To test the DNA samples for the presence of *T. gondii* DNA, the B1 gene amplification method was developed. A 2.5% agarose gel electrophoresis methodology was used for B1 nested PCR products as the amplicon size was small. The expected amplicon length for B1 was 96bp. While the function of the 35-fold repetitive B1 gene is unknown, it is highly conserved, which means that it is ideal for use in PCR amplification. In Figure 4.2, the primary wood mice DNA samples, given by Prof Geoff Hide, which were experimentally tested, show that B1 genomic DNA could not be found. The negative control suggested no signs of contamination, and the positive control indicated that the B1 gene sequence was present at the expected bp length (96bp). In Figure 4.3, (Prevalence of 23.8% ,95% confidence interval: 10.2; 45.5%) of the wood mice samples (five out of 21) – 475, 482, 485, 486 and 488 – successfully amplified the B1 gene. When the second round PCR resulted in the predicted 96bp band, the field samples were judged as positive via B1-PCR. This study, however, does not confirm this result. Instead, improved amplification of parasite DNA was achieved using other markers, which proved to be more reliable.

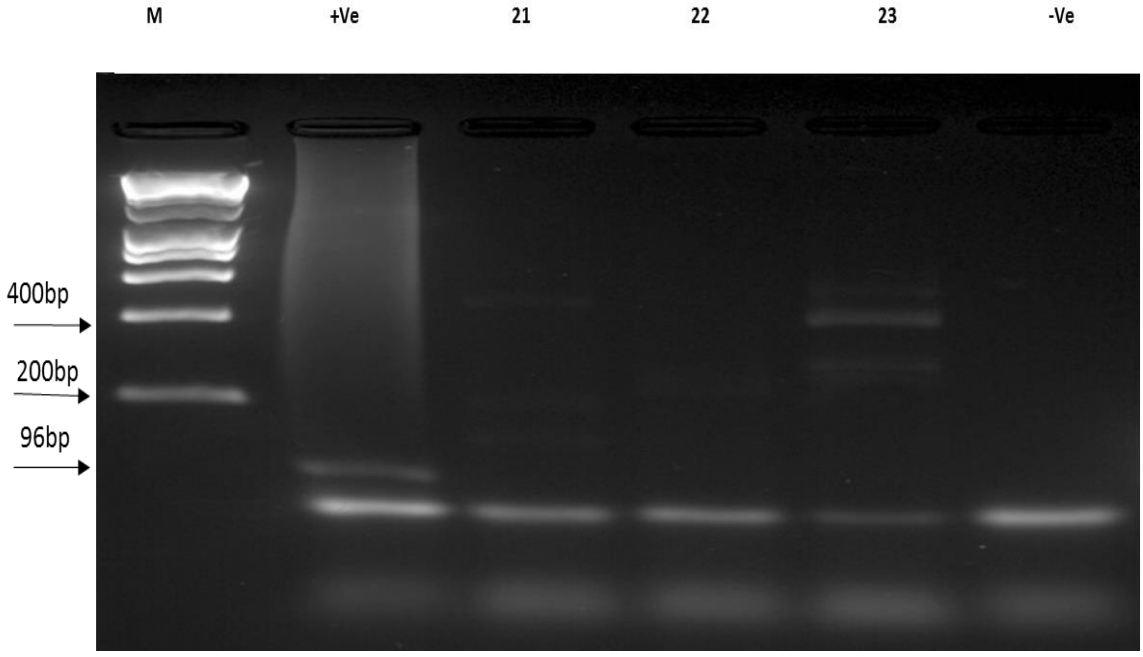


Figure 4 2: B1 PCR analysis of DNA from *A. sylvaticus* for the detection of *T. gondii* in 2.5%

agarose gel. Lane 2: RH positive control (*T.gondii* DNA). Lanes 3–6: DNA samples (*A. sylvaticus*). Lane 7: negative control (water). M: Hyperladder 1Kb (Invitrogen, UK).

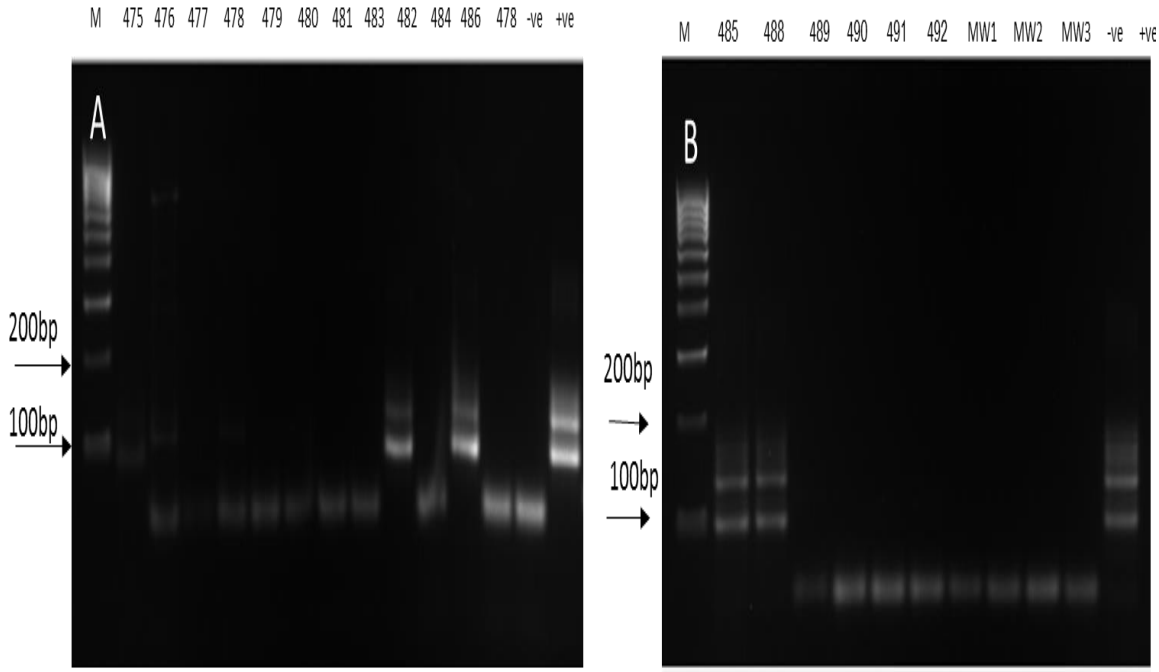


Figure 4 3: B1 PCR analysis of DNA from *A. sylvaticus* for the detection of *T. gondii* in 2.5% agarose gel. A) Lanes 2–13: DNA samples (*A. sylvaticus*). Lane 14: negative control (water). Lane 15: RH positive control (*T. gondii* positive DNA). M: Hyperladder 1Kb (Invitrogen, UK). B1) PCR analysis of DNA for the detection of *T. gondii* in 2.5% agarose gel. Lanes 2-10: DNA samples (*A. sylvaticus*). Lane 11: negative control (water). Lane 12: RH positive control (*T. gondii* positive DNA). M: Hyperladder 1Kb (Invitrogen, UK).

4.3.3 SAG1 PCR Amplification

To test DNA samples for the presence of *T. gondii* DNA, SAG1 gene amplification and SAG1 nested PCR were run in 1.5% agarose gel electrophoresis. The expected amplicon length for SAG1 was 522bp. Figure 4.4A shows the first attempt to detect SAG1 positive

control amplicons. As with α-tubulin (Figure 4.1A), SAG1 expression was not readily visualised, possibly due to have incorrectly programmended in the wrong cycle conditions recommended for PCR reaction.

In Figure 4.4B, the test results for the primary experimental mice DNA samples, given by Prof Geoff Hide, are also shown. In order to get better results, PCR optimation was performed at Tm of 63°C. SAG1 genomic DNA was not detected in any of the samples loaded, and no contamination was detected (negative control). However, the positive PCR control did indicate the presence of the SAG1 gene sequence at the expected bp length (522bp).

The research was initially conducted with samples from the brains of 21 wood mice. It was found that five samples (Prevalence of 23.8% ,95% confidence interval: 10.2; 45.5%) – 475, 482, 485, 486 and 488 – were infected with *T. gondii*, as shown by the SAG1-PCR assay. Figure 4.5 depicts an example of successful *T. gondii* detection. After the first round of PCR employing the external primers, a product of 914bp was amplified. In the second round of the PCR assay, the product was used to attain a highly specific product of 522bp.

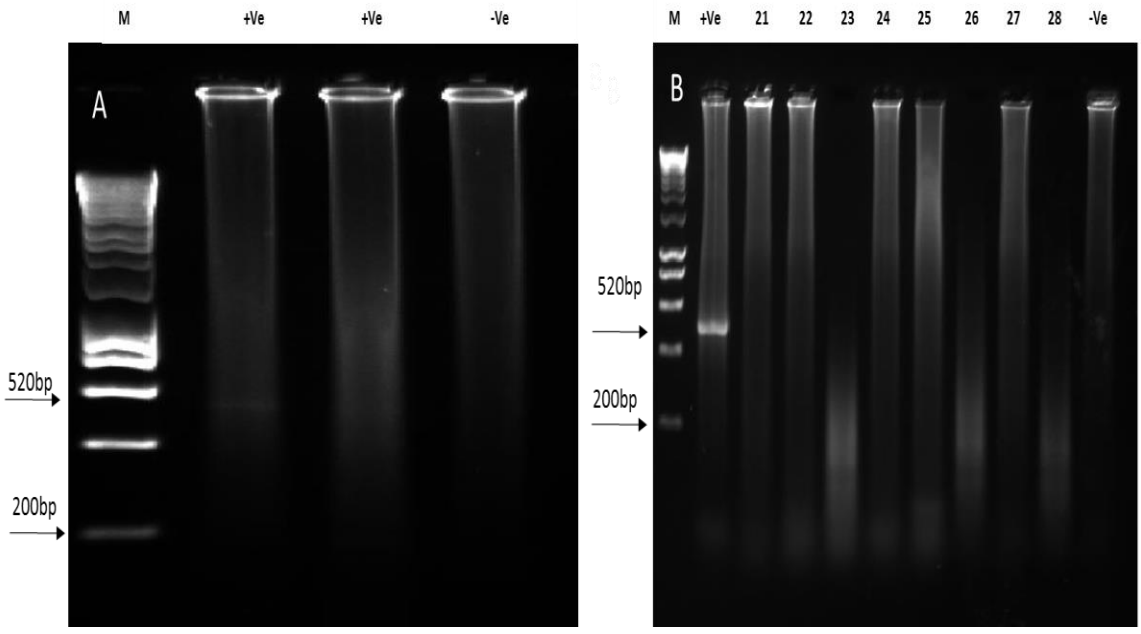


Figure 4 4: SAG1 PCR analysis of DNA from *A. sylvaticus* for the detection of *T. gondii* in 1.5% agarose gel. A) Lanes 2–3: RH positive control (*T.gondii* positive DNA). Lane 4: negative control (water). M: Hyperladder 1Kb (Invitrogen, UK). B) SAG1 PCR analysis of DNA from *A. sylvaticus* for the detection of *T. gondii* in 1.5% agarose gel. Lane 2: RH positive control (*T.gondii* positive

DNA). Lanes 3–10: DNA samples (*A. sylvaticus)*. Lane 11: negative control (water). M: Hyperladder 1Kb (Invitrogen, UK).

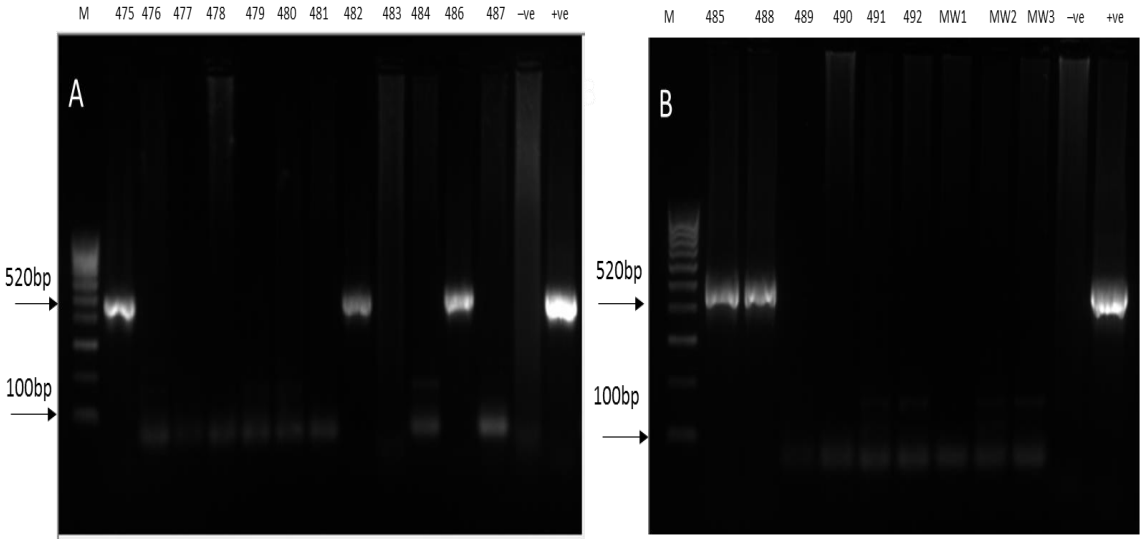


Figure 4 5: SAG1 PCR analysis of DNA from A. sylvaticus for the detection of *T. gondii* in 1.5% agarose gel. Lanes 2–13: DNA samples (*A. sylvaticus*). A) Lane 14: negative control (water). Lane 15: positive PCR control (*T. gondii* positive DNA). M: Hyperladder 1Kb (Invitrogen, UK). B) SAG1 PCR analysis of DNA from *A. sylvaticus* for the detection of *T. gondii* on 1.5% agarose gel. Lanes 2–10: DNA samples (*A. sylvaticus*). Lane 11: negative control (water). Lane 12: RH positive control (*T. gondii* positive DNA). M: Hyperladder 1Kb (Invitrogen, UK).

4.3.4 SAG2 PCR Amplification

To test the DNA samples for the presence of *T. gondii* DNA, SAG2 gene amplification was conducted via 2% agarose gel electrophoresis in SAG2 nested PCR products. The expected amplicon length for SAG2 was 222bp. Figure 4.6 shows the results of tests conducted for signs of the amplification of the SAG2 gene in the total sample of 21 wood mice brain tissue specimens. Howe et al. (1997) state that the SAG2 locus has one polymorphic site at the 3/ end for type II identification. To establish the 222bp product, primers were chosen to separately amplify the 3/ end of the *T. gondii* SAG2 locus. All five

positive tissue samples (Prevalence of 23.8% ,95% confidence interval: 10.2; 45.5%) – 475, 482, 485, 486 and 488 – indicated that the amplification of the 3/ end of the SAG2 gene was successful, and the negative controls remained free of amplified products.

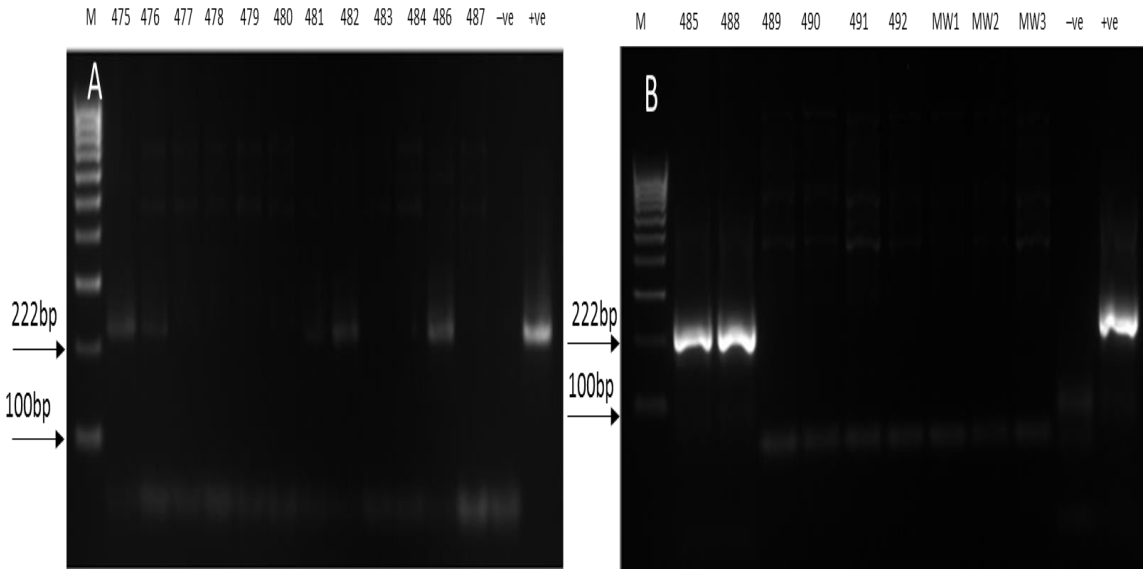


Figure 4.6: SAG2 PCR analysis of DNA from A. sylvaticus for the detection of *T. gondii* in 2% agarose gel. A) Lanes 2-13: DNA sample (*A. sylvaticus)*. A) Lane 14: negative control (water). Lane 15: RH positive control (*T. gondii* positive DNA). M: Hyperladder 1Kb (Invitrogen, UK). B) SAG2 PCR analysis of DNA from *A. sylvaticus* for the detection of *T. gondii* in 2% agarose gel. Lanes 2–10: DNA samples (*A. sylvaticus*). Lane 11: negative control (water). Lane 12: RH positive control (*T. gondii* positive DNA). M: Hyperladder 1Kb (Invitrogen, UK).

Summary of prevalence

Overall prevalence of *T. gondii* prevalence by nested PCR. In total 5 samples gave positive reactions with three *T. gondii* specific markers B1, SAG1 and SAG2.Thus an infection rate of 23.8% (95% confidence interval: 10.2; 45.5%) was found. In total, out of 5 positive mice from Malham Tarn, were PCR positive for *T. gondii* (Table 4.1).

Table 4.2 The PCR analysis of the brain tissue from *A. sylvaticus* for detection of *T. gondii* by PCR with 3 different markers, B1, SAG1 and SAG2. Key: "+" = positive result, "-" =negative result.

PCR Marks

Sample NO	B1	SAG1	SAG2	*T.gondii*
475	+	+	+	+
476	-	-	-	-
477	-	-	-	-
478	-	-	-	-
479	-	-	-	-
480	-	-	-	-
481	-	-	-	-
482	+	+	+	+
483	-	-	-	-
484	-	-	-	-
485	+	+	+	+
486	+	+	+	+
487	-	-	-	-
488	+	+	+	+
489	-	-	-	-
490	-	-	-	-
491	-	-	-	-
492	-	-	-	-
MW1	-	-	-	-
MW2	-	-	-	-
MW3	-	-	-	-

4.4 Discussion

This study sought to obtain a collection of wood mice (*Apodemus sylvaticus*), the *T. gondii* infection status of which was known. This was intended to provide brain tissue samples, enabling the researchers to compare the expression of important inflammatory genes in infected and uninfected animals. This collection was taken from an area relatively free of cats. The results showed that 23.8% (95% confidence interval: 10.2; 45.5%) of the mice, a comparatively high number, were infected with the *T. gondii* parasite. This is surprising because the mice were chosen from an area with a low presence of the definitive *T. gondii* host: the cat (Hughes et al., 2008).The results obtained from these wild wood mice showed that *T. gondii* is readily present in wild rodents, making them potential intermediate hosts for infecting the definitive host, the cat. Previous reports suggest that it is possible for mice to act as a mutation hub for the evolution of new, virulent *T. gondii* strains (Dubey et al., 2013; Dubey et al., 2010). These results contrast with those of a German study (Hermann et al., 2012), which, surprisingly, found a 0% prevalence of *T. gondii* in voles, shrews and field mice. This study was carried out using serological testing.

In all, this study concluded that wood mice may serve as intermediate hosts for *T. gondii*. In general, apart from feline excrements being a potential route through which humans may contract toxoplasmosis, wild mice could also pose a risk for human health. In fact, recent reports have shown that *Apodemus sylvaticus* and the urban mouse, *Mus domesticus*, are hosts for *T. gondii* (Bajnok et al., 2015; Marshall et al., 2004; Thomasson et al., 2011). According to Banjok et al., the prevalence of *T. gondii* seems to be linked to the host's genotype and not with the geographical location (Bajnok et al., 2015), while the presence of cats, cohabiting with mice, is not considered a factor for *T. gondii* prevalence in the murine population (Thomasson et al., 2011). However, in the current research, the prevalence of *T. gondii* infection among the sampled mice was lower than that observed by Bajnok et al. (2015) (34.92%) and by Thomasson et al. (2011) (40.78%), the latter of whom conducted their study in the same locality near Malham Tarn

Depending on the country of origin of the research and species in question, *T. gondii* prevalence in mammals varies greatly across Europe. A recent PCR-based study by Fuehrer (2010) has determined that 0.7% of Austrian *Microtus arvalis* tested positive. A serological

study by Alfonso et al. (2007) shows a high presence of *T. gondii* in the natural populations of many small mammals in and around Ardennes, France; the study has also found a 60% prevalence in shrews (*Sorex sp.*), 39% in moles (*Talpa europaea*) and 39% in water voles (*Arvicola terrestris*). A North American study by Dabritz et al. (2008) illustrates a higher prevalence in a specific species – *Peromyscus sp.* – of around 23%–31%, as opposed to the negative results from *Mus musculus* and *Microtus californicus*. Four studies in Europe show a high seroprevalence in wild carnivores across Europe as a whole: 33% in polecats (*Mustela putorius*), 18% in stone martens (*Martes foina*), 17% in pine martens (*Martes martes*) (Hejlicek et al., 1997) and 43% in arctic foxes (*Vulpes lagopus*) (Prestrud et al., 2008). Foxes have also presented an 84.7% seropositivity for *T. gondii* in Saxony-Anhalt and a 74.5% seropositivity in Brandenburg (Hermann et al., 2012). Due to the cumulative effect when consuming infected prey, these species are expected to have higher *T. gondii* prevalence. Infections among mice are most likely caused by oocysts in water, food (grains and insects), contaminated soil and vertical transmission, via either milk or the placenta. Studies by Eichenwald (1948), Thiermann (1957), Beverley (1959), Remington et al. (1961), De Roever-Bonnet (1969), Owen and Trees (1998) and Duncanson et al. (2001) reveal that *T. gondii* can be vertically transmitted through a variety of hosts, including humans. Duncanson et al. (2001) state that, even in a non-feline environment, other species, such as sheep, can be intermediate hosts for the parasite. The high prevalence in artic foxes could result from vertical transmission, according to Prestrud et al. (2007), considering that the ecosystem may be the most important host when there is no definitive host present. It is likely that the prime transmission route in voles and Svalbard reindeer, which have nil prevalence, is not the presence of oocysts.

It would be logical to suppose that there would be a higher incidence of *T. gondii* infections in older individuals, as the infection is more likely to be contracted over time, if oocysts in the environment caused the infection in the Malham Tarn area. This, however, was not evident in the present study, as there was no significant age prevalence and no greater infection prevalence in the older age group. Conversely, young adults and juveniles had a higher prevalence compared to adults. Nevertheless, age prevalence in larger animals, which have longer lifespans, may be better established than in mice, which are relatively short-lived.

Conventional PCR is more popular than nested PCR. Although it does not have the same level of sensitivity, it does not require two PCR amplification steps. Nested PCR is also a more time consuming, expensive and intensive procedure and has a higher risk of contamination. However, nested PCR is essential for detecting *T. gondii* DNA, as the total amount of DNA from the pathogen among samples varies and, in some cases, appears in extremely small amounts. The limitations caused by the sample sizes of animal tissues also create a major problem for detecting *T. gondii* as it is possible that the parasite is present in the tissue that is not examined.

Because the infective state of *T. gondii* lasts for approximately two weeks, and because all infective oocytes must be released during that time, there is an element of doubt as to the part cats play in the parasite's transmission. This study has indicated that a higher number of cats may actually have been present in the research area as mixed infections were found, or there may have been one cat infected with a sexual recombination or multiple strains of *T. gondii,* which could have resulted in the generation of new genotype combinations. Still, there is no strong evidence to suggest that wood mice and cats are linked concerning *T. gondii* infection, particularly when there is a low density of cats in a given area. Congenital transmission of *T. gondii* is a likely explanation, according to Tenter et al. (2000), despite its comparative rarity, particularly as rodents cannot be infected by the disease through carnivory. Despite an absence of felid-derived oocysts, in theory, *T. gondii* infection levels could still persist in a mouse population. For instance, other research has shown the occurrence of congenital transmission at high levels (61%) in sheep (Duncanson et al., 2001) and in urban mice (Marshall et al., 2004). This indicates that *T. gondii*, evidenced by its highly clonal structure, has succeeded in generating asexual transmission cycles, bypassing the definitive host (Aspinall et al., 2001) and being transmitted in food or, according to Marshall et al. (2004), by vertical transmission. A recent study considering the Malham Tarn mice populations has indicated that this is probably the case as different mice families had different levels of prevalence for *T. gondii* infections (Bajnok et al. 2015).

A modern theory put forward by a number of scientists is that infected men pass toxoplasmosis to non-infected women during unprotected intercourse (Flegr et al., 2014).

It is argued that seminal fluid carries tachyzoites in humans and in the testes tissue of various animals. Some females have reportedly been infected via the semen of infected men through artificial insemination (Flegr et al., 2014). However, the known risk factors do not explain the *T. gondii* infections in two-thirds of pregnant women. This sexual transmission supposition, however, does provide the soundest explanation for the high number of mixed infections in tested mice, though it has not been proven. In the Malham Tarn area, the *T. gondii* prevalence in mice was confirmed to be 23.8%. The oocyst route of *T. gondii* contraction is unlikely in this environment due to the lack of felines; nevertheless, the parasite still seems to spread. Congenital transmission could be a possible explanation for this. This study shows that *T. gondii* presence in mammals is high and can be spread, potentially congenitally, without the need for cats. Future research requires a wider sample to conduct more in-depth analysis of the effects on family-based populations of *Apodemus sylvaticus*. PCR-based diagnostics have been used in this study, as they are a very specific method for identifying the presence of parasite DNA and could be usefully employed to address these future questions.

Chapter 5: Development of *Apodemus sylvaticus* as a model organism for population studies on the interaction of *Toxoplasma gondii* infection and autophagy and inflammation gene expression in brain tissue: identification of human gene homologues in *Apodemus*.

5.1 Introduction

Autophagy and inflammation have been shown to contribute to AD, as have immune dysregulation and *Toxoplasma* prevalence, in mammals (Babu et al., 2008; Cappelletti et al., 2014; Jiang & Mizushima, 2014; Michiorri et al., 2010; Fujikake, Shin and Shimizu, 2018; O'Brien and Wong, 2011).

It is, therefore, essential to investigate whether these regulatory mechanisms affect AD onset and progression in relation to *Toxoplasma* infection status. In a similar manner, the inflammatory genes involved in innate immune pathogen recognition are also important in toxoplasmosis (Denkers, 2003; Hunter & Sibley, 2012; Pollard, Knoll & Mordue, 2009; Yarovinsky, 2014). The role of amyloid deposits in AD, and in relation to the aforementioned diseases and mechanisms, may also reveal novel pathways for understanding AD onset and progression.

In Chapter 3, this study has illustrated that the availability of human brain samples was insufficient for investigating the research questions. Chapter 4 has shown that, within a population of wood mice (*A. sylvaticus*), if there are animals infected or uninfected by *T. gondii*, these mice could be used to investigate differences in the expressions of inflammation-related and autophagy genes. However, to conduct research from this standpoint, it is necessary to determine whether these genes are present in *A. sylvaticus*. Additionally, as these genes have not been characterised in *A. sylvaticus*, it is also necessary to determine whether their sequences are homologous enough to the human equivalents that homologous human tools (antibodies) may be used to detect the genes' expressions.

First, the aim was to look for the presence or absence of autophagy and inflammation gene homologues in *A. sylvaticus*. The chosen genes were those encoding the microtubule-associated protein 1 light chain 3 beta (LC3) protein (MAL1LC3B gene), Beclin (Becn1), LAMP2A, TLR2, APP and Tau. These genes are all important requirements for producing the proteins involved in autophagy, inflammation and AD in humans. Thus, further investigating them offers the opportunity to develop tools to explore the relationships between *Toxoplasma* infection and these processes in natural populations of wood mice.

Objectives:

1- To design PCR primers for gene homologues of autophagy/inflammation genes in *Apodemus sylvaticus* using comparative bioinformatics with homologous genes from *Mus musculus*, *Rattus norvegicus* and *Apodemus sylvaticus*.

2- To investigate the presence of key autophagy genes in wood mice (MAP1LC3, Beclin1, LAMP2) and Inflammation related genes (TLR2, Tau and APP) using PCR amplification.

3- Confirm the identity of the genes by sequencing and generate novel information on the homology of *Apodemus sylvaticus* genes with other mammalian homologues using DNA and derived protein sequence data.

5.2 Materials and Methods

DNA was extracted from brain tissue samples from *A.sylvaticus* using Qiagen's DNeasy Blood & Tissue kit (as described in Chapter 2), in order to investigate the presence of homologous genes via conventional polymerase chain reaction (PCR). Specific primers for amplifying specific gene segments were designed for use after post-PCR product clean-up and PCR optimisation. Primer design was performed using information from the GeneBank

Database at NCBI (http//www.ncbi.nlm.gov/) and Ensembl (http://ensembl.org/indeex.html) DNA variants selected were subjected to ClustalW alignment of the European Bioinformatics Institute (EB)(http://www.ebi.ac.uk/Tools/msa/clustalo). There is a draft *A.sylvaticus* genome available which can be used to get sequence data for comparison of *Mus musculus* and *Rattus norvegicus* genes with the *A.sylvaticus* genes. Primer pairs were eventually designed using the Primer3 online software tool (http://primer3.ut.ee/). Selected primer pairs were ordered and amplicons were sequenced as described in Chapter 2.

5.3 Results

5.3.1 DNA sequence alignment of mouse and rat genomic sequences to identify conserved regions for autophagy and inflammation genes

The National Center for Biotechnology Information (NCBI) was used to obtain the genome for each of the species of rodents. There is a draft preliminary genome assembly on NCBI for *A.sylvaticus (*European woodmouse) (ASM130590v1). However, prior to this study there were no annotations or publications that have described the DNA sequence of any autophagy- or inflammation-related genes for this genome or for this species of rodent. In order to identify homologous genes, annotated genomes from related rodents were employed such as *Mus musculus* (house mouse, NCBI assembly GRCm38.p6) and *Rattus norvegicus* (Norway rat, NCBI assembly Rnor_6.0). In order to identify relevant genes for *A.sylvaticus*, these two genomes were used to identify and design primers from identified and annotated existing gene information. Once suitable DNA sequences had been identified for the mouse and rat species, of the DNA sequences were aligned using the program ClustalW. ClustalW finds the best alignment over the full sequence submitted with the symbol (*) identifying identical amino acid residues in pairwise samples.

A search of the NCBI was carried out that combined the terms *Mus musculus* and *Rattus norvegicus* with Map1LC3, the first gene of interest. Both species showed a number of variations within the MapLc3 DNA sequence, and therefore several of these sequence variations from both species were aligned in order to establish the location of similarities between them. This procedure is illustrated in table 5.1. Primers were then designed based on those regions of the MapLc3 DNA sequence where there was shown to be similarity between the two species.

Table 5.1 A) MapLc3 DNA sequence (variant 1, exon 4) from *Mus musculus*, B) Lc3 DNA sequence (variant 1, exon 4) from *Rattus norvegicus*, C) Clustal W alignment of variants of the Map1Lc3 gene from *Mus musculus* and *Rattus norvegicus*.

A

GGCGCTTGCAGCTCAATGCTAACCAAGCCTTCTTCCTCCTGGTGAATGGGCACAGCATGGTGAGTGTGTCCACTCCCATCTCCGAAG
TGTACGAGAGTGAGAGAGATGAAGACGGCTTCCTGTACATGGTTTATGCCTCGCAGGAGACATTCGGGACAGCAATGGCTGTGTAAG
ACTCCAACAAAGCCAATGGTTGTTAAGCCCTTACCAAGGCAAAAAGGGATGTTACCAGCGGACGCTGGACGGCTCACCACCCACAGA
TGAGAACGTAGGCACCCACATAGGGTATTAGGAACTGTTCATCAGCCAGAAACTGAGCTCCATGCAAGTGCACTCAGCTTGGAAACT
CGTCTAAACTAGGCTATTTTGTGTTCAAACTTTAGAAGTTAAAAATAAAATACTTTTTGCATCCTAAGTTGCCAATAAAAAGATCAA
GTTATTTTGACATTTTTCTCCCTAGTAGGGACTTAAAACAGAAGAGCAGTGTCAGGGGCAGTCTCTTCCTGGGACCTGCTGCCTTCT
CTAAAAGGGCTCCCACTCAGCAGAGAAGCCCCTCTGAAGGCCCAGGTTGCCTAGCAGAGGCCCCCAGAGGGCCCTGGCTTTGCCCTA
AGCAGGTCTGGTGTTGTAGGATATAGCTCTAAGCCGGGTCCTGGTCTTGACCCCAGCCACACCCTTTCACTCAGCAGTGCAGGGACA
TGCAGCTCGCTGCTGTCTAGATGTTACCATACGCCCTTCTGCTGAGGCCAGGTCTCCGCTTGTCCAGGGGTTGCTGACCCAGCTTAA
GCGACTGGAGAGCTGTTTCTCTCCTGGTTTGAATTCTGTCTGGTCCATGCCTGCTTCAGGGAGAGCAAGCGCTGGCCTTAGGATGTG
GTTGTCAAGTGGTAGACTGCAAGTCCAATGCTCCAGACTTGGCTCACAGCTCTTTGTTGGTGTGTAACTGTCTCTGTAAGGGTGGTT
CTGACAGCTGTTGGGATTTCCCCATTTCATGTCAGTACATACATGTCAGCTGTGAGGACAACAGCAACAGAAGACATGCTTTTGATA
GACATGCCACTTCAGCTAAAGCTAGCCTCTGCTTCCTGCATGTGGCCCGCTGTGACACCAGGCCATCCCTGGTCACTCACTCACTCG
TGGTCTGAGGATCTCAGAGCTGCCTGCTGTCATGGACTGAAGCCAGCATAGCCACCTCCCACCTAGCTCCCCACAGGCACCCACCTT
CCCTGCATGCAGCTGTCTTTGCTAACCCTCAATGTTATTTTACACTGTAAATTCTCACTGCTCTGTCTTGTGTAGGTTGTATACGTC
ATTATGTCCCTGGTTTTTATAACTATGGTGCGATCAGTAAGGATTCCTGTAATACTGCTTTAAAGATGCTGCTCAGAGGGCGGGCGG
CCTCCATTATAGGACACCTGTACACTCTGATGCACTAATAAAGGCACAACGAACCTGT

B

AGGCGCCTGCAGCTCAATGCTAACCAAGCCTTCTTCCTCCTGGTGAATGGGCACAGCATGGTGAGTGTGTCCACACCCATCTCTGAA
GTGTACGAGAGCGAGAGAGATGAAGACGGCTTCCTGTACATGGTCTATGCCTCCCAGGAGACGTTCGGGACAGCACTGGCTGTGTAA
GACTAGAACAATACCTAATGTTTGTTAAGCCCCTACCAAGGCAAAAAGGGACGTTACCAGCGGAAGCTGGTCAGCTCACCACCCCAC
AGATCAGAACGTAGGCACCCACGTAGGGGATTAGAAACTATCTCAGCCAGAAACTGAGTGCCATGCAAGTGCATTTGGCTTGGAAAC
TCATCTAAACTAGACTATCCTGTGTTCAAACTTTAGTTTAAAAATAAAATACTTTGCATCCTAAGTTGCCAATAAAAAGACCAAGTT
ATTTTGACATTTTCTCCCTAGTAGGGACTTGAAATAGAAGAGCAGTGTCAGGGGCGGCCTCTTCCTGGGACCTGCTGCCTTCTCTAA
AAGGGCTTCTACTCAGAGAAGTCCCTGGAAACAGTCCCTCGCCCAGGTTGCCTAGCAGAGGTCTCCAGAGGGCTCTGGCTTTGCCCG
AAACAGGTCAGGTGTATAGGACATAGCTCTAAGGACCCACATGGGCCCTGGTCCTGAACCCCAGCCATTTCACCTCAGCAGTGTGGG
ACGCTCACCCGCTGCTGTGTAGACATTACCACATGCACCACTGCTCAGAGGCCAGTTCCAACCCTGTCCAGGGGTTGCTTGGTCCAG
CTTCTTAAGAAACTGCAAGGTGTTTTTCTCCTGGTTTGGACTTCTCTGGTCCATGCCTGCTTCAGGTGTGCAATGCTGGCCTTCCCG
TGCAGCTGTCCAGTGGAAGGCTGAAGTCCAAGTGCACTCGCCTTGTACGTGGTTCACACCTACTTGTCAGTGCACACCCACAGTCTT
TGTAAGGGCGGTTCTGACAGCTGTTGGGGTTTCCCCATTTCATGCCAGTACATACATGTCAGCTCTGAAGGCAACAGCAACAGGAAG
AGAGCCATGCTTGTGACAGACATACAGCCACTTCCAACTAAAGCAAGCCTCTGCTTCCTGCTACCTGCATGGAGCCCACTGTGACAC
TCAGACCATCCCCGGTCACTCACTCGTGTCTGAGAATCTCAGTGAGAGCTGCCTCTGTCACGGACGGAAGCCAACACAGCCACCTCT
CGACCTGGCTCCCCACAGCACCCACCCTCCCTGCATGCAGCTGTCCCTGCTAACCCCCAATGTTATGTTACACTGTGTAAATCCCCA
CTGCTGCCGTGTGTGGGTTGTGTACGTCGTCATGTCCCTGGTTTATAACTATGGTGCGGTCGGGAAGGATTCCTGTAATGCTGCTCT
AAGATGCTGCTCAGGCAGCCATTGTAGGACACCTGTACTCTGATGCACTAAGTCAATAAAGGCACAACTGGACCTGT

C

```
mouse      -GGCGCTTGCAGCTCAATGCTAACCAAGCCTTCTTCCTCCTGGTGAATGGGCACAGCATG      59
rat        AGGCGCCTGCAGCTCAATGCTAACCAAGCCTTCTTCCTCCTGGTGAATGGGCACAGCATG      60
            ***** **************************************************

mouse      GTGAGTGTGTCCACTCCCATCTCCGAAGTGTACGAGAGTGAGAGAGATGAAGACGGCTTC      119
rat        GTGAGTGTGTCCACACCCATCTCTGAAGTGTACGAGAGCGAGAGAGATGAAGACGGCTTC      120
           ************** ******** ************** ********************

mouse      CTGTACATGGTTTATGCCTCGCAGGAGACATTCGGGACAGCAATGGCTGTGTAAGACTCC      179
rat        CTGTACATGGTCTATGCCTCCCAGGAGACGTTCGGGACAGCACTGGCTGTGTAAGACTAG      180
           *********** ******** ******** ************ ***************

mouse      AACAAAGCC-AATGGTTGTTAAGCCCTTACCAAGGCAAAAAGGGATGTTACCAGCGGACG      238
rat        AACAATACCTAATGTTTGTTAAGCCCCTACCAAGGCAAAAAGGGACGTTACCAGCGGAAG      240
           *****  ** **** *********** ****************** ************ *

mouse      CTGGACGGCTCACCACC-CACAGATGAGAACGTAGGCACCCACATAGGGTATTAGGAACT      297
rat        CTGGTCAGCTCACCACCCCACAGATCAGAACGTAGGCACCCACGTAGGGGATTAGAAAC-      299
           **** * ********** ******* ***************** ***** ***** ***

mouse      GTTCATCAGCCAGAAACTGAGCTCCATGCAAGTGCACTCAGCTTGGAAACTCGTCTAAAC      357
rat        -TATCTCAGCCAGAAACTGAGTGCCATGCAAGTGCATTTGGCTTGGAAACTCATCTAAAC      358
            *   ****************  ************* *  ************ *******

mouse      TAGGCTATTTTGTGTTCAAACTTTAGAAGTTAAAAATAAAATACTTTTTGCATCCTAAGT      417
rat        TAGACTATCCTGTGTTCAAACTTTAGTTTAAAAAT----AAAATACTTTGCATCCTAAGT      414
           *** ****  ****************     ***     ** *   **************

mouse      TGCCAATAAAAAGATCAAGTTATTTTGACATTTTTCTCCCTAGTAGGGACTTAAAACAGA      477
rat        TGCCAATAAAAAGACCAAGTTATTTTGACA-TTTTCTCCCTAGTAGGGACTTGAAATAGA      473
```

```
          ************** *************** ******************** *** ***

mouse     AGAGCAGTGTCAGGGGCAGTCTCTTCCTGGGACCTGCTGCCTTCTCTAAAAGGGCTCCCA     537
rat       AGAGCAGTGTCAGGGGCGGCCTCTTCCTGGGACCTGCTGCCTTCTCTAAAAGGGCTTCTA     533
          ***************** * ************************************ * *

mouse     CTCAGCAGAGAAGCCCCT-----CTGAAGGCCCAGGTTGCCTAGCAGAGGCCCCCAGAGG     592
rat       CTCAGAGAAGTCCCTGGAAACAGTCCCTCGCCCAGGTTGCCTAGCAGAGGTCTCCAGAGG     593
          *****   **   *               ******************** * *******

mouse     GCCCTGGCTTTGCCCTAAGCAGGTCTGGTGTTGTAGGATATAGCTCTAAGCCGGGTCCTG     652
rat       GCTCTGGCTTTGCCCGAAACAGGTCAGGTGTATAGGACA-TAGCTCTAAGGACCCACATG     652
          ** ************ ** ****** *****    *    **********      * **

mouse     GTCT-T-GACCCCAGCCACAC--CCTTTCACTCAGCAGTGCAGGGACATGCAGCTCGCTG     708
rat       GGCCCTGGTCCTGAACCCCAGCCATTTCACCTCAGCAGTGTGGGAC--GCTCACCCGCTG     710
          * *  * * **  * ** **     **   **********  **         * *****

mouse     CTGTCTAGATGTTACCATACGCCC--TTCTGCTGAGGCCAGGTCTCCGCTTGTCCAGGGG     766
rat       CTGTGTAGACATTACCACATGCACCACTGCTCAGAGGCCAGTTCCAACCCTGTCCAGGGG     770
          **** ****  ****** * ** *   *   * ******** **    * **********

mouse     TTGCTGACCCAGC----TTAAGCGACTGGAGAGCTGTTTCTCTCCTGGTTTGAATTCTGT     822
rat       TTGCTTGGTCCAGCTTCTTAAGAAAC-TGCAAGGTGTTTTTCTCCTGGTTTGG-ACTTCT     828
          *****    *       *****  **  *  ** ***** ************     * *

mouse     CTGGTCCATGCCTGCTTCAGGGAGAGCAAGCGCTGGCCTTAGGATGTGGTTGTCAAGTGG     882
rat       CTGGTCCATGCCTGCTTCAGGTGTGCA--ATGCTGGCCTTCCCGTGCAGCTGTCCAGTGG     886
          **********************          *********    **  * **** *****

mouse     TAGACTGCAAGTCCAATGCTCCAG------ACTTGGCTCAC---AGCTCTTTGTTGGTGT     933
rat       AAGGCTGAAGTCCAAGTGCACTCGCCTTGTACGTGGTTCACACCTACTTGTCAGTGCACA     946
           ** *** *   * * *** *  *      ** *** ****     **  *   **

mouse     GTAACTGTCTCTGTAAGGGTGGTTCTGACAGCTGTTGGGATTTCCCCATTTCATGTCAGT     993
rat       CCCACAGTCTTTGTAAGGGCGGTTCTGACAGCTGTTGGGGTTTCCCCATTTCATGCCAGT     1006
             ** **** ******** ****************** *************** ****

mouse     ACATACATGTCAGCTGTGAGGACAACAGCAACAGAAGACATGCTTTTG-----ATAGACA     1048
rat       ACATACATGTCAGCTCTGAAGGCAACAGCAACAGGAAGAGAGCCATGCTTGTGACAGACA     1066
          *************** *** * ************ *     **  *       * *****

mouse     T----GCCACTTCAGCTAAAGCTAGCCTCTGCTTCCTGCAT-------GTGGCCCGCTGT     1097
rat       TACAGCCACTTCCAACTAAAGCAAGCCTCTGCTTCCTGCTACCTGCATGGAGCCCACTGT     1126
          *     *   * ** ******* ****************         *  **** ****

mouse     GACACCAG-GCCATCCCTGGTCACTCACTCACTCGTGGTCTGAGGATCTCAG---AGCTG     1153
rat       GACACTCAGACCATCCCCGGTCACTCACTCGT-----GTCTGAGAATCTCAGTGAGAGCT     1181
          *****     ******* ************       ******* *******

mouse     CCTGCTGTCATGGACTGAAGCCAGCATAGCCACCTCCCACCTAGCTCCCCACAGGCACCC     1213
rat       GCCTCTGTCACGGACGGAAGCCAACACAGCCACCTCTCGACCTGGCTCCCCACAGCACCC     1241
           *  ****** **** ******* ** ********* *  *  *   ***    ******

mouse     ACCTTCCCTGCATGCAGCTGTCTTTGCTAACCCTCAATGTTATTTTACACTGTAAATTCT     1273
rat       ACCCTCCCTGCATGCAGCTGTCCCTGCTAACCCCCAATGTTATGTTACACTGTGTAAATC     1301
          *** ******************  ********* ********* *********  *

mouse     CACTGCTCTGTCTTGTGTAGGTTGTATACGTCATTATGTCCCTGGTTTTTATAACTATGG     1333
rat       CCCACTGCTGCCGTGTGTGGGTTGTGTACGTCGTCATGTCCCTGGTTTA--TAACTATGG     1359
          * *    *** * ***** ****** ****** * *************   *********

mouse     TGCGATCAGTAAGGATTCCTGTAATACTGCTTTAAAGATGCTGCTCAGAGGGCGGGCGGC     1393
rat       TGCGGTCGGGAAGGATTCCTGTAATGCTGCTCTAAGATGCTGCTC-AG----------GC     1408
          **** ** * *************** ***** ***           **          **

mouse     CTCCATTATAGGACACCTGTACACTCTGATGCACTAATAAAGGCACAACGAACCT-----     1448
rat       AGCCATTGTAGGACACCTGTACTCT--GATGCACTAAGTCAATAAAGGCACAACTGGACC     1466
            ***** ************** **  **********   *   *   *  * **

mouse     -GT     1450
```

```
rat         TGT     1469
```

Also, A search of the NCBI directory that combined the terms *Mus musculus* and *Rattus norvegicus with* Beclin1, Lamp2a, TLR2, Tau and APP the all genes of interest. In order to locate regions of similarity between the two species, several variations of the genes DNA sequence from both species were aligned. This process is illustrated in Tables 1- 5. Once these specific regions of similarity had been identified, they were chosen to design the primers.

5.3.2 Alignment of conserved regions of *Rattus norvegicus* and *Mus musculus* genes with potential sequences in the *Apodemus sylvaticus* draft genome and design of PCR primers.

The lack of prior database information on autophagy genes in the genome of *A.sylvaticus* was resolved by aligning the two aforementioned rodent genomes with DNA sequences obtained from a draft genome sequencing project, produced in Liverpool, and located on the NCBI database. In order to find primer pairs able to bind to gene regions of interest of all three genomes, the conserved regions previously identified in rats and mice were used to locate the homologous sequences in the *A.sylvaticus* genome. The aim of this was to minimise the possibility of primer mismatches due to sequence variation related to the evolution of these different species. These alignments were used for the design of primer pairs for the six genes described previously. The primers were chosen from certain positions using a number of criteria. For example, some positions were more stable than others and contained a higher G and C content. Also, the difference between the melting temperatures of the forward and reverse primers needs to be low, which will be helpful during the PCR reaction. The aligned sequences produced the primer pairs shown in Table 5.8. In particular, the ClustalW methodology successfully identified highly conserved regions in the *Mus musculus* and *Rattus norvegicus* through which the primers could be identified and sourced and enables further investigation into autophagy and inflammation genes in *A. sylvaticus* (Tables 5.2-5.8).

Table 5.2 Locations of the forward and reverse primers for the Beclin1 gene from *Apodemus sylvaticus.* Three Beclin1 related sequences were aligned using Clustal W: Rat, *Rattus norvegicus*, Beclin1, *Apodemus sylvaticus*, Mouse, *Mus musculus*. Numbers down the left-hand side refer to the base positions relative to that start of the relevant sequence. Yellow shaded areas indicate the selected forward and reverse primes.

```
CLUSTAL O(1.2.4) multiple sequence alignment


Rat         ----------------------------------------ACCTGCAGGATGGACGTGG     19
Beclin1     GACTTAGGTTGGCTCTGTTACTAATGAGCTCTGCTATGTCCACCTGCAGGATGGACGTGG     60
mouse       GACTTAGGTTGGCTCTGTTACTAATGAGCTCTGCTATGTCCACCTGCAGGATGGACGTGG     60
                                                  ******************

Rat         AGAAAGGCAAGATTGAAGACACTGGAGGCAGTGGCGGCTCCTATTCCATCAAAACCCAGT     79
Beclin1     AGAAAGGCAAGATTGAAGACACTGGAGGCAGTGGCGGCTCCTATTCCATCAAAACCCAGT     120
mouse       AGAAAGGCAAGATTGAAGACACTGGAGGCAGTGGCGGCTCCTATTCCATCAAAACCCAGT     120
            ************************************************************

Rat         TTAACTCTGAGGAGCAGTGGACAAAGGCGCTCAAGTTCATGCTGACGAATCTCAAGTGGG     139
Beclin1     TTAACTCGGAGGAGCAGTGGACGAAAGCGCTCAAGTTCATGCTGACGAATCTCAAGTGGG     180
Mouse       TTAACTCGGAGGAGCAGTGGACAAAAGCGCTCAAGTTCATGCTGACCAATCTCAAGTGGG     180
            ******* ************** ** ******************* *************



Rat         GTCTTGCTTGGGTGTCCTCACAGTTCTATAACAAGTGACTTGCTCCTTAGGGGATATTTG     199
Beclin1     GTCTTGCCTGGGTGTCCTCACAGTTTTATAACAAGTGACTTGCTCCTTAGGGGATCTTTG     240
Mouse       GTCTTGCCTGGGTGTCCTCACAGTTCTATAACAAGTGACTTGCTCCTTAGGGGATGTTTG     240
            ******* ***************** ***************************** ****

Rat         CCTTTAAGGTTTTACATTTTGTTTGGTTTGGAAAGATGCTTTAAATTAAATTTGGGTAAT     259
Beclin1     CCTTTAAGGTTTAACACTTT----GGTTTGGAAAGATGCTTTAAATTAAATTTGGGTAAT     296
Mouse       CCTTTAAGGTTTTATACTTTGTTTGGTTTGGAAAGATGCTTTAAATTAAATTTGGGTAAT     300
            ************ * * ***    ************************************

Rat         ATTAAACCACATGTTTACAATACCAGAATCCACAAAAGCTACTTTATTTTACAATATGAC     319
Beclin1     ATTAAACCACATGTTTACAATACCAA-ATCCACAAAAGCTACTTTATTTTCAAATATGAC     355
Mouse       ATTAAACCACATGTTTACAATACCAAAATCCACAAAAGCTACTTTATTTTCAAATATGAC     360
            ************************  ***********************  ********

Rat         AGTTTTC---AGAGCACAGCACGCCATGTATAGCAAAGAGCCCTGCCGTAGTTTGGCTCA     376
Beclin1     AGATAGTTTCCAGAGTTGACACGACGTGTAGAGCAAATACCCCTGCCACAGTTTTGACTC     415
Mouse       AGATAGTTTCCAGAGTACGC----CATGTATAGCAAAGAACCCTGCCATAGTTTTGACTC     416
            ** *               *    * **** ****** * *******  ***** *

Rat         ACCCCATGCTGTCCTTTCCCTCTTCCCTGGAAACAACTAATTTAAATTTGCTTTGTTTTC     436
Beclin1     AGCCCCATGCTGGC-CTCTCCCTCTCCTGAAAACAACTAATTTAATTTTGCTTTGTTTTC     474
Mouse       AGCCCCATGCATCCTTTCCCTCTTTCCTGAAAACAACTAATTTAAATTTGCTTTGTTTTC     476
            * ***        *  ** * **  **** *************** **************

Rat         TTTTTTAAGTTGAATTGACATTAATGTGTTTTCACTGGATTTTATCTCTCTCAACTTCCT     496
Beclin1     TTTTTTAAGTACAATTGACATTAATGTGTTTTCACTGGATTTTATCTCTCTCAACTTCCT     534
Mouse       TTTTTTAAGTTGAATTGACGTTAATGTGTTTTCACTGGATTTTATCTCTCTCAACTTCCT     536
            **********  ******* ****************************************

Rat         GTACTTGAA-------ACAGAAAAGTTTTGAGATGAGATGCTTGTGGCACACAGTTGGGT     549
Beclin1     GCACTTAAAACATGAAACAGAAGAGTTTTGAAATGAGATGTTTGTGGCACACAATTGGGT     594
Mouse       GCACTTAAAATTTGAAACAGCAAAGGTTTGAGATGAGATGCTTGTGGCACACAGTTGGGT     596
            * **** **       **** * ** ***** ******** ************ ******

Rat         AATGTGGGGAAGGGACAAGA--------GGTAAAGGTTTAACTCTGTCCTCACTTCTAGT     601
```

```
Beclin1    GATATGGGGAAAGGACACCAGGTTAGGAGTTGCAAGTTTAACTCCATCCTCACTTCTAGC     654
Mouse      GATGTGGGGAAAGGACACCGGGTCAGGAGTTGCAAGTTTAACTCCGTCCTCACTTGTAGC     656
            ** ******* *****           * *  * *********  ********* ***

Rat        ATTGAACGCTTACTGTGCTAACTATTGGAATTACAAAATGCTGTTTGATACTGTTTGAGA     661
Beclin1    ATTGAATGCCTACTGTGCTATCTATTGGAATTACAAAATGCTGTTTGATACTGTTTGAGA     714
Mouse      ATTGAATGCCTCCTGTGCTGTCTAGTGGGACTACAGAATGCTGTTTGATACTGTGTGCGA     716
           ****** ** * *******  *** *** * **** ****************** ** **

Rat        TGTGGAAGGATTTAATTATTTGTAATAAAGGATTTGCTATGGTCTATTAATTA   714
Beclin1    CATGGAGAGATTTAATTATTTGTAATAAAGGATTTGCTATGGTCTGTTAATTA   767
Mouse      CGTGGAGAGATTTAATTATTTGTAATAAAGGATTTGCTATGGTCTATTAATTA   769
             ****  ************************************ *******
```

Table 5.3 Locations of the forward and reverse primers for the Lamp2a gene from *Apodemus sylvaticus.* Three Lamp2a related sequences were aligned using Clustal W: Rat, *Rattus norvegicus*, Lamp2a, *Apodemus sylvaticus*, Mouse, *Mus musculus*. Numbers down the left-hand side refer to the base positions relative to that start of the relevant sequence. Yellow shaded areas indicate the selected forward and reverse primes.

```
CLUSTAL O(1.2.4) multiple sequence alignment


Rat        ---TCAAGACTGCAGTGCAGATGAAGACAACTTCCTTGTGCCCATAGCGGTGGGAGCAGC     57
Lamp2      AGCTCAAGACTGCAGTGCAGATGAAGACAACTTCCTTGTGCCCATAGCAGTGGGAGCGGC     60
mouse      AGCTCAAGACTGCAGTGCAGATGAAGACAACTTCCTTGTGCCCATAGCGGTGGGAGCAGC     60
              ****************************************** ******** **

Rat        CCTGGGAGGAGTACTTATTCTAGTGTTGCTGGCTTATTTTATTGGTCTCAAGCGCCATCA     117
Lamp2      CCTGGGAGGAGTACTTATTCTAGTGTTGCTGGCTTATTTTATTGGTCTCAAGCGCCATCA     120
mouse      TCTGGGAGGAGTACTTATTCTAGTGTTGCTGGCTTATTTTATTGGTCTCAAGCGCCATCA     120
            ********************************************************

Rat        TACTGGATATGAGCAATTTTAGCACCTACAATCTGATTGAATATATTACAAAATACATAC     177
Lamp2      TACTGGATATGAGCAGTTTTAGCTCCCGCAATCTGATTGATTATATTACAAAATACATAC     180
mouse      TACTGGATATGAGCAATTTTAGTAACTGCAATCTGATTGATTATATTACAGAATACATAC     180
           *************** ******   *  ************ ********* *********

Rat        AAATAA-CAAAGTTGTCTTACCTGTCAGTTTATGAAACACTTTGCTACTTAAGACAGACT     236
Lamp2      AAAAAC--CAAAGTTGTCAACCTGTCAGTGTATGAAGCACTTTGCTCCTTAAGACAGACA     238
mouse      AAAAAAACAAAGTTGTCTTACCTGTCAGTGTATGAAGCACTTTGCTCCTTAAGACAGACA     240
           *** *    **  *     ********** ****** ********* ************

Rat        CCTTGGAACTTTTATTTGGAAATCAGTCCCCACAATTTGAGATAAGGCTTTTTTTGAAAA     296
Lamp2      CCTTGGAACCTTCATTCAGAAATCAGTCCCCACATTTTGACATAAGTCTTTTATTGAACA     298
mouse      CCTTGGAACTTTCATTCGGAAATTAGTCCCCGAAGTTTGAGATAAGTCTTTTTTTGAATT     300
           ********* ** ***  ***** *******  * ***** ***** ***** *****

Rat        AAAAAACAAAAAACAACAAAAAAACAAAAAACAAAAAAATCATTCTCTGATCTGCTTAAA     356
Lamp2      A-------------------------------------AAATATCTCTGATCTGCTTAAA     321
mouse      T-------------------------------------TTTTTTCTCTGATCTGCTTAAA     323
                                                  *****************

Rat        ATGCAGACTGGCTTTAAAAAAAGGAGAAAATGAATACACGTGTGTGATGTTTTAAGGTCT     416
Lamp2      ATGCAGACTGGCCTTAAAAAAAAAAGAAAGTGAACACATAC-------CTGCAAAGGTCT     374
mouse      ATGCAGATTGGCTTTAAAAAGAAAAGAAAACAAACACACATATGCAATGTTTTAAGGTCT     383
           ******* **** ******* *  *****   ** ***           *   *******
```

```
Rat         GTCTTAAGAAGCTTTGGCCAAATTGGGATCCTAACCTA-AATGCCTTAAACTTATTAACA     475
Lamp2       GTCTTAAGAAGCTTTGGCCAAATTGGGATCCTAACCTAAGATGCCTTAAACTTATTAACA     434
mouse       GTCTTAAGAAGCTTTGGCCAAATTGGGATCCTAACCTAAGATGCCTTAAACTTATTAACA     443
            ************************************  ********************

Rat         TGACCGTTATAAGAAAAGATATATGGAGTTGTATCTTACTGGAATTAATAAACACTGCTT     535
Lamp2       TGACCATTATAAGAAAAGTTATATGGAGTTGTATCTTATTGGAATTAATAAACACTGCTT     494
mouse       TGACCATTATAAGAAAAGATATATGGAGTTGTATCTTACTGGAATTAATAAACACTGCTT     503
            ***** ************ ****************** ********************

Rat         CACCACC--------------------542
Lamp2       CACCACTGGTGTTCTGTTTCAATTTGT521
mouse       GACCACCGGTGTTCTGTTTCAATTTGT530
                         *******
```

Table 5.4: Locations of the forward and reverse primers for the TLR2 gene from *Apodemus sylvaticus*. Three TLR2 related sequences were aligned using Clustal W: Rat, Rattus norvegicus, TLR2, *Apodemus sylvaticus*, Mouse, *Mus musculus*. Numbers down the left-hand side refer to the base positions relative to that start of the relevant sequence. Yellow shaded areas indicate the selected forward and reverse primes.

```
CLUSTAL O(1.2.4) multiple sequence alignment


Rat         -------TGCTTCGTTTGTTTTCAGGTCAAATCTCAGAGGATGCTGCAAGCTCTTTGGCT      53
TLR2        --------------TTTGTTTTAAGGTCAAGTCTCAGAGGATGCTACGAGCTCTTTGGAT      46
mouse       TCACATGAGCGTCATTTGTTTTAAGGTCAAATCTCAGAGGATGCTACGAGCTCTTTGGCT      60
                      ******** ******* ************** * ********** *

Rat         CTTCTGGATCTTGATGGCTGTGATAGGCCTCTCAAGGGAAGGCCATTCTGCCCAGGCATC      113
TLR2        CTTCTGGCTCTTGGTGGCCATGACCGGCCTCTCCAGCAAAGACTGTTCTGCTCAGGTGTC      106
mouse       CTTCTGGATCTTGGTGGCCATAACAGTCCTCTTCAGCAAACGCTGTTCTGCTCAGGAGTC      120
            ******* ***** ****  * *  * *****  **  **  *  ****** ****  **

Rat         TCTGTCATGTGATGCTGCTGGTGTGTGTGATGGCAGCTCCAGGTCTTTCACCTCTATTCC      173
TLR2        TCTGTCATGTGATGCTGCTGGTGTGTGTGATGGCCGCTCCAGATCTTTCACCTCTATTCC      166
mouse       TCTGTCATGTGATGCTTCTGGGGTGTGTGATGGCCGCTCCAGGTCTTTCACCTCTATTCC      180
            ***************** **** ************ ******* *****************

Rat         CTCGGGACTCACAGCAAACACAAAGAAGCTTGACCTGTCTTTCAACAAGATCACCTACAT      233
TLR2        CTCCGGACTCACAGCTGCCATGAAGAGCCTAGACCTGTCTTTCAACAAGATCACCTCCAT      226
mouse       CTCCGGACTCACAGCAGCCATGAAAAGCCTTGACCTGTCTTTCAACAAGATCACCTACAT      240
            *** ************   **  ** *  ** ************************ ***

Rat         TGGCCATGGTGACTTGCGAGCCTGTGTGAACCTCCGGGTTCTGACATTGGAGTCCAGCGG      293
TLR2        TGGCCATGGTGACCTGCGGCCGTGTGTGAACCTCCAGGTTCTGATATTGGAGTCGAGCGG      286
mouse       TGGCCATGGTGACCTCCGAGCGTGTGCGAACCTCCAGGTTCTGATGTTGAAGTCCAGCAG      300
            ************* * **  * **** ******** ********  *** **** *** *

Rat         AATCAACACAATAGAGGGAGATGCCTTTTATTCTCTGGGCAGTCTTGAACACTTGGACTT      353
TLR2        AGTCGATACCATAGAGGAAGACGCCTTTTATTCTCTGGGCAGTCTTGAACATTTGGACTT      346
mouse       AATCAATACAATAGAGGGAGACGCCTTTTATTCTCTGGGCAGTCTTGAACATTTGGATTT      360
            * ** * ** ******* *** ***************************** ***** **

Rat         GTCTAATAATCACCTATCTAGTTTATCTTCCTCCTGGTTCAGGCCCCTTTCCTCTTTGAA      413
TLR2        GTCTGATAATCACCTATCTAGTTTATCTTCCTCCTGGTTCAGGCCCCTTTCCTCTTTGAA      406
mouse       GTCTGATAATCACCTATCTAGTTTATCTTCCTCCTGGTTCGGGCCCCTTTCCTCTTTGAA      420
            **** ********************************** ******************

Rat         ATACTTAAACTTAATGGGAAATCCTTACAGGACACTGGGGGAAACATCACTGTTCTCCAA      473
TLR2        ATACTTAAACTTAATGGGAAATCCTTACCGGACACTGGGGGAAACATCACTCTTTTCCAG      466
```

```
mouse       ATACTTAAACTTAATGGGAAATCCTTACCAGACACTGGGGGTAACATCGCTTTTTCCCAA     480
            ****************************  *********** ****** ** **  ***

Rat         TCTCACAAATTTACAAAACCTTAGGGTAGGAAATGTTGACACTTTCAGTGAGATAAGGAG     533
TLR2        TCTCACAAACTTACAGACCCTCAGGGTAGGAAATGTGGACACCTTCAGTGAGATAAGGAG     526
mouse       TCTCACAAATTTACAAACCCTCAGGATAGGAAATGTAGAGACTTTCAGTGAGATAAGGAG     540
            ********* ***** * *** *** ********** ** ** *****************

Rat         AATAGATTTTGCTGGGCTGACCTCTCTCAACGAACTTGAAATTCAGGTATTAAGTCTCGG     593
TLR2        AAGAGATTTTGCTGGGCTGACTTCTCTCAATGAACTTGAAATTAAGGCAACAAGTCTCTG     586
mouse       AATAGATTTTGCTGGGCTGACTTCTCTCAATGAACTTGAAATTAAGGCATTAAGTCTCCG     600
            ** ****************** ******** ************ *** *  ******* *

Rat         CAACTATGAGTCCCGAAGTCTACAGTCAATTAGAGACATCTATCACCTGACCCTGCACTT     653
TLR2        GAATTATCAGTCCCAAAGTCTAGAGTCGTTTAGAGACATCTATCACCTGACGCTTCACTT     646
mouse       GAATTATCAGTCCCAAAGTCTAAAGTCGATCCGCGACATCCATCACCTGACTCTTCACTT     660
             ** *** ****** ******* ****  *  * ****** ********** ** *****

Rat         GAGCGAGTCTGCTTTCCTGCTGGGGATTTTTGCAGATATTCTGAGTTCCGTGAGATATTT     713
TLR2        GAGCAAGTCTGATTTCCTGCTGGGGATTTTTGCAGATATTCTGAGTTCTGTGAGGTATTT     706
mouse       AAGCGAGTCTGCTTTCCTGCTGGAGATTTTTGCAGATATTCTGAGTTCTGTGAGATATTT     720
             *** ****** *********** ********************** ***** *****

Rat         AGAACTAAGAGATACTAACTTGGCTAGGTTCCAATTTTCTGAACTGTCTGTAGACGAAAT     773
TLR2        AGAACTAAGAGATACTAACTTGACCAGTTTCCGATTTTCAGAACTTCCCGTAGCTGAAAT     766
mouse       AGAACTAAGAGATACTAACTTGGCCAGGTTCCAGTTTTCACCACTGCCCGTAGATGAAGT     780
            ********************** * ** ****  *****   ***  * ****  *** *

Rat         CAATTCGCCAATGAAGAAGCTGGCATTCCGGAATGCAGATCTCACCGATAAAAGTTTTAA     833
TLR2        CAACTCACCGATGAAGAAGCTGGCATTCCGAAATGCAGATCTCACTGATGAAAGCTTTAA     826
mouse       CAGCTCACCGATGAAGAAGCTGGCATTCCGAGGCTCGGTTCTCACTGATGAAAGCTTTAA     840
            **  ** ** *********************     * * ****** *** **** *****

Rat         TGAACTCCTGAAGCTGTTGCGTTACATCTTGGAACTGATGGAGGTGGAGTTTGATCACTG     893
TLR2        TGAACTCCTGAAGCTGTTGCGTTATATCTTGGAACTGTTGGAAGTAGAATTTGATGACTG     886
mouse       CGAGCTCCTGAAGCTGTTGCGTTACATCTTGGAACTGTCGGAGGTAGAGTTCGACGACTG     900
             ** ******************** ************  *** ** ** ** **  ****

Rat         CACCCTCAATGGGGTTGGTAATTTCAACCCCTCAGAGTCAGACGTAGTGAGGGAGCTAGG     953
TLR2        TACCCTCAACGGGGTCGGCGACTTCAACCCCTCGGAGTCAGACATAGTGAGAGAGCTAGG     946
mouse       TACCCTCAATGGGCTCGGCGATTTCAACCCCTCGGAGTCAGACGTAGTGAGCGAGCTGGG     960
             ******** *** * **  * *********** ********* ******* ***** **

Rat         TAAAGTAGAAACGGTAACAATACGGAGCCTGCACATCCCCCAGTTCTATTTGTTCTATGA    1013
TLR2        TAAAGTAGAGACGGTAACAATACGGAGGTTGCACATCCCCCAGTTCTATTCATTTTATGA    1006
mouse       TAAAGTAGAAACAGTCACTATCCGGAGGTTGCATATCCCCCAGTTCTATTTGTTTTATGA    1020
            ********* ** ** ** ** *****  **** ****************  ** *****

Rat         TCTGAGCACTGTGTATTCCCTCCTGGAGAAAGTGAAGCGAATCACAGTAGAGAACAGTAA    1073
TLR2        TCTGAGTACTGTCTATTCCCTCCTGGAGAAGGTGAAGCGAATCACCGTGGAGAACAGTAA    1066
mouse       CCTGAGTACTGTCTATTCCCTCCTGGAGAAGGTGAAGCGAATCACAGTAGAGAACAGCAA    1080
             ***** ***** ***************** ************** ** ******** **

Rat         GGTCTTTCTGGTTCCCTG-CTCTTTCTCACAGCATTTAAAATCATTAGAGTTCTTAGACC    1132
TLR2        GGTCTTCCTGGTCCCCTGCCTC-TTCTCGCAGCATTTAAAATCATTAGAATTCTTAGACC    1125
mouse       GGTCTTCCTGGTTCCCTG-CTCGTTCTCCCAGCATTTAAAATCATTAGAATTCTTAGACC    1139
            ****** ***** ***** *** ***** ******************** **********

Rat         TCAGCGAAAATCTGATGGTTGAAGAGTATTTGAAAAACTCAGCCTGTGAGGGTGGCTGGC    1192
TLR2        TCAGCGAAAATCTGATGGTTGAAGAATATTTGAAATACTCAGCATGTGAGGGCGGCTGGC    1185
mouse       TCAGCGAAAATCTGATGGTTGAAGAATATTTGAAGAACTCAGCCTGTAAGGGAGCCTGGC    1199
            ************************ ********  ******* *** **** * *****

Rat         CTTCTCTACAAAGCTTGGTTTTGAGTCAGAATCATTTGAGATCAATACGAAAAACTGCTG    1252
TLR2        CTTCTTTACAAACCTTAGTTTTGAGCCAGAATCATTTGAGATCAATACAAAAAACGGGAG    1245
mouse       CTTCTCTACAAACCTTAGTTTTGAGCCAGAATCATTTGAGATCAATGCAAAAAACAGGAG    1259
            ***** ****** *** ******** ******************** * ****** *  *

Rat         AGATTTTGCTGACTCTGAAAAACCTGACAGCCCTTGACATCAGCAAGAACAGTTTTCAGC    1312
TLR2        AGATTTTGCTGACTCTGAAAAACCTGACAGCCCTTGACATCAGCAGGAACGGGTTTCATC    1305
mouse       AGATTTTGCTGACTCTGAAAAACCTGACCTCTCTTGACATCAGCAGGAACACTTTTCATC    1319
```

```
           ***************************  * ************* ****   ***** *

Rat        CTATGCCCGACAGTTGTCAGTGGCCAGGAAAGATGCGCTTCCTGAACTTGTCCAGTACAG     1372
TLR2       CGATGCCCGACCGTTGTCAGTGGCCAGGAAAGATGCGCTTCCTGAACTTGTCCAGTACAG     1365
mouse      CGATGCCCGACAGCTGTCAGTGGCCAGAAAAGATGCGCTTCCTGAATTTGTCCAGTACAG     1379
           * ********* * ************* ***************** *************

Rat        GGATACAGGCCGTCAAGACATGCATTCCTCAGACCCTGGAGGTGTTGGATGTTAGTAACA     1432
TLR2       GGATACAGGCGGTAAGAGCATGCCTTCCTCAGACGCTGGAGGTGTTGGATGTTAGTAACA     1425
mouse      GGATCCGGGTGGTAAAAACGTGCATTCCTCAGACGCTGGAGGTGTTGGATGTTAGTAACA     1439
           **** * **  ** *   * *** ********** ************************

Rat        ACAATCTCGATTCATTTTCTTTGTTTTTGCCTCGGCTCCAAGAGCTGTATATTTCCAGAA     1492
TLR2       ACAATCTTGAGTCATTTTCTTTGTTTTTGCCTCGACTGCAAGAGCTCTATATTTCCAGAA     1485
mouse      ACAATCTTGACTCATTTTCTTTGTTCTTGCCTCGGCTGCAAGAGCTCTATATTTCCAGAA     1499
           ******* ** ************** ******** ** ******** *************




Rat        ATAAACTGAAGACACTCCCAGAAGCTTCATTGTTCCCTGTGTTACAGGTCATGAAGATCA     1552
TLR2       ATAAGCTGAAAATGCTCCCAGAAGCTTCCTTGTTCCCCGTGTTACTCGTCATGAGGATCA     1545
mouse      ATAAGCTGAAAACACTCCCAGATGCTTCGTTGTTCCCTGTGTTGCTGGTCATGAAAATCA     1559
           **** ***** *  ******** ***** ******** ***** *  *******  ****

Rat        GAGAGAACGCAATAAGTACTTTCTCTAAAGACCAGCTTGGTTCTTTTCCCAAACTGGAGA     1612
TLR2       GAGAGAACGCAATAAGTACTTTCTCTAAAGACCAACTTGATTCTTTTCCCAAACTGGAGA     1605
mouse      GAGAGAATGCAGTAAGTACTTTCTCTAAAGACCAACTTGGTTCTTTTCCCAAACTGGAGA     1619
           ******* *** ********************** **** ********************

Rat        CTCTGGAAGCAGGTGACAACCATTTCATCTGCTCCTGTGAACTCCTGTCCTTCATTCTGG     1672
TLR2       CTCTGGAAGCAGGTGACAACCACTTCATCTGCTCCTGTGAACTCCTGTCCTTCACTACGG     1665
mouse      CTCTGGAAGCAGGCGACAACCACTTTGTTTGCTCCTGCGAACTCCTATCCTTTACTATGG     1679
           ************* ******** **  * ******** ******** ***** * *  **

Rat        AGAGGCCAGCCCTGGTCCATGTCCTGGTTGACTGGCCAGACAGCTACCTGTGTGATTCTC     1732
TLR2       AGAGGCCAGCCCTGGCCCATGTCCTGGTTGACTGGCCAGACAGCTACCTGTGTGACTCTC     1725
mouse      AGACGCCAGCTCTGGCTCAAATCCTGGTTGACTGGCCAGACAGCTACCTGTGTGACTCTC     1739
           *** ****** ****  **  ********************************** ****

Rat        CGCCTCGCCTGCATGGCCAGAGGCTTCAGGATGCCCGGCCCTCAGTCTTGGAGTGTCACC     1792
TLR2       CGCCTCGCCTGCATGGCCAGAGGCTTCAGGATGCCCGGCTCTCTGTCTTGGAGTGTCACC     1785
mouse      CGCCTCGCCTGCACGGCCACAGGCTTCAGGATGCCCGGCCCTCCGTCTTGGAATGTCACC     1799
           ************* ***** ****************** *** ******** *******

Rat        AGGCCGCACTGGTCTCTGGAGTCTGCTGTGCCCTTCTCCTGTTGATCCTGCTCTTAGGCG     1852
TLR2       AGGCAGCCCTGGTGTCTGGAATCTGCTGTGCCCTTCTCCTGTTGATCCTGCTCATAGGTG     1845
mouse      AGGCTGCACTGGTGTCTGGAGTCTGCTGTGCCCTTCTCCTGTTGATCTTGCTCGTAGGTG     1859
           **** ** ***** ****** ************************** ***** **** *

Rat        CCCTGTGTTACCATTTCCATGGGCTGTGGTATCTGAGAATGATGTGGGCGTGGCTCCGGG     1912
TLR2       TCTTGTGCCACCATTTCCATGGGCTGTGGTACCTCAGAATGATGTGGGCGTGGCTCCAGG     1905
mouse      CCCTGTGCCACCATTTCCACGGACTGTGGTACCTGAGAATGATGTGGGCGTGGCTCCAGG     1919
            * ****  ********** ** ******** ** ********************** **

Rat        CCAAGAGGAAGCCCAAGAAAGCTCCTTGCAGGGACCTTTGCTATGATGCCTTTGTTTCCT     1972
TLR2       CCAAGAGGAAGCCCAAGAAAGCTCCCTGCAGGGACGTTTGCTATGATGCCTTTGTTTCTT     1965
mouse      CCAAGAGGAAGCCCAAGAAAGCTCCCTGCAGGGACGTTTGCTATGATGCCTTTGTTTCCT     1979
           ************************ ********* ********************** *


Rat        ACAGCGAGCAGGATTCCTATTGGGTGGAGAACCTCATGGTCCAGCAGCTGGAGAACTCTG     2032
TLR2       ACAGTGAACAGGATTCCTACTGGGTGGAGAACCTCATGGTCCAGCAGCTGGAGCACTCTG     2025
mouse      ACAGTGAGCAGGATTCCCATTGGGTGGAGAACCTCATGGTCCAGCAGCTGGAGAACTCTG     2039
           **** ** ********* * ******************************** ******

Rat        ACCCACCCTTCAAGCTGTGTCTCCACAAGCGGGACTTTGTTCCGGGCAAATGGATCATTG     2092
TLR2       ACCCGCCCTTCAAGCTGTGTCTCCACAAGCGGGACTTTGTTCCGGGCAAATGGATCATTG     2085
mouse      ACCCGCCCTTTAAGCTGTGTCTCCACAAGCGGGACTTCGTTCCGGGCAAATGGATCATTG     2099
           **** ***** ************************* **********************
```

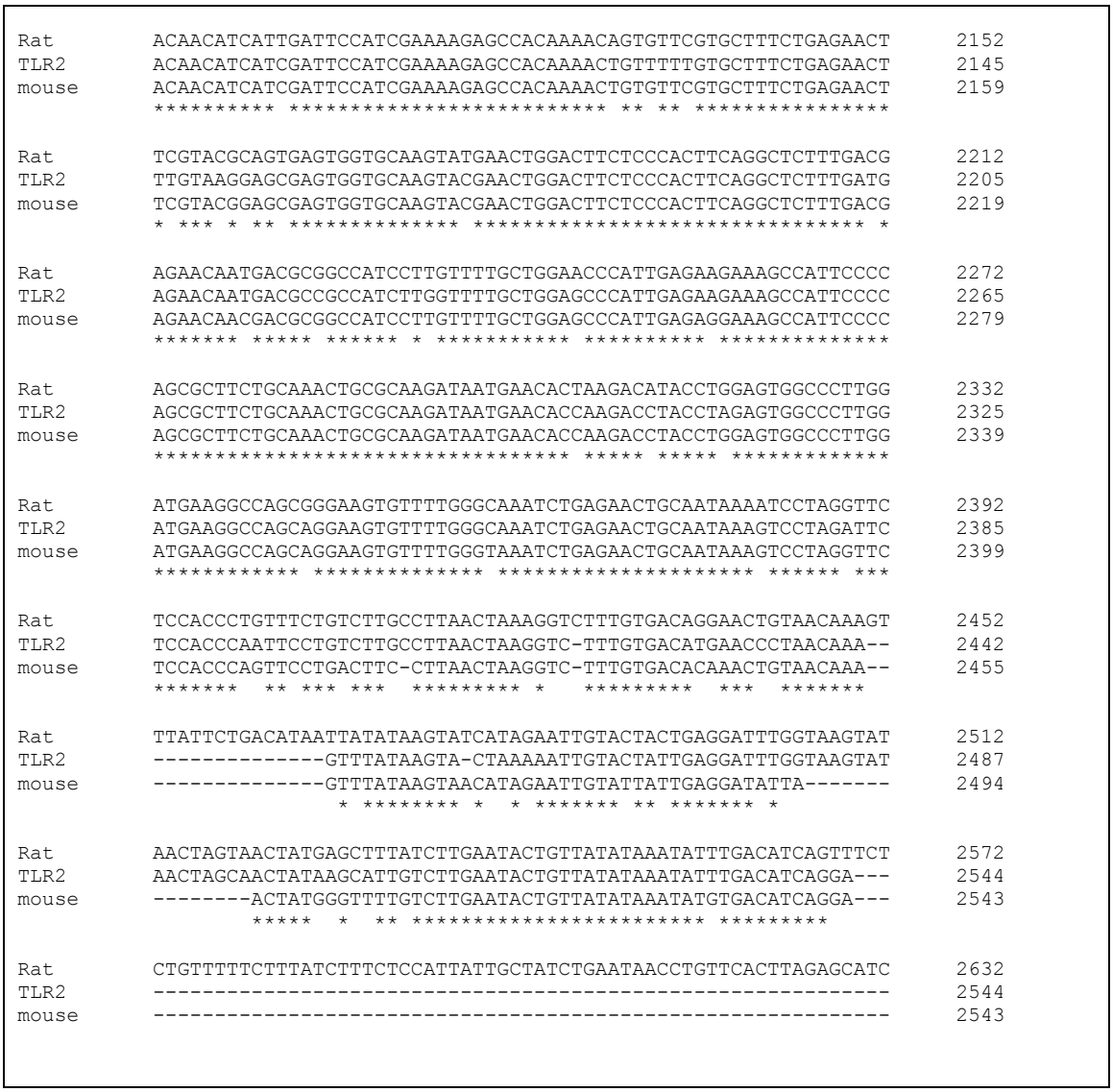
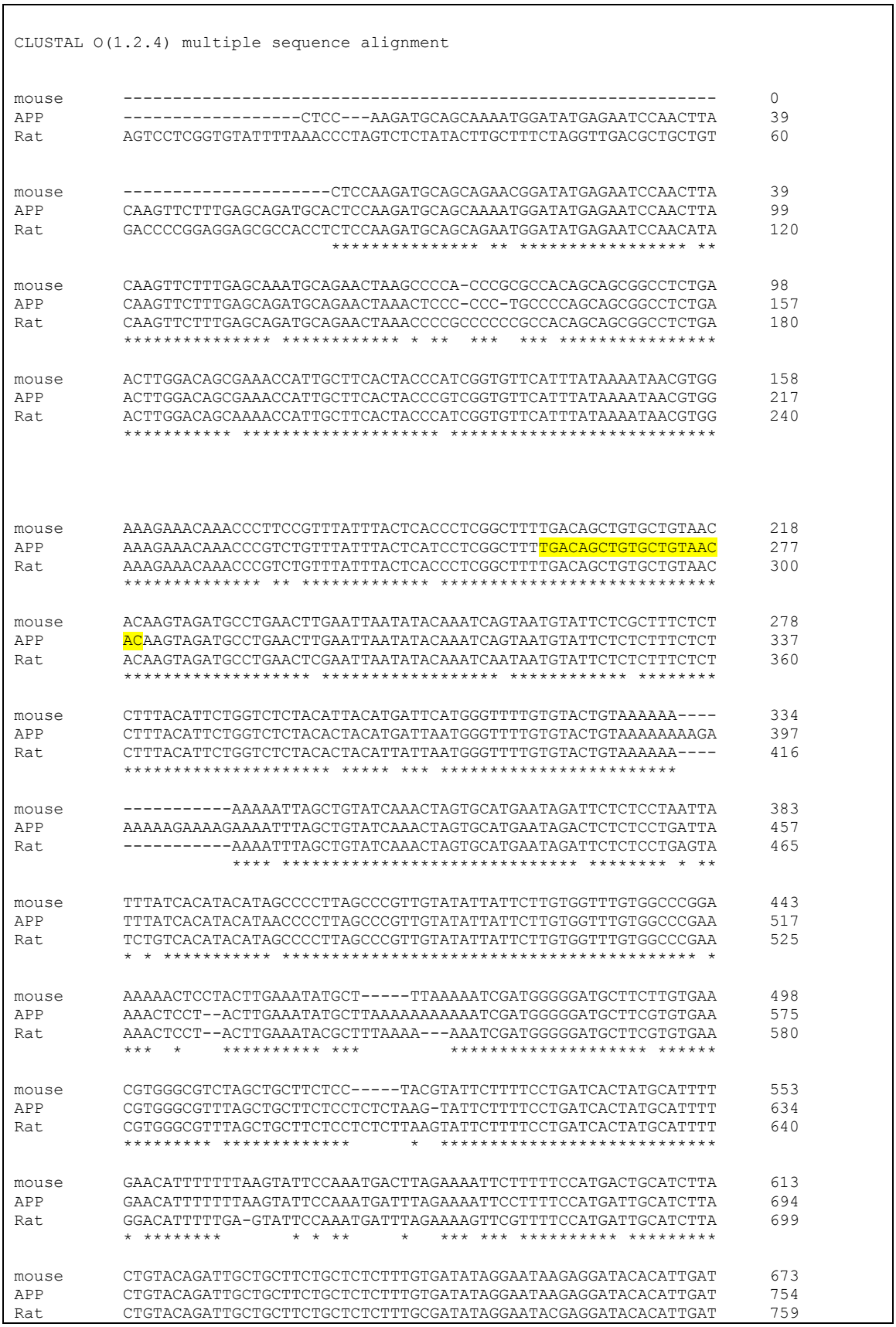


Table 5.5: Locations of the forward and reverse primers for the APP gene from *Apodemus sylvaticus*. Three APP related sequences were aligned using Clustal W: Rat, *Rattus norvegicus*, APP, *Apodemus sylvaticus*, Mouse, *Mus musculus*. Numbers down the left-hand side refer to the base positions relative to that start of the relevant sequence. Yellow shaded areas indicate the selected forward and reverse primes.



```
CLUSTAL O(1.2.4) multiple sequence alignment


mouse      ------------------------------------------------------------      0
APP        ------------------CTCC---AAGATGCAGCAAAATGGATATGAGAATCCAACTTA      39
Rat        AGTCCTCGGTGTATTTTAAACCCTAGTCTCTATACTTGCTTTCTAGGTTGACGCTGCTGT      60


mouse      ---------------------CTCCAAGATGCAGCAGAACGGATATGAGAATCCAACTTA      39
APP        CAAGTTCTTTGAGCAGATGCACTCCAAGATGCAGCAAAATGGATATGAGAATCCAACTTA      99
Rat        GACCCCGGAGGAGCGCCACCTCTCCAAGATGCAGCAGAATGGATATGAGAATCCAACATA     120
                           *************** ** ***************** **

mouse      CAAGTTCTTTGAGCAAATGCAGAACTAAGCCCCA-CCCGCGCCACAGCAGCGGCCTCTGA      98
APP        CAAGTTCTTTGAGCAGATGCAGAACTAAACTCCC-CCC-TGCCCCAGCAGCGGCCTCTGA     157
Rat        CAAGTTCTTTGAGCAGATGCAGAACTAAACCCCGCCCCCCGCCACAGCAGCGGCCTCTGA     180
           *************** ************ * **  ***  *** ****************

mouse      ACTTGGACAGCGAAACCATTGCTTCACTACCCATCGGTGTTCATTTATAAAATAACGTGG      158
APP        ACTTGGACAGCGAAACCATTGCTTCACTACCCGTCGGTGTTCATTTATAAAATAACGTGG      217
Rat        ACTTGGACAGCAAAACCATTGCTTCACTACCCATCGGTGTTCATTTATAAAATAACGTGG      240
           *********** ******************** ***************************




mouse      AAAGAAACAAACCCTTCCGTTTATTTACTCACCCTCGGCTTTTGACAGCTGTGCTGTAAC      218
APP        AAAGAAACAAACCCGTCTGTTTATTTACTCATCCTCGGCTTTTGACAGCTGTGCTGTAAC      277
Rat        AAAGAAACAAACCCGTCTGTTTATTTACTCACCCTCGGCTTTTGACAGCTGTGCTGTAAC      300
           ************** ** ************* ****************************

mouse      ACAAGTAGATGCCTGAACTTGAATTAATATACAAATCAGTAATGTATTCTCGCTTTCTCT      278
APP        ACAAGTAGATGCCTGAACTTGAATTAATATACAAATCAGTAATGTATTCTCTCTTTCTCT      337
Rat        ACAAGTAGATGCCTGAACTCGAATTAATATACAAATCAATAATGTATTCTCTCTTTCTCT      360
           ****************** ***************** ************ ********

mouse      CTTTACATTCTGGTCTCTACATTACATGATTCATGGGTTTTGTGTACTGTAAAAAA----      334
APP        CTTTACATTCTGGTCTCTACACTACATGATTAATGGGTTTTGTGTACTGTAAAAAAAAGA      397
Rat        CTTTACATTCTGGTCTCTACACTACATTATTAATGGGTTTTGTGTACTGTAAAAAA----      416
           ******************** ***** *** ************************

mouse      -----------AAAAATTAGCTGTATCAAACTAGTGCATGAATAGATTCTCTCCTAATTA      383
APP        AAAAAGAAAAGAAAATTTAGCTGTATCAAACTAGTGCATGAATAGACTCTCTCCTGATTA      457
Rat        -----------AAAATTTAGCTGTATCAAACTAGTGCATGAATAGATTCTCTCCTGAGTA      465
                     **** ****************************** ******** * **

mouse      TTTATCACATACATAGCCCCTTAGCCCGTTGTATATTATTCTTGTGGTTTGTGGCCCGGA      443
APP        TTTATCACATACATAACCCCTTAGCCCGTTGTATATTATTCTTGTGGTTTGTGGCCCGAA      517
Rat        TCTGTCACATACATAGCCCCTTAGCCCGTTGTATATTATTCTTGTGGTTTGTGGCCCGAA      525
           * * *********** ****************************************** *

mouse      AAAAACTCCTACTTGAAATATGCT-----TTAAAAATCGATGGGGGATGCTTCTTGTGAA      498
APP        AAACTCCT--ACTTGAAATATGCTTAAAAAAAAAAATCGATGGGGGATGCTTCGTGTGAA      575
Rat        AAACTCCT--ACTTGAAATACGCTTTAAAA---AAATCGATGGGGGATGCTTCGTGTGAA      580
           ***  *    ********** ***         ******************** ******

mouse      CGTGGGCGTCTAGCTGCTTCTCC-----TACGTATTCTTTTCCTGATCACTATGCATTTT      553
APP        CGTGGGCGTTTAGCTGCTTCTCCTCTCTAAG-TATTCTTTTCCTGATCACTATGCATTTT      634
Rat        CGTGGGCGTTTAGCTGCTTCTCCTCTCTTAAGTATTCTTTTCCTGATCACTATGCATTTT      640
           ********* *************      *  ****************************

mouse      GAACATTTTTTTAAGTATTCCAAATGACTTAGAAAATTCTTTTTCCATGACTGCATCTTA      613
APP        GAACATTTTTTTAAGTATTCCAAATGATTTAGAAAATTCCTTTTCCATGATTGCATCTTA      694
Rat        GGACATTTTTGA-GTATTCCAAATGATTTAGAAAAGTTCGTTTTCCATGATTGCATCTTA      699
           * ********       * * **     *   *** *** ********** *********

mouse      CTGTACAGATTGCTGCTTCTGCTCTCTTTGTGATATAGGAATAAGAGGATACACATTGAT      673
APP        CTGTACAGATTGCTGCTTCTGCTCTCTTTGTGATATAGGAATAAGAGGATACACATTGAT      754
Rat        CTGTACAGATTGCTGCTTCTGCTCTCTTTGCGATATAGGAATACGAGGATACACATTGAT      759
```

```
           ***************************** ************ ***************

mouse      TTCTTTGTGCCTGTTTTATGTGCACACATTAGGCATTGAGAATTTGAACATTTTTTTT--     731
APP        TTCTTTGTGCCTGTTTTATGTGCACACATTAGGCATTGAGAATCTGAACTTTTTTTTTT     814
Rat        TTCTTTGTGCCTGTTTTATGTGCACACATTAGGCATTGAGGATTTGAACTTTTTTTGTC     819
           ************************************* ** ***** *******

mouse      -GTCCATGTATCTTTGGATCTTTGATAAAAAAA-AATTAAAAAAAAAATTATCCCTGTTCA     789
APP        TGTCCATGTATCTTTGGATCTTTGATAAAAAAT--AAAATAAAATAAATATCCCTGTTCA     872
Rat        ----CATGTATCTTTGGATCTTTGATAAAAAAAAAATTTAAAAAAAAAAATCCCTGTTCA     875
               ***************************   *    **** **  ***********

mouse      TCATAAGCACTTTTACGGGTGGGGGGAGGGAGTGTTCTGCTGGTCTCCAATTACCAAGAA     849
APP        TCGTAAGCACTTTTACGGGTGGGGGGAGGGAGTGTTCTGCTGGTCTCCAATTACCAAGAA     932
Rat        TCGTAAGCACTTTTACGGGTGGGGGGAGGGAGTGTTCTGCTGGTCTCCAATTACCAAGAA     935
           ** ********************************************************

mouse      TTCTCCAAAAATTAATTTTCTGCAGGATGATTGTACAGAATCATTGCTTATGCCATGATA     909
APP        TTCTCCAAAAATAATTTTCTGCAGGATGATTGTACAGAAATCAATGCTTATGCCATGATA     992
Rat        TTCTCCAAAAATAATTTTCTGCAGGATGATTGTACAGA-ATCATTGCTTATGCCATGATA     994
           ************ * ***      *     *        **** ****************

mouse      GCTTTCTACACTGTATTACATAAATAAATTAAATAAAATAACTCCAGGCAAGACGTTTCT     969
APP        GCTTTCTACACTGTATTACATAAATAAATTAAATAAAATAACTCCAGGCAAGACGTTTCT     1052
Rat        GCTTTCTACACTGTATTACATAAATAAATTAAATTAAATAACTCCA--------------     1040
           ********************************** ***********

mouse      TTGAAGGGTGATTGTGTGGGA----------GGGAAAAGGCAAGATCCCTGCATTTATCA     1019
APP        TTGAAGGGTGATTGTGAACGTTGTGTGGGAGGGGGAAAGGCAGGATCCTTGCATTTATCA     1112
Rat        ------------------------------------------------------------     1040
```

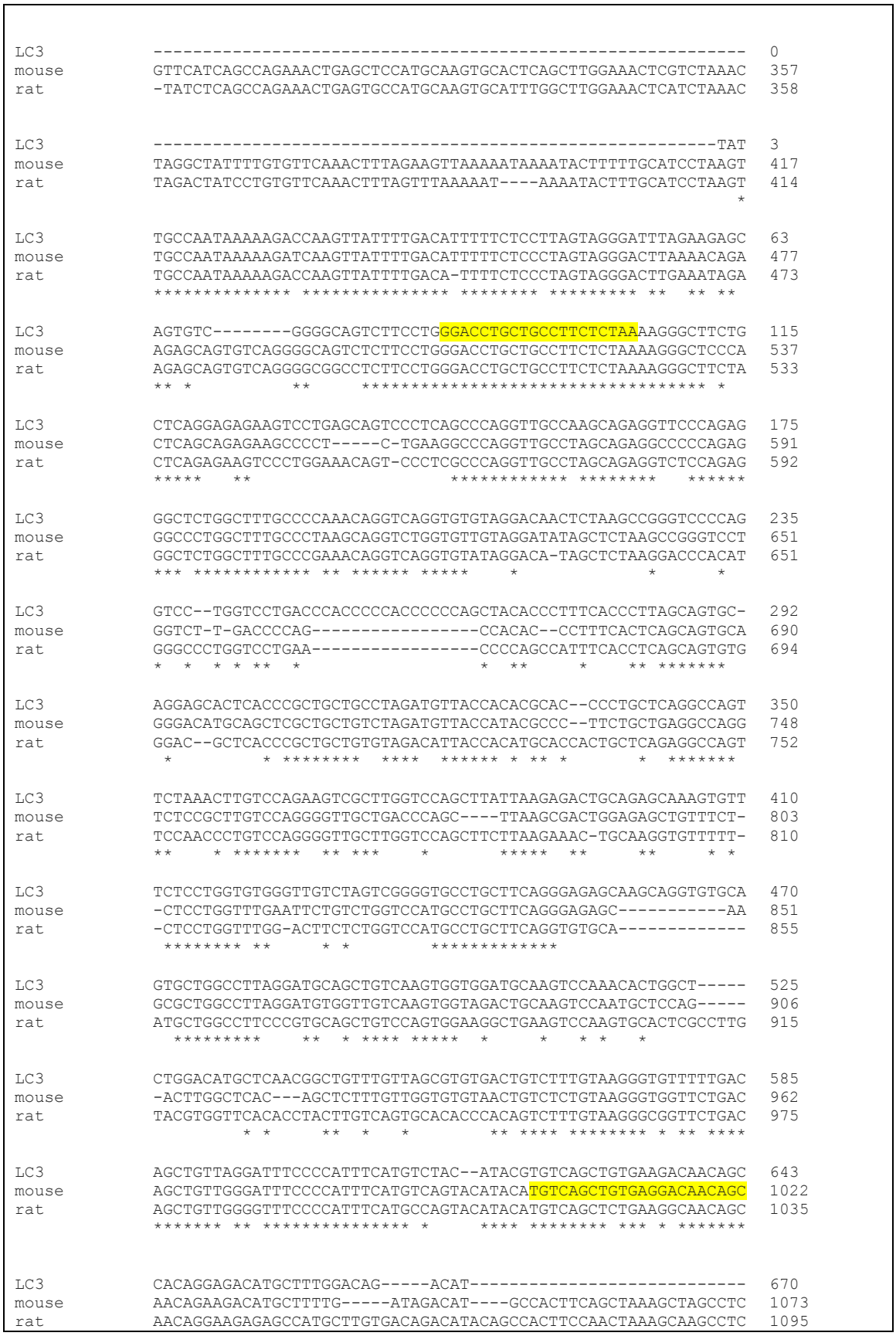
Table 5.6 : Locations of the forward and reverse primers for the Tau gene from *Apodemus sylvaticus*. Three Tau related sequences were aligned using Clustal W: Rat, *Rattus norvegicus*, Tau, *Apodemus sylvaticus*, Mouse, *Mus musculus*. Numbers down the left-hand side refer to the base positions relative to that start of the relevant sequence. Yellow shaded areas indicate the selected forward and reverse primes.

```
CLUSTAL O(1.2.4) multiple sequence alignment


Rat        ATTGAAACCCACAAGCTGACCTTCAGGGAGAATGCCAAAGCCAAGACAGACCATGGAGCA      60
Mouse      ATTGAAACCCACAAGCTGACCTTCAGGGAGAATGCCAAAGCCAAGACAGACCATGGAGCA      60
Tau        ATTGAAACCCACAAGCTGACCTTCAGGGAGAATGCCAAAGCCAAGACAGACCACGGAGCG      60
           ************************************************** *****

Rat        GAAATCGTGTACAAGTCACCTGTGGTGTCTGGGGACACATCTCCACGGCACCTCAGCAAC      120
Mouse      GAAATTGTGTATAAGTCACCCGTGGTGTCTGGGGACACATCTCCACGGCACCTCAGCAAT      120
Tau        GAAATCGTGTACAAGTCTCCCGTGGTGTCTGGGGACACATCGCCACGGCACCTCAGCAAT      120
           ***** ***** ***** ** ******************** ****************

Rat        GTCTCCTCCACGGGCAGCATCGACATGGTGGACTCTCCACAGCTTGCCACGTTAGCCGAT      180
Mouse      GTGTCTTCCACGGGCAGCATCGACATGGTGGACTCACCACAGCTTGCCACACTAGCCGAT      180
Tau        GTGTCGTCCACGGGCAGCATCGACATGGTGGACTCACCACAGCTTGCCACACTCGCCGAT      180
           ** ** ***************************** **************  * ******

Rat        GAAGTGTCCGCCTCTTTGGCCAAGCAGGGTTTGTGATCAGGCCCCTGGGGCCGTCACTGA      240
Mouse      GAAGTGTCTGCTTCCTTGGCCAAGCAGGGTTTGTGATCAGGCTCCCAGGGCAGTCAATAA      240
Tau        GAAGTGTCTGCGTCCTTGGCCAAGCAGGGTTTGTGATCAGGCTCCCAGGGTAGTCAATAA      240
           ******** ** ** ************************** **  ***  **** * *
```

```
Rat         TCATGGAGAGAAGAGAGAGTGAGAGTGTGGAAAAAAAAAAAAAAAAAAAGAATGACCTGGC      300
Mouse       TCATGGAGAGAAGAGAGAGTGAGAGTGTGGAAAAAAAAAA-AAAAAAAGAATGATCTGGC      299
Tau         TCATGGAGAGAAGAGAGAGTGAGAGTGTGGAAGAAAAAAA-TAG-AAAGAATGATCTGGC      298
            ******************************** *******  *  ********* *****

Rat         CCCTCACCCTCTGCCCTCCCCGCTGCTCCTCATAGACAGGCTGACCAGCTTGTCACCTAA      360
Mouse       CCCTTGCCCTCTGCCCTCCCCGCTGCTCCTCATAGACAGGCTGACCTGCTTGTCACCTAA      359
Tau         CCCTCGCCCTCTG-CCCTCCCGCTGCTCCTCACAGGCAGGCTGACCCGCTTGTCACCTAA      357
            ****  ******* **  ************** ** ********** *************

Rat         CCTGCTTTTGTGGCTCGGGTTTGGCTCGGGACTTCAAAATCAGTGATGGGAAAAAG--TA      418
Mouse       CCTGCTTTTGTGGCTCGGATTTGGCTCGGGACTTCAAAATCAGTGATGGGAAAAG---TA      416
Tau         CCTACTTTTGTGGCTTGGCTTTGGCTTGGGACTTCAAATTCAGTGATGGGAAAAAGAGTA      417
            *** *********** ** ******* *********** ***************    **

Rat         AATTTCATCTTTCCAAATTGATTTGTGGGCTAGTAATAAAATATTTTTAAGGAAGGAAAA      478
Mouse       CATTTCATCTTTCCAAATTGATTTGTGGGCTAAAAATAAAACATATTTAAGGGAAAAAAA      476
Tau         AATTTCATCTTTCCAAATTGACTTGTGGGCTAGTAATAAATTTTTTTTGAAA---AAAAA      474
             ******************** **********  ******   * *** *      ****

Rat         AAAAAAAAACACGTAAAACCATGGCCAAACAAAACCCAACATT------TCCTTGGCAAT      532
Mouse       AACATGTAAAA-------ACATGGCCAAA-------AAATTTCCTTGGGCAATTGCTAAT      522
Tau         AATATGTAAAA-------ACATGGCCAAAAAACCCAACATTTCCTTGGGCGATTGATAAT      527
            ** *   ** *        **********         *  *          ***  ***

Rat         T--------GTTATTGACCCCGCCCCCCCCCTCTGAGTTTTAGAGGGTGAAGGAGGCTTT      584
Mouse       TGATTTCCCCCCCCTGACCCCGCCCTCCCTCTCTGAGTATTAGAGGGTGAAGA----AGG      578
Tau         TGATTCCCCCCCCC----------------------------------------------      541
            *
```

Table 5.7: Locations of the forward and reverse primers for the LC3 gene from *Apodemus sylvaticus*. Three LC3 related sequences were aligned using Clustal W: Rat, *Rattus norvegicus*, LC3, *Apodemus sylvaticus*, Mouse, *Mus musculus*. Numbers down the left-hand side refer to the base positions relative to that start of the relevant sequence. Yellow shaded areas indicate the selected forward and reverse primes.

```
CLUSTAL O(1.2.4) multiple sequence alignment


LC3           ------------------------------------------------------------  0
mouse         -GGCGCTTGCAGCTCAATGCTAACCAAGCCTTCTTCCTCCTGGTGAATGGGCACAGCATG  59
rat           AGGCGCCTGCAGCTCAATGCTAACCAAGCCTTCTTCCTCCTGGTGAATGGGCACAGCATG  60


LC3           ------------------------------------------------------------  0
mouse         GTGAGTGTGTCCACTCCCATCTCCGAAGTGTACGAGAGTGAGAGAGATGAAGACGGCTTC  119
rat           GTGAGTGTGTCCACACCCATCTCTGAAGTGTACGAGAGCGAGAGAGATGAAGACGGCTTC  120


LC3           ------------------------------------------------------------  0
mouse         CTGTACATGGTTTATGCCTCGCAGGAGACATTCGGGACAGCAATGGCTGTGTAAGACTCC  179
rat           CTGTACATGGTCTATGCCTCCCAGGAGACGTTCGGGACAGCACTGGCTGTGTAAGACTAG  180


LC3           ------------------------------------------------------------  0
mouse         AACAAAGCC-AATGGTTGTTAAGCCCTTACCAAGGCAAAAAGGGATGTTACCAGCGGACG  238
rat           AACAATACCTAATGTTTGTTAAGCCCCTACCAAGGCAAAAAGGGACGTTACCAGCGGAAG  240


LC3           ------------------------------------------------------------  0
mouse         CTGGACGGCTCACCACC-CACAGATGAGAACGTAGGCACCCACATAGGGTATTAGGAACT  297
rat           CTGGTCAGCTCACCACCCCACAGATCAGAACGTAGGCACCCACGTAGGGGATTAGAAAC-  299
```


LC3 ---------- 0
mouse GTTCATCAGCCAGAAACTGAGCTCCATGCAAGTGCACTCAGCTTGGAAACTCGTCTAAAC 357
rat -TATCTCAGCCAGAAACTGAGTGCCATGCAAGTGCATTTGGCTTGGAAACTCATCTAAAC 358
LC3 ----------TAT 3
mouse TAGGCTATTTTGTGTTCAAACTTTAGAAGTTAAAAATAAAATACTTTTTGCATCCTAAGT 417
rat TAGACTATCCTGTGTTCAAACTTTAGTTTAAAAAT----AAAATACTTTGCATCCTAAGT 414
LC3 TGCCAATAAAAAGACCAAGTTATTTTGACATTTTTCTCCTTAGTAGGGATTTAGAAGAGC 63
mouse TGCCAATAAAAAGATCAAGTTATTTTGACATTTTTCTCCCTAGTAGGGACTTAAAACAGA 477
rat TGCCAATAAAAAGACCAAGTTATTTTGACA-TTTTCTCCCTAGTAGGGACTTGAAATAGA 473
LC3 AGTGTC--------GGGGCAGTCTTCCTGGGACCTGCTGCCTTCTCTAAAAGGGCTTCTG 115
mouse AGAGCAGTGTCAGGGGCAGTCTCTTCCTGGGACCTGCTGCCTTCTCTAAAAGGGCTCCCA 537
rat AGAGCAGTGTCAGGGGCGGCCTCTTCCTGGGACCTGCTGCCTTCTCTAAAAGGGCTTCTA 533
LC3 CTCAGGAGAGAAGTCCTGAGCAGTCCCTCAGCCCAGGTTGCCAAGCAGAGGTTCCCAGAG 175
mouse CTCAGCAGAGAAGCCCCT-----C-TGAAGGCCCAGGTTGCCTAGCAGAGGCCCCCAGAG 591
rat CTCAGAGAAGTCCCTGGAAACAGT-CCCTCGCCCAGGTTGCCTAGCAGAGGTCTCCAGAG 592
LC3 GGCTCTGGCTTTGCCCCAAACAGGTCAGGTGTGTAGGACAACTCTAAGCCGGGTCCCCAG 235
mouse GGCCCTGGCTTTGCCCTAAGCAGGTCTGGTGTTGTAGGATATAGCTCTAAGCCGGGTCCT 651
rat GGCTCTGGCTTTGCCCGAAACAGGTCAGGTGTATAGGACA-TAGCTCTAAGGACCCACAT 651
LC3 GTCC--TGGTCCTGACCCACCCCCACCCCCCAGCTACACCCTTTCACCCTTAGCAGTGC- 292
mouse GGTCT-T-GACCCCAG----------CCACAC--CCTTTCACTCAGCAGTGCA 690
rat GGGCCCTGGTCCTGAA----------CCCCAGCCATTTCACCTCAGCAGTGTG 694
LC3 AGGAGCACTCACCCGCTGCTGCCTAGATGTTACCACACGCAC--CCCTGCTCAGGCCAGT 350
mouse GGGACATGCAGCTCGCTGCTGTCTAGATGTTACCATACGCCC--TTCTGCTGAGGCCAGG 748
rat GGAC--GCTCACCCGCTGCTGTGTAGACATTACCACATGCACCACTGCTCAGAGGCCAGT 752
LC3 TCTAAACTTGTCCAGAAGTCGCTTGGTCCAGCTTATTAAGAGACTGCAGAGCAAAGTGTT 410
mouse TCTCCGCTTGTCCAGGGGTTGCTGACCCAGC----TTAAGCGACTGGAGAGCTGTTTCT- 803
rat TCCAACCCTGTCCAGGGGTTGCTTGGTCCAGCTTCTTAAGAAAC-TGCAAGGTGTTTTT- 810
LC3 TCTCCTGGTGTGGGTTGTCTAGTCGGGGTGCCTGCTTCAGGGAGAGCAAGCAGGTGTGCA 470
mouse -CTCCTGGTTTGAATTCTGTCTGGTCCATGCCTGCTTCAGGGAGAGC----------AA 851
rat -CTCCTGGTTTGG-ACTTCTCTGGTCCATGCCTGCTTCAGGTGTGCA---------- 855
LC3 GTGCTGGCCTTAGGATGCAGCTGTCAAGTGGTGGATGCAAGTCCAAACACTGGCT----- 525
mouse GCGCTGGCCTTAGGATGTGGTTGTCAAGTGGTAGACTGCAAGTCCAATGCTCCAG----- 906
rat ATGCTGGCCTTCCCGTGCAGCTGTCCAGTGGAAGGCTGAAGTCCAAGTGCACTCGCCTTG 915
LC3 CTGGACATGCTCAACGGCTGTTTGTTAGCGTGTGACTGTCTTTGTAAGGGTGTTTTTGAC 585
mouse -ACTTGGCTCAC---AGCTCTTTGTTGGTGTGTAACTGTCTCTGTAAGGGTGGTTCTGAC 962
rat TACGTGGTTCACACCTACTTGTCAGTGCACACCCACAGTCTTTGTAAGGGCGGTTCTGAC 975
LC3 AGCTGTTAGGATTTCCCCATTTCATGTCTAC--ATACGTGTCAGCTGTGAAGACAACAGC 643
mouse AGCTGTTGGGATTTCCCCATTTCATGTCAGTACATACATGTCAGCTGTGAGGACAACAGC 1022
rat AGCTGTTGGGGTTTCCCCATTTCATGCCAGTACATACATGTCAGCTCTGAAGGCAACAGC 1035
LC3 CACAGGAGACATGCTTTGGACAG-----ACAT---------- 670
mouse AACAGAAGACATGCTTTTG-----ATAGACAT----GCCACTTCAGCTAAAGCTAGCCTC 1073
rat AACAGGAAGAGAGCCATGCTTGTGACAGACATACAGCCACTTCCAACTAAAGCAAGCCTC 1095

```
                **** *     **  *          ****

LC3             ------------------------------------------------------------  670
mouse           TGCTTCCTGCAT-------GTGGCCCGCTGTGACACCAG-GCCATCCCTGGTCACTCACT  1125
rat             TGCTTCCTGCTACCTGCATGGAGCCCACTGTGACACTCAGACCATCCCCGGTCACTCACT  1155
```

Table 5.8 The primers shown in here were designed to amplify the regions of the Map1Lc3, Lamp2a, Bcn1, Tlr2, App and Tau genes of *Apodemus sylvaticus*.

Primers	**Melting Temperature (Tm)**	**Molecular Weight**
Map1LC3 FOR 5’ GGACCTGCTGCCTTCTCTAA 3’	**60** °C	559bp
Map1LC3 REV 5’ GCTGTTGTCTTCACAGCTGACA 3’	60°C	
Beclin1 FOR 5’ GCAGTGGCGGCTCCTATTCC 3’	65°C	517bp
Beclin1 REV 5’ TGTGTGCCACAAGCATCTCA 3’	60°C	
Lamp2 FOR 5’TGGTCTCAAGCGCCATCATA 3’	59°C	317bp
Lamp2 REV 5’ GGCATCTTAGGTTAGGATCCCA 3’	59°C	
APP FOR 5’ TGACAGCTGTGCTGTAACAC 3’	58°C	679bp
APP REV 5’ TGGAGACCAGCAGAACACTC 3’	60°C	
Tau FOR 5’ GCTGACCTTCAGGGAGAATG 3’	58°C	210bp
Tau REV 5’ GAGCCTGATCACAAACCCTG 3’	55°C	

5.3.3 PCR amplification of potential autophagy and inflammation genes from *Apodemus sylvaticus*

In order to confirm the presence of homologues of inflammation and autophagy genes in *A. sylvaticus*, designed PCR primers were used to amplify these genes from *A. sylvaticus.* DNA and PCR Amplifications were carried out as described in Chapter 2. During the PCR on samples of *A.sylvaticus* DNA the amplifications were optimised using various melting temperatures until the expected the sizes of the bands were found. The melting temperatures used were different according to the type of primer and were optimised around the calculated Tm values shown in Table 5.8. Also, during optimisation of the PCR reactions, temperature gradient and magnesium gradient PCR was repeated three times to ensure consistent. Amplified PCR products were allowed to run on a 1% agarose gel in order to assess the presence of the genes of interest in the *A. sylvaticus* genome. All primer pairs designed for *A. sylvaticus* using other reference genomes amplified gene regions of interest successfully. As illustrated in figure 5.1, DNA samples from the brain tissue of *A. sylvaticus* were investigated for the presence of the Map1LC3 gene using the designed primers at an annealing temperature of 59°C. The size of the amplificated bands was 559bp.

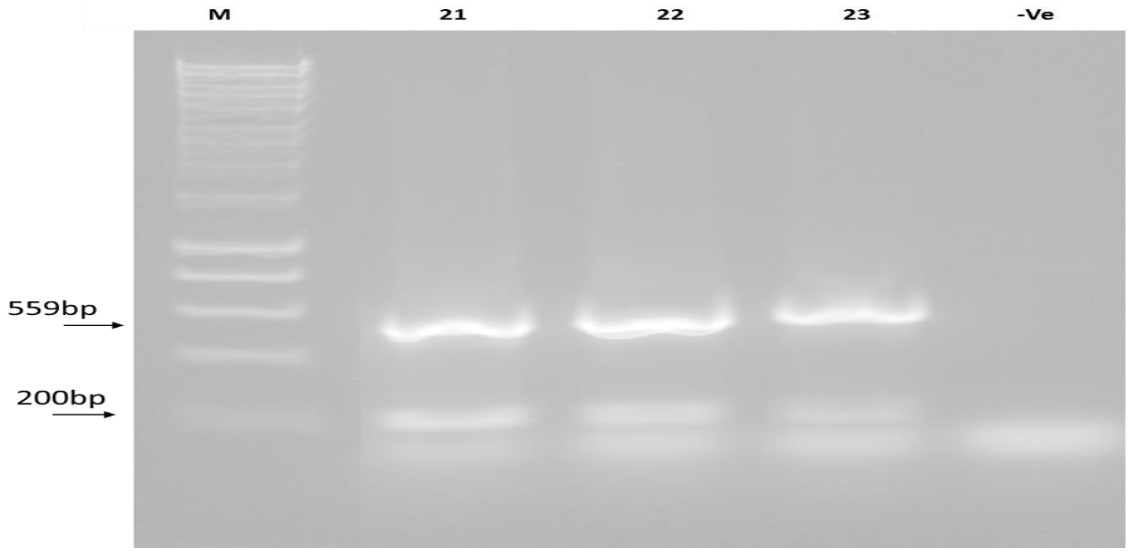


Figure 5. 1. Representative agarose gel (1%) image showing PCR amplification of *Apodemus sylvaticus* Map1Lc3 fragments. Lanes 2-5: Test samples for *Apodemus sylvaticus* lane 6, negative control (distilled water). M: Hyperladder 1Kb (Invitrogen, UK).

Figure 5.2 shows the *A. sylvaticus* DNA samples that were assessed for the primer of Beclin1 at an annealing temperature of 57°C. The band size of the targeted fragments was 517bp.

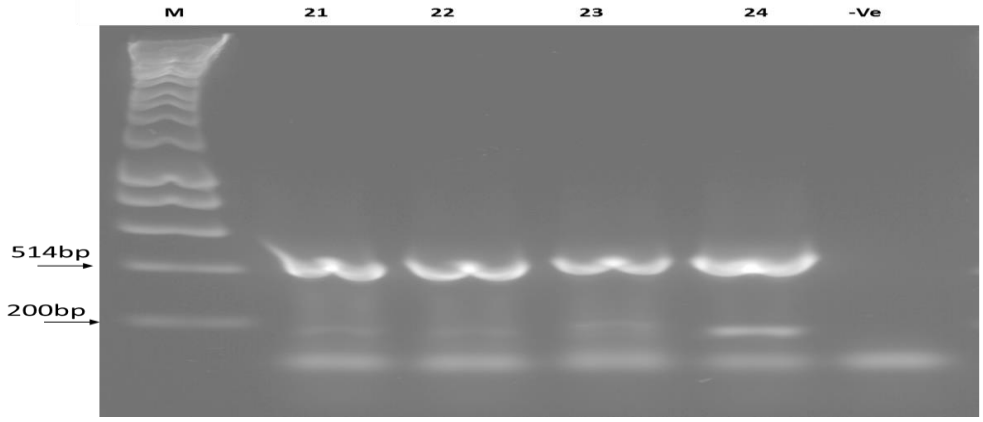


Figure 5.2. Representative agarose gel (1%) image showing PCR amplification of *Apodemus sylvaticus* Beclin1 fragments. Lane 6 Negative control, Lane 2-5 Test samples *Apodemus sylvaticus*. M: Hyperladder 1Kb (Invitrogen, UK).

As illustrated in figure 5.3, the presence of the primer of Lamp2 was tested on DNA samples of *A. sylvaticus* with an annealing temperature of *57°C.* Successfully amplified primers of the gene were detected with a band size of 317bp.

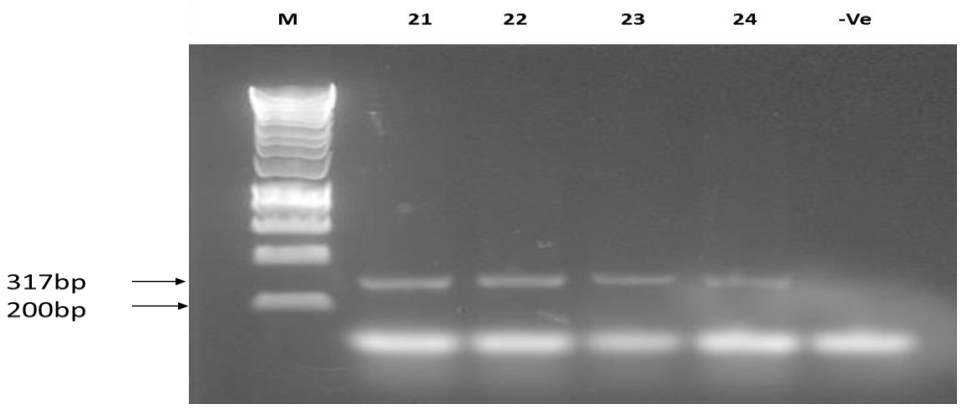


Figure 5.3. Representative agarose gel (1%) image showing PCR amplification of *Apodemus sylvaticus* Lamp2 fragments. Lane 6 Negative control, Lane 2-5 Test samples *Apodemus sylvaticus*. M: Hyperladder 1Kb (Invitrogen, UK).

The primer for the APP gene was successfully amplified in a DNA samples from *A.sylvaticus* at an annealing temperature of 58°C. As seen in figure 5.4, the band size of the targeted fragment was 679bp.

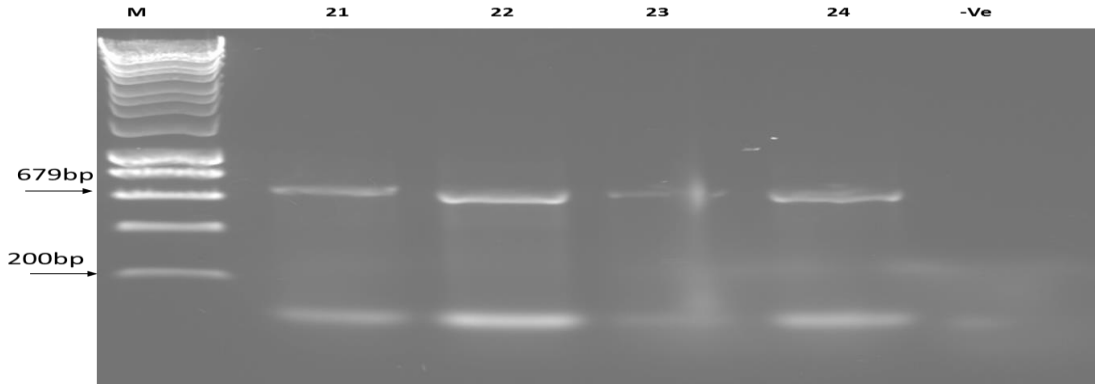


Figure 5.4. Representative agarose gel (1%) image showing PCR amplification of *Apodemus sylvaticus* APP fragments. Lanes 2-7 test samples, Negative control. M: Hyperladder 1Kb (Invitrogen, UK).

Figure 5.5 shows successful detection of the primer for the Tau gene, in DNA samples taken from *A. sylvaticus*. The amplificated primers had a band size of 210pb, and were tested at an annealing temperature of 58°C.

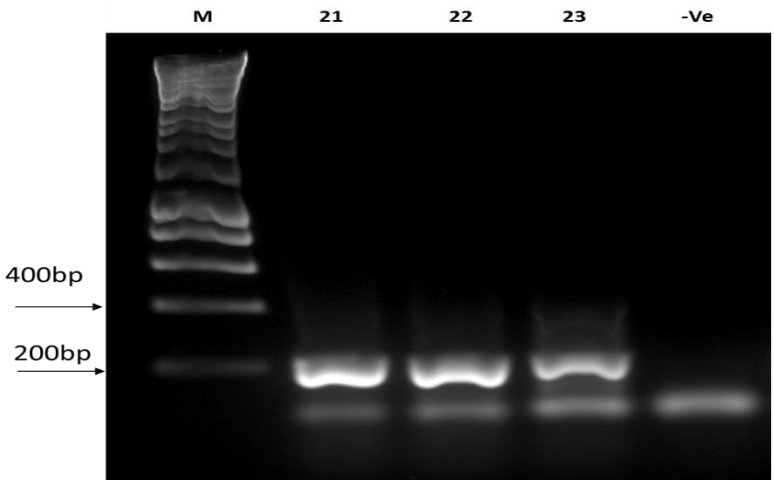


Figure 5.5. Representative agarose gel (1%) image showing PCR amplification of *Apodemus sylvaticus* Tau fragments. Lanes 2,3,4,6 Test samples, lane 5, negative control (water). M: Hyperladder 1Kb (Invitrogen, UK).

DNA samples from *A. sylvaticus* were tested for the primer of the Tlr2 gene at an annealing temperature of 59°C. Figure 5.6 shows that the band size of the successfully amplified primers was 373bp.

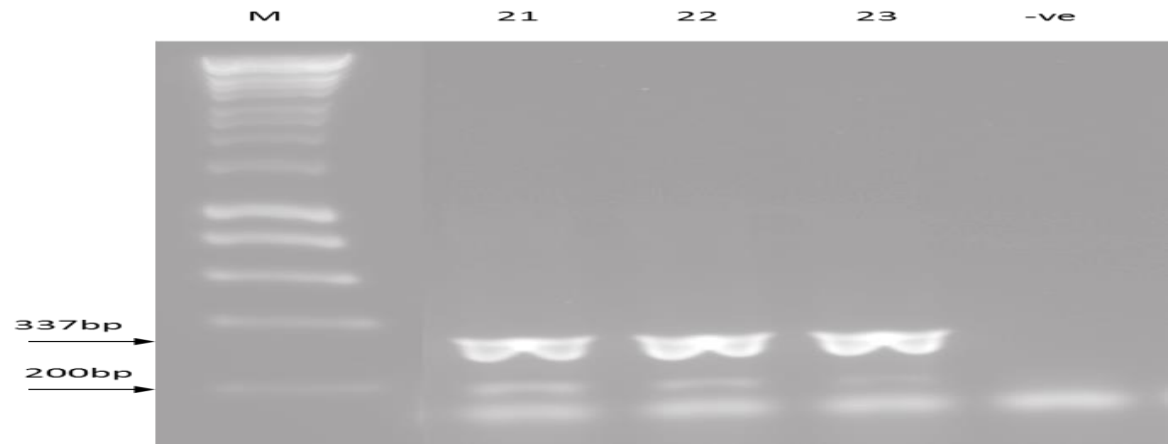


Figure 5.6. Representative agarose gel (1%) image showing PCR amplification of *Apodemus sylvaticus* Tlr2 fragments. Lane 2-4 test samples for *Apodemus sylvaticus*, Negative control. M: Hyperladder 1Kb (Invitrogen, UK).

Once the DNA was amplified it was then sent for sequence analysis. The PCR products of samples were sent for sequence analysis utilising the methodology of preparing the correct ratios of DNA to buffer to reverse and forward primers as outlined in the materials and methods. In addition to the

outlined methodology prior to sequencing the samples were also cleaned in a 5-stage process prior to PCR as in both the *A. sylvaticus* samples both forward and reverse initial sequences were unreliable. By checking all the sequenced DNA data, *A. sylvaticus* samples DNA had been generated.

Since the primer pairs used in *A. sylvaticus* were successful, the amplified DNA sequences needed to be sequenced in order to obtain a consensus for all six genes. Sequencing results provided with unprocessed readouts sometimes contained unassigned nucleotides (Ns). As a rule, these unassigned nucleotides were corrected by comparing the forward and reverse sequences. This manual chromatogram check also validated and improved the accuracy. The sequences were also analysed for the presence of any single nucleotide polymorphisms (SNPs) and heterozygotes (Tables 5.9-5.14).

This was carried out by Clustal W alignment to double-check the complementarity of both forward and reverse sequences. The analysis steps are explained in Tables 5.9A-5.114A, which shows the unreliable DNA sequence of *A. sylvaticus* including forward and reverse complement sequences.

These sequence figures show the correction of all N's in order to make the sequences clearer and enabled the consensus sequences to be generated. In both cases for both forward and reverse sequences the initially unreliable sequences were corrected to reduce error and increase comparison potential. All unassigned bases at the start and the end of the sequences were deleted from FinchTV software manually (Tables 5.9B-5.114B).

These results highlight that the sequences are improved after multiple alignment and this should be conducted in order to detect the consensus sequence. Clustal W alignment between forward sequence and reverse complement sequence of genes were performed on both *A.sylvaticus* samples (Tables 5.9C-5.14C) following successful PCR reactions and visualisation using electrophoresis. By investigating the similarity, Forward and reverse complement sequences results in high similarity. It can be concluded that both CLUSTALW alignment sequences are matched and conserved and as a result can be considered consensus sequence.

Table 5.9. A) Raw TLR2 sequence sample – forward and reverse sequences. In yellow unreliable sequence is depicted. B) Corrected Tlr2 sequence C) Clustal W alignment of Tlr2 for forward and reverse Complement sequences

A Forward

NNNNNNNNGTNNNNNNNGTACNATACNGAGGTTGCACATCCCCCAGTTCTATTCATTTTATGATCTGAGTACTGTCTATTCCCTCCTGGA
GAAGGTGAAGCGAATCACCGTGGAGAACAGTAAGGTCTTCCTGGTCCCCTGCCTCTTCTCGCAGCATTTAAAATCATTAGAATTCTTAGA
CCTCAGCGAAAATCTGATGGTTGAAGAATATTTGAAATACTCAGCATGTGAGGGCGGCTGGCCTTCTTTACAAACCTTAGTTTTGAGCCA
GAATCATTTGAGATCAATACAAAAAACGGGAGAGATTTTGCTGACTCTGAAAAACCTGACAGCCCTTGACATA

A Reverse

NNNNNNNNNNTNNNGNNNNNNNGNNNTNNTNNNNNNGATTCTGGCTCAAAACTAAGGTTTGTAAAGAAGGCCAGCCGCCCTCACATGCTG
AGTATTTCAAATATTCTTCAACCATCAGATTTTCGCTGAGGTCTAAGAATTCTAATGATTTTAAATGCTGCGAGAAGAGGCAGGGGACCA
GGAAGACCTTACTGTTCTCCACGGTGATTCGCTTCACCTTCTCCAGGAGGGAATAGACAGTACTCAGATCATAAAATGAATAGAACTGGG
GGATGTGCAACCTCCGAATTGTTACCGTCTCTACTTTACCTAGCTCTCTCACTATGTCTGATTCCGAGGGG

B Forward

AGGTTGCACATCCCCCAGTTCTATTCATTTTATGATCTGAGTACTGTCTATTCCCTCCTGGAGAAGGTGAAGCGAATCACCGTGGAGAAC
AGTAAGGTCTTCCTGGTCCCCTGCCTCTTCTCGCAGCATTTAAAATCATTAGAATTCTTAGACCTCAGCGAAAATCTGATGGTTGAAGAA
TATTTGAAATACTCAGCATGTGAGGGCGGCTGGCCTTCTTTACAAACCTTAGTTTTGAGCCAGAATCATTTGAGATCAATACAAAAAACG
GGAGAGATTTTGCTGACTCTGAAAAACCTGACAGCCCTTGACATA

B Reverse Complement

CCCCTCGGAATCAGACATAGTGAGAGAGCTAGGTAAAGTAGAGACGGTAACAATTCGGAGGTTGCACATCCCCCAGTTCTATTCATTTTA
TGATCTGAGTACTGTCTATTCCCTCCTGGAGAAGGTGAAGCGAATCACCGTGGAGAACAGTAAGGTCTTCCTGGTCCCCTGCCTCTTCTC
GCAGCATTTAAAATCATTAGAATTCTTAGACCTCAGCGAAAATCTGATGGTTGAAGAATATTTGAAATACTCAGCATGTGAGGGCGGCTG
GCCTTCTTTACAAACCTTAGTTTTGAGCCAGAATC

C

```
F       ------------------------------------------------------------AG        2
R       CCCCTCGGAATCAGACATAGTGAGAGAGCTAGGTAAAGTAGAGACGGTAACAATTCGGAG        60
                                                                  **

F       GTTGCACATCCCCCAGTTCTATTCATTTTATGATCTGAGTACTGTCTATTCCCTCCTGGA         62
R       GTTGCACATCCCCCAGTTCTATTCATTTTATGATCTGAGTACTGTCTATTCCCTCCTGGA         120
        ************************************************************

F       GAAGGTGAAGCGAATCACCGTGGAGAACAGTAAGGTCTTCCTGGTCCCCTGCCTCTTCTC        122
R       GAAGGTGAAGCGAATCACCGTGGAGAACAGTAAGGTCTTCCTGGTCCCCTGCCTCTTCTC        180
        ************************************************************

F       GCAGCATTTAAAATCATTAGAATTCTTAGACCTCAGCGAAAATCTGATGGTTGAAGAATA        182
R       GCAGCATTTAAAATCATTAGAATTCTTAGACCTCAGCGAAAATCTGATGGTTGAAGAATA        240
        ************************************************************

F       TTTGAAATACTCAGCATGTGAGGGCGGCTGGCCTTCTTTACAAACCTTAGTTTTGAGCCA        242
R       TTTGAAATACTCAGCATGTGAGGGCGGCTGGCCTTCTTTACAAACCTTAGTTTTGAGCCA        300
        ************************************************************

F       GAATCATTTGAGATCAATACAAAAAACGGGAGAGATTTTGCTGACTCTGAAAAACCTGAC        302
R       GAATC-------------------------------------------------------        305
        *****

F       AGCCCTTGACATA       315
R       -------------       305
```

Table 5.10. A) Raw APP sequence sample – forward and reverse sequences. In yellow unreliable sequence is depicted. B) Corrected App sequence C) Clustal W alignment of App for forward and reverse Complement sequences.

A Forward

NNNNNNNNNNNNNNNNTNNNNNNNNNNNNNNNNNNNNTATTCTCTCTTTCTCTCTTTACATTCTGGTCTCTACACTACATGATTAATGGG
TTTTGTGTACTGTAAAAAAAAGAAAAAAGAAAAGAAAATTTAGCTGTATCAAACTAGTGCATGAATAGACTTTTTCCTGATTATTTA
TCACATACATAACCCCTTAGCCCGTTGTATATTATTCTTGTGGTTTGTGGCCCGAAAAACTCCTACTTGAAATATGCTTAAAAAAAA
AAATCGATGGGGGATGCTTCGTGTGAACGTGGGCGTTTAGCTGCTTTTCCTCTCTAAGTATTCTTTTCCTGATCACTATGCATTTTG
AACATTTTTTTAAGTATTCCAAATGATTTAAAAAATTCCTTTTCCATGATTGCATCTTACTGTACAGATTGCTGCTTCTGCTCTCTT
TGTGATATAGGAATAAAAGGATACACATTGATTTCTTTGTGCCTGTTTTATGTGCACACATTAGGCATTGAGAATCTGAACTTTTTT
TTTTGTCCATGTATCTTTGGATCTTTGATAAAAAATAAAATAAAATAAATATCCCTGTTCATCGTAAGCACTTTTACGGGGGGGGGG
AGGGAGTGTTCTGCTGGTCTCCAA

A Reverse

NNNNNNNNNNNNNNNNNNNNNANNANANNNNGNNNNANNNANTTTATTTTATTTTTTATCAAAGATCCAAAGATACATGGACAAAAA
AAAAAGTTCAAATTCTCAATGCCTAATGTGTGCACATAAAACAGGCACAAAGAAATCAATGTGTATCCTTTTATTCCTATATCACAA

AGAGAGCAGAAGCAGCAATCTGTACAGTAAGATGCAATCATGGAAAAGGAATTTTTTAAATCATTTGGAATACTTAAAAAAATGTTC
AAAATGCATAGTGATCAGGAAAAGAATACTTAGAGAGGAAAAGCAGCTAAACGCCCACGTTCACACGAAGCATCCCCCATCGATTTT
TTTTTTTAACGATATTTCAAGTAGGAGTTTTTCGGGCCACAAACCACAAGAATAATATACAACGGGCTAAGGGGTTATGTATGTGAT
AAATAATCAGGAAAAAGTCTATTCATGCACTAGTTTGATACAGCTAGATTTTCTTTTCTTTTTTCTTTTTTTTACAGTACACAAAAC
CCATTAATCATGTAGGGTAAAAACCAAAATGTAAAGAGAGAAAAAAAAAATACATTACTGATTTGTATATTAATTCAAATTCAGGCA
TCTACTTGTGTTACACCACACCTGTAA

B Forward

TATTCTCTCTTTCTCTCTTTACATTCTGGTCTCTACACTACATGATTAATGGGTTTTGTGTACTGTAAAAAAAAGAAAAAAGAAAAG
AAAATTTAGCTGTATCAAACTAGTGCATGAATAGACTTTTTCCTGATTATTTATCACATACATAACCCCTTAGCCCGTTGTATATTA
TTCTTGTGGTTTGTGGCCCGAAAAACTCCTACTTGAAATATGCTTAAAAAAAAAAAATCGATGGGGGATGCTTCGTGTGAACGTGGGC
GTTTAGCTGCTTCTCCTCTCTAAGTATTCTTTTCCTGATCACTATGCATTTTGAACATTTTTTTAAGTATTCCAAATGATTTAAAAA
ATTCCTTTTCCATGATTGCATCTTACTGTACAGATTGCTGCTTCTGCTCTCTTTGTGATATAGGAATAAAAGGATACACATTGATTT
CTTTGTGCCTGTTTTATGTGCACACATTAGGCATTGAGAATCTGAACTTTTTTTTTTGTCCATGTATCTTTGGATCTTTGATAAAAA
ATAAAATAAAATAAATATCCCTGTTCATCGTAAGCACTTTTACGGGGGGGGGGAGGGAGTGTTCTGCTGGTCTCCAA

B Reverse complement

TTACAGGTGTGGTGTAACACAAGTAGATGCCTGAATTTGAATTAATATACAAATCAGTAATGTATTTTTTTTTTCTCTCTTTACATT
TTGGTTTTTACCCTACATGATTAATGGGTTTTGTGTACTGTAAAAAAAAGAAAAAAGAAAAGAAAATTTAGGTGTATCAAAATAGTG
CATGAATAGACTTTTTCCTGATTATTTATCACATACATAACCCCTTAGCCCGTTGTATATTATTTTTGTGGTTTGTGGCCCGAAAAA
CTCCTACTTGAAATATGGTTAAAAAAAAAAATCGATGGGGGATGCTTCGTGTGAACGTGGGCGTTTAGCTGCTTTTCCTCTCTAAGT
ATTCTTTTCCTGATCACTATGCATTTTGAACATTTTTTTAAGTATTCCAAATGATTTAGAAAATTCCTTTTCCATGATTGCATCTTA
CTGTACAGATTGCTGCTTTTGCTCTCTTTGTGATATAGGAATAAGAGGATACACATTGATTTCTTTGTGCCTGTTTTATGTGCACAC
ATTAGGCATTGAGAATTTGAACTTTTTTTTTTGTCCATGTATCTTTGGATCTTTGATAAAAAAT

C

```
F       ------------------------------------------------------------          0
R       TTACAGGTGTGGTGTAACACAAGTAGATGCCTGAATTTGAATTAATATACAAATCAGTAA          60


F       ----------------------------------------CTACATGATTAATGGGTTTTG          21
R       TGTATTTTTTTTTTCTCTCTTTACATTTTGGTTTTTACCCTACATGATTAATGGGTTTTG         140
                                                 **********************

F       TGTACTGTAAAAAAAAGAAAAAAGAAAAGAAAATTTAGCTGTATCAAACTAGTGCATGAA          81
R       TGTACTGTAAAAAAAAGAAAAAAGAAAAGAAAATTTAGCTGTATCAAACTAGTGCATGAA         180
        ************************************************************

F       TAGACTTTTTCCTGATTATTTATCACATACATAACCCCTTAGCCCGTTGTATATTATTCT          142
R       TAGACTTTTTCCTGATTATTTATCACATACATAACCCCTTAGCCCGTTGTATATTATTCT          240
        ************************************************************

F       TGTGGTTTGTGGCCCGAAAAACTCCTACTTGAAATATGCTTAAAAAAAAAAAATCGATGGG          202
R       TGTGGTTTGTGGCCCGAAAAACTCCTACTTGAAATATGCTTAAAAAAAAAAAATCGATGGG          300
        ************************************************************

F       GGATGCTTCGTGTGAACGTGGGCGTTTAGCTGCTTTTCCTCTCTAAGTATTCTTTTCCTG          262
R       GGATGCTTCGTGTGAACGTGGGCGTTTAGCTGCTTTTCCTCTCTAAGTATTCTTTTCCTG          360
        ************************************************************

F       ATCACTATGCATTTTGAACATTTTTTTAAGTATTCCAAATGATTTAAAAAATTCCTTTTC          322
R       ATCACTATGCATTTTGAACATTTTTTTAAGTATTCCAAATGATTTAAAAAATTCCTTTTC          420
        ************************************************************

F       CATGATTGCATCTTACTGTACAGATTGCTGCTTCTGCTCTCTTTGTGATATAGGAATAAA          482
R       CATGATTGCATCTTACTGTACAGATTGCTGCTTCTGCTCTCTTTGTGATATAGGAATAAA          480
        ************************************************************

F       AGGATACACATTGATTTCTTTGTGCCTGTTTTATGTGCACACATTAGGCATTGAGAATCT          442
R       AGGATACACATTGATTTCTTTGTGCCTGTTTTATGTGCACACATTAGGCATTGAGAATCT          540
        ************************************************************

F       GAACTTTTTTTTTTGTCCATGTATCTTTGGATCTTTGATAAAAAATAAAATAAAATAAAT          502
R       GAACTTTTTTTTTTGTCCATGTATCTTTGGATCTTTGATAAAAAAT--------------          586
        *******************************************

F       ATCCCTGTTCATCGTAAGCACTTTTACGGGGGGGGGGAGGGAGTGTTCTGCTGGTCTCCA          562
R       ------------------------------------------------------------          593
```

```
F       A  599
R                -           586
```

Table 5.11. A) Raw Beclin1 sequence sample – forward and reverse sequences. In yellow unreliable sequence is depicted. B) Corrected Beclin1 sequence C) Clustal W alignment of Beclin1 for forward and reverse Complement sequences.

A Forward

NNNNNNNNNNNNNNNGCNGTGGACNNAAGCGCTCAAGATCACNCTGACGANTCTCAAGTGGGGTCTTGCCTGGGTGTCCTCACAGTTTTA
TAACAAGCGACTTCCTCCTTGGGGGAATTTTTCCTTTTAGGGTTTTCCCCTTGGTTTGGATAGAAGAGATGCTTTAAATTTGGGTAATAT
TAAACCACATGTTTACAATCCCAATCCCCCAAAACCTCTTTTTTTTTCCAATATGACCCAAAGTTTCCCGAATTCGCCCCATGTAGAGCAG
AGGACCCCCCCGTAGCTTTGACTCCTCCGGCCCCTGCCCTTTCCCCCCCCCCCGAAAACCACCAAATTAAATTCTGCTTGGTTTCCTCTT
TTTTTTACAATTGACTTTAATTTGTTTTCTCTGGATTGTATTTCTCTCAACTTCCTGCCCTTAAAACAAAAAACAAAAAAGATTTGATAT
GGGATGCTTGNNNCGNNCAANCAAGG

A Reverse

NNNNNNNNNNNNNNTNANTGCAGGAAGTTNGNNNNGAGAGAAAATCCAGTGNNNNGCATGAATGTCAATTGTACTTAAAAAAGAAAACAA
AACAAAATTAAATTAAATTGTTTGAGGAGAGGGAGAGGCCGAAGGGGGGCTCAGGCAAAACTGTGGCAGGGGTATTTGCTCTACACGTTG
TGTCAACTCTGGAAACTATATGTCATATTTGAAAATAAAATAGATTTTGTGGATTTGGTTTCGTAAACATGTGGTTTGATTTAACCTAAC
CTAAATTTAAAGCATCTTTCCAAACCAAAGTGTTGAACCTTAATGGCGGAGATCCCCTCCCCAGCAAGGCACTTGTTTTAAAACTGTGAG
GACAGCCAGGCAAGACCCCACTTGAGATTCGACAGCATGAACTTGAGCGCTTTCGTCCACTGCNCCTCCCAGTNAAATTGGNNNNNGNTG
NAATGGNNNNNNCCCCTGCANNNAA

B Forward

TCTCAAGTGGGGTCTTGCCTGGGTGTCCTCACAGTTTTATAACAAGCGACTTCCTCCTTGGGGGAATTTTTCCTTTTAGGGTTTTCCCCT
TGGTTTGGATAGAAGAGATGCTTTAAATTTGGGTAATATTAAACCACATGTTTACAATCCCAATCCCCCAAAACCTCTTTTTTTTTCCAAT
ATGACCCAAAGTTTCCCGAATTCGCCCCATGTAGAGCAGAGGACCCCCCCCGTAGCTTTGACTCCTCCGGCCCCTGCCCTTTCCCCCCCCC
CCGAAAACCACCAAATTAAATTCTGCTTGGTTTCCTCTTTTTTTTTACAATTGACTTTAATTTGTTTTCTCTGGATTGTATTTCTCTCAAC
TTCCTGCCCTTAAAACAAAAAACAAAAAAGATTTGATATGGGATGCTTG

B Reverse complement

GCAGTGGACGAAAGCGCTCAAGTTCATGCTGTCGAATCTCAAGTGGGGTCTTGCCTGGCTGTCCTCACAGTTTTAAAACAAGTGCCTTGC
TGGGGAGGGGATCTCCGCCATTAAGGTTCAACACTTTGGTTTGGAAAGATGCTTTAAATTTAGGTTAGGTTAAATCAAACCACATGTTTA
CGAAACCAAATCCACAAAATCTATTTTATTTTCAAATATGACATATAGTTTCCAGAGTTGACACAACGTGTAGAGCAAATACCCCTGCCA
CAGTTTTGCCTGAGCCCCCCTTCGGCCTCTCCCTCTCCTCAAACAATTTAATTTAATTTTGTTTTGTTTTCTTTTTTAAGTACAATTGAC
ATTCATGC

C

```
F       ------------------------------------TCTCAAGTGGGGTCTTGCCTGGGT          24
R       GCAGTGGACGAAAGCGCTCAAGTTCATGCTGTCGAATCTCAAGTGGGGTCTTGCCTGGCT          60
                                            ********************** *

F       GTCCTCACAGTTTTATAACAAGCGACTTCCTCCTTGGGGGAATTTTTCCTTTTAGGGTTT          84
R       GTCCTCACAGTTTTAAAACAAGTGCCTTGCTGGGGAGGGGATCTCCGCCATTAAGGTTCA          120
        *************** ****** * *** **     *****  *   ** ** *** *
```

```
F       TCCCCTTGGTTTGGATAGAAGAGATGCTTTAAATTTGGGTAATATTAAACCACATGTTTA          144
R       ACACTTTGGTTTGGAAAGATGCTTTAAATTTAGGTTAGGTTAAATCAAACCACATGTTTA          180
         * * ********** *** *   *   ** *  ** *** * ** **************

F       CAATCCCAATCCCCCAAAACCTCTTTTT-TTTCCAATATGACCCAAAGTTTCCCGAATTC          203
R       CGAAACCAAATCCACAAAATCTATTTTATTTTCAAATATGACATATAGTTTCCAGAGTTG          240
        * *  ****  ** ***** ** ****  **** ********  * ******* ** **

F       GCCCCATGTAGAGCAGAGG--ACCCCCCCCGTAGCTTTGACTCCTCCGGCCCCTGCCCTTT          261
R       ACACAACGTGTAGAGCAAATACCCCTGCCACAGTTTTGCCTGAGCCCCCCTTCGGCCT--          298
         * * * **  **   *     ***  **  ** **** **   **  **   * ***

F       CCCCCCCCCCCCGAAAACCACCAAATTAAATTCTGCTTGGTTTCCTCTTTTTTTTACAATT          321
R       -CTCCCTCTCCTCAAACAATTTAATTTAATTTTGTTTTGTTTTCTTTTTTAAGTACAATT          357
         * *** * **  **** *   **** **** ** ** **** ** ****   *******

F       GACTTTAATTTGTTTTCTCTGGATTGTATTTCTCTCAACTTCCTGCCCTTAAAACAAAAA          381
R       GACATTCATGC-------------------------------------------------          368
        *** ** **

F       ACAAAAAAGATTTGATATGGGATGCTTG   409
R       ---------------------------   368
```

Table 5.12. A) Raw Lamp2 sequence sample – forward and reverse sequences. In yellow unreliable sequence is depicted. B) Corrected Lamp2 sequence C) Clustal W alignment of Lamp2 for forward and reverse Complement sequences.

A Forward

```
NNNNNNNNNNNNNNNNNNNCATTTGATTGATTATATTACAAAATACATACAAAAACCAAAGTTGTCAACCTGTCAGTGTATGAAGCA
CTTTGCTCCTTAAGACAGACACCTTGGAACCTTCATTCAGAAATCAGTCCCCACATTTTGACATAAGTCTTTTATTGAACAAAAAT
ATCTCTGATCTGCTTAAAATGCAGACTGGCCTTAAAAAAAAAAGAAAGTGAACACATACCTGCAAAGGTCTGTCTTAAGAAGCTTT
GGCCAAATTGGGATCCTAACCTAAAATGC
```

B Reverse

```
NNNNNNNNNNNNNNACCTTTGCAGGTATGTGTTCACTTTCTTTTTTTTTTAAGGCCAGTCTGCATTTTAAGCAGATCAGAGATATTT
TTGTTCAATAAAAGACTTATGTCAAAATGTGGGGACTGATTTCTGAATGAAGGTTCCAAGGTGTCTGTCTTAAGGAGCAAAGTGCT
TCATACACTGACAGGTTGACAACTTTGGTTTTTGTATGTATTTTGTAATATAATCAATCAAATTGCGGGAGCTAAAACTGCTCATA
TCCAGTATGATGGCGCTTGAGACCAA
```

B Forward

```
CATTTGATTGATTATATTACAAAATACATACAAAAACCAAAGTTGTCAACCTGTCAGTGTATGAAGCACTTTGCTCCTTAAGACAG
ACACCTTGGAACCTTCATTCAGAAATCAGTCCCCACATTTTGACATAAGTCTTTTATTGAACAAAAATATCTCTGATCTGCTTAAA
ATGCAGACTGGCCTTAAAAAAAAAAGAAAGTGAACACATACCTGCAAAGGTCTGTCTTAAGAAGCTTTGGCCAAATTGGGATCCTA
ACCTAAAATGC
```

B Reverse Complement

```
TTGGTCTCAAGCGCCATCATACTGGATATGAGCAGTTTTAGCTCCCGCAATTTGATTGATTATATTACAAAATACATACAAAAACC
AAAGTTGTCAACCTGTCAGTGTATGAAGCACTTTGCTCCTTAAGACAGACACCTTGGAACCTTCATTCAGAAATCAGTCCCCACAT
TTTGACATAAGTCTTTTATTGAACAAAAATATCTCTGATCTGCTTAAAATGCAGACTGGCCTTAAAAAAAAAAGAAAGTGAACACA
TACCTGCAAAGGT
```

C

```
F       ------------------------------------------------CATTTGATTGAT        12
R       TTGGTCTCAAGCGCCATCATACTGGATATGAGCAGTTTTAGCTCCCGCAATTTGATTGAT        60
                                                         ***********

F       TATATTACAAAATACATACAAAAACCAAAGTTGTCAACCTGTCAGTGTATGAAGCACTTT        72
R       TATATTACAAAATACATACAAAAACCAAAGTTGTCAACCTGTCAGTGTATGAAGCACTTT        120
        ************************************************************

F       GCTCCTTAAGACAGACACCTTGGAACCTTCATTCAGAAATCAGTCCCCACATTTTGACAT        132
```

```
R       GCTCCTTAAGACAGACACCTTGGAACCTTCATTCAGAAATCAGTCCCCACATTTTGACAT        180
        ************************************************************

F       AAGTCTTTTATTGAACAAAAATATCTCTGATCTGCTTAAAATGCAGACTGGCCTTAAAAA        192
R       AAGTCTTTTATTGAACAAAAATATCTCTGATCTGCTTAAAATGCAGACTGGCCTTAAAAA        240
        ************************************************************

F       AAAAAGAAAGTGAACACATACCTGCAAAGGTCTGTCTTAAGAAGCTTTGGCCAAATTGGG        252
R       AAAAAGAAAGTGAACACATACCTGCAAAGGT------------------------------        271
        *******************************

F       ATCCTAACCTAAAATGC   269
R       ----------------   271
```

Table 5.13. A) Raw Map1Lc3 sequence sample – forward and reverse sequences. In yellow unreliable sequence is depicted. B) Corrected Map1Lc3 sequence C) Clustal W alignment of Lc3 for forward and reverse Complement sequences.

A Forward

NNNNNNNNNNANTNNTGNNNAGTNNTCANCNNNNNNNNNNNNCANANGTTCCCANAGGGNTCNGGCTTTGCCCCNNNCANGTCAAGT
GTGTAGGACAACTCTAAGCCGGGTCCCCAGGTCCAGGTCCTGACCCACCCCCACCCCCCAGCTACACCCTTTCACCCTTAGCAGTGC
AGGAGCACTCACCCGCTGCTGCCTAGATGTTACCACACGCACCCCTGCTCAGGCCAGTTCTAAACTTGTCCAGAAGTCGCTTGGTCC
AGCTTATTAAGAGACTGCAGAGCAAAGTGTTTCTCCTGGTGTGGGTTGTCTAGTCGGGGTGCCTGCTTCAGGGAGAGCAAGCAGGTG
TGCAGTGCTGGCCTTAGGATGCAGCTGTCAAGTGGTGGATGCAAGTCCAAACACTGGCTCTGGACATGCTCAATGGCTGTTTGTTAG
CGTGTGACTGTCTTTGTAAGGGTGTTTTTGACAGCTGTTAGGATTTCCCCATTTCATGTCTACATACGTGTCAGCTGTGAGAACAAC
AGA

A Reverse

NNNNNNNNNNNNNNNNNNNNNGCTGTCANNNCACNCNNNCNNGACNGTCACACGCTAACAAACAGCCATTGAGCATGTCCAGAGCCA
GTGTTTGGACTTGCATCCACCACTTGACAGCTGCATCCTAAGGCCAGCACTGCACACCTGCTTGCTCTCCCTGAAGCAGGCACCCCG
ACTAGACAACCCACACCAGGAGAAACACTTTGCTCTGCAGTCTCTTAATAAGCTGGACCAAGCGACTTCTGGACAAGTTTAGAACTG
GCCTGAGCAGGGGTGCGTGTGGTAACATCTAGGCAGCAGCGGGTGAGTGCTCCTGCACTGCTAAGGGTGAAAGGGTGTAGCTGGGGG
GTGGGGGTGGGTCAGGACCTGGACCTGGGGACCCGGCTTAGAGTTGTCCTACACACTTGACCTGTTTGGGGCAAAGCCAGAGCCCTC
TGGGAACCTCTGCTTGGCAACCTGGGCTGAGGGACTGCTCAGGACTTCTCTCCTGAGCAGAAGCCCTTTTAGAGAAGGCAGCAGGTC
C

B Forward

GTCAAGTGTGTAGGACAACTCTAAGCCGGGTCCCCAGGTCCAGGTCCTGACCCACCCCCACCCCCCAGCTACACCCTTTCACCCTTA
GCAGTGCAGGAGCACTCACCCGCTGCTGCCTAGATGTTACCACACGCACCCCTGCTCAGGCCAGTTCTAAACTTGTCCAGAAGTCGC
TTGGTCCAGCTTATTAAGAGACTGCAGAGCAAAGTGTTTCTCCTGGTGTGGGTTGTCTAGTCGGGGTGCCTGCTTCAGGGAGAGCAA
GCAGGTGTGCAGTGCTGGCCTTAGGATGCAGCTGTCAAGTGGTGGATGCAAGTCCAAACACTGGCTCTGGACATGCTCAATGGCTGT
TTGTTAGCGTGTGACTGTCTTTGTAAGGGTGTTTTTGACAGCTGTTAGGATTTCCCCATTTCATGTCTACATACGTGTCAGCTGTGA
GAACAACAGCA

B Reverse Complement

GGACCTGCTGCCTTCTCTAAAAGGGCTTCTGCTCAGGAGAGAAGTCCTGAGCAGTCCCTCAGCCCAGGTTGCCAAGCAGAGGTTCCC
AGAGGGCTCTGGCTTTGCCCCAAACAGGTCAAGTGTGTAGGACAACTCTAAGCCGGGTCCCCAGGTCCAGGTCCTGACCCACCCCCA
CCCCCCAGCTACACCCTTTCACCCTTAGCAGTGCAGGAGCACTCACCCGCTGCTGCCTAGATGTTACCACACGCACCCCTGCTCAGG
CCAGTTCTAAACTTGTCCAGAAGTCGCTTGGTCCAGCTTATTAAGAGACTGCAGAGCAAAGTGTTTCTCCTGGTGTGGGTTGTCTAG
TCGGGGTGCCTGCTTCAGGGAGAGCAAGCAGGTGTGCAGTGCTGGCCTTAGGATGCAGCTGTCAAGTGGTGGATGCAAGTCCAAACA
CTGGCTCTGGACATGCTCAATGGCTGTTTGTTAGCGTGTGAC

C

```
F       ------------------------------------------------------------          0
R       GGACCTGCTGCCTTCTCTAAAAGGGCTTCTGCTCAGGAGAGAAGTCCTGAGCAGTCCCTC          60

F       ------------------------------------------------------GTCAAG          6
R       AGCCCAGGTTGCCAAGCAGAGGTTCCCAGAGGGCTCTGGCTTTGCCCCAAACAGGTCAAG          120
                                                               ******

F       TGTGTAGGACAACTCTAAGCCGGGTCCCCAGGTCCAGGTCCTGACCCACCCCCACCCCCC          66
R       TGTGTAGGACAACTCTAAGCCGGGTCCCCAGGTCCAGGTCCTGACCCACCCCCACCCCCC          180
        ************************************************************

F       AGCTACACCCTTTCACCCTTAGCAGTGCAGGAGCACTCACCCGCTGCTGCCTAGATGTTA          126
R       AGCTACACCCTTTCACCCTTAGCAGTGCAGGAGCACTCACCCGCTGCTGCCTAGATGTTA          240
        ************************************************************

F       CCACACGCACCCCTGCTCAGGCCAGTTCTAAACTTGTCCAGAAGTCGCTTGGTCCAGCTT          186
R       CCACACGCACCCCTGCTCAGGCCAGTTCTAAACTTGTCCAGAAGTCGCTTGGTCCAGCTT          300
        ************************************************************

F       ATTAAGAGACTGCAGAGCAAAGTGTTTCTCCTGGTGTGGGTTGTCTAGTCGGGGTGCCTG          246
R       ATTAAGAGACTGCAGAGCAAAGTGTTTCTCCTGGTGTGGGTTGTCTAGTCGGGGTGCCTG          360
        ************************************************************


F       CTTCAGGGAGAGCAAGCAGGTGTGCAGTGCTGGCCTTAGGATGCAGCTGTCAAGTGGTGG          306
R       CTTCAGGGAGAGCAAGCAGGTGTGCAGTGCTGGCCTTAGGATGCAGCTGTCAAGTGGTGG          420
        ************************************************************

F       ATGCAAGTCCAAACACTGGCTCTGGACATGCTCAATGGCTGTTTGTTAGCGTGTGACTGT          366
R       ATGCAAGTCCAAACACTGGCTCTGGACATGCTCAATGGCTGTTTGTTAGCGTGTGAC---          477
        ********************************************************

F       CTTTGTAAGGGTGTTTTTGACAGCTGTTAGGATTTCCCCATTTCATGTCTACATACGTGT          426
R       ------------------------------------------------------------          477


F       CAGCTGTGAGAACAACAGCA  446
R       --------------------  477
```

Table 5.14. A) Raw Tau sequence sample – forward and reverse sequences. In yellow unreliable sequence is depicted. B) Corrected Tau sequence C) Clustal W alignment of Tau for forward and reverse Complement sequences.

A Forward

NNNNNNNNNGNNNNGNNANCGTGTACAGTCTCCCGTGGTGTCTGGGGACACATCGCCACGGCACCTCAGCAATGTGTCGTCCACGGG
CAGCATCGACATGGTGGACTCACCACAGCTTGCCACACTCGCCGATGAAGTGTCTGCGTCCTTGGCCAAGCAGGGTTTGTGATCAGG
CTCA

A Reverse

NNNNNNNNNNNNNNNNGNCGAGTGTGGCAGCTGTGGTGAGTCCACCATGTCGATGCTGCCCGTGGACGACACATTGCTGAGGTGCCG
TGGCGATGTGTCCCCAGACACCACGGGAGACTTGTACACGATTTCCGCTCCGTGGTCTGTCTTGGCTTTGGCATTCTCCCTGAAGGT

CAGCAATGTGTNGTNNNNGGGNNNNNNGNNNNTNNNNNNNNCNNNNNNNCTTGTNNCNNTCGCNGGTNGGNNNNNNNNNNNNNTGGC
CCTNNNNNNNNCCCTGTNCNTCNNCGGNNNNNNNNNNNTNNNNNNNNNNNNNNNNG

B Forward

AGTCTCCCGTGGTGTCTGGGGACACATCGCCACGGCACCTCAGCAATGTGTCGTCCACGGGCAGCATCGACATGGTGGACTCACCAC
AGCTTGCCACACTCGCCGATGAAGTGTCTGCGTCCTTGGCCAAGCAGGGTTTGTGATCAGGCTCA

B Reverse Complement

ACACATTGCTGACCTTCAGGGAGAATGCCAAAGCCAAGACAGACCACGGAGCGGAAATCGTGTACAAGTCTCCCGTGGTGTCTGGGG
ACACATCGCCACGGCACCTCAGCAATGTGTCGTCCACGGGCAGCATCGACATGGTGGACTCACCACAGCT

C

```
F       ------------------------------------------------------------          0
R       ACACATTGCTGACCTTCAGGGAGAATGCCAAAGCCAAGACAGACCACGGAGCGGAAATCG          60


F       AGTCTCCCGTGGTGTCTGGGGACACATCGCCACGGCACCTCAGCAATGTGTCGT 54
R       AGTCTCCCGTGGTGTCTGGGGACACATCGCCACGGCACCTCAGCAATGTGTCGT      109
        **************************************************

F       CCACGGGCAGCATCGACATGGTGGACTCACCACAGCTTGCCACACTCGCCGATGAAGTGT          114
R       CCACGGGCAGCATCGACATGGTGGACTCACCACAGCT-----------------------          167
        **********************************

F       CTGCGTCCTTGGCCAAGCAGGGTTTGTGATCAGGCTCA   152
R       ----------------------------------------   167
```

Overall, the peaks were very well spaced with some ambiguities (N). In sequencing data, bases marked with N means that the software was unable to identify which of the four bases occupied that position in the genome (Figures 5.7A-5.12A). These events occur more often at the beginning of a sequence, where base tag-related chemistry has not been stabilised and considered ambiguous. Similarly, a streak of N bases at the end of a sequence may indicate the end of sequencing data analysis. As a rule, regions with a high N number are often A/T- or G/C- rich and may indicate secondary or hairpin structures that may be difficult to read due to its two-dimensional structure (Balkwill, Williams, & Searle, 2007; Miao et al., 1993). The results show that the most similar alignment sequences that appeared (figure 5.7B-5.12B) are genes fragments from other mouse species. These species include *Mus musculus*, with over 91% similarity between them. Unfortunately, on this occasion Beclin1 sequencing has failed to produce any data (figure 5.12). We do not believe this to be a concentration issue as when DNA was originally PCR cleaned, it was optimised and the reactions both times were performed with the concentration at 10ng/ul which the recommended concentration for a PCR product. Also, dGTP chemistry was used for this reaction but has not worked. Having investigated

the data however there do appear to be small regions of GC rich areas. The suggestion we would make is to redesign the primer to bind to a different region as this could be a primer binding issue.

B

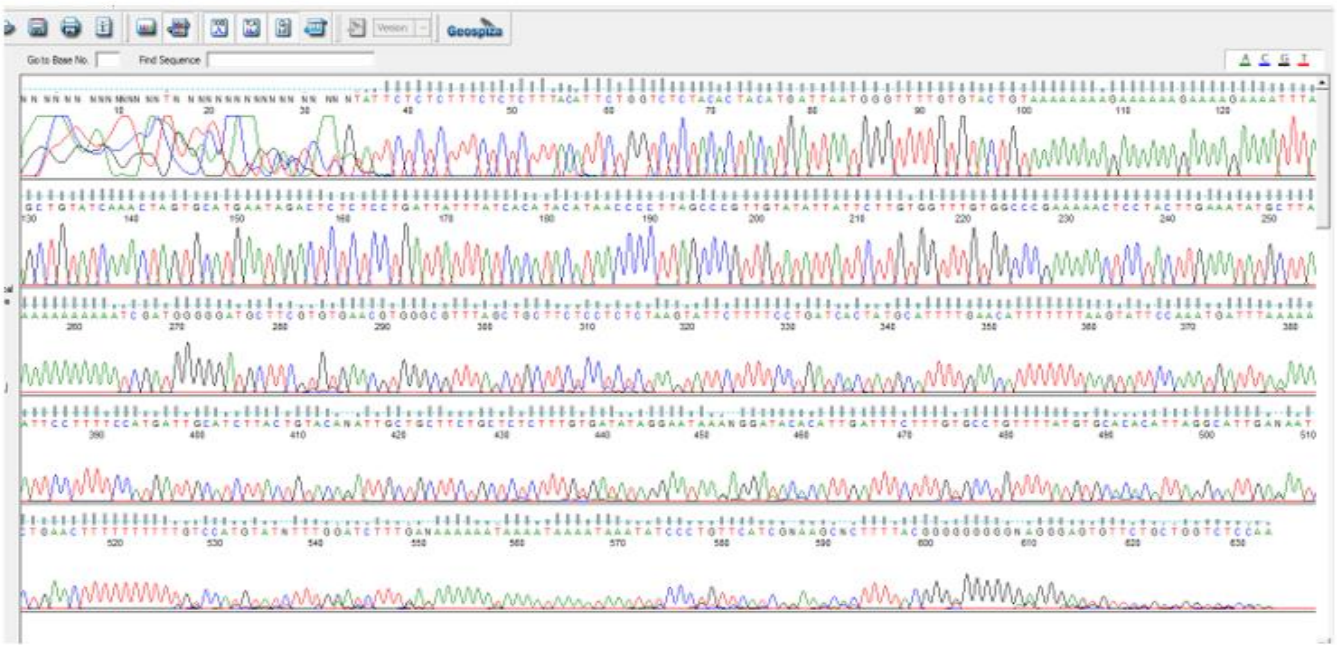
Sequences producing significant alignments Download Manage Columns Show 100

select all 36 sequences selected GenBank Graphics Distance tree of results

Description	Max Score	Total Score	Query Cover	E value	Per Ident	Accession
Apodemus flavicollis haplotype 3 toll-like receptor 2 (TLR2) gene, partial cds	580	580	99%	1e-161	100.00%	JN674551.1
Apodemus flavicollis haplotype 2 toll-like receptor 2 (TLR2) gene, partial cds	580	580	99%	1e-161	100.00%	JN674550.1
Apodemus flavicollis haplotype 1 toll-like receptor 2 (TLR2) gene, partial cds	580	580	99%	1e-161	100.00%	JN674549.1
Apodemus sylvaticus toll-like receptor 2 (TLR2) gene, complete cds	580	580	99%	1e-161	100.00%	HM215602.1
Apodemus flavicollis toll-like receptor 2 (TLR2) gene, complete cds	580	580	99%	1e-161	100.00%	HM215601.1
Apodemus alpicola toll-like receptor 2 (TLR2) gene, complete cds	575	575	99%	5e-160	99.68%	HM215600.1
PREDICTED: Mastomys coucha toll like receptor 2 (Tlr2), transcript variant X2, mRNA	472	472	99%	6e-129	93.91%	XM_031374200.1
PREDICTED: Mastomys coucha toll like receptor 2 (Tlr2), transcript variant X1, mRNA	472	472	99%	6e-129	93.91%	XM_031374199.1
PREDICTED: Mus caroli toll like receptor 2 (Tlr2), transcript variant X2, mRNA	470	470	99%	2e-128	93.65%	XM_029474778.1
PREDICTED: Mus caroli toll like receptor 2 (Tlr2), transcript variant X1, mRNA	470	470	99%	2e-128	93.65%	XM_021158462.1
Micromys minutus toll-like receptor 2 (TLR2) gene, complete cds	459	459	99%	5e-125	92.99%	HM215603.1
Mus musculus Toll-like receptor 2 mRNA, complete cds	459	459	99%	5e-125	93.02%	AF216289.1
Mus musculus NOD-derived CD11c +ve dendritic cells cDNA, RIKEN full-length enriched library, clone:F630040N12 product:toll-like recep	459	459	99%	5e-125	93.02%	AK154504.1
Mus musculus toll-like receptor 2 mRNA, complete cds	459	459	99%	5e-125	93.02%	AY179346.1
Mus musculus toll-like receptor 2 (Tlr2) mRNA, complete cds	459	459	99%	5e-125	93.02%	AF165189.1
Mus musculus Toll-like receptor 2 (Tlr2) mRNA, complete cds	459	459	99%	5e-125	93.02%	AF185284.1
Mus musculus Toll-like receptor 2 (Tlr2) mRNA, complete cds	459	459	99%	5e-125	93.02%	AF124741.1
PREDICTED: Mus musculus toll-like receptor 2 (Tlr2), transcript variant X2, mRNA	453	453	99%	2e-123	92.70%	XM_030252611.1
PREDICTED: Mus pahari toll like receptor 2 (Tlr2), mRNA	453	453	98%	2e-123	92.93%	XM_021196861.2
PREDICTED: Mus musculus toll-like receptor 2 (Tlr2), transcript variant X1, mRNA	453	453	99%	2e-123	92.70%	XM_006501460.3
Mus musculus targeted non-conditional, lacZ-tagged mutant allele Tlr2 tm1e(EUCOMM)Hmgu, transgenic	453	453	99%	2e-123	92.70%	JN953366.1
Mus musculus targeted KO-first, conditional ready, lacZ-tagged mutant allele Tlr2 tm1a(EUCOMM)Hmgu, transgenic	453	453	99%	2e-123	92.70%	JN945473.1
Mus musculus toll-like receptor 2 (Tlr2), mRNA	453	453	99%	2e-123	92.70%	NM_011905.3
Mus musculus toll-like receptor 2, mRNA (cDNA clone MGC:11394 IMAGE:3966249), complete cds	453	453	99%	2e-123	92.70%	BC014693.1

Figure 5.7. A) Sequencing of the TLR2 amplicon from *Apodemus sylvaticus* sample. B) BLASTed (NCBI) sequence shows high similarity (92%) to *Mus musculus* (Accession number: AF216289.1).

A

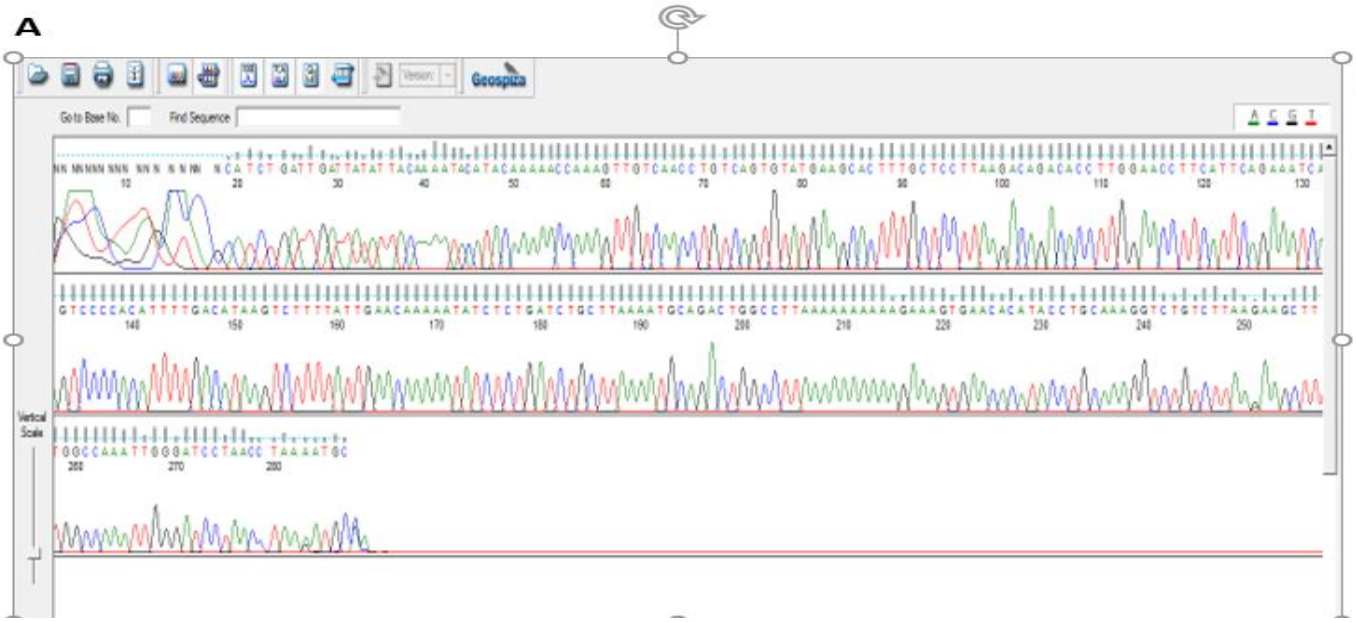


B

PREDICTED: Rattus norvegicus amyloid beta precursor protein (App), transcript variant X1, mRNA	813	813	100%	0.0	91.71%	XM_006248008.3
PREDICTED: Grammomys surdaster amyloid beta precursor protein (App), transcript variant X6, mRNA	811	811	100%	0.0	91.86%	XM_028757206.1
PREDICTED: Grammomys surdaster amyloid beta precursor protein (App), transcript variant X5, mRNA	811	811	100%	0.0	91.86%	XM_028757205.1
PREDICTED: Grammomys surdaster amyloid beta precursor protein (App), transcript variant X4, mRNA	811	811	100%	0.0	91.86%	XM_028757204.1
PREDICTED: Grammomys surdaster amyloid beta precursor protein (App), transcript variant X3, mRNA	811	811	100%	0.0	91.86%	XM_028757203.1
PREDICTED: Grammomys surdaster amyloid beta precursor protein (App), transcript variant X2, mRNA	811	811	100%	0.0	91.86%	XM_028757202.1
PREDICTED: Grammomys surdaster amyloid beta precursor protein (App), transcript variant X1, mRNA	811	811	100%	0.0	91.86%	XM_028757201.1
PREDICTED: Mus caroli amyloid beta precursor protein (App), transcript variant X6, mRNA	808	808	100%	0.0	91.69%	XM_029470477.1
PREDICTED: Mus caroli amyloid beta precursor protein (App), transcript variant X5, mRNA	808	808	100%	0.0	91.69%	XM_029470476.1
PREDICTED: Mus caroli amyloid beta precursor protein (App), transcript variant X4, mRNA	808	808	100%	0.0	91.69%	XM_029470475.1
PREDICTED: Mus caroli amyloid beta precursor protein (App), transcript variant X3, mRNA	808	808	100%	0.0	91.69%	XM_029470474.1
PREDICTED: Mus caroli amyloid beta precursor protein (App), transcript variant X2, mRNA	808	808	100%	0.0	91.69%	XM_029470473.1
PREDICTED: Mus caroli amyloid beta precursor protein (App), transcript variant X1, mRNA	808	808	100%	0.0	91.69%	XM_021184768.2
PREDICTED: Mus musculus amyloid beta (A4) precursor protein (App), transcript variant X1, mRNA	793	793	100%	0.0	91.21%	XM_006522873.3
Mus musculus amyloid beta (A4) precursor protein (App), transcript variant 6, mRNA	793	793	100%	0.0	91.21%	NM_001198826.1
Mus musculus amyloid beta (A4) precursor protein (App), transcript variant 5, mRNA	793	793	100%	0.0	91.21%	NM_001198825.1
Mus musculus amyloid beta (A4) precursor protein (App), transcript variant 3, mRNA	793	793	100%	0.0	91.21%	NM_001198824.1
Mus musculus amyloid beta (A4) precursor protein (App), transcript variant 2, mRNA	793	793	100%	0.0	91.21%	NM_007471.3
Mus musculus amyloid beta (A4) precursor protein (App), transcript variant 1, mRNA	793	793	100%	0.0	91.21%	NM_001198823.1
Mus musculus amyloid beta (A4) precursor protein, mRNA (cDNA clone MGC:95575 IMAGE:5708093), complete cds	793	793	100%	0.0	91.21%	BC070409.1
Mus musculus cDNA fis, clone TRACH3014885, highly similar to Alzheimer's disease amyloid A4 protein homolog precursor	793	793	100%	0.0	91.21%	AK128971.1
Mus musculus osteoclast-like cell cDNA, RIKEN full-length enriched library, clone:I420011K11 product:amyloid beta (A4) prec	793	793	100%	0.0	91.21%	AK159245.1
Mus musculus cDNA, RIKEN full-length enriched library, clone:M5C1069M13 product:amyloid beta (A4) precursor protein, fu	793	793	100%	0.0	91.21%	AK147543.1
Mouse DNA sequence from clone RP23-99P18 on chromosome 16, complete sequence	793	793	100%	0.0	91.21%	CT010505.18

Figure 5.8**.** A) Sequencing of the APP amplicon from *Apodemus sylvaticus* sample. B) BLASTed (NCBI) sequence shows high similarity (91%) to *Mus musculus* (Accession number: XM_006522873.3).

A



B

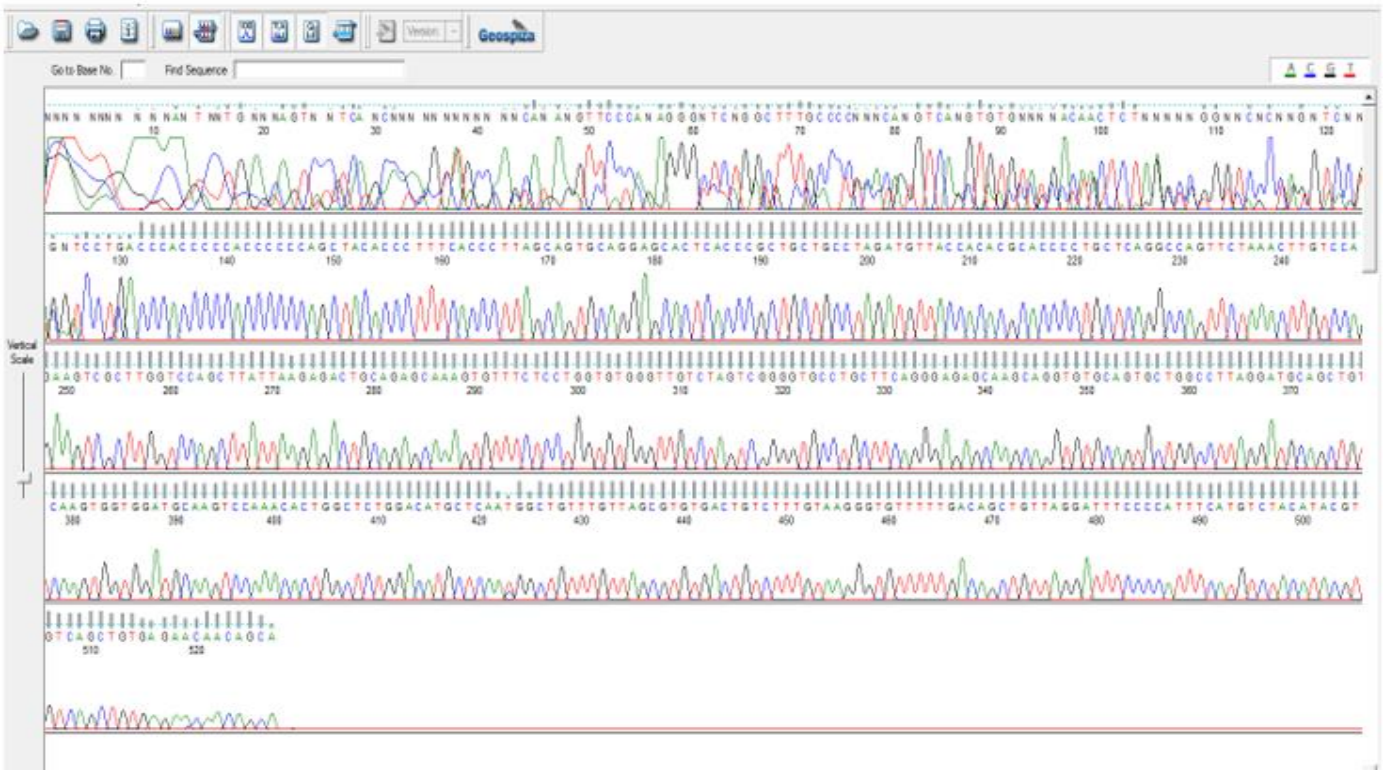
Sequences producing significant alignments Download Manage Columns Show 100

select all *19 sequences selected* GenBank Graphics Distance tree of results

	Description	Max Score	Total Score	Query Cover	E value	Per. Ident	Accession
☑	PREDICTED: Mus caroli lysosomal associated membrane protein 2 (Lamp2), transcript variant X2, mRNA	322	322	98%	6e-84	88.45%	XM_021152776.1
☑	PREDICTED: Grammomys surdaster lysosomal associated membrane protein 2 (Lamp2), transcript variant X1, mRNA	294	294	99%	1e-75	86.32%	XM_028770286.1
☑	Mus musculus lysosomal-associated membrane protein 2 (Lamp2), transcript variant 4, non-coding RNA	292	292	99%	4e-75	86.28%	NR_152733.1
☑	Mus musculus lysosomal-associated membrane protein 2 (Lamp2), transcript variant 1, mRNA	292	292	99%	4e-75	86.28%	NM_001017959.2
☑	Mus musculus osteoclast-like cell cDNA, RIKEN full-length enriched library, clone:I420029E15 product:lysosomal membrane glyc	292	292	99%	4e-75	86.28%	AK159731.1
☑	Mus musculus osteoclast-like cell cDNA, RIKEN full-length enriched library, clone:I420013J24 product:lysosomal membrane glyc	292	292	99%	4e-75	86.28%	AK159272.1
☑	Mus musculus 17 days pregnant adult female amnion cDNA, RIKEN full-length enriched library, clone:I920047M01 product:lysos	292	292	99%	4e-75	86.28%	AK146731.1
☑	Mouse DNA sequence from clone RP23-193O17 on chromosome X, complete sequence	292	292	98%	4e-75	86.50%	AL513356.8
☑	Mouse lysosomal membrane glycoprotein type B (lgp-B) mRNA, 3' end	292	292	99%	4e-75	86.28%	M32017.1
☑	PREDICTED: Mastomys coucha lysosomal associated membrane protein 2 (Lamp2), transcript variant X2, mRNA	281	281	99%	1e-71	85.92%	XM_031353619.1
☑	Mouse lysosomal membrane glycoprotein (LAMP-2) mRNA, complete cds	281	281	99%	1e-71	85.56%	J05287.1
☑	PREDICTED: Mus pahari lysosomal associated membrane protein 2 (Lamp2), transcript variant X6, mRNA	215	215	99%	1e-51	81.29%	XM_021187918.2
☑	PREDICTED: Mus pahari lysosomal associated membrane protein 2 (Lamp2), transcript variant X3, mRNA	215	215	99%	1e-51	81.29%	XM_029534029.1

Figure 5.9. A) Sequencing of the Lamp2 amplicon from *Apodemus sylvaticus* sample. B) BLASTed (NCBI) sequence shows high similarity (86.2%) to *Mus musculus* (Accession number: NM_001017959.2).

A



B

Sequences producing significant alignments

Download | Manage Columns | Show 100

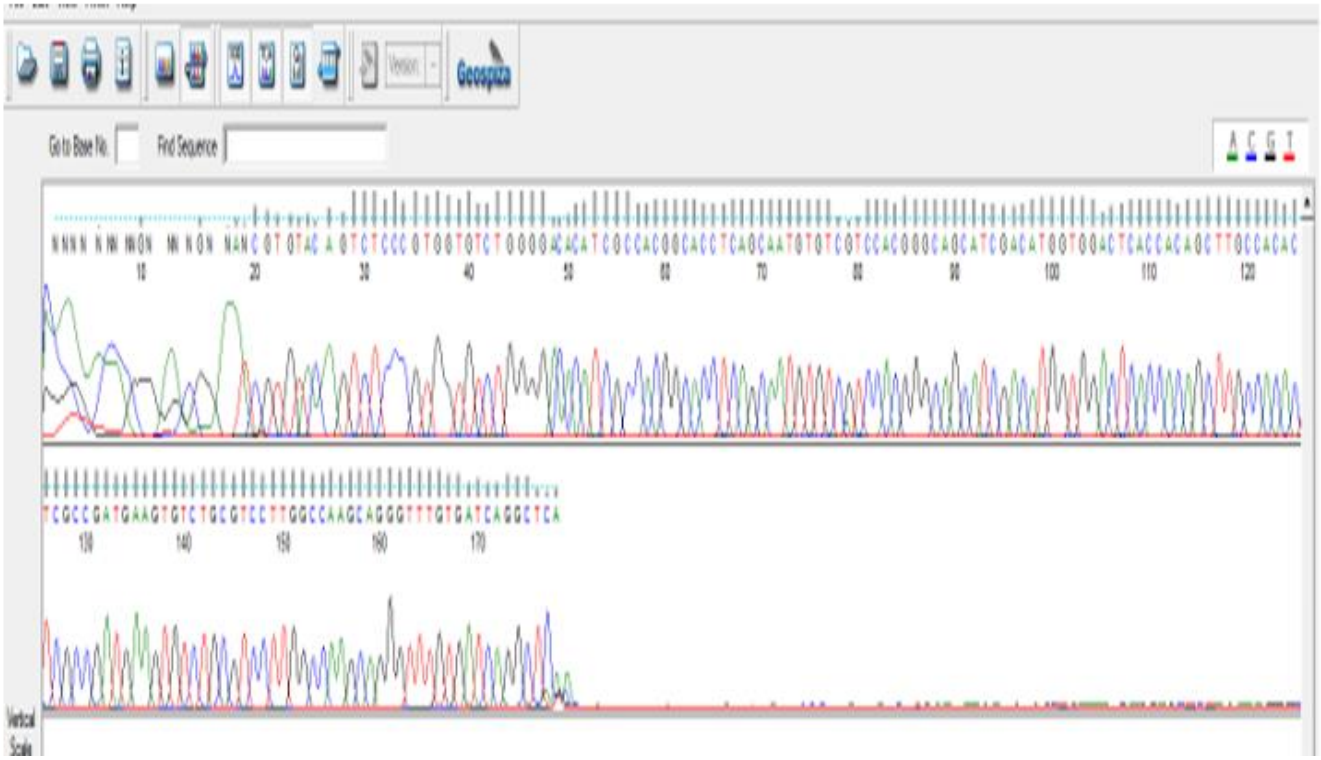
select all 5 sequences selected

GenBank | Graphics | Distance tree of results

Description	Max Score	Total Score	Query Cover	E value	Per. Ident	Accession
PREDICTED: Mus caroli microtubule associated protein 1 light chain 3 beta (Map1lc3b), mRNA	329	329	86%	6e-86	82.78%	XM_021169817.2
Mus musculus predicted gene 5612 (Gm5612), pseudogene on chromosome 9	327	327	86%	2e-85	82.65%	NG_005234.4
Mus musculus microtubule-associated protein 1 light chain 3 copy 1 (Map1lc3) pseudogene, complete sequence	327	327	86%	2e-85	82.65%	AY392028.1
Mus musculus BAC clone RP23-78N5 from chromosome 9, complete sequence	327	327	86%	2e-85	82.65%	AC162941.5
Mus musculus BAC clone RP23-216L13 from chromosome 9, complete sequence	327	327	86%	2e-85	82.65%	AC154519.2

Figure 5.10. A) Sequencing of the Map1Lc3 amplicon from *Apodemus sylvaticus* sample. B) BLASTed (NCBI) sequence shows high similarity (83%) to *Mus musculus* (Accession number: AY392028.1).

A



B

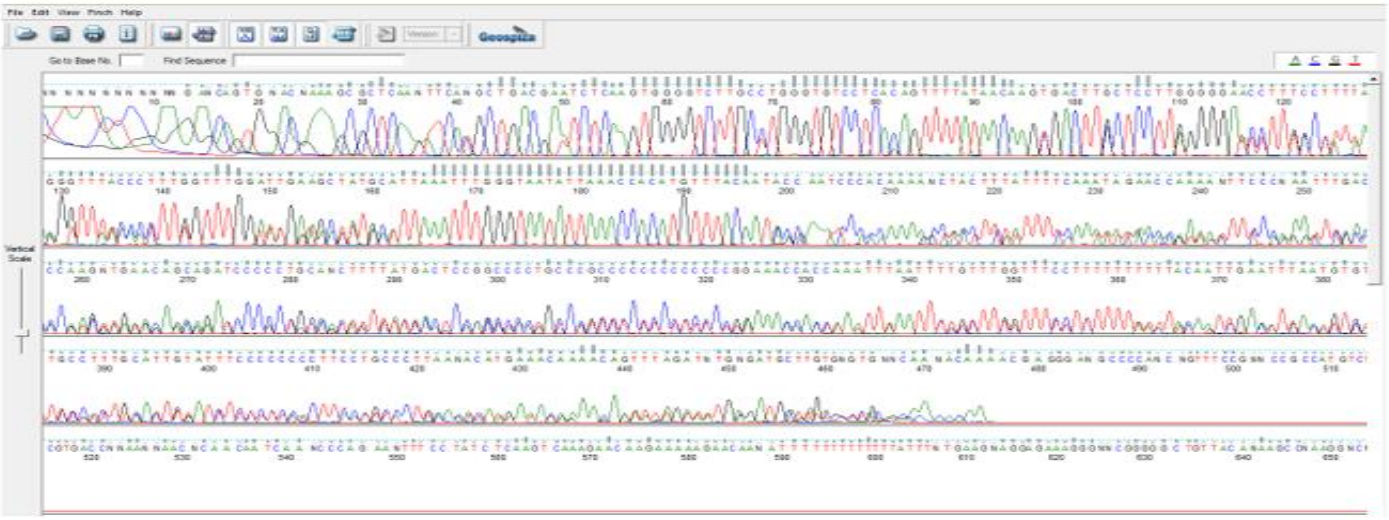
Sequences producing significant alignments Download Manage Columns Show 100

select all *0 sequences selected* GenBank Graphics Distance tree of results

Description	Max Score	Total Score	Query Cover	E value	Per. Ident	Accession
PREDICTED: Mus musculus microtubule-associated protein tau (Mapt), transcript variant X5, mRNA	252	252	99%	4e-63	96.69%	XM_030245631.1
PREDICTED: Mus musculus microtubule-associated protein tau (Mapt), transcript variant X4, mRNA	252	252	99%	4e-63	96.69%	XM_006532409.3
PREDICTED: Mus musculus microtubule-associated protein tau (Mapt), transcript variant X3, mRNA	252	252	99%	4e-63	96.69%	XM_006532408.4
PREDICTED: Mus musculus microtubule-associated protein tau (Mapt), transcript variant X2, mRNA	252	252	99%	4e-63	96.69%	XM_006532407.4
PREDICTED: Mus musculus microtubule-associated protein tau (Mapt), transcript variant X1, mRNA	252	252	99%	4e-63	96.69%	XM_017314315.2
Mus musculus microtubule-associated protein tau (Mapt), transcript variant 4, mRNA	252	252	99%	4e-63	96.69%	NM_001285455.1
Mus musculus microtubule-associated protein tau (Mapt), transcript variant 1, mRNA	252	252	99%	4e-63	96.69%	NM_001038609.2
Mus musculus microtubule-associated protein tau (Mapt), transcript variant 5, mRNA	252	252	99%	4e-63	96.69%	NM_001285456.1
Mus musculus microtubule-associated protein tau (Mapt), transcript variant 2, mRNA	252	252	99%	4e-63	96.69%	NM_010838.4
Mus musculus microtubule-associated protein tau, mRNA (cDNA clone MGC:25379 IMAGE:4504850), complete cds	252	252	99%	4e-63	96.69%	BC014748.1
Mus musculus adult male brain UNDEFINED_CELL_LINE cDNA, RIKEN full-length enriched library, clone:M5C1097G21 pro	252	252	99%	4e-63	96.69%	AK147636.1
Mus musculus adult male olfactory brain cDNA, RIKEN full-length enriched library, clone:6430400E08 product:microtubule-as	252	252	99%	4e-63	96.69%	AK078167.1
Mus musculus chromosome 11 map 11 64cM Band E1 complete sequence	252	252	99%	4e-63	96.69%	AC091629.1

Figure 5.11. A) Sequencing of the Tau amplicon from *Apodemus sylvaticus* sample. B) BLASTed (NCBI) sequence shows high similarity (97%) to *Mus musculus* (Accession number: XM_017314315.2).

A



B

Sequences producing significant alignments

Download | Manage Columns | Show 100

select all 0 sequences selected

GenBank | Graphics | Distance tree of results

	Description	Max Score	Total Score	Query Cover	E value	Per Ident	Accession
☐	Mus musculus beclin 1, autophagy related (Becn1), transcript variant 3, mRNA	281	281	100%	2e-71	79.52%	NM_001359820.1
☐	Mus musculus beclin 1, autophagy related (Becn1), transcript variant 2, mRNA	281	281	100%	2e-71	79.52%	NM_001359819.1
☐	Mus musculus beclin 1, autophagy related (Becn1), transcript variant 1, mRNA	281	281	100%	2e-71	79.52%	NM_019584.4
☐	Mus musculus targeted non-conditional, lacZ-tagged mutant allele Cnot1 tm1e(KOMP)Wtsi, transgenic	281	281	100%	2e-71	79.52%	JN954190.1
☐	Mus musculus targeted non-conditional, lacZ-tagged mutant allele Becn1 tm1e(KOMP)Wtsi, transgenic	281	281	100%	2e-71	79.52%	JN945696.1
☐	Mus musculus targeted KO first, conditional ready, lacZ-tagged mutant allele Becn1 tm1a(KOMP)Wtsi, transgenic	281	281	100%	2e-71	79.52%	JN945695.1
☐	Mus musculus beclin 1, autophagy related, mRNA (cDNA clone MGC:6843 IMAGE:2650059), complete cds	281	281	100%	2e-71	79.52%	BC005770.1
☐	Mus musculus bone marrow macrophage cDNA, RIKEN full-length enriched library, clone:I830031G17 product:beclin 1 (coiled-c	281	281	100%	2e-71	79.52%	AK151531.1
☐	Mus musculus 12 days embryo spinal ganglion cDNA, RIKEN full-length enriched library, clone:D130011M20 product:beclin 1 (c	281	281	100%	2e-71	79.52%	AK083800.1
☐	Mus musculus adult male liver tumor cDNA, RIKEN full-length enriched library, clone:C730037L14 product:beclin 1 (coiled-coil, r	281	281	100%	2e-71	79.52%	AK050329.1
☐	Mus musculus adult male olfactory brain cDNA, RIKEN full-length enriched library, clone:6430407H14 product:beclin 1 (coiled-c	281	281	100%	2e-71	79.52%	AK032176.1
☐	Mus musculus 6 days neonate head cDNA, RIKEN full-length enriched library, clone:5430417M23 product:beclin 1 (coiled-coil, n	281	281	100%	2e-71	79.52%	AK077392.1
☐	Mus musculus 13 days embryo male testis cDNA, RIKEN full-length enriched library, clone:6030442J23 product:beclin 1 (coiled-	281	281	100%	2e-71	79.52%	AK031501.1
☐	Mus musculus 10 days neonate skin cDNA, RIKEN full-length enriched library, clone:4732462H16 product:beclin 1 (coiled-coil, n	281	281	100%	2e-71	79.52%	AK028851.1
☐	Mouse DNA sequence from clone RP23-281C18 on chromosome 11, complete sequence	281	281	100%	2e-71	79.52%	AL590969.12
☐	PREDICTED: Mastomys coucha beclin 1 (Becn1), transcript variant X2, mRNA	265	265	100%	2e-66	78.72%	XM_031351336.1

Figure 5.12. A) Sequencing of the Beclin1 amplicon from *Apodemus sylvaticus* sample. B) BLASTed (NCBI) sequence shows high similarity (79%) to *Mus musculus* (Accession number: NM_019584.4).

5.5 Discussion

In order to investigate whether there are links between chronic toxoplasmosis and autophagy/inflammation, in the module *Apodemus* system, it was necessary to identify whether the target genes could be found in *A.sylvaticus*. Specifically, three important genes that are related to normal autophagic mechanism, Beclin1, Lc3 and Lamp2 were investigated (Huang et al., 2015; Li et al., 2017; Michiorri et al., 2010; Pajares et al., 2018).

The experimental model chosen was the wood mouse, which is considered to be a suitable natural model for investigating toxoplasmosis and could be used for investigating the relation between infection and autophagy. Considerable previous *T. gondii* infection data has been collected on *A. sylvaticus* and in particular in mice collected from the same samples site as used for this study (Bajnok et al., 2015; Owen & Trees, 1998; Thomasson et al., 2011). Yet, the genome for the wood mouse is not currently readily available and properly annotated in public genome databases and thus it was necessary to design primer pairs for genes of interest and to test whether the wood mouse genome is similar to two closely related rodent species, *Mus musculus and Rattus norvegicus.*

All three genomes were aligned to assess the similarity of the three autophagy genes of interest. Indeed, the primer pairs designed based on the non-wood mouse genome templates successfully amplified the gene regions relating to Beclin1, Lc3 and Lamp2, producing the expected base-pair (bp) lengths. These findings indicate that the genes are present in the wood mouse and from the specific tissue of interest (brain).

In a similar manner, TLR2, a gene paramount for activating innate immunity in mammals, has also been implicated in toxoplasmosis regulation and perhaps in AD in humans and in correlation to autophagy (Arroyo et al., 2013; Into, Inomata, Takayama, & Takigawa, 2012; Mun et al., 2003; Oliveira et al., 2016). The same method for designing primer pairs was applied for this genomic region in woodmouse as mentioned earlier. The TLR2 genomic region was successfully detected in the species of interest, indicating the presence of a TLR-related immune response. Finally, Tau and APP genes implicated in AD onset and progression were able to be detected using primer pairs designed using the *Mus musculus and Rattus norvegicus* as genomic templates, on *A. sylvaticus.* These results pave the way for using the

wood mouse as a potential experimental model for investigating *T. gondii* infection status, not due to demographics but due to population genetics that may be essential for understanding how degenerative disease(s) occur. Therefore, it can be used for investigating AD onset and autophagy/inflammation.

In this results chapter, the researcher investigated the presence of genes involved in autophagy and inflammation on *A.sylvaticus*, a possible mouse model for investigating rodent toxoplasmosis. There was a draft *A.sylvaticus* genome but that this has not been annotated and therefore could not directly identify the genes wanted – thus we have to identify the genes from *Mus musculus* and *Rattus norvegicus* and use that by homology to get the correct sequence from *the A.sylvaticus* genome in order to design primer pairs for PCR or to carry out any molecular investigation. This obstacle was successfully overcome by employing two well-recorded species, *Mus musculus* and *Rattus norvegicus*. Their genomes were used as templates once aligned. For the autophagy genes, Map1Lc3, Beclin-1 and Lamp2, primer pairs successfully amplified a genomic region from the wood mouse tissue samples that the amplicons were of the same expected bp length (as for the other two species). Similar findings were shown for the TLR2 genomic DNA amplicon as well for the AD-related genes APP and Tau. Using the online tool BLAST, the similarity for all 6 genomic regions was over 79%. Future work should include one important milestone, namely protein detection, for example, using bulk expression via Western blot analysis. At the moment, there are commercial antibodies able to tag autophagy and inflammation genes, such as Beclin 1, LAMP2a, LC3, Tau and APP and Tlr2 antibody. Yet, these antibodies have not been tested if they can detect the aforementioned peptides on the *A. sylvaticus* and it may be essential to raise new clones from cell lines or primary tissues extracted from that specific experimental animal. Using the same rationale, either the commercially available or the newly developed antibody clones will be used comparing autophagy and inflammation protein expression levels in *T. gondii*-infected animals against uninfected animals. This will be to assess if toxoplasmosis, autophagy and innate immune function are interlinked.

Chapter 6: Analysis of host brain proteins and *Toxoplasma* infection in *Apodemus Sylvaticus* brain tissue.

6.1 Introduction

The aim of this research is to investigate expression of genes involved in autophagy and inflammation in animals naturally infected with *T. gondii* and to determine the potential impact of *Toxoplasma* infection on neurodegenerative diseases associated porteins, samples of infected and uninfected *Apodemus sylvaticus* brain tissues were analysed by western blotting. The level of the following proteins were recorded: Toll-like receptor 2 (TLR2), Microtubule-associated proteins 1A/1B light chain 3B (LC3), Lysosome-associated membrane protein 2 (Lamp2), P62, Beclin1, N-methyl-d-aspartate receptor (NMDAR)-1,Tau, Amyloid precursor protein (APP), Suppressor of cytokine signalling 1 (Socs1), Arginase (Arg)-1, and iNOS. The aim of these experiments was to determine the level of these proteins in both infected and uninfected mouse brain tissue. These experiments were designed to provide data on the potential physiological effect of *Toxoplasma* infection on neurodegenerative disease associated brain proteins.

Chapter 4 describes the collection of wood mice and the process through which they were tested for *Toxoplasma* infection so that these mice could be used to address the research question. In Chapter 5, the researcher has determined that the autophagy genes are present in *A. sylvaticus*, are highly similar to the human *A. sylvaticus* genes, and, therefore, it is likely that human antibodies will recognise these proteins. Thus, the combined results of Chapters 4 and 5 have set up a naturally infected animal system in which the expression of the studied genes may be investigated in infected/uninfected animals. Currently, there are no data on how these genes are expressed in the brain tissues of animals (including humans) naturally infected with *Toxoplasma*. Therefore, this is an important knowledge gap. Having identified this gap, the aims of the chapter are to address their overall scientific question – whether *Toxoplasma* infection affects the expression of any of these autophagy/inflammation genes – and pursue specific research objectives. For the purpose of this section, an overview of the former proteins is provided below.

Toll-like receptors, such as TLR-2 are a heterogenous group of receptors, that within the central nervous system, are expressed by neurons, astrocytes and microglia and function to activate the adaptive immune system in response to pathogen-associated molecular patterns (Fiebich *et al.*, 2018). Evidence has begun to reveal that TLRs, including TLR-2, -4 and -9, are involved in neurodegeneration with their expression being heightened in microglial cells and are thought to contribute to AD through cross-talk with deleterious cellular stress responses, although other evidence have revealed that activation of TLR-2 can increase amyloid-beta uptake (Chen *et al.*, 2006; Guillot-Sestier and Town, 2018). In AD, TLRs, including TLR-2, have been found to optimise the recognition of fibrillary amyloid-beta by microglial cells through co-interactions with other expression molecules, such as CD36 and SR-A, and in turn, promoting enhanced uptake/degradation (Bamberger et al., 2003). However, other authors have found a paradoxical effect with TLRs and TLR complexes having the ability to stimulate the release of pro-inflammatory mediators and reactive species in the presence of amyloid-beta, which can induce and exacerbate neuronal toxicity (Reed-Geaghan et al., 2009). LC3 are a family of proteins involved in the autophagocytic degradation and clearance of waste metabolic and cellular products and it has been implicated in neurodegenerative diseases including Alzheimer's, although its specific role in the pathogenesis of the disease remains unclear (Fujikake, Shin and Shimizu, 2018). Recently however, Heckmann et al. (2019) showed that LC3, in addition to Rab5, clathrin and endosome-containing beta-amyloid, exerted a unique non-canonical function in mice, which involved stringent regulation of immune-mediated endocytosis and aggregate clearance within microglia. Notably, in mice who were deficient for this specific function, the rate of neurodegeneration was markedly attenuated, suggesting that LC3 is critical to protecting host brains from the deleterious accumulation of beta-amyloid (Heckmann et al., 2019). The LC3 pathway is one of the more widely studied ubiquitin-like processes of autophagy with the protein having to be converted to its phosphatidylethanol-conjugated LC3-II form from LC3-I and whilst increased expression of LC3-II, as well as Beclin-1, have been implicated in amyotrophic lateral sclerosis, this has not been confirmed for AD (Chen et al., 2015; Fujikake, Shin and Shimizu, 2018). Lamp2 is a lysosomal-based protein that plays also plays a role in immunity and autophagy and is thought to act as the receptor for chaperone-mediated autophagy (Rothaug *et al.*, 2015). Deficiency of Lamp2 has been

observed within the hippocampus of AD brains and other neurodegenerative diseases and is thought to exacerbate neuroinflammatory processes, as well as impair lysosomal and autophagy function to lead to the accumulate of toxic waste products (Rothaug *et al.*, 2015). p62 is a protein with a multitude of functions that can bind to specific motifs of ubiquitinated proteins, in order to control their aggregation and elimination/clearance either through autophagy or proteasomal degradation (Salminen et al., 2012). Indeed, reduced levels of p62 has been observed within the intracellular inclusions of various tauopathies, including AD where such decreases in peptide abundance it tends to co-exist with hyperphosphorylated tau and ubiquitin (Babu et al., 2008). Moreover, loss of p62 has been correlated with memory and psychiatric symptomology related to AD, suggesting that it plays a crucial role in optimising tau solubility to mediate degradation and thus, prevent aggregation (Salminen et al., 2012). Beclin1 is also intrinsically involved with tau-reducing autophagic processing and correlations with AD in states of reduced expression or function (Jaeger and Wyss-Coray, 2010). Precisely, Beclin1 is known to be involved in the initiation and progression of autophagy and functions at the core component of a large proteinaceous complex, which can alter its subunit conformation to regulate autophagy in various ways (He and Levine, 2010). The association between Beclin1 and AD has arisen from murine observations where mutant Beclin1 has been found to lead to increases in amyloid-beta deposition and lysosomal dysfunction to exacerbate neuronal toxicity and cell death (Pickford et al., 2008; Jaeger et al., 2010). Other proteins mentioned formerly are involved in non-autophagic cell signalling processes. Socs1 – one of a group of suppressor of cytokine signalling proteins, is reported to exert an anti-inflammatory regulatory function over the JAK-STAT pathway, which become expressed in response to JAK-STAT activation (a complex signalling pathway with control over immune function, cell survival and tumour development) (Kershaw et al., 2013). Socs1 is thought to interact with various JAK-STAT receptors to confer negative feedback upon pro-inflammatory cytokines but in addition, the protein is also known to stimulate proteasomal degradation of its receptor but may also promote elimination of other peptides involved in neurodegenerative tauopathies (Trengove and Ward, 2013). Evidence has shown that Socs1 is usually present in elevated levels within AD brains but this may simply represent the usual physiological response to perturbing amyloid-beta and tau induced-mediated

inflammation (Walker, Whetzel and Lue, 2015). Arg-1 is a molecular known to be involved in the mediation of collagen formation to stimulate and ensure repair to injurious tissue but has also been shown to promote uptake of amyloid-beta (Clayton, Van Enoo and Ikezu, 2017). In the context of AD, murine studies have shown that the over-expression of Arg-1 can lead to protective anti-inflammatory effects from hyperphosphorylated tau through decreasing pro-inflammatory mediators and cytokines (Hunt et al., 2013; Hunt et al., 2015b). In keeping with the chronic inflammatory course of AD, iNOS as a biomarker of neuroinflammation was previously thought to exacerbate inflammatory signalling and neurotoxicity, although recent evidence has begun to reveal that iNOS may be neuroprotective through increase neuro-excitability to protect synapses from destruction (Balez and Ooi, 2016). APP and Tau are the longest recognised proteins with pathological contributions to AD with APP acting as the precursor for beta-amyloid and tau representing the core component of neurofibrillary tangles with the accumulation and hyperphosphorylation of the respective proteins causing direct neurotoxicity (O'Brien and Wong, 2011). Finally, NMDAR1 is a receptor playing a role in the synaptic transmission and synaptic plasticity thought to underlie learning and memory function; it is central, not only to the development and function of the nervous system, but also to neurotoxicity (Paoletti et al., 2013; Kodis et al., 2018). NMDAR 1 has been implicated in Alzheimer's pathogenesis related to synaptic dysfunction. Infection with *T. gondii* shows that the expression of iNOS, Arg-1, Socs1, Lamp2, Beclin1, TLR-2, LC3, p62, NMDAR-1, APP and Tau can be altered, but such aberrations have not been consistently or directly correlated in models with coexisting neurodegenerative disease. This is an important knowledge gap (Silva et al., 2009; Hwang et al., 2018; Subauste, 2019; Torres et al., 2018: Zhao et al. 2013).

Objectives:

1. Optimization of Western Blotting in *Apodemus sylvaticus* brain tissue using antibodies against human brain derived proteins.

2. Investigation of variation in LC3 levels between *Toxoplasma* infected and none infected *Apodemus sylvaticus* brain tissue.

3. Investigation of variation in SQSTM1/P62 levels between *Toxoplasma* infected and none infected *Apodemus sylvaticus* brain tissue.

4. Investigation of variation in Beclin1 levels between *Toxoplasma* infected and none infected *Apodemus Sylvaticus* brain tissue.

5. Investigation of variation in LAMP2a levels between *Toxoplasma* infected and none infected *Apodemus sylvaticus* brain tissue.

6. Investigation of variation in APP and Tau levels between *Toxoplasma* infected and none infected *Apodemus sylvaticus* brain tissue.

7. Investigation of variation in NMADR1 levels between *Toxoplasma* infected and none infected *Apodemus sylvaticus* brain tissue.

8. Investigation of variation in TLR2 levels between *Toxoplasma* infected and none infected *Apodemus sylvaticus* brain tissue.

9. Investigation of variation in SOCS1 levels between *Toxoplasma* infected and none infected *Apodemus sylvaticus* brain tissue.

10. Investigation of variation in Arg1 and iNOS levels between *Toxoplasma* infected and none infected *Apodemus sylvaticus* brain tissue.

6.2 Materials and Methods

Studies have suggested that mouse brain tissue could shed light on the effects of *Toxoplasma* infection upon neurodegenerative disease associated protein (as described in chapter 1). The present chapter describes a comparative investigation of protein expression in *Toxoplasma* infected and uninfected mouse brain tissue. The brain tissue for protein extraction were collected using a ratio of 0.1g of tissue to 1000L of using the N-PER neuronal tissue protein extraction reagent (Thermo Scientific)and stored −80 °C. Proteins were extracted (see Chapter 2 for detail) and examined via Western Blotting with various antibodies in order to identify the levels of APP, Arg1, Beclin1, iNOS, Lamp2, LC3, NMDAR, Socs1, Tau, and TLR2.The ImageJ software was used to quantify the protein intensities and the GraphPad prism software was employed for the statistical analysis. As a loading control, housekeeping Glycerol-3-phosphate dehydrogenase (GPDH) was used to normalize the semi-quantified levels of all the investigated proteins. In the present study, 16 samples were obtained from various mouse brain tissue samples, including 5 *Toxoplasma* infected tissue samples and 11 uninfected brain tissue samples.

6.3. Results

6.3.1 Optimisation of Western Blotting

The aim of this set of experiments was to develop Western Blotting techniques for use in the investigation of expression of proteins in relation *Toxoplasma* infection. The antibodies to be optimized were associated with neurodegeneration, inflammation and infection in brain mouse tissue. The optimal percentage of gel, quantity of protein load, as well as identify the ideal temperature. The proteins of interest for each marker was most evident when the gel was 12%. The concentration of each antibody loaded was 20 μg, as this generated superior signal. As the success of western blotting is partly affected by temperature, this environmental factor was regulated to be constant. When gels were run under non-optimized conditions, the variable room temperature produced bands, which were indistinct or broken. However, this issue was resolved by running all the gels in the cold room, under a constant temperature of 4° C. The proteins were transferred onto the membrane, which was also at 4° C. Duration of exposure was the final parameter that was modulated after reading the membranes in the G-box to obtain the best staining results. A sequence of exposure durations ranging from a few seconds to 10 min were evaluated for all antibodies. Optimal intensity of the protein bands was determined to be 5 min. Optimization of western blotting (Figure 6.1).

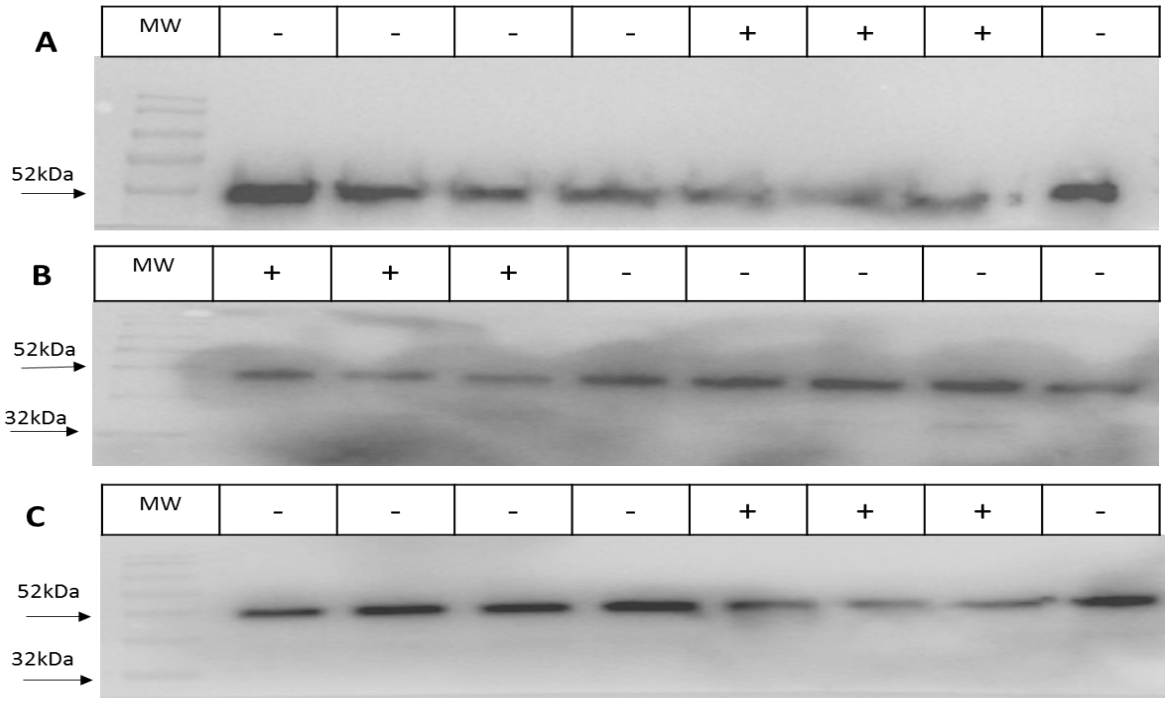


Figure 6. 1 Example of an optimized Western blotting using the Beclin 1. Different gel percentages and Various protein concentrations were tested in order to achieve better intensity for optimal bands: A) 10% and 30ug. B) 10% and 10µg and C) 12% and 20µg (bottom) on beclin1 antibody. Different exposure times of Beclin1 bands: A) 3 mins and B) 5 mins. MW=molecular weight ladder, - =Uninfected samples, += Infected samples.

After numerous and extensive optimisation experiments, optimal results were chosen to represent the autophagy and inflammation marker levels of expression in infected and uninfected *A. sylvaticus* brain tissues.

6.3.2 Exploring the impact of *Toxoplasma* infection on expression of autophagy markers in naturally infected *Apedemus sylvaticus*

6.3.2.1 Expression of LC3 in infected and uninfected *Apodemus sylvaticus* brain tissue

The aim of this experiment was to investigate the expression of LC3 in mouse brain tissues to determine whether or not there is differential expression of this protein in infected and uninfected brain tissues. Western blotting was carried out on infected and uninfected mouse brain tissue using LC3 antibody. This antibody designed to detect the human LC3 protein. LC3 is a marker of apparatus to create autophagosomes. The western blot experiment demonstrated that LC3 in infected tissues was significantly-higher than in uninfected tissues (p<0.04). suggest increased autophagy or the capacity to form the autophagosomes (Figure 6.2).

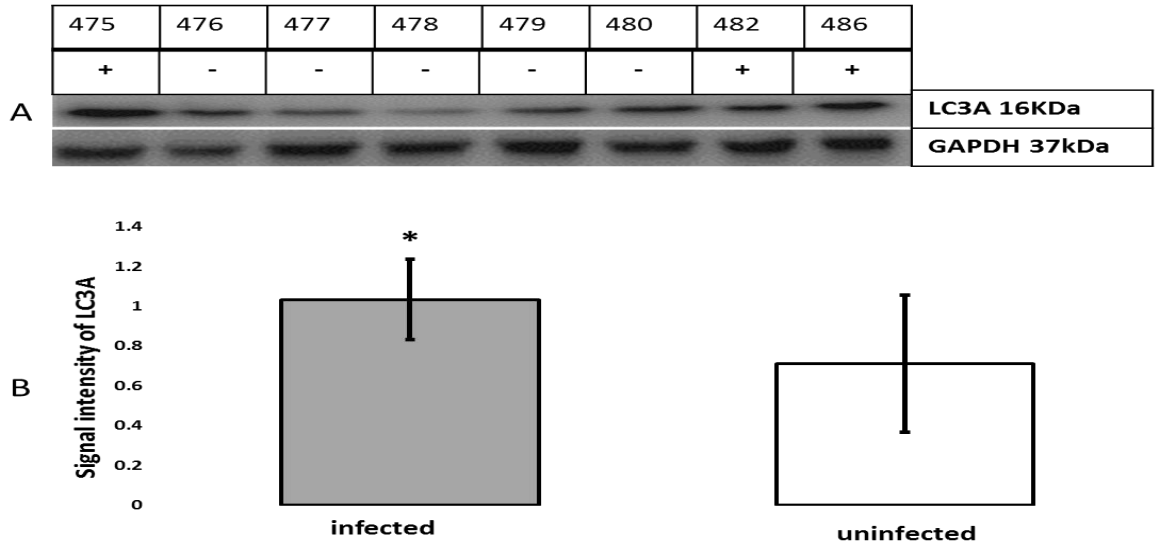


Figure 6. 2 LC3 Protein levels in *Toxoplasma*-infected and uninfected mouse brain tissues. (A) Western blot of protein intensities of LC3 and the loading control protein (GPDH) in infected (+) or uninfected (-) mouse brain tissues and mouse numbers for each of the lanes are shown in each panel (B) Mean intensity of LC3 in infected versus uninfected tissues. Error bars represent the standard deviation from the mean. The p-value, derived from a student *t*-test of 3 independent experiments is shown. Error bars represent ± SD. The values were normalised to the GAPDH intensity for each sample. Indicates a significant difference (p<0.04).

6.3.2.2 Expression of Lamp2 in infected and uninfected *Apodemus sylvaticus* brain tissues

This experiment was conducted to examine the Lamp2 expression in the brain tissues of mice to establish the possibility of this protein having a differential expression in infected and uninfected tissues. The Lamp2a antibody, which assists in finding the human Lamp2a protein, was used in western blotting for both infected and uninfected mouse brain tissues. Lamp2 is a glycoprotein associated with the membrane of lysosomes and is marker for autophagy in the tissue. Lamp2 expression was found to be significantly-lower in infected than in uninfected tissues (p<0.02) Suggesting an infection-associated decrease in expression (Figure 6.3).

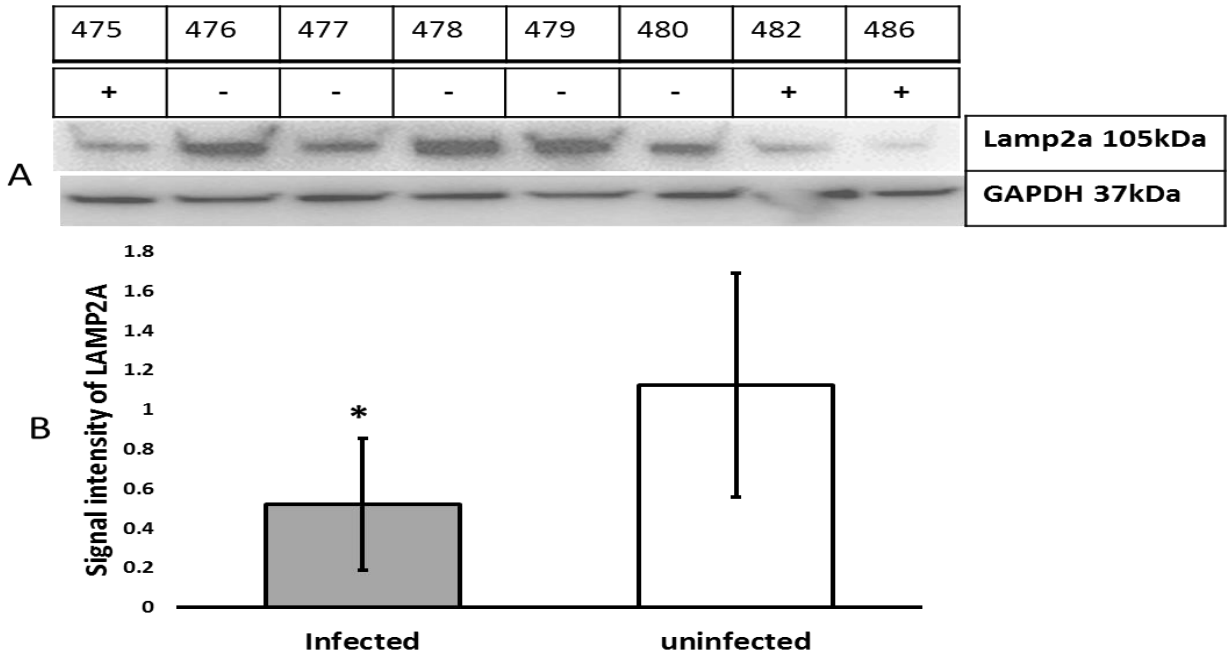


Figure 6.3 Lamp2a Protein levels in *Toxoplasma*-infected and uninfected mouse brain tissues. (A) Western blot of protein intensities of Lamp2 and the loading control protein (GPDH) in infected (+) or uninfected (-) mouse brain tissues and mouse numbers for each of the lanes are shown in each panel (B) Mean intensity of Lamp2 in infected versus uninfected tissues. Error bars represent the standard deviation from the mean. The p-value, derived from a student *t*-test of 3 independent experiments is shown. Error bars represent ± SD. The values were normalised to the GAPDH intensity for each sample. Indicates a significant difference (p<0.02).

6.3.2.3 Expression of P62 in infected and uninfected *Apodemus sylvaticus* brain tissue

The objective of this test was to examine the P62 expression in mouse brain tissues to establish the likelihood of a differential expression existing in infected and uninfected tissues. Both infected and uninfected mouse brain tissues were submitted to the western blotting procedure through the P62 antibody, which is designed to recognise the human P62 protein. P62 is a cell-signalling protein, with an important function in autophagy. The level of P62 was found to be statically-significantly lower in infected tissues versus uninfected tissues (Figure 3.4). Although significant (p<0.003), a large degree of variation was observed in the samples (Figure 3.4B). suggesting an infection-associated decrease in expression. The important role of P62 in autophagy suggests that the protein would be decreased in response to an infection.

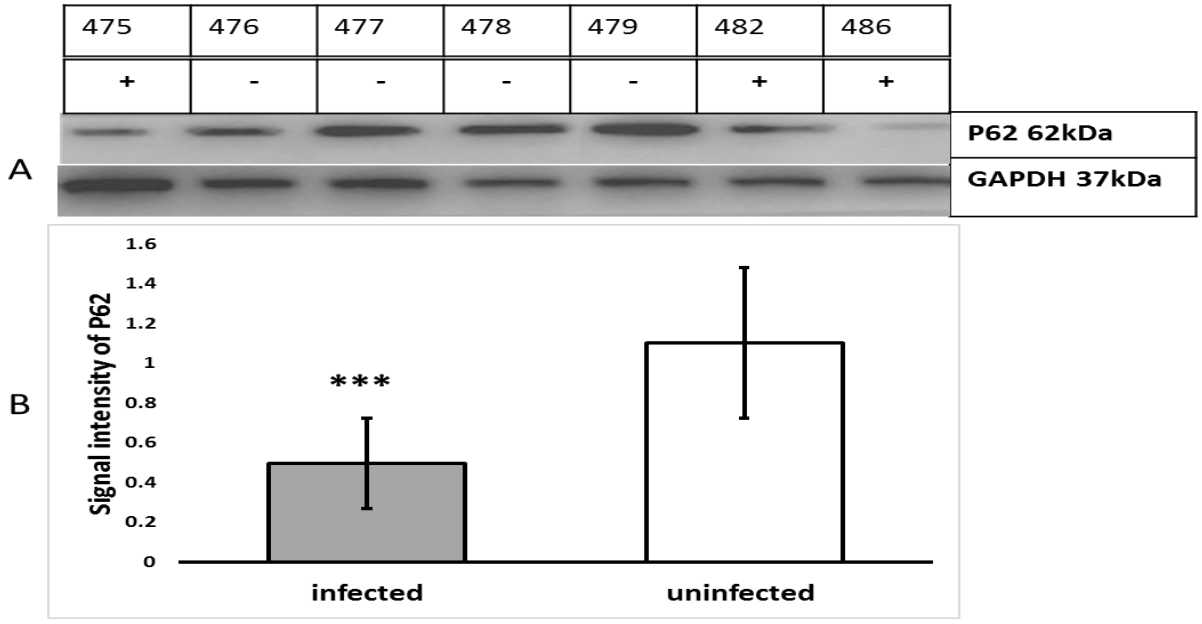


Figure 6.4 P62 Protein levels in *Toxoplasma*-infected and uninfected mouse brain tissues. (A) Western blot of protein intensities of SQSTM1/P62 and the loading control protein (GPDH) in infected (+) or uninfected (-) mouse brain tissues and mouse numbers for each of the lanes are shown in each panel (B) Mean intensity of P62 in infected versus uninfected tissues. Error bars represent the standard deviation from the mean. The p-value derived from a student *t*-test of 3 independent experiments is shown. Error bars represent ± SD. The values were normalised to the GAPDH intensity for each sample. Indicates a significant difference (p<0.003).

6.3.2.4 Expression of Beclin1 in infected and uninfected *Apodemus sylvaticus* brain tissue

The goal of this experiment was to study Beclin 1 expression in mouse brain tissues, with the objective of ascertaining whether or not infected and uninfected tissues carry a differential expression of this protein. The Beclin1 antibody, which serves to recognise the human Beclin1 protein, was used to conduct western blotting on infected and uninfected mouse tissues. Beclin1 was another protein that was investigated as a marker for autophagy. The results from the present study (Figure 6.5) showed that Beclin1 levels were significantly decreased in infected tissues, compared with uninfected tissues (p<0.01). suggesting a process whereby sequestration, degradation or down-regulation of Beclin-1 acts as a defence mechanism against infection.

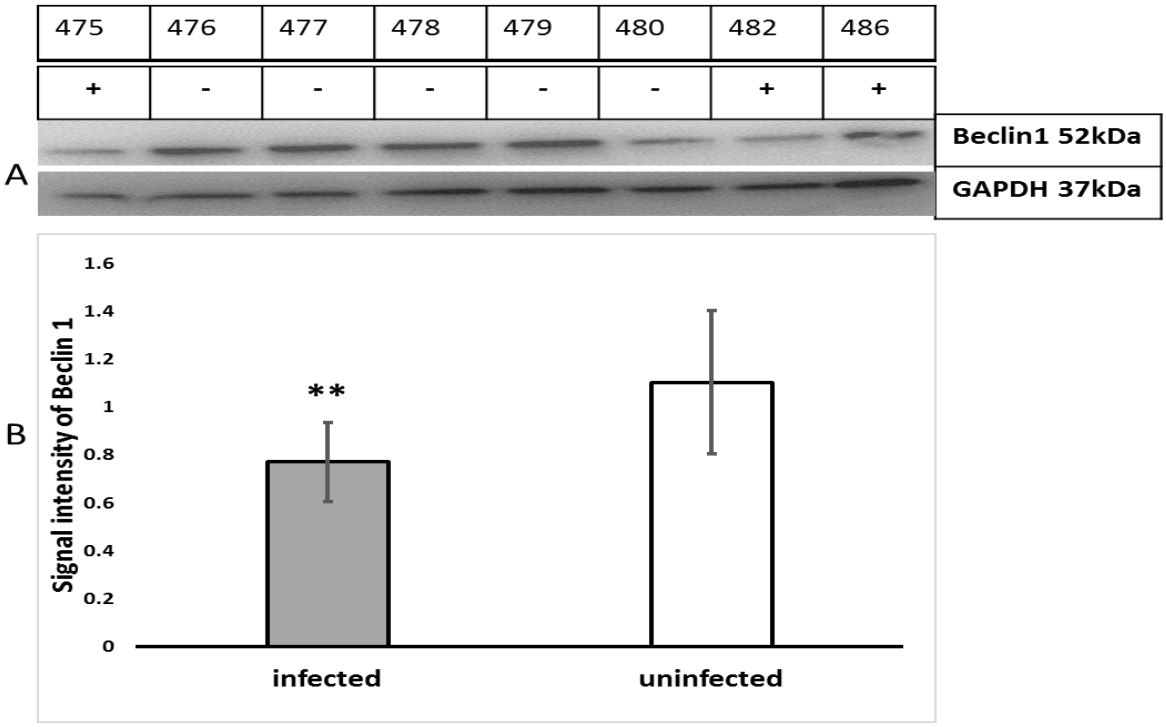


Figure 6. 5 Beclin1 Protein levels in *Toxoplasma*-infected and uninfected mouse brain tissues. (A) Western blot of protein intensities of Beclin1 and the loading control protein (GPDH) in infected (+) or uninfected (-) mouse brain tissues and mouse numbers for each of the lanes are shown in each panel (B) Mean intensity of Beclin1 in infected versus uninfected tissues. Error bars represent the standard deviation from the mean. The p-value, derived from a student *t*-test of 3 independent experiments is shown. Error bars represent ± SD. The values were normalised to the GAPDH intensity for each sample. Indicates a significant difference (p<0.01)

6.3.3 Exploring the impact of *Toxoplasma* infection on expression of AD associated proteins in naturally infected *Apodemus sylvaticus*

6.3.3.1 Expression of Tau and APP in infected and uninfected *Apodemus sylvaticus* brain tissue

This investigation was conducted to analyse the Tau and APP expressions in mouse brain tissues. This was intended to test for the presence of a differential expression of this protein in infected and uninfected tissues. Tissues, both infected and uninfected, were subjected to the western blotting process by means of Tau and APP antibodies, which were used to find human Tau and APP proteins. In the current study, the level of protein was investigated, Both Tau and APP protein levels were not significantly different between infected and uninfected tissues (p=0.8 and p=0.6, respectively), as measured by western blotting (Figure 6.6).

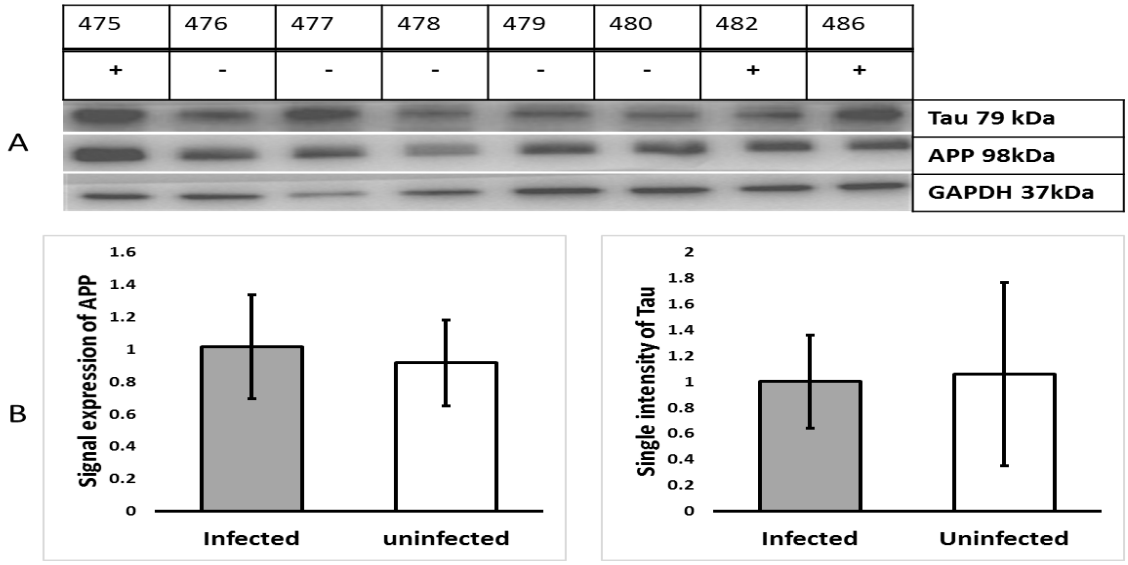


Figure 6.6 Tau and APP Protein levels in *Toxoplasma*-infected and uninfected mouse brain tissues. (A) Western blot of protein intensities of Tau and APP, and the loading control protein (GPDH) in infected (+) or uninfected (-) mouse brain tissues and mouse numbers for each of the lanes are shown in each panel (B) Mean intensity of Tau and APP in infected versus uninfected tissues. Error bars represent the standard deviation from the mean. The p-value derived from a student t-test of 3 independent experiments is shown. Error bars represent ± SD. The values were normalised to the GAPDH intensity for each sample. Indicates no significant difference (p=0.8 and p=0.6, respectively).

6.3.4 Exploring the impact of *Toxoplasma* infection on markers of inflammatory markers in naturally infected *Apodemus sylvaticus*

6.3.4.1 Expression of NMDAR1 in infected and uninfected *Apodemus sylvaticus* brain tissue

This experiment sought to analyse NMADR1 expression in mouse brain tissues to determine whether infected and uninfected tissues contain a differential expression of this protein. Both infected and uninfected mouse brain tissues underwent western blotting, through the application of the NMADR1 antibody, which functions to recognise the human NMADR1 protein. NMDAR1 is one of the proteins involved in forming ion channels in the mammalian brain and is a marker for neuroinflammation. The western blot experiment (Figure 6.7) demonstrated that the concentration of NMDAR1 was significantly lower in infected tissues (p<0.04). suggesting that the *Toxoplasma* infection resulted in inflammation and a reduction of this important protein.

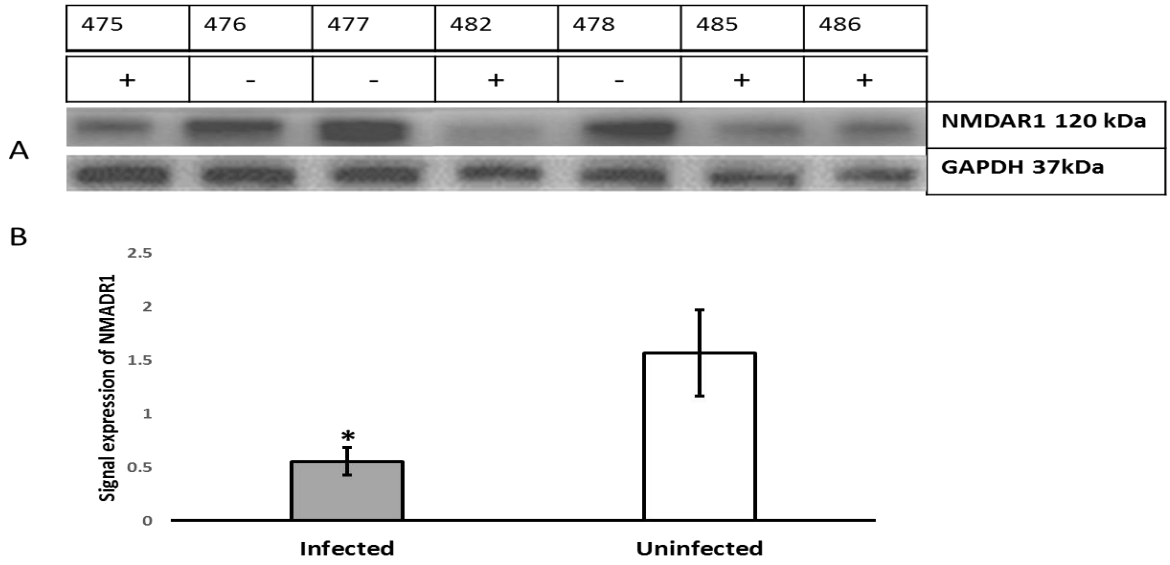


Figure 6.7 NMDAR1 Protein levels in *Toxoplasma*-infected and uninfected mouse brain tissues. (A) Western blot of protein intensities of NMDAR1 and the loading control protein (GPDH) in infected (+) or uninfected (-) mouse brain tissues and mouse numbers for each of the lanes are shown in each panel (B) Mean intensity of NMDAR1 in infected versus uninfected tissues. Error bars represent the standard deviation from the mean. The p-value, derived from a student *t*-test of 3 independent experiments is shown. Error bars represent ± SD. The values were normalised to the GAPDH intensity for each sample. Indicates a significant difference (p<0.04).

6.3.4.2 Expression of TLR2 in infected and uninfected *Apodemus sylvaticus* brain tissue

This test was performed to study the TLR2 expression associated with brain tissues in mice. The purpose of this experiment was to ascertain the possibility of there being a differential expression of this protein within tissues, both infected and uninfected. These tissues were subjected to western blotting through the application of the TLR2 antibody, whose function is to locate the human TLR2 protein. TLR2 is a protein, which is involved in the immunological response to infection and was used as a proxy for the induction of an immune response in infected tissues. The expression of TLR2 in infected tissues was significantly-higher than in uninfected tissues ($p<0.01$). As Figure 6.8A indicates, an important result in this section is that some of the uninfected mice do not express TLR2 at all. When the investigation was repeated, this complete absence of TLR2 remained consistent for each mouse in each replicated experiment. Suggesting that TLR2 was expressed in response to the *Toxoplasma* infection (Figure 6.8). Table 6.1 Mouse tissues listed according to infection status and according to whether they showed the presence (+) or absence (-) of TLR2 for each replicated experiment

Table 6.1: Presence or absence TLR2 in infected and uninfected *A.sylvaticus* brain tissue

Sample NO	TLR2 expressed	*T.gondii*
475	+	+
476	+	-
477	-	-
478	+	-
479	+	-
480	+	-
481	+	-
482	+	+

483	+	-
484	+	-
485	+	+
486	+	+
487	+	-
488	+	+
489	+	-
490	+	-
491	+	-
492	+	-
MW1	+	-
MW2	-	-

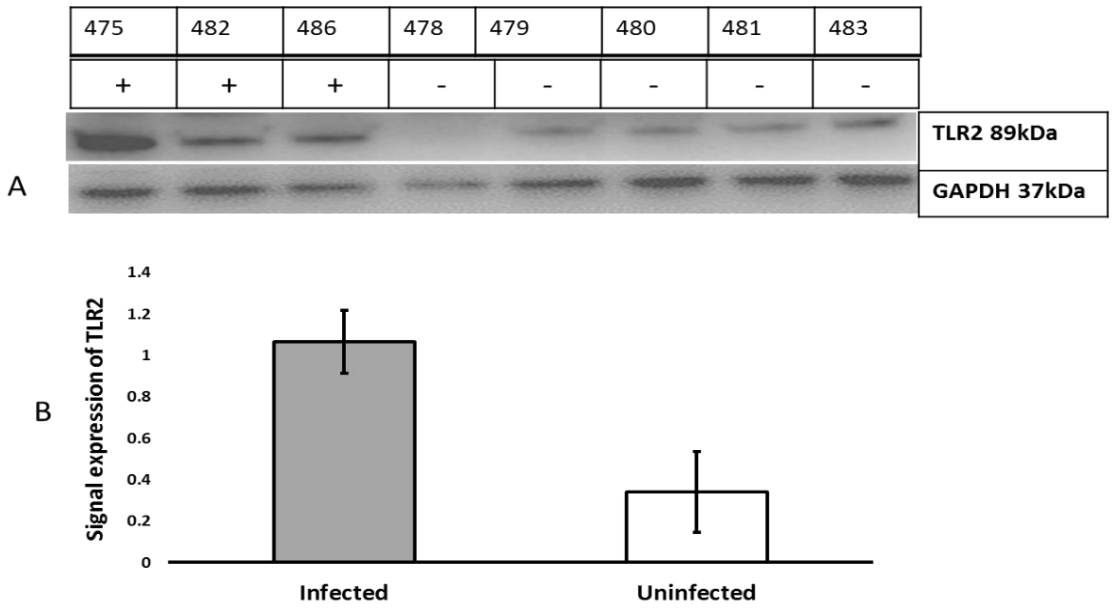


Figure 6.8 TLR2 Protein levels in *Toxoplasma*-infected and uninfected mouse brain tissues.
(A) Western blot of protein intensities of TLR2 and the loading control protein (GPDH) in infected (+) or uninfected (-) mouse brain tissues and mouse numbers for each of the lanes are shown in each panel (B) Mean intensity of TLR2 in infected versus uninfected tissues. Error bars represent the standard deviation from the mean. The p-value derived from a student *t*-test of 3 independent experiments is shown. Error bars represent ± SD. The values were normalised to the GAPDH intensity for each sample. Indicates a significant difference ($p<0.001$).

6.3.4.3 Expression of SOCS1 in infected and uninfected *Apodemus sylvaticus* brain tissue

An examination of the SOCS1 expression in mouse brain tissues was conducted to identify the presence of any differential protein in tissues, both infected and uninfected. The SOCS1 antibody, which is designed to identify the human SOCS1 protein, was applied in western blotting to test these tissues. In the current study, Socs1 was present at significantly higher concentrations in infected tissues, compared with uninfected tissues (p<0.03) as measured by western blotting (figure 6.9). The presence of higher levels of Socs1 in infected tissues suggests that the inflammation-induced protein was up-regulated in response to the *Toxoplasma* infection.

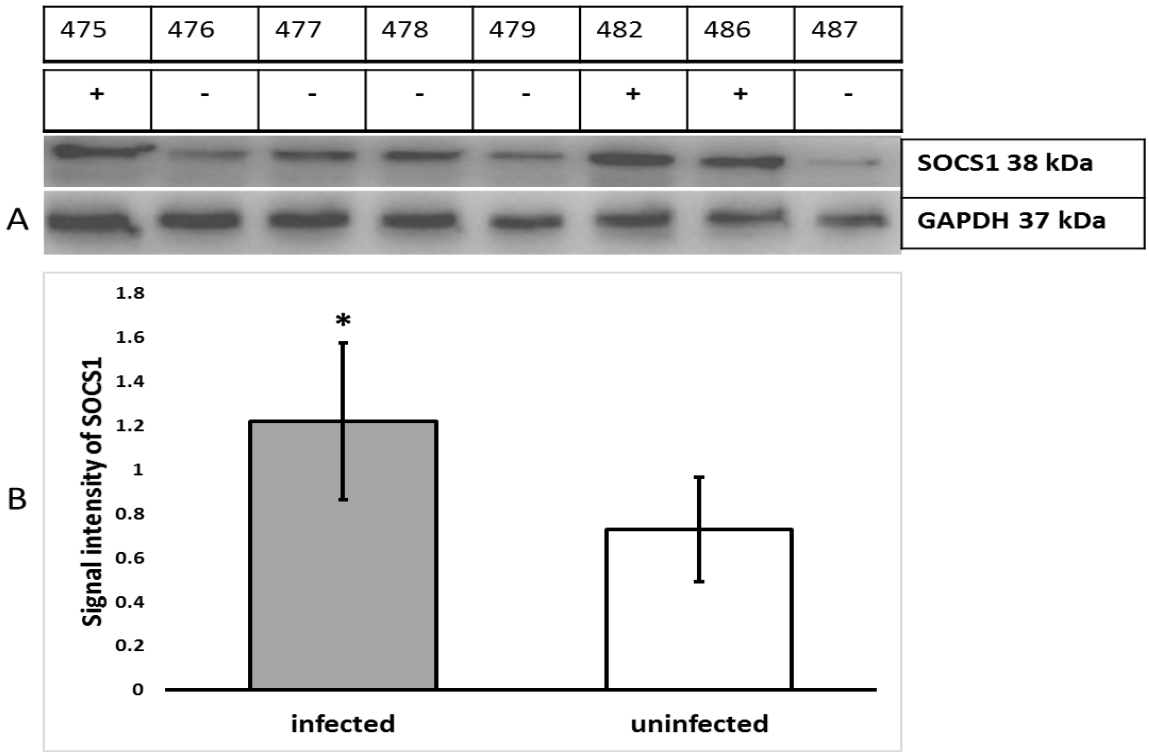


Figure 6.9 SOCS1 Protein levels in *Toxoplasma*-infected and uninfected mouse brain tissues. (A) Western blot of protein intensities of Socs1 and the loading control protein (GPDH) in infected (+) or uninfected (-) mouse brain tissues and mouse numbers for each of the lanes are shown in each panel (B) Mean intensity of Socs1 in infected versus uninfected tissues. Error bars represent the standard deviation from the mean. The p-value, derived from a student *t*-test of 3 independent experiments is shown. Error bars represent ± SD. The values were normalised to the GAPDH intensity for each sample. Indicates a significant difference (p<0.03).

6.3.4.4 Expression of iNOS and Arg1 in infected and uninfected *Apodemus sylvaticus* brain tissue

This experiment was undertaken to examine the iNOS and Arg1 expressions in mouse brain tissues. The test was conducted to determine if a differential expression of the proteins was present in the tissues, both infected and uninfected. The Arg1 and iNOS antibodies were used for western blotting among these tissues. These antibodies assist in locating the human Arg1 and iNOS proteins. Arg1 and iNOS were investigated, due to their opposite modes of action in response to infection, either pathogen-killing or tissue repair. In the current study, the western blot experiment (Figure 6.10) demonstrated that Arg1 was present at a significantly-increased concentration ($p<0.006$), compared with the uninfected tissues. Arg1 was present at the highest concentration of all the infection-associated proteins. By contrast, iNOS was more highly-expressed in the uninfected tissues than the infected tissues ($p<0.002$). As depicted in figure 6.10 C, higher levels of Arg1 were correlated with lower expressions of iNOS. Spearman's correlation analysis between Arg1 and iNOS revealed a significant negative association ($r = -0.93$). Suggesting an infection-associated decrease in iNOS expression and increase in Arg1 expression.

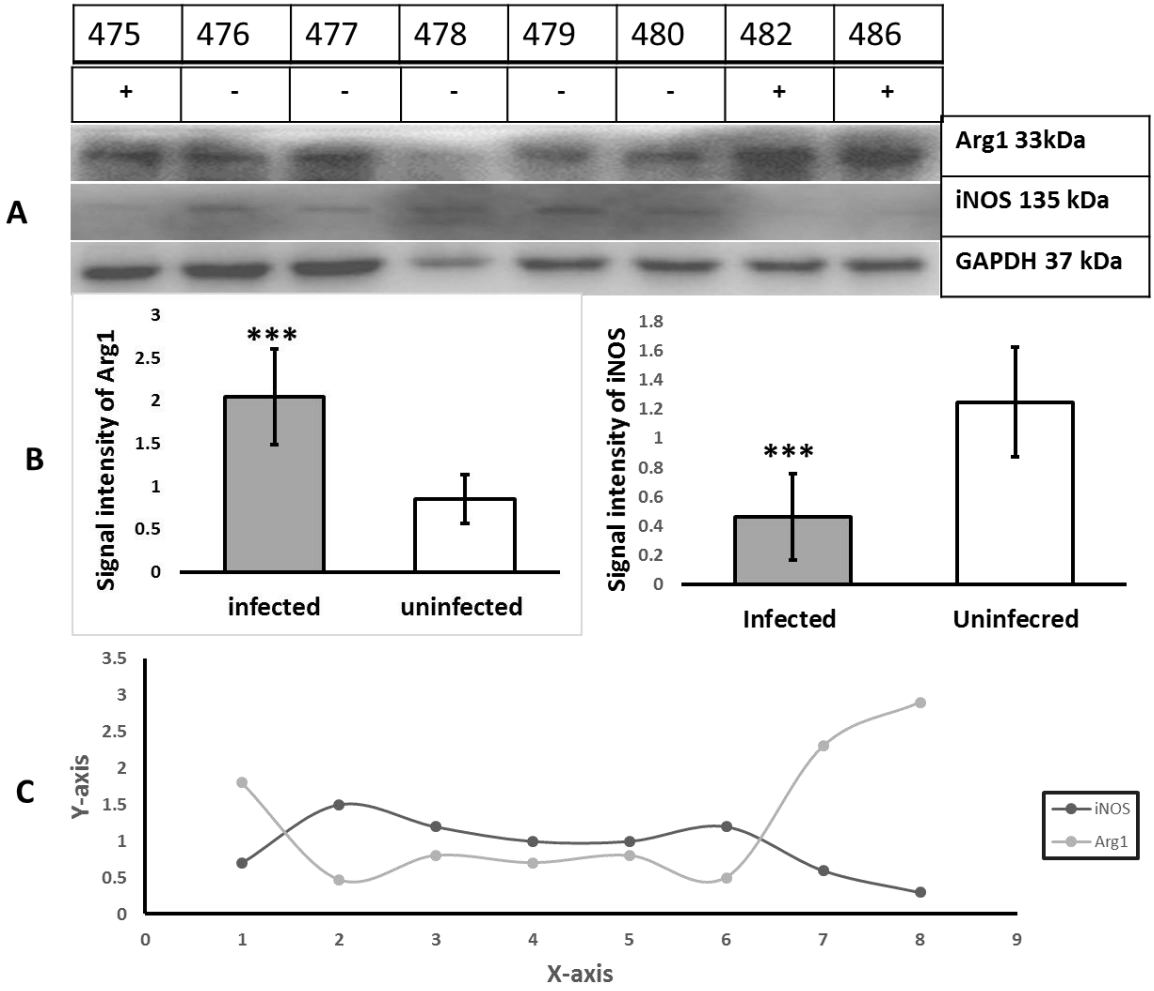


Figure 6.10 iNOS and Arg1 Protein levels in *Toxoplasma*-infected and uninfected mouse brain tissues. (A) Western blot of protein intensities of iNOS and Arg1, and the loading control protein (GPDH) in infected (+) or uninfected (-) mouse brain tissues and mouse numbers for each of the lanes are shown in each panel (B) Mean intensity of iNOS and Arg1 in infected versus uninfected tissues. (C) Protein expression of iNOS and Arg1 had negative correlations. The numbers along the X-axis refer to the 8 mice depicted in panel A. Error bars represent the standard deviation from the mean. The p-value, derived from a student *t*-test of 3 independent experiments is shown. Error bars represent ± SD. The values were normalised to the GAPDH intensity for each sample. Indicates a significant difference ($p<0.006$ and $p<0.002$, respectively).

Summary of expression

This study has investigated the expressions of proteins, which are involved in autophagy, inflammation and AD and are associated with *T. gondii* Infected *A. sylvatica* brain tissue. The results of this analysis are depicted in Table 6.2 below.

Table 6.2: Summary of protein expression in animals naturally infected with *T. gondii*

Autophagy Markers	**Expression**
LC3A	Increased
Beclin1	Decreased
Lamp2a	Decreased
P62	Decreased
Inflammation Markers	**Expression**
NMADR1	Decreased
TLR2	Increased
SOCS1	Increased
iNOS	Decreased
Arg1	Increased
Alzheimer's diseases proteins	**Expression**
Tau	Stay same
APP	Stay same

6.4 Discussion

The results from the protein analysis in mouse brain tissue, either infected or uninfected with *Toxoplasma*, provided information on the host response to infection and the potential for infection-related neurodegenerative diseases. While most rodent studies are carried out on experimentally infected lab strains of mice, this research is unique because it has sampled mice that have been infected with *T. gondii* through natural routes at natural doses. These mice have also evolved to cope with the rigours of 'life in the wild', which means that they are genetically diverse, as compared to inbred lab strains. Results obtained from these naturally infected mice can be compared with data from experimentally infected animals. Such studies are not possible among humans since experimental infection is not permissible; therefore, a model natural infection system is required. In this model system, has been possible to study the naturally infected animals at real stages in their lifecycles. Comparable studies in humans rely on post-mortem research and are, therefore, often biased towards elderly/sick/trauma subjects.

In summary, of the proteins investigated, TLR2, LC3, Socs1, and Arg1 were all significantly-increased in infected tissues, compared with uninfected tissues. Conversely, NMDAR1, Lamp2, P62, Beclin-1 and iNOS were present at significantly lower levels in the infected tissues. Both Tau and APP were not present at significantly different levels between the infected and uninfected tissues.

However, given that a murine model lacking AD was employed in this study, the value of these observations is somewhat limited as in accordance with the evidence in the introduction section, expression levels may vary to either a greater extent or opposing effect. In addition, given the minimal volume and anatomical site of brain tissue sampled from the mice, inferences about whether such findings were expected are difficult to evaluate.

Tau has a function in maintaining the stability of microtubules in neurons, and is therefore a key component of the central nervous system (Kametani and Hasegawa, 2018). APP is

an integral membrane protein in various tissues including the synapses of neurons. The function of APP is not fully-understood but is thought to be related to synapse formation and is therefore a critical part of neuronal signalling (Kametani and Hasegawa, 2018). These proteins are important, as some neurodegenerative conditions, including Parkinson's disease are associated with defective Tau-proteins (Lei et al., 2010), whereas APP is a known pre-cursor of amyloid plaques, which are often found in the brains of patients suffering from AD (Meadowcroft, Connor, Smith, & Yang, 2009). In healthy persons, APP is abundantly present both within and outside of the central nervous system and undergoes processing through the secretory pathway that involves post-translational modifications, such as glycosylation, phosphorylation and sulfation, and ultimately generating amyloid-beta and p3 protein products through secretase enzymes (Zheng and Koo, 2011). A previous study identified that β-amyloid plaque formation in the brain, mediated by APP was reduced during infection with *T. gondii* suggesting that *Toxoplasma* brain infections may slow the spread of AD among sufferers (Jung *et al.*, 2012). Tau concentrations have been shown previously to increasing markedly in immunocompromised patients suffering from, for example, HIV/AIDS (Andersson, Blennow, Fuchs, Svennerholm, & Gisslén, 1999). Although it has been hypothesised that *Toxoplasma* infections, which commonly occur in immunocompromised individuals, would be associated with AD, at least one study has found no significant association between *Toxoplasma gondii* infection and AD (Rashno *et al.*, 2016).

NMDAR1 regulates the neuronal synapse function (Ehrenreich, 2017) and, in neurodegenerative conditions, chronic neuroinflammation is associated with the loss of NMDAR1 (Kempuraj *et al.*, 2016; Rosi, Ramirez-Amaya, Hauss-Wegrzyniak, & Wenk, 2004).The results presented here suggest that neuroinflammation was occurring in the infected tissue, which has been linked to *Toxoplasma gondii* infections in a recent study (French *et al*., 2019). After infection, *Toxoplasma* is known to cause persistent subclinical neuroinflammation, which has been associated with psychiatric and neurodegenerative disorders (Hurley & Taber, 2012). Interestingly, it has been shown that a *Toxoplasma* infection of mice results in the loss of NMDAR-1 expression, resulting in signs of AD,

specifically through the formation of hyperphosphorylated Tau protein (Torres, Robinson, Kim, Yan, Cleland, & Bynoe, 2018).

Inflammation was also highlighted by the increase in concentration of Socs1, which is present in various mammalian tissues and has been previously associated as a regulator of inflammatory human neurodegenerative diseases (Davey, Heath, & Starr, 2006). Evidence has shown that chronic infection with *Toxoplasma gondii* can lead to increases in Socs1 expression and in turn, enhance M1 polarisation of microglia and the levels of inflammatory cytokines (Hwang *et al.*, 2018). Moreover, Socs1 can also enhance the levels of iNOS, which may lead to a perpetuating cycle of neuroinflammation in the presence of *Toxoplasma gondii* (Hwang *et al.*, 2018).

The present study indicates that an immune response was elicited, mediated by TLR2, a membrane-bound receptor present in immune cells, including monocytes, macrophages, B-cells, and T-cells (Komai-Koma, Jones, Ogg, Xu, & Liew, 2004), where it triggers an immune response (Heldwein *et al.*, 2003). The expression of TLR2 has been shown previously to increase in response to *Toxoplasma* infections (Denkers, Kim, & Butcher, 2003). Using mouse models, Mun et al. (2003) have argued that TLR2 is critical for the immune response against *T. gondii* because TLR2-deficient mice fail to survive against *T. gondii* infection. In contrast, Debierre-Grockiego et al. (2007) have indicated that the survival of infected mice is unaffected by TLR2 knockout; they also suggest that, in the context of *T. gondii* infection, the functions served by TLRs are determined by murine genetics, infective inocula and parasitic strain. Astrocytes, microglia and neurons have all been associated with TLR2 expression. Crack et al. (2007) have proposed that, in these structures, the receptor may contribute to the onset and modulation of CNS inflammation, neurodegeneration and trauma. Nevertheless, there are still substantial gaps in knowledge regarding how neurological conditions, triggered by *T. gondii* infection, and the role of TLR2 in the brain correlate. In regard to neurodegeneration, TLR-2 has been implicated in AD following observations that it can function as a key receptor to detecting neuronal cell death and even promoting latency of *Toxoplasma gondii* infection (Ihara *et al.*, 2019; Lalancette-Hebert *et al.*, 2009)

The presence of inflammation is supported by the divergent concentrations of Arg1 and iNOS, which suggest a clear mechanism of repair and the induction of anti-inflammatory signalling pathways. The iNOS protein is typically present in M1 macrophage cells where it produces nitric oxide from L-arginine(Yang & Ming, 2014). Nitric oxide inhibits cell proliferation and kills invading pathogens. By contrast, Arg1 converts L-arginine to L-ornithine, which forms part of the cell proliferation and collagen-forming pathways, involved in tissue repair and the production of anti-inflammatory compounds from M2 macrophages (Yang & Ming, 2014). Decreased nitric oxide production has been observed previously in mice brain cells that were infected with *T. gondii*, compared with uninfected cells (Rozenfeld *et al.*, 2003). The mechanism was shown to be mediated through inhibition of iNOS and resulted in the avoidance of neuron injury (Rozenfeld *et al.*, 2005). A similar mechanism has been identified elsewhere, demonstrating that the suppression of iNOS during *Toxoplasma* infection is accompanied by an increased activity of Arg1, highlighting the switch in pathway from nitric oxide production to tissue repair (Hwang *et al.*, 2018). Empirical research on murine models has revealed a correlation between *T. gondii* infection and the equilibrium between the expression levels of iNOS and Arg-1 (Li et al., 2012); more specifically, resistance has been related to high iNOS and low Arg-1, while sensitivity has been related to low iNOS and high Arg-1. Zhao et al. (2013) has notably observed high iNOS and low Arg-1 levels in the peritoneal macrophages of inbred rat lines, indicating resistance to *T. gondii*, while the low iNOS and high Arg-1 levels in the alveolar macrophages of identical rat lines point to sensitivity to *T. gondii*. Furthermore, studies in laboratory rodents have shown that anti-inflammatory drugs, like glucocorticoids, reduce iNOS expression but increase susceptibility to *T. gondii* infection (Wang et al., 2014). Gao et al. (2015) have shown that iNOS/Arg-1 expression balance is linked to congenital infection with *T. gondii*, and, more generally, iNOS is important in resistance to parasite infections. For example, a study published in in PNAS. This indicates that iNOS expression is also important for resistance to schistosome infections (Shen et al., 2017). as iNOS knockout rats are much more susceptible to infection. In the *Apodemus sylvaticus* study carried out here, the differences in detected iNOS and Arginase levels raises the possibility that individual animals in the wild may have inherent resistance or susceptibility to

infection. Those expression low levels of iNOS were correlated with those animals that were infected. A alternative possibility is, of course, that infection is linked to a decrease in the expression of iNOS and consequent increase of Arginase caused by the immune response to infection.

At the same time as the inflammation, the process of autophagy was triggered through LC3, which is confirmed by the lower concentration of P62 in infected tissue, than in uninfected tissue. P62 is a target of autophagy so it is expected that the protein would be present at lower levels in infected cells (Deng *et al.*, 2017). However, it is difficult to correlate P62 concentration with autophagy specifically, as P62 is known to play a wide range of roles in human health and disease (Sánchez-Martín & Komatsu, 2018). In regard to *Toxoplasma gondii* infection, research suggests that the levels and/or function of p62 may be affected, although no evidence has confirmed whether p62 observes reduced or heighten expression in chronic infection (Lee *et al.*, 2015; Subauste, 2019). LC3 is a cytoplasmic protein that is involved in the development of autophagosomes (Weidberg *et al*., 2011), which are key organelles required for the non-selective clearance of proteins from the cell, via autophagy (Tanida, Ueno, & Kominami, 2008).There is growing evidence that autophagy is an important component of the innate immune system to degrade infected cells and to defend against invading pathogens (Hu *et al.*, 2019). LC3 has been shown to be required for *Toxoplasma* infections in the past, due to the protein's role in targeting effector proteins to the parasite-containing vacuole that allows *Toxoplasma* to subvert the immune response (Park *et al.*, 2016). The importance of LC3 in various host immune responses to infection, including autophagy, make this protein a crucial target for both *Toxoplasma* and for novel therapeutics to combat *Toxoplasma* infections (Mehta, Henault, Kolbeck, & Sanjuan, 2014). However, there can be methodological difficulty in interpreting the results of LC3 immunoblotting. In this regard, LC3-II correlates well with the abundance of autophagosomes, however, as LC3-II undergoes degradation by autophagy, the quantification and interpretation of this proteins levels can be misleading (Mizushima and Yoshimori, 2007). Thus, research suggests that the ratio of LC3-I to LC3-II should be determined, in order to provide more reliable information concerning autophagy flux (Mizushima and Yoshimori, 2007). Therefore, the findings observed in this study may be limited due to the lack of deriving the LC3-I/II ratio.

Beclin1 is a protein that has a defined role in autophagic programmed cell death, through the binding with other proteins in large complexes. Alongside other autophagic proteins, Beclin1 is an important component for the degradation of infected cells, although the precise mechanism of altered expression remains unclear (Zhong *et al.*, 2009; Chu et al.,2017). The results presented in this thesis support previously published results, which have shown that low Beclin-1 levels are associated with increased resistance to infection by intracellular pathogens (Niu, Xiong, Yamamoto, Hayashi-Nishino, & Rikihisa, 2012).

Lamp2 has been implicated in the maintenance of lysosome integrity, specifically through the protection of the membrane from auto-digestion (Eskelinen, 2006). In the current study, Lamp2 was decreased in infected tissue, likely resulting in a compromised lysosome integrity – a marker for autophagy in the cells. This has been previously supported by Florimond et al. (2018). Decreased Lamp2a has been observed in tissues infected with *T. gondii*, although how the infection leads to altered expression has yet to be elucidated. The process of autophagy is a classic response to an infection, suggesting an attempt to clear the infection in the brain tissue. It has been shown that *Toxoplasma* depends on autophagy in the brain to persist in the environment (Di Cristina *et al.*, 2017). There is a strong link between autophagy and inflammation, through mediating the survival of pro-inflammatory immune cells. It has been hypothesised that modulating autophagy may result in the management of inflammatory diseases (Qian, Fang, & Wang, 2017). Currently, there are few studies that have demonstrated a role for *Toxoplasma* in mediating host autophagy, and further research is required in this area.

The results of the current study have provided evidence of an immune response to a *Toxoplasma* infection. The interaction between the parasite and the immune system, and the potential as a cause for neurodegenerative disorders is a relative new field. It is important to consider the limitations of the current study, when evaluating the overall impact on inflammation and neurodegenerative diseases. while the study identified changes in protein concentration, it did not seek to evaluate the activity of the proteins, nor the presence of the myriad proteins involved in post-translational regulation of these

proteins. Furthermore, while it is tempting to consider these proteins in relation to their impact on inflammation, many of these proteins have pleiotropic effects in the body and their increase or decrease in concentration cannot be reliably linked to a single process. With these caveats in mind, it is possible to consider some tentative early hypotheses based on the protein concentrations, which can guide further work. There remains much to elucidate with regards to the role of *Toxoplasma* in hijacking normal cellular processes to enable persistence in the brain, and the impact of long-term infection on neurological health, brain chemistry and the development of psychological disorders. Further studies looking at the role of the proteins, identified in this study, are required and subsequent investigations may result in novel pharmacological targets for treatments of neurodegenerative conditions in the future. There are challenges associated with studying these detailed interactions. They can be examined in a controlled manner in experimental lab systems, but such systems miss out on the genetic diversity of hosts found in wild systems and on simulating natural infection routes in wild systems. The uniqueness of this project, therefore, is that the researcher has been able to investigate the expression of key proteins in these natural systems.

Chapter 7: General Discussion

In response to the growing body of research that has begun to implicate *T.gondii* infection in the pathogenesis of dementia, this research aimed to investigate whether the organism would be readily detectable in cadaveric brain tissue of patients who had been previously diagnosed with the most common cause of dementia that is AD (Mahami-Oskouei *et al.*, 2016). However, among brain tissue taken from the frontal lobe and hippocampal regions of cadavers, *T.gondii* or related cysts could not be detected, although it is likely that this was compounded by the small sample size used in the study and the testing of small brain tissue volumes.

In effect, this was a weakness in the design that could represent a false-negative result, however, whilst obtaining larger volumes of brain tissue could enhance the sensitivity of *T. gondii* detection, such volumes of brain tissue are not readily available to researchers. Indeed, epidemiological studies have reported that *T.gondii* has infected more than a third of the global population and as a similar prevalence has been observed in the UK (23-33%) (Advisory Committee on the Microbiological Safety of Food, 2012; Pappas, Roussos, & Falagas, 2009) and coupled with the markedly high prevalence of AD (1.5%: 850,000 persons aged >65 years), it was expected that a proportion of this population group would have had detectable *T. gondii* within their brain tissue (Alzheimer's Society, 2019; Pappas, Roussos, & Falagas, 2009). However, the true prevalence of *T. gondii* has been difficult to ascertain given that the majority of cases (approximately 80%) are asymptomatic, that infection can be non-specific and thereby mimic other infectious diseases, and as it confers an unpredictable natural course with active infection occurring at any age post-colonisation (Aguirre *et al.*, 2019). Notably, the brain is the principal organ infected by *T. gondii* following systemic dissemination, which occurs due to insufficient immune control over the highly proliferative tachyzoites, although once infected, the parasite transition into slowly proliferative bradyzoites, which ultimately leads to the formation of cysts and defining chronic infection/latency (Kusbeci, Miman, Yaman, Aktepe, & Yazar, 2011). More specifically, neuropathological research has shown that *T. gondii* can infect a range of central nervous system cells, such as astrocytes, glial cells, neurons and microglia, but particularly those within the subcortical white matter and basal ganglia – sites where cystic

change is prevalent and therefore, these were appropriate brain regions to sample in this study, albeit failing to detect the pathogen or cystic malformations (Mendez & Koshy, 2017).

In regard to the correlation with AD, chronic or latent infection with *T. gondii* was not previously thought to be of clinical concern, although in the past decade, serological studies have shown that there is an increasing association with various neurodegenerative and neuropsychiatric disease with reports that it has been responsible for triggering clinical manifestations and thus, accounting for a large proportion of neurological disease burden (Flegr, Prandota, Sovickova, & Israili, 2014). If it is not the parasite itself causing/inducing AD, perhaps the parasite causes inflammation, autophagy or other processes in the brain. The secondary effect of this might be to induce AD or other neurological diseases. Therefore, an association would be observed between *Toxoplasma* serology and AD or other neurological diseases, but it would not necessarily be directly causative. The role *T. gondii* plays in several human neurological and psychiatric conditions has been the focus of numerous studies (Torrey et al., 2007; Arling et al., 2009; Groer et al., 2011; Hinze-Selch et al., 2010; Prandota, 2014; Sutterland et al., 2015). One association that appears to be quite robust is between the parasite and schizophrenia (Torrey et al., 2007), the evidence of which is particularly convincing in the meta-analysis undertaken by Sutterland et al. (2015). Other associations that have been deemed probable include personality disorders (Hinze-Selch et al., 2010) and attempted suicide (Arling et al., 2009). The meta-analysis from Sutterland et al. (2015) has also indicated that *T. gondii* may play a role in addiction, bipolar disorder and obsessive-compulsive disorder, though no association has been detected between *T. gondii* and major depression. This latter result is consistent with findings from studies conducted by Gale et al. (2014, 2016) and Suvisaari et al. (2017). None of this research has found a link or quantitative association between depression scores and the titres of *T. gondii* IgG antibodies (Nourollahpour Shiadeh et al., 2016; Gao et al., 2019). Thus, it is evident that, in some cases, *Toxoplasma* has an effect, and, in others, it has no effect. Studies exploring the aetiological contribution of *T. gondii* infection to AD have been numerous but have generated mixed results. For example, in a case-control study

of 75 persons with AD and 75 healthy controls, found that the prevalence of serum anti-*Toxoplasma* immunoglobulin G (IgG) was comparable between groups (61.3% v. 62.6%, p>0.05), indicating previous exposure, although no subjects had detectable anti-*Toxoplasma* IgM that would have indicated active infection. In contrast, another case-control study found that there was a significantly greater seropositive rate for anti-*T. gondii* IgG among persons with AD, compared with healthy controls (44.1% v. 24.3%, p=0.005) (Kusbeci *et al.*, 2011). Moreover, in a recent systematic review and meta-analysis of eight serological studies comprising more than 3,000 subjects, Nayeri Chegeni *et al.* (2019) sought to ascertain whether there was a link between *T. gondii* and AD. The results showed that persons with prior exposure to toxoplasmosis, as defined by the presence of serum anti-*Toxoplasma gondii* IgG, observed a significant 53% greater risk of AD as compared to controls (OR 1.53; 95% CI 1.07, 2.18), which overall, suggests that *Toxoplasma* infection does play a role in the pathogenesis of AD. However, these serological findings have yet to be supported or validated by histopathological studies of post-mortem brain tissue and thus, the association between *T. gondii* and Alzheimer's remains unanswered. Notably, the absence of *T. gondii* within brain tissue samples in this study may have represented a false-negative finding given the restricted analysis of tissue from two discrete brain regions with a possibility that *Toxoplasma* infection was present among other sites of these tissues or among other brain regions. Unfortunately, the samples could not be correlated with serological investigations of anti-*T. gondii* IgG or IgM, due to the nature of the study design. Indeed, evidence from the UK has found that the prevalence of *T.gondii* infection in only 3g of brain tissue can be as high as 24.9% (mean 17.9%, range 12.1-24.9%) but the authors were unable to correlate this evidence with correlates of AD or the results of *Toxoplasma* serology (Burrells *et al.*, 2016). Notably, the methods employed by Burrells *et al.* (2016) highlight the limitations of this study in regard to sample size and site, where the authors analysed 3g of brain tissue (0.2% of total brain volume) taken from the hind brain (pons, cerebellum and medulla) and detected *T.gondii* within 27/151 samples, which equated to a prevalence of 17.9%. In contrast, this study analysed 0.126 ml of brain tissue taken from the frontal lobe and hippocampus, with each sample approximating 0.0001% of brain volume, which is 2000-fold lower than that used by Burrells *et al.* (2016) and therefore, it is not surprising that *T. gondii* was undetectable.

To design a study addressing this question, it would be necessary to use frozen or paraffin-embedded brain slices from human cadavers, divided into at least two different groups; the first group would be derived from *T. gondii*-negative and positive brains, 60 years of age and above. The second group would be derived from age-matched *T. gondii*-negative and positive brains with confirmed AD. The statuses of these samples would necessarily be confirmed by PCR, immunofluorescence and western blotting. Although there are several subtypes of the *T. gondii* parasite, it would be beneficial to examine brains infected with the same genotype. If this was not possible, then comparable cyst size and distribution in the *T. gondii* groups would be preferred. In the present case, a human study was evidently not feasible, which necessitated an alternative study. However, this also offered an opportunity to investigate the expression of genes linked to AD (e.g., inflammation and autophagy genes) in cases of *T. gondii* infection. Future work could build on this by investigating the expression of AD linked genes in other animal models, such as lab rats and mice, experimentally infected with *Toxoplasma*. However, while other researchers have already examined such experimentally infected animals, these models may not be truly representative because they make use of unnatural infection routes and unnatural doses of the infectious agent. This, then, necessitates a model involving naturally infected animals and the expression of AD linked genes in this system. Therefore, given the limitations of human brain tissue sampling for the detection of *T. gondii*, the present study has also explored the feasibility of a method for detecting *T. gondii* within murine brain tissue (*A.sylvaticus*) and has found a prevalence of 23.8% ,95% confidence interval: 10.2; 45.5%, which was considered to be high given that the sample was taken from an area of low feline density – Malham Tarn. The results have essentially shown that rodents can act as an intermediary host for *T. gondii* and, thus, contribute to its broader spread to other intermediate hosts, such as the predators of mice and carrion feeders. However, the mice used in this study were taken from an area known to be endemic for *Toxoplasma gondii* and thus, further research should sample non-endemic areas, in order to derive a more representative prevalence of *T. gondii* in the UK. Despite this, a similar prevalence of *T.gondii* infection among *A.sylvaticus* has been supported by other studies with reported prevalence rates of 40.8% (Thomasson *et al.*, 2011) and 41.1% (Bajnok *et al.*, 2015), whilst

the prevalence among other murine species (59%: *Mus domesticus*) has been found to be even higher despite the samples being taken from areas of relatively low feline density, suggesting that the sustained prevalence may be attributed to feline-independent factors (Marshall *et al.*, 2004). The feline species are the established definitive host and vector of *T. gondii* with contaminated excrement being responsible for human transmission, although it has been increasingly recognised that human exposure can also occur through incidental hosts, such as marine mammals, and intermediate hosts - generally the prey of felids, such as mice and birds, but this group also includes livestock and larger mammals (Aguirre *et al.*, 2019). The reported prevalence of *T. gondii* infections across these incidental and intermediate hosts has been found to vary between 0.7% and 84.7%, and given reports that human infection can occur following contact with or ingestion of oocysts or cysts from various species, toxoplasmosis appears to be a complex zoonotic infection with significant neurological implications that could render it a major health problem in the near future (Fuhrer, Schneider, Walochnik, & Auer, 2010; Herrmann *et al.*, 2012).

Whilst this study found a comparable prevalence of *T. gondii* among mice, the use of nested PCR for detection could have overestimated this prevalence, given the risk of cross-sample contamination, although negative controls and replicated testing minimised this risk. Nevertheless, the risk *T. gondii* poses to public health persists in the UK, as well as in other nations. This study also explored whether *A. sylvaticus* is a suitable model for exploring the relationship between *T. gondii* infection and autophagy in the context of AD. This involved ascertaining whether several key autophagy and neuroinflammatory genes (Beclin1, Lc3, Lamp2, Tlr2, Tau and APP) could be detected using primers that had been previously used to amplify such gene sequences among other murine models (*Mus musculus and Rattus norvegicus*) and indeed, the results generated strong and accurate matches for each of the autophagy and inflammation genes .Therefore, as *A. sylvaticus* was found to check for the presence of these genes and compare their homology with their counterpart homologues in humans and mice of Beclin1, Lc3 and Lamp2, TLR2, Tau and APP and represents an ideal model for *T.gondii* infection, this mouse species could be used

to guide further research into the neuroprotective or neuro-exacerbatory effects of toxoplasmosis.

Further to exploring the expression potential of *A. sylvaticus* in regard to autophagy and neuroinflammation, this research also found that the levels of various immune and autophagy proteins involved in the former processes (TLR2, LC3, Socs1, Arg1) were increased in the brain tissue of *T. gondii*-infected mice, as compared to non-infected controls, but in contrast, levels of NMDAR1, Lamp2, P62, Beclin-1 and iNOS were significantly lower among infected tissue, than non-infected tissue. Both Tau and APP were not present at significantly different levels between the infected and uninfected tissues, which may simply reflect the absence of overt AD within the murine model. From a technical perspective, the results demonstrate the potential of *A.sylvaticus*, as a common wood mouse, to facilitate Western blotting investigations into the genetic expression of proteins related to AD. From a molecular perspective, the findings suggest that the *Toxoplasma*-infected tissues were undergoing neuroinflammation and that the immune responses to the parasite were being mediated by a milieu of proteins, including TLR2, LC3, Socs1, Arg1, APP, Tau, NMDAR1, Lamp2, P62, Beclin-1 and iNOS, which are processes that have been previously supported across various studies (Butcher *et al.*, 2011; Cabral, McGovern, MacDonald, Franco, & Koshy, 2017; Denkers, 2010; Hwang *et al.*, 2018; Stutz, Kessler, Kaschel, Meissner, & Dalpke, 2012; Subauste, 2019; Torres *et al.*, 2018). However, a limitation of this study was that no comparisons were used to demonstrate differences in protein/gene expression between *T. gondii* infected sensitive versus and resistance mice and this affects the ability to interpret the results found herein. Moreover, it was not possible to ascertain the resistance profiles of mice to *T. gondii* infection and thus, variances in resistance/susceptibility to infection could account for inter-mouse differences in protein and gene expression. One solution to this problem for future research could be to explore the protein and gene expression of younger and thus, *Toxoplasma*-naive/sensitive mice and compare this to older mice who may have been exposed to the organism and developed immune resistance.

Notably, the importance of autophagy in AD cannot be undermined with the process having capacity to degrade neurotoxic aggregates of amyloid-beta and tau proteins through phagosomal engulfment and destruction that is mediated by lysozyme (Uddin et al., 2018). A number of genes common to autophagy and AD have been characterised and of those evaluated in this study, Beclin1 is known to play a crucial role in the initiation of autophagy and in the formation of autophagosomes with deficiency or reduced expression having been detected in human post-mortem brain tissue and having been found to impair them former functions in murine models (Lucin et al., 2013; Pickford et al., 2008; Xue et al., 2013).

Similarly, LC3 is involved in the initiation of autophagy with the encoding protein being a component required to form autophagosomes from elongated autophagy membranes, whilst the third gene (Lamp2) is involved in the shuttling or translocation of targeted proteins into autophagosomes for lysozyme-mediated degradation (Zare-Shahabadi, Masliah, Johnson, & Rezaei, 2015). Notably, recent evidence has begun to reveal the inter-relatedness between *Toxoplasma* infection, autophagy and AD where host T-helper 1 immune responses mediate binding to *Toxoplasma* -infected cells via CD40, which is a potent inducer of autophagy and this facilitates protection from *T. gondii* (Subauste, 2019). More specifically, CD40 ligation has been found to enhance the conversion of LC3-I into LC3-II to increase autophagosome formation, as well as facilitating increases in autophagy flux – processes of which depend upon genes, such as Beclin1, Lc3, Lamp2, as were investigated in this study (Liu, Lopez Corcino, Portillo, Miao, & Subauste, 2016; Subauste, 2019).

However, some host cells that become infected with *T. gondii* and are subsequently tagged with CD40 for autophagy-mediated destruction, fail to undergo autophagosomal chaperoning and lysozyme-induced degradation, which has been attributed to *T. gondii* hijacking of cell signalling processes to facilitate its evasion and continued survival (Subauste, 2019). In this regard, *T.gondii* has been shown to stimulate the phosphorylation

of host epidermal growth factor receptors (EGFRs) at various tyrosine residues, which is able to inhibit autophagic processing through downstream activation of the phosphoinositide 3-kinase pathway (Muniz-Feliciano et al., 2013). In turn, this leads to activation of the mTORC1 pathway and this helps to preserve the integrity of the parisitophorous vacuole, that encases *T. gondii*, by preventing fusion with autophagy-related peptides (Kim & Denkers, 2006).

iNOS and Arginase genes have been shown to be involved in resistance and sensitivity to *Toxoplasma* infection (Li et al., 2012; Gao et al., 2015). Therefore, in this study, it was hypothesised that iNOS and Arg1 could determine the resistance and sensitivity levels in natural populations of *A. sylvaticus* wood mice. When the iNOS and Arginase protein expressions were measured, uninfected *A. sylvaticus* samples evidenced a significantly higher ratio of iNOS/Arginase expression compared to infected samples. Thus, interesting differences between infected and uninfected animals were observed, which could potentially be linked to expression differences in iNOS/Arginase proteins.

Notably, the reduced expression of NMDAR1 has been previously evident in mice with AD and this combined with *T. gondii* infection, has been associated with enhanced beta-amyloid immunoreactivity and greater tau phosphorylation – pathological hallmarks of the disease, which in turn, conferred greater neuronal loss, than compared to non-infected mice (Torres *et al.*, 2018).

Studies in lab mouse systems have shown this effect, but, importantly, in this research, *Toxoplasma* also depressed NMDAR1 expression in a naturally infected wild population of mice. This is important because so many lab-based studies among mice populations are never verified in nature. Moreover, low expression of NMDAR1 has also been linked with impaired regulation of synaptic function and in turn, this has correlated with neurodegenerative diseases that observe a chronic inflammatory pathogenesis, as with Ad (Zhang, Li, Feng, & Wu, 2016). However, the levels of the proteins identified in this study should be interpreted in light of some methodological limitations, which were that no

attempts to assess protein function were employed and interpreting increases and decreases in such proteins has limited value given the pleiotropic effects and as overall activity is often influenced by the microenvironmental molecular or cytokine milieu (Soni & Singh, 2019). Despite this, it is emerging that *T. gondii* infection exacerbates neuronal degeneration in AD through altering inflammatory and immune molecular responses. For example, in a study that investigated the effect of inhibiting neuroinflammation induced by toxoplasmosis among Tg2576 mice, a well-established model for AD, Jung *et al.* (2012) showed that there was considerable reductions in amyloid-beta deposition within infected mice, than compared to non-infected mice. Notably, the enhanced pathological deposits within infected mice was found to correlate with a greater rate and degree of neurodegeneration, than non-infected mice. However, in contrast to the former study, Mohle et al. (2016) have found that chronic *T. gondii* infections in C57BL/6J and 5XFAD mice (other models for AD) lead to significant reductions in amyloid-beta plaque load, which is attributed to enhanced autophagic capacity and degradation of the deposits. This result is similar to that found in the present work, where enhanced autophagy involved an increase in LC3 protein in the brain tissue of *T. gondii*-infected mice. However, the results in both studies demonstrate that there is still some uncertainty as to whether toxoplasmosis is protective or promotive of AD in humans (Jung et al., 2012; Mohle et al., 2016).

Considering the findings of this study and comparison with the wider literature, it is imperative that future research explores several avenues pertaining to *T. gondii* infection and AD. Firstly, whilst a number of studies have demonstrated an association between toxoplasmosis and AD, these have been limited by their case-control and animal model designs and therefore, it is imperative for future research to conduct prospective longitudinal research if *T. gondii* is to be causally implicated in the neurodegenerative process. Secondly, research should also advance an understanding of the suitability of *A.sylvaticus* as a model for exploring the relationship between toxoplasmosis and AD by further investigating the expression of genes associated with disease pathogenesis and autophagy. This could involve taking a larger and longitudinal sample of brain tissue from human tissue banks, similar to that reported by Burrells *et al.* (2016), and among *T. gondii* infected samples, analysing whether the gene/protein expression profiles are comparable to that found in *A. sylvaticus* in this study. However, given the relative scarcity of human

brain tissue available and particularly, tissue taken from persons that had diagnosed AD and thus possible *T.gondii* colonisation or infection, it may take several years or even decades to acquire sufficient and valid data. However, this is an important area of research as it may help to further validate the utility and potential of *A.sylvaticus* as a representative research model for studying AD in humans and thus, this research should be a high priority. Thirdly, the field would also benefit from studies that can better characterise the gene and protein expression profiles of *T. gondii* infected mice and clarify how this translates into a mechanism that promotes or exacerbates neurodegenerative disease and most notably, AD. Differential gene expression in infected versus uninfected hosts could elucidate novel pathways affected by toxoplasmosis and, importantly, whether these are related to AD proteins and onset, or if they offer a protective advantage against degenerative disease. Future work should also include next-generation RNA-sequencing, which may provide alterations in transcriptional networks that could help decipher potential links between AD and *T. gondii* infection. In addition, it would be useful to further characterise the levels of expressed neuroinflammatory, immune system and autophagy-related proteins in *T. gondii* infected mice, in order to better understand the molecular processes perpetuating neurodegeneration and toxicity. Finally, research should also develop and explore the safety and efficacy of anti-protozoal drugs specific to *T. gondii* as this may hold significant potential in ameliorating the risk of AD in colonised humans. At present, the primary agents used to treat toxoplasmosis, that include pyrimethamine and sulfadiazine, have markedly high failure rates and are associated with excess adverse effects, which are particularly prominent in elderly persons with compromised immune systems – an at-risk group for AD (Dunay, Gajurel, Dhakal, Liesenfeld, & Montoya, 2018). Therefore, it is important that research seeks to advance understanding of the pathogenicity of *Toxoplasma*.

References

Abdel Hameed, D. M., & Helmy, H. (2004). Avidity IgG: diagnosis of primary Toxoplasma gondii infection by indirect immunofluorescent test. *J Egypt Soc Parasitol, 34*(3), 893-902.

Abdel Razek, A. A., Watcharakorn, A., & Castillo, M. (2011). Parasitic diseases of the central nervous system. *Neuroimaging Clin N Am, 21*(4), 815-841, viii. doi:10.1016/j.nic.2011.07.005.

Afonso, E., Lemoine, M., Poulle, M. L., Ravat, M. C., Romand, S., Thulliez, P., … Gilot-Fromont, E. (2008). Spatial distribution of soil contamination by Toxoplasma gondii in relation to cat defecation behaviour in an urban area. *International Journal for Parasitology, 38*(8–9), 1017–1023. https://doi.org/10.1016/j.ijpara.2008.01.004

Afonso, E., P. Thulliez, and E. Gilot-Fromont, 2006: Transmission of Toxoplasma gondii in an urban population of domestic cats (Felis catus). Int. J. Parasitol. 36, 1373–1382.

Afonso, E.; Poulle, M.L.; Lemoine, M.; Villena, I.; Aubert, D. and Gilot-Fromont, E. (2007). Prevalence of *Toxoplasma gondii* in small mammals from the Ardennes region, France. Folia Parasitologica (Praha). 54 (4): 313 – 314.

Aguirre, A. A., Longcore, T., Barbieri, M., Dabritz, H., Hill, D., Klein, P. N., . . . Sizemore, G. C. (2019). The One Health Approach to Toxoplasmosis:

Epidemiology, Control, and Prevention Strategies. *EcoHealth, 16*(2), 378-390. doi:10.1007/s10393-019-01405-7.

Al-Hussainy, N. H., Al-saedi, A. M., Al-lehaibi, J. H., Al-lehaibi, Y. A., Al-Sehli, Y. M., & Afifi, M. A. (2015). Serological evidences link toxoplasmosis with schizophrenia and major depression disorder. *Journal of Microscopy and Ultrastructure*, *3*(3), 148–153. https://doi.org/10.1016/j.jmau.2015.03.006

Aliberti, J. (2005). Host persistence: Exploitation of anti-inflammatory pathways by Toxoplasma gondii. *Nature Reviews Immunology*, *5*(2), 162–170. https://doi.org/10.1038/nri1547

Alvarado-Esquivel, C., Liesenfeld, O., Burciaga-Lopez, B. D., Ramos-Nevarez, A., Estrada-Martinez, S., Cerrillo-Soto, S. M., . . . Ruiz-Martinez, M. M. (2012). Seroepidemiology of Toxoplasma gondii infection in elderly people in a northern Mexican city. *Vector Borne Zoonotic Dis*, *12*(7), 568-574. doi:10.1089/vbz.2011.0875.

Alvarado-Esquivel, C., Mendez-Hernandez, E. M., Salas-Pacheco, J. M., Ruano-Calderon, L. A., Hernandez-Tinoco, J., Arias-Carrion, O., . . . Ramos-Nevarez, A. (2017). Toxoplasma gondii exposure and Parkinson's disease: a case-control study. *BMJ Open*, *7*(2), e013019. doi:10.1136/bmjopen-2016-013019.

Alvarado-Esquivel, C., Sánchez-Anguiano, L. F., Hernández-Tinoco, J., Arreola-Cháidez, E., López, J., Salcido-Meraz, K. I., . . . Liesenfeld, O. (2015). High Seroprevalence of Toxoplasma Gondii Infection in Female Sex Workers: A Case-

Control Study. *European journal of microbiology & immunology*, *5*(4), 285-292. doi:10.1556/1886.2015.00039

Alzheimer's Society. (2019). Statistics on dementia. Retrieved from https://www.alzheimers.org.uk/about-us/news-and-media/facts-media

Amano, A., Nakagawa, I., & Yoshimori, T. (2006). Autophagy in innate immunity against intracellular bacteria. *J Biochem, 140*(2), 161-166. doi: 10.1093/jb/mvj162

Andersson, L.-M., Blennow, K., Fuchs, D., Svennerholm, B., & Gisslén, M. (1999). Increased cerebrospinal fluid protein tau concentration in neuro-AIDS. *Journal of the Neurological Sciences, 171*(2), 92-96. doi: https://doi.org/10.1016/S0022-510X(99)00253-1

Andrade, R. M., Wessendarp, M., Gubbels, M. J., Striepen, B., & Subauste, C. S. (2006). CD40 induces macrophage anti-Toxoplasma gondii activity by triggering autophagy-dependent fusion of pathogen-containing vacuoles and lysosomes. *J Clin Invest, 116*(9), 2366-2377. doi: 10.1172/JCI28796animals to humans. *Int J Parasitol,* 30, 1217-1258.Argentina. *Clin. Microbiol. Infect.,* 5 (Suppl. 3).

Arling, T.A., Yolken, R.H., Lapidus, M., Langenberg, P., Dickerson, F.B., Zimmerman, S.A.,et al., 2009. Toxoplasma gondii antibody titers and history of suicide attempts in patients with recurrent mood disorders. J. Nerv. Ment. Dis. 197 (12), 905–908.

Arroyo, D. S., Soria, J. A., Gaviglio, E. A., Garcia-Keller, C., Cancela, L. M., Rodriguez-Galan, M. C., . . . Iribarren, P. (2013). Toll-like receptor 2 ligands promote microglial cell death by inducing autophagy. *FASEB J, 27*(1), 299-312. doi: 10.1096/fj.12-214312

Baruch, K., Rosenzweig, N., Kertser, A., Deczkowska, A., Sharif, A. M., Spinrad, A., … Schwartz, M. (2015). Breaking immune tolerance by targeting Foxp3+ regulatory T cells mitigates Alzheimer's disease pathology. *Nature Communications, 6*, 1–12. https://doi.org/10.1038/ncomms8967

Aspinall, T.; V.; Marlee, D.; Hyde, J.E.; Sims, P.F. (2002). Prevalence of Toxoplasma gondii in commercial meat products as monitored by polymerase chain reaction--food for thought? International Journal of Parasitology. 32(9): 1193-1199.

Ayala, P., Lin, L., Hopper, S., Fukuda, M., & So, M. (1998). Infection of Epithelial Cells by Pathogenic Neisseriae Reduces the Levels of Multiple Lysosomal Constituents. *Infection and Immunity*, *66*(10), 5001.

Babu, J. R., Lamar Seibenhener, M., Peng, J., Strom, A. L., Kemppainen, R., Cox, N., Zhu, H., Wooten, M. C., Diaz-Meco, M. T., Moscat, J. and Wooten, M. W. (2008) Genetic inactivation of p62 leads to accumulation of hyperphosphorylated tau and neurodegeneration. *J Neurochem,* 106 (1)**,** 107-120.

Bajnok, J., Boyce, K., Rogan, M. T., Craig, P. S., Lun, Z. R., & Hide, G. (2015). Prevalence of Toxoplasma gondii in localized populations of Apodemus

sylvaticus is linked to population genotype not to population location. *Parasitology, 142*(5), 680-690. doi:10.1017/s0031182014001760

Balez, R. and Ooi, L. (2016) Getting to NO Alzheimer's Disease: Neuroprotection versus Neurotoxicity Mediated by Nitric Oxide. Oxidative medicine and cellular longevity, 2016 (1)**,** 1-10.

Balfour, A. H., Bridges, J. B., & Harford, J. P. (1980). An evaluation of the ToxHA test for the detection of antibodies to Toxoplasma gondii in human serum. *J Clin Pathol, 33*(7), 644-647.

Balkwill, G. D., Williams, H. E., & Searle, M. S. (2007). Structure and folding dynamics of a DNA hairpin with a stabilising d(GNA) trinucleotide loop: influence of base pair mis-matches and point mutations on conformational equilibria. *Org Biomol Chem, 5*(5), 832-839. doi: 10.1039/b616820e

Barragan, A., & Sibley, L. D. (2002). Transepithelial migration of Toxoplasma gondii is linked to parasite motility and virulence. *J Exp Med, 195*(12), 1625-1633.

Barragan, A., Brossier, F., & Sibley, L. D. (2005). Transepithelial migration of Toxoplasma gondii involves an interaction of intercellular adhesion molecule 1 (ICAM-1) with the parasite adhesin MIC2. *Cell Microbiol, 7*(4), 561-568. doi:10.1111/j.1462-5822.2005.00486.x

Berdoy, M., Webster, J. P., & Macdonald, D. W. (2000). Fatal attraction in rats infected with Toxoplasma gondii. *Proc Biol Sci, 267*(1452), 1591-1594. doi:10.1098/rspb.2000.1182

Berenreiterova, M., Flegr, J., Kubena, A. A., & Nemec, P. (2011). The distribution of Toxoplasma gondii cysts in the brain of a mouse with latent toxoplasmosis:

implications for the behavioral manipulation hypothesis. *PLoS ONE, 6*(12), e28925. doi:10.1371/journal.pone.0028925

Beste, C., Getzmann, S., Gajewski, P. D., Golka, K., & Falkenstein, M. (2014). Latent Toxoplasma gondii infection leads to deficits in goal-directed behavior in healthy elderly. *Neurobiol Aging, 35*(5), 1037-1044. doi:10.1016/j.neurobiolaging.2013.11.012

Besteiro, S. (2012). Which roles for autophagy in Toxoplasma gondii and related apicomplexan parasites? *Mol Biochem Parasitol, 184*(1), 1-8. doi: 10.1016/j.molbiopara.2012.04.001

Beyreuther, K., & Masters, C. L. (1991). Amyloid precursor protein (APP) and beta A4 amyloid in the etiology of Alzheimer's disease: precursor-product relationships in the derangement of neuronal function. *Brain Pathol, 1*(4), 241-251.

Biedler, J. L., Helson, L., & Spengler, B. A. (1973). Morphology and Growth, Tumorigenicity, and Cytogenetics of Human Neuroblastoma Cells in Continuous Culture. *Cancer Research*, *33*(11), 2643–2652.

Biswas, A., French, T., Düsedau, H. P., Mueller, N., Riek-Burchardt, M., Dudeck, A., … Dunay, I. R. (2017). Behavior of neutrophil granulocytes during Toxoplasma gondii infection in the central nervous system. *Frontiers in Cellular and Infection Microbiology*, *7*(JUN), 1–13. https://doi.org/10.3389/fcimb.2017.00259

Blackston, C. R., Dubey, J. P., Dotson, E., Su, C., Thulliez, P., Sibley, D., & Lehmann, T. (2001). High-resolution typing of Toxoplasma gondii using microsatellite loci. *J*

Parasitol, 87(6), 1472-1475. doi:10.1645/0022-3395(2001)087[1472:HRTOTG]2.0.CO;2

Blader, I. J., Manger, I. D., & Boothroyd, J. C. (2001). Microarray analysis reveals previously unknown changes in Toxoplasma gondii-infected human cells. *J Biol Chem, 276*(26), 24223-24231. doi:10.1074/jbc.M100951200

Blalock, E. M., Chen, K. C., Stromberg, A. J., Norris, C. M., Kadish, I., Kraner, S. D., … Landfield, P. W. (2005). Harnessing the power of gene microarrays for the study of brain aging and Alzheimer's disease: Statistical reliability and functional correlation. *Ageing Research Reviews, 4*(4), 481–512. https://doi.org/10.1016/j.arr.2005.06.006

Boland, B., Kumar, A., Lee, S., Platt, F. M., Wegiel, J., Yu, W. H., & Nixon, R. A. (2008). Autophagy induction and autophagosome clearance in neurons: relationship to autophagic pathology in Alzheimer's disease. *J Neurosci, 28*(27), 6926-6937. doi: 10.1523/JNEUROSCI.0800-08.2008

Bouvier, D. S., & Murai, K. K. (2015). Synergistic actions of microglia and astrocytes in the progression of Alzheimer's disease. *Journal of Alzheimer's Disease, 45*(4), 1001–1014. https://doi.org/10.3233/JAD-143156

Boya, P., Reggiori, F., & Codogno, P. (2013). Emerging regulation and functions of autophagy. *Nat Cell Biol, 15*(7), 713-720. doi: 10.1038/ncb2788

Braak, H., & Braak, E. (1991). Neuropathological stageing of Alzheimer-related changes. *Acta Neuropathol, 82*(4), 239-259.

Braak, H., & Braak, E. (1995). Staging of Alzheimer's disease-related neurofibrillary changes. *Neurobiol Aging, 16*(3), 271-278; discussion 278-284.

Braak, H., Braak, E., Yilmazer, D., Schultz, C., de Vos, R. A., & Jansen, E. N. (1995). Nigral and extranigral pathology in Parkinson's disease. *J Neural Transm Suppl, 46*, 15-31.

Burg, J. L., Grover, C. M., Pouletty, P., & Boothroyd, J. C. (1989). Direct and sensitive detection of a pathogenic protozoan, Toxoplasma gondii, by polymerase chain reaction. *J Clin Microbiol, 27*(8), 1787-1792.

Burrells, A., Opsteegh, M., Pollock, K. G., Alexander, C. L., Chatterton, J., Evans, R., … Katzer, F. (2016). The prevalence and genotypic analysis of Toxoplasma gondii from individuals in Scotland, 2006-2012. Parasites and Vectors, 9(1), 1–12. https://doi.org/10.1186/s13071-016-1610-6

Butcher, B. A., Fox, B. A., Rommereim, L. M., Kim, S. G., Maurer, K. J., Yarovinsky, F., . . . Denkers, E. Y. (2011). Toxoplasma gondii rhoptry kinase ROP16 activates STAT3 and STAT6 resulting in cytokine inhibition and arginase-1-dependent growth control. *PLoS Pathog, 7*(9), 1-8. doi:10.1371/journal.ppat.1002236

Cabral, C. M., McGovern, K. E., MacDonald, W. R., Franco, J., & Koshy, A. A. (2017). Dissecting Amyloid Beta Deposition Using Distinct Strains of the Neurotropic Parasite Toxoplasma gondii as a Novel Tool. *ASN neuro, 9*(4), 1-12. doi:10.1177/1759091417724915

Cabral, C. M., Tuladhar, S., Dietrich, H. K., Nguyen, E., MacDonald, W. R., Trivedi, T., . . . Koshy, A. A. (2016). Neurons are the Primary Target Cell for the Brain-

Tropic Intracellular Parasite Toxoplasma gondii. *PLoS Pathog, 12*(2), e1005447. doi:10.1371/journal.ppat.1005447

Canu, N., Amadoro, G., Triaca, V., Latina, V., Sposato, V., Corsetti, V., . . . Calissano, P. (2017). The Intersection of NGF/TrkA Signaling and Amyloid Precursor Protein Processing in Alzheimer's Disease Neuropathology. *Int J Mol Sci, 18*(6). doi: 10.3390/ijms18061319.

Cao, A., Liu, Y., Wang, J., Li, X., Wang, S., Zhao, Q., . . . Zhou, H. (2015). Toxoplasma gondii: Vaccination with a DNA vaccine encoding T- and B-cell epitopes of SAG1, GRA2, GRA7 and ROP16 elicits protection against acute toxoplasmosis in mice. *Vaccine, 33*(48), 6757-6762. doi:10.1016/j.vaccine.2015.10.077

Cappelletti, C., Galbardi, B., Kapetis, D., Vattemi, G., Guglielmi, V., Tonin, P., . . . Bernasconi, P. (2014). Autophagy, inflammation and innate immunity in inflammatory myopathies. *Plos One, 9*(11), e111490. doi: 10.1371/journal.pone.0111490

Celik, T., Kamisli, O., Babur, C., Cevik, M. O., Oztuna, D., & Altinayar, S. (2010). Is there a relationship between Toxoplasma gondii infection and idiopathic Parkinson's disease? *Scand J Infect Dis, 42*(8), 604-608. doi:10.3109/00365541003716500

Chang, S., Shan, X., Li, X., Fan, W., Zhang, S. Q., Zhang, J., … Mao, Z. (2015). Toxoplasma gondii rhoptry protein ROP16 mediates partially SH-SY5Y cells apoptosis and cell cycle arrest by directing ser15/37 phosphorylation of p53. *International Journal of Biological Sciences*, *11*(10), 1215–1225. https://doi.org/10.7150/ijbs.10516

Charoenthongtrakul, S., Zhou, Q., Shembade, N., Harhaj, N. S., & Harhaj, E. W. (2011). Human T cell leukemia virus type 1 Tax inhibits innate antiviral signaling via NF-kappaB-dependent induction of SOCS1. *J Virol, 85*(14), 6955-6962. doi: 10.1128/JVI.00007-11.

Chen, K., Iribarren, P., Hu, J., Chen, J., Gong, W., Cho, E. H., Lockett, S., Dunlop, N. M. and Wang, J. M. (2006) Activation of Toll-like receptor 2 on microglia promotes cell uptake of Alzheimer disease-associated amyloid beta peptide. *J Biol Chem,* 281 (6)**,** 3651-3659.

Chu, J. Q., Jing, K. P., Gao, X., Li, P., Huang, R., Niu, Y. R., … Quan, J. H. (2017). Toxoplasma gondii induces autophagy and apoptosis in human umbilical cord mesenchymal stem cells via downregulation of Mcl−1. *Cell Cycle*, *16*(5), 477–486. https://doi.org/10.1080/15384101.2017.1281484

Clayton, K. A., Van Enoo, A. A. and Ikezu, T. (2017) Alzheimer's Disease: The Role of Microglia in Brain Homeostasis and Proteopathy. *Frontiers in Neuroscience,* 11 (680)**,** 1-8.

Clough, B., Wright, J. D., Pereira, P. M., Hirst, E. M., Johnston, A. C., Henriques, R., & Frickel, E. M. (2016). K63-Linked Ubiquitination Targets Toxoplasma gondii for Endo-lysosomal Destruction in IFNgamma-Stimulated Human Cells. *PLoS Pathog, 12*(11), e1006027. doi: 10.1371/journal.ppat.1006027.

Cohen-Kaplan, V., Ciechanover, A., & Livneh, I. (2016). p62 at the crossroad of the ubiquitin-proteasome system and autophagy. *Oncotarget, 7*(51), 83833-83834. doi: 10.18632/oncotarget.13805

Collins, A. T., & Cromwell, L. D. (1980). Computed tomography in the evaluation of congenital cerebral toxoplasmosis. *J Comput Assist Tomogr, 4*(3), 326-329.

Combs, C. K. (2009). Inflammation and microglia actions in alzheimer's disease. *Journal of Neuroimmune Pharmacology*, *4*(4), 380–388. https://doi.org/10.1007/s11481-009-9165-3

Conejero-Goldberg, C., Torrey, E. F. and Yolken, R. H. (2003) Herpesviruses and Toxoplasma gondii in orbital frontal cortex of psychiatric patients. *Schizophr Res,* 60 (1)**,** 65-69.

Cosgrove, K., Mazure, C., & Staley, J. (2007). Evolving knowledge of sex differences in brain structure, function, and chemistry. Biological Psychiatry, 62(8), 847–855. https://doi.org/10.1016/j.biopsych.2007.03.001.Evolving

Courret, N., Darche, S., Sonigo, P., Milon, G., Buzoni-Gatel, D., & Tardieux, I. (2006). CD11c- and CD11b-expressing mouse leukocytes transport single Toxoplasma gondii tachyzoites to the brain. *Blood, 107*(1), 309-316. doi:10.1182/blood-2005-02-0666.

Cowen, D. & Wolf, A. 1950b. Experimental congenital toxoplasmosis. II. Transmission of toxoplasmosis to the placenta and fetus following vaginal infection in the pregnant mouse. *J Exp Med,* 92, 403-16.

Cowen, D. & Wolf, A. (1951). Experimental congenital toxoplasmosis. IV. Genital and secondary lesions in the mouse infected with toxoplasma by the vaginal route. *J Neuropathol Exp Neurol,* 10, 1-15.

Crack, P. J., & Bray, P. J. (2007). Toll-like receptors in the brain and their potential roles in neuropathology. *Immunology and Cell Biology, 85*(6), 476–480. https://doi.org/10.1038/sj.icb.7100103

Cuyvers, E., van der Zee, J., Bettens, K., Engelborghs, S., Vandenbulcke, M., Robberecht, C., . . . of the, E. U. E. O. D. C. (2015). Genetic variability in SQSTM1 and risk of early-onset Alzheimer dementia: a European early-onset dementia consortium study. *Neurobiol Aging, 36*(5), 2005 e2015-2022. doi: 10.1016/j.neurobiolaging.2015.02.014

Da Gama, L. M., Ribeiro-Gomes, F. L., Guimaraes, U., Jr., & Arnholdt, A. C. (2004). Reduction in adhesiveness to extracellular matrix components, modulation of adhesion molecules and in vivo migration of murine macrophages infected with Toxoplasma gondii. *Microbes Infect, 6*(14), 1287-1296. doi:10.1016/j.micinf.2004.07.008

Dabritz, H.A.; Miller, M.A.; Gardner, I.A.; Packham, A.E.; Atwill, E.R.; Conrad, P.A. (2008). Risk factors for Toxoplasma gondii infection in wild rodents from central coastal California and a review of T. gondii prevalence in rodents. Journal of Parasitology 94(3): 675-683.

Dansokho, C., Ait Ahmed, D., Aid, S., Toly-Ndour, C., Chaigneau, T., Calle, V., . . . (2016). Regulatory T cells delay disease progression in Alzheimer-like pathology. *Brain, 139*(Pt 4), 1237-1251. doi:10.1093/brain/awv408

Davey, G. M., Heath, W. R., & Starr, R. (2006). SOCS1: a potent and multifaceted regulator of cytokines and cell-mediated inflammation. *Tissue Antigens, 67*(1), 1-9. doi: 10.1111/j.1399-0039.2005.00532.x

Debierre-Grockiego, F., Campos, M. A., Azzouz, N., Schmidt, J., Bieker, U., Resende, M. G., … Schwarz, R. T. (2007). Activation of TLR2 and TLR4 by Glycosylphosphatidylinositols Derived from Toxoplasma gondii . *The Journal of Immunology*, *179*(2), 1129–1137. https://doi.org/10.4049/jimmunol.179.2.1129

Dendrou, C. A., Fugger, L., & Friese, M. A. (2015). Immunopathology of multiple sclerosis. *Nature Reviews Immunology*, *15*(9), 545–558. https://doi.org/10.1038/nri3871

Deng, Z., Purtell, K., Lachance, V., Wold, M. S., Chen, S., & Yue, Z. (2017). Autophagy Receptors and Neurodegenerative Diseases. *Trends Cell Biol*, *27*(7), 491-504. doi: 10.1016/j.tcb.2017.01.001

Denkers, E. Y. (2003). From cells to signaling cascades: manipulation of innate immunity by Toxoplasma gondii. *FEMS Immunol Med Microbiol*, *39*(3), 193-203.

Denkers, E. Y. (2010). Toll-like receptor initiated host defense against Toxoplasma gondii. *J Biomed Biotechnol*, *2010*(1), 1-10. doi:10.1155/2010/737125

Denkers, E. Y., & Gazzinelli, R. T. (1998). Regulation and function of T-cell-mediated immunity during Toxoplasma gondii infection. *Clin Microbiol Rev*, *11*(4), 569-588.

Denkers, E. Y., Butcher, B. A., Del Rio, L., & Bennouna, S. (2004). Neutrophils, dendritic cells and Toxoplasma. *Int J Parasitol*, *34*(3), 411-421. doi:10.1016/j.ijpara.2003.11.001

Denkers, E. Y., Kim, L., & Butcher, B. A. (2003). In the belly of the beast: subversion of macrophage proinflammatory signalling cascades during *Toxoplasma gondii*

infection. *Cellular Microbiology, 5*(2), 75-83. doi: 10.1046/j.1462-5822.2003.00258.x

Denkers, E. Y., Schneider, A. G., Cohen, S. B., & Butcher, B. A. (2012). Phagocyte responses to protozoan infection and how Toxoplasma gondii meets the challenge. *PLoS Pathog, 8*(8), e1002794. doi:10.1371/journal.ppat.1002794

Deretic, V., & Levine, B. (2018). Autophagy balances inflammation in innate immunity. *Autophagy, 14*(2), 243-251. doi: 10.1080/15548627.2017.1402992.

Desmonts, G., Naot, Y., & Remington, J. S. (1981). Immunoglobulin M-immunosorbent agglutination assay for diagnosis of infectious diseases: diagnosis of acute congenital and acquired Toxoplasma infections. *J Clin Microbiol, 14*(5), 486-491. determined by polymerase chain reaction. *Parasitology, 116*(4), 299-304.

Di Cristina, M., Dou, Z., Lunghi, M., Kannan, G., Huynh, M.-H., McGovern, O. L., . . . Carruthers, V. B. (2017). Toxoplasma depends on lysosomal consumption of autophagosomes for persistent infection. *Nature Microbiology, 2*(8), 17096. doi: 10.1038/nmicrobiol.2017.96

Di Cristina, M., Marocco, D., Galizi, R., Proietti, C., Spaccapelo, R., & Crisanti, A. (2008). Temporal and spatial distribution of Toxoplasma gondii differentiation into Bradyzoites and tissue cyst formation in vivo. *Infect Immun, 76*(8), 3491-3501. doi:10.1128/IAI.00254-08.

Draberova, E., Stegurova, L., Sulimenko, V., Hajkova, Z., Draber, P., & Draber, P. (2013). Quantification of alpha-tubulin isotypes by sandwich ELISA with signal amplification through biotinyl-tyramide or immuno-PCR. *J Immunol Methods, 395*(1-2), 63-70. doi:10.1016/j.jim.2013.07.001

Drubin, D. G., & Kirschner, M. W. (1986). Tau protein function in living cells. *The Journal of cell biology*, 103(6), 2739-2746.

Du, Y., Wooten, M. C., Gearing, M., & Wooten, M. W. (2009). Age-associated oxidative damage to the p62 promoter: implications for Alzheimer disease. *Free Radic Biol Med, 46*(4), 492-501. doi: 10.1016/j.freeradbiomed.2008.11.003

Dubey, J. P. (1998). Advances in the life cycle of Toxoplasma gondii. *Int J Parasitol, 28*(7), 1019-1024.

Dubey, J. P. (2009). History of the discovery of the life cycle of Toxoplasma gondii. *Int J Parasitol, 39*(8), 877-882.

Dubey, J. P., Alvarado-Esquivel, C., Herrera-Valenzuela, V. H., Ortiz-Diaz, J. J., Oliveira, S., Verma, S. K., . . . Su, C. (2013). A new atypical genotype mouse virulent strain of Toxoplasma gondii isolated from the heart of a wild caught puma (Felis concolor) from Durango, Mexico. *Vet Parasitol, 197*(3-4), 674-677. doi:10.1016/j.vetpar.2013.06.005

Dubey, J. P., Ferreira, L. R., Alsaad, M., Verma, S. K., Alves, D. A., Holland, G. N., & McConkey, G. A. (2016). Experimental Toxoplasmosis in Rats Induced Orally with Eleven Strains of Toxoplasma gondii of Seven Genotypes: Tissue Tropism, Tissue Cyst Size, Neural Lesions, Tissue Cyst Rupture without Reactivation, and Ocular Lesions. *PLoS ONE, 11*(5), e0156255. doi:10.1371/journal.pone.0156255

Dubey, J. P., Lindsay, D. S. and Speer, C. A. (1998) Structures of Toxoplasma gondiiTachyzoites, Bradyzoites, and Sporozoites and Biology and Development of Tissue Cysts. *Clinical Microbiology Reviews,* 11 (2)**,** 267-275.

Dubey, J. P., Lindsay, D. S., & Lappin, M. R. (2009). Toxoplasmosis and other intestinal coccidial infections in cats and dogs. *Vet Clin North Am Small Anim Pract, 39*(6), 1009-1034, v. doi:10.1016/j.cvsm.2009.08.001

Dubey, J. P., Rajendran, C., Ferreira, L. R., Kwok, O. C., Sinnett, D., Majumdar, D., & Su, C. (2010). A new atypical highly mouse virulent Toxoplasma gondii genotype isolated from a wild black bear in Alaska. *J Parasitol, 96*(4), 713-716. doi:10.1645/GE-2429.1

Dubey, J. P., Velmurugan, G. V., Alvarado-Esquivel, C., Alvarado-Esquivel, D., Rodriguez-Pena, S., Martinez-Garcia, S., . . . Su, C. (2009). Isolation of Toxoplasma gondii from animals in Durango, Mexico. *J Parasitol, 95*(2), 319-322. doi:10.1645/ge-1874.1

Duncanson, P., Terry, R. S., Smith, J. E., & Hide, G. (2001). High levels of congenital transmission of Toxoplasma gondii in a commercial sheep flock. *Int J Parasitol, 31*(14), 1699-1703.

Ehrenreich, H. (2017). Autoantibodies against the N-Methyl-d-Aspartate Receptor Subunit NR1: Untangling Apparent Inconsistencies for Clinical Practice. *Frontiers in immunology, 8,* 181-181. doi: 10.3389/fimmu.2017.00181

Eiamprapai, P., Matsumura, Y., Hiraumi, H., Yamamoto, N., Takakura, S., & Ito, J. (2013). Rapid detection of bacterial DNA in mastoid granulation tissue with nested-PCR technique. *J Med Assoc Thai, 96*(4), 460-466.

Eichenwald, H. (1948). Experimental toxoplasmosis. I. Transmission of the infection in utero and through the milk of lactating female mice. American journal of diseases of children. 76(3): 307 -315.

Elahy, M., Jackaman, C., Mamo, J. C. L., Lam, V., Dhaliwal, S. S., Giles, C., … Takechi, R. (2015). Blood-brain barrier dysfunction developed during normal aging is associated with inflammation and loss of tight junctions but not with leukocyte recruitment. *Immunity and Ageing*, *12*(1), 1–9. https://doi.org/10.1186/s12979-015-0029-9

Elnagar, M. R., Walls, A. B., Helal, G. K., Hamada, F. M., Thomsen, M. S., & Jensen, A. A. (2017). Probing the putative alpha7 nAChR/NMDAR complex in human and murine cortex and hippocampus: Different degrees of complex formation in healthy and Alzheimer brain tissue. *Plos One, 12*(12), e0189513. doi: 10.1371/journal.pone.0189513

Esch, K. J., & Petersen, C. A. (2013). Transmission and epidemiology of zoonotic protozoal diseases of companion animals. *Clin Microbiol Rev, 26*(1), 58-85. doi:10.1128/CMR.00067-12

Escotte-Binet, S., Ajzenberg, D., Darde, M. L., Durand, B., Boireau, P. and Villena,

Eskelinen, E. L. (2006). Roles of LAMP-1 and LAMP-2 in lysosome biogenesis and autophagy. *Mol Aspects Med, 27*(5-6), 495-502. doi: 10.1016/j.mam.2006.08.005

Esshili, A., Thabet, S., Jemli, A., Trifa, F., Mechri, A., Zaafrane, F., … Bel Hadj Jrad, B. (2016). Toxoplasma gondii infection in schizophrenia and associated clinical features. *Psychiatry Research*, *245*, 327–332. https://doi.org/10.1016/j.psychres.2016.08.056

Estupinan-Diaz, B. O., Morales-Chacon, L. M., Garcia-Maeso, I., Lorigados-Pedre, L., Baez-Martin, M., Garcia-Navarro, M. E., . . . Grupo Interdisciplinario de Cirugia

de Epilepsia, C. I. d. R. N. (2015). Corpora amylacea in the neocortex in patients with temporal lobe epilepsy and focal cortical dysplasia. *Neurologia, 30*(2), 90-96. doi:10.1016/j.nrl.2013.06.008Fabis, M. J., Scott, G. S., Kean, R. B., Koprowski, H., & Hooper, D. C. (2007). Loss of blood-brain barrier integrity in the spinal cord is common to experimental allergic encephalomyelitis in knockout mouse models. *Proceedings of the National Academy of Sciences of the United States of America*, *104*(13), 5656–5661. https://doi.org/10.1073/pnas.0701252104

Fan, W., Chang, S., Shan, X., Gao, D., Zhang, S. Q., Zhang, J., … Mao, Z. (2016). Transcriptional profile of SH-SY5Y human neuroblastoma cells transfected by Toxoplasma rhoptry protein 16. *Molecular Medicine Reports*, *14*(5), 4099–4108. https://doi.org/10.3892/mmr.2016.5758

Ferguson, D. J., & Hutchison, W. M. (1987a). The host-parasite relationship of Toxoplasma gondii in the brains of chronically infected mice. *Virchows Arch A Pathol Anat Histopathol, 411*(1), 39-43.

Ferguson, D. J., & Hutchison, W. M. (1987b). An ultrastructural study of the early development and tissue cyst formation of Toxoplasma gondii in the brains of mice. *Parasitol Res, 73*(6), 483-491.

Feustel, S. M., Meissner, M., & Liesenfeld, O. (2012). Toxoplasma gondii and the blood-brain barrier. *Virulence, 3*(2), 182-192. doi:10.4161/viru.19004

Fiala, M., Liu, P. T., Espinosa-Jeffrey, A., Rosenthal, M. J., Bernard, G., Ringman, J. M., … Cashman, J. (2007). Innate immunity and transcription of MGAT-III and Toll-like receptors in Alzheimer's disease patients are improved by

bisdemethoxycurcumin. *Proceedings of the National Academy of Sciences of the United States of America*, *104*(31), 12849–12854. https://doi.org/10.1073/pnas.0701267104

Fiebich, B. L., Batista, C. R. A., Saliba, S. W., Yousif, N. M. and de Oliveira, A. C. P. (2018) Role of Microglia TLRs in Neurodegeneration. *Frontiers in cellular neuroscience,* 12 (1)**,** 329-339.

Fiedler K., Hulsse C., Straube W. & Briese V. (1999). Toxoplasmosis-antibody seroprevalence in Mecklenburg-Western Pomerania (In German). Zentralbl. Gynakol. 121, 239–243.

Filipcik, P., Cente, M., Ferencik, M., Hulin, I., & Novak, M. (2006). The role of oxidative stress in the pathogenesis of Alzheimer's disease. *Bratisl Lek Listy, 107*(9-10), 384-394.

Fischer, H. G., Bonifas, U., & Reichmann, G. (2000). Phenotype and functions of brain dendritic cells emerging during chronic infection of mice with Toxoplasma gondii. *J Immunol, 164*(9), 4826-4834.

Fischer, H. G., Nitzgen, B., Reichmann, G., Gross, U., & Hadding, U. (1997). Host cells of Toxoplasma gondii encystation in infected primary culture from mouse brain. *Parasitol Res, 83*(7), 637-641.

Flegr, J. (2007). Effects of toxoplasma on human behavior. *Schizophr Bull, 33*(3), 757-760. doi:10.1093/schbul/sbl074

Flegr, J., Klapilova, K., & Kankova, S. (2014). Toxoplasmosis can be a sexually transmitted infection with serious clinical consequences. Not all routes of

infection are created equal. *Med Hypotheses, 83*(3), 286-289. doi:10.1016/j.mehy.2014.05.019

Flegr, J., Prandota, J., Sovickova, M., & Israili, Z. H. (2014). Toxoplasmosis--a global threat. Correlation of latent toxoplasmosis with specific disease burden in a set of 88 countries. *PLoS ONE, 9*(3), 1-12. doi:10.1371/journal.pone.0090203

Flegr, J.; Klapilova, K.; Kankova, S. (2014). Toxoplasmosis can be a sexually transmitted infection with serious clinical consequences. Not all routes of infection are created equal. Medical Hypotheses. 83: 289-289.

Florimond, C., Liu, T., Menendez, M., Lippl, K., Christopher, J., Blader, I. J., & Road, M. (2018). TRAPPING LYSOSOMAL DEGRADATION OF PROLYL-HYDROXYLASE 2.

Folch, J., Ettcheto, M., Petrov, D., Abad, S., Pedros, I., Marin, M., . . . Camins, A. (2018). Review of the advances in treatment for Alzheimer disease: Strategies for combating beta-amyloid protein. *Neurologia, 33*(1), 47-58. doi: 10.1016/j.nrl.2015.03.012

Foster, T. C., Kyritsopoulos, C., & Kumar, A. (2017). Central role for NMDA receptors in redox mediated impairment of synaptic function during aging and Alzheimer's disease. *Behav Brain Res, 322*(Pt B), 223-232. doi: 10.1016/j.bbr.2016.05.012

Franck, J., Garin, Y.J. and Dumon, H. (2008). LDBio-Toxo II immunoglobulin G Western blot confirmatory test for anti-*Toxoplasma* antibody detection. *J Clin Microbiol.* 46(7), pp.2334-2338.

French, T., Düsedau, H. P., Steffen, J., Biswas, A., Ahmed, N., Hartmann, S., . . . Dunay, I. R. (2019). Neuronal impairment following chronic Toxoplasma gondii infection

is aggravated by intestinal nematode challenge in an IFN-γ-dependent manner. *Journal of Neuroinflammation, 16*(1), 159. doi: 10.1186/s12974-019-1539-8

Fuehrer, H. P., Blöschl, I., Siehs, C., & Hassl, A. (2010). Detection of Toxoplasma gondii, Neospora caninum, and Encephalitozoon cuniculi in the brains of common voles (Microtus arvalis) and water voles (Arvicola terrestris) by gene amplification techniques in western Austria (Vorarlberg). *Parasitology Research*, *107*(2), 469–473. https://doi.org/10.1007/s00436-010-1905-z

Fuhrer, H. P., Schneider, R., Walochnik, J., & Auer, H. (2010). Extraintestinal helminths of the common vole (Microtus arvalis) and the water vole (Arvicola terrestris) in Western Austria (Vorarlberg). *Parasitol Res, 106*(4), 1001-1004. doi:10.1007/s00436-010-1753-x.

Fuentes, I.; Rubio, J.M.; Ramírez, C. and Alvar, J. (2001). Genotypic Characterization of *Toxoplasma gondii* Strains Associated with Human Toxoplasmosis in Spain: Direct Analysis from Clinical Samples. Journal of Clinical Microbiology 39(4): 1566–1570.

Fujikake, N., Shin, M. and Shimizu, S. (2018) Association Between Autophagy and Neurodegenerative Diseases. *Frontiers in Neuroscience,* 12 (255-265).

Fulton, J. D., & Turk, J. L. (1959). Direct agglutination test for Toxoplasma gondii. *Lancet, 2*(7111), 1068-1069.

Gale, S.D., Brown, B.L., Berrett, A., Erickson, L.D., Hedges, D.W., 2014. Association between latent toxoplasmosis and major depression, generalised anxiety disorder and panic disorder in human adults. Folia Parasitol. (Praha) 61 (4), 285–292.

Gambuzza, M., Sofo, V., Salmeri, F., Soraci, L., Marino, S., & Bramanti, P. (2014). Toll-Like Receptors in Alzheimer's Disease: A Therapeutic Perspective. *CNS & Neurological Disorders - Drug Targets*, *13*(9), 1542–1558. https://doi.org/10.2174/1871527313666140806124850

Gao, D., Zhang, J., Zhao, J., Wen, H., Pan, J., Zhang, S., . . . Wang, S. (2014). Autophagy activated by Toxoplasma gondii infection in turn facilitates Toxoplasma gondii proliferation. *Parasitol Res, 113*(6), 2053-2058. doi: 10.1007/s00436-014-3853-5

Gao, J-M, He, Z-H, Xie, Y-T, Hide, G , Lai, D-H and Lun, Z-R (2019),.'The association between Toxoplasma gondii infection and postpartum blues' , Journal of Affective Disorders, 250 , pp. 404-409. https://doi.org/10.1016/j.jad.2019.03.013

Gatkowska, J., Wieczorek, M., Dziadek, B., Dzitko, K., & Dlugonska, H. (2012). Behavioral changes in mice caused by Toxoplasma gondii invasion of brain. *Parasitol Res, 111*(1), 53-58. doi:10.1007/s00436-011-2800-y

Gazzinelli, R. T., Amichay, D., Sharton-Kersten, T., Grunwald, E., Farber, J. M., & Sher, A. (1996). Role of macrophage-derived cytokines in the induction and regulation of cell-mediated immunity to Toxoplasma gondii. *Current Topics in Microbiology and Immunology*, *219*, 127–139. https://doi.org/10.1007/978-3-642-51014-4_12

Ghosh, D., Walton, J. L., Roepe, P. D., & Sinai, A. P. (2012). Autophagy is a cell death mechanism in Toxoplasma gondii. *Cell Microbiol, 14*(4), 589-607. doi: 10.1111/j.1462-5822.2011.01745.x

Giovannoni, G., Heales, S. J. R., Land, J. M., & Thompson, E. J. (1998). The potential role of nitric oxide in multiple sclerosis. *Multiple Sclerosis*, *4*(3), 212–216. https://doi.org/10.1177/135245859800400323

Glass, C. K., Saijo, K., Winner, B., Marchetto, M. C., & Gage, F. H. (2010). Mechanisms underlying inflammation in neurodegeneration. *Cell, 140*(6), 918-934. doi:10.1016/j.cell.2010.02.016

Gonzalez, L.E., Rojnik, B., Urrea, F., Urdaneta, H., Petrosino, P., Colasante, C., Pino, S. and Hernandez, L. (2007). *Toxoplasma gondii* infection lower anxiety as 118 measured in the plus-maze and social interaction tests in rats: A behavioral analysis. *Behavioural Brain Research.* **177**(1), pp.70-79.

Graille, M., Stura, E. A., Bossus, M., Muller, B. H., Letourneur, O., Battail-Poirot, N., … Ducancel, F. (2005). Crystal structure of the complex between the monomeric form of Toxoplasma gondii surface antigen 1 (SAG1) and a monoclonal antibody that mimics the human immune response. *Journal of Molecular Biology*, *354*(2), 447–458. https://doi.org/10.1016/j.jmb.2005.09.028

Groer, M.W., Yolken, R.H., Xiao, J.C., Beckstead, J.W., Fuchs, D., Mohapatra, S.S., et al.,(2011). Prenatal depression and anxiety in Toxoplasma gondii-positive women. Am. J.Obstet. Gynecol. 204 (5), 431–433.

Guillot-Sestier, M. V. and Town, T. (2018) Let's make microglia great again in neurodegenerative disorders. *J Neural Transm,* 125 (5)**,** 751-770.

Gutierrez, J., Rodriguez, M., Piedrola, G., & del Carmen Maroto, M. (1997). Detection of IgA and low-avidity IgG antibodies for the diagnosis of recent active toxoplasmosis. *Clin Microbiol Infect, 3*(6), 658-662.

De Roever-Bonnet,H. (1969). Congenital *Toxoplasma* infections in mice and hamsters infected with avirulent and virulent strains Trop. Geogr. Med., 21 (1969), pp. 443-450

Hamidinejat, H., Ghorbanpoor, M., Hosseini, H., Alavi, S. M., Nabavi, L., Jalali, M. H. R., … Mohammadaligol, S. (2010). Toxoplasma gondii infection in first-episode and inpatient individuals with schizophrenia. *International Journal of Infectious Diseases*, *14*(11), e978–e981. https://doi.org/10.1016/j.ijid.2010.05.018

Haass, C., & Selkoe, D. J. (2007). Soluble protein oligomers in neurodegeneration: lessons from the Alzheimer's amyloid beta-peptide. *Nat Rev Mol Cell Biol*, *8*(2), 101-112. doi:10.1038/nrm2101

Halonen, S. K. (2009). Role of autophagy in the host defense against Toxoplasma gondii in astrocytes. *Autophagy*, *5*(2), 268-269.

Halonen, S. K., Lyman, W. D., & Chiu, F. C. (1996). Growth and development of Toxoplasma gondii in human neurons and astrocytes. *J Neuropathol Exp Neurol*, *55*(11), 1150-1156.

Halonen, S. K., Taylor, G. A., & Weiss, L. M. (2001). Gamma interferon-induced inhibition of Toxoplasma gondii in astrocytes is mediated by IGTP. *Infect Immun*, *69*(9), 5573-5576.

Hamidinejat, H., Ghorbanpoor, M., Hosseini, H., Alavi, S. M., Nabavi, L., Jalali, M.

Hara, T., Nakamura, K., Matsui, M., Yamamoto, A., Nakahara, Y., Suzuki-Migishima, R., . . . Mizushima, N. (2006). Suppression of basal autophagy in neural cells

causes neurodegenerative disease in mice. *Nature, 441*(7095), 885-889. doi: 10.1038/nature04724

Halos, L., Thébault, A., Aubert, D., Thomas, M., Perret, C., Geers, R., … Villena, I. (2010). An innovative survey underlining the significant level of contamination by Toxoplasma gondii of ovine meat consumed in France. *International Journal for Parasitology*, *40*(2), 193–200. https://doi.org/10.1016/j.ijpara.2009.06.009

Harold, D., Abraham, R., Hollingworth, P., Sims, R., Gerrish, A., Hamshere, M. L., … Williams, J. (2009). Genome-wide association study identifies variants at CLU and PICALM associated with Alzheimer's disease. *Nature Genetics*, *41*(10), 1088–1093. https://doi.org/10.1038/ng.440

Haroon, F., Händel, U., Angenstein, F., Goldschmidt, J., Kreutzmann, P., Lison, H., Fischer, K.-D., Scheich, H., Wetzel, W., Schlüter, D. and Budinger, E. (2012) Toxoplasma gondii Actively Inhibits Neuronal Function in Chronically Infected Mice. *PLOS ONE,* 7 (4)**,** 1-8.

Harris, S. A., & Harris, E. A. (2015). Herpes Simplex Virus Type 1 and Other Pathogens are Key Causative Factors in Sporadic Alzheimer's Disease. *J Alzheimers Dis*, *48*(2), 319-353. doi:10.3233/JAD-142853

Harrison, Paul J.; Freemantle, Nick; Geddes, John R. (2003). Meta-analysis of brain weight in schizophrenia. Schizophrenia Research. 64 (1): 25–34.

He, C. and Levine, B. (2010) The Beclin 1 interactome. *Curr Opin Cell Biol*, 22 (2)**,** 140-149.

HE, X. L., GRIGG, M. E., BOOTHROYD, J. C. & GARCIA, K. C. 2002. Structure of the immunodominant surface antigen from the Toxoplasma gondii SRS superfamily. *Nat Struct Biol*, 9, 606-11.

Heldwein, K. A., Liang, M. D., Andresen, T. K., Thomas, K. E., Marty, A. M., Cuesta, N., . . . Fenton, M. J. (2003). TLR2 and TLR4 serve distinct roles in the host immune response against Mycobacterium bovis BCG. *Journal of leukocyte biology*, *74*(2), 277-286.

Herrmann, D. C., Maksimov, P., Maksimov, A., Sutor, A., Schwarz, S., Jaschke, W., . . . Schares, G. (2012). Toxoplasma gondii in foxes and rodents from the German Federal States of Brandenburg and Saxony-Anhalt: seroprevalence and genotypes. *Vet Parasitol, 185*(2-4), 78-85. doi:10.1016/j.vetpar.2011.10.030

Hickman, S. E., Allison, E. K., & El Khoury, J. (2008). Microglial dysfunction and defective β-amyloid clearance pathways in aging alzheimer's disease mice. *Journal of Neuroscience, 28*(33), 8354–8360. https://doi.org/10.1523/JNEUROSCI.0616-08.2008

Hinze-Selch, D., Daubener, W., Eggert, L., Erdag, S., Stoltenberg, R., & Wilms, S. (2007). A controlled prospective study of toxoplasma gondii infection in individuals with schizophrenia: beyond seroprevalence. *Schizophr Bull*, *33*(3), 782-788. doi:10.1093/schbul/sbm010

Hinze-Selch, D., Daubener, W., Erdag, S., Wilms, S., 2010. The diagnosis of a personality disorder increases the likelihood for seropositivity to Toxoplasma gondii in psychiatric patients. Folia Parasitol. (Praha) 57 (2), 129–135.

Holt, S. V., Wyspianska, B., Randall, K. J., James, D., Foster, J. R., & Wilkinson, R. W. (2011). The development of an immunohistochemical method to detect the autophagy-associated protein LC3-II in human tumor xenografts. *Toxicol Pathol, 39*(3), 516-523. doi: 10.1177/0192623310396903

Hsiao, K., Chapman, P., Nilsen, S., Eckman, C., Harigaya, Y., Younkin, S., . . . Cole, G. (1996). Correlative memory deficits, Abeta elevation, and amyloid plaques in transgenic mice. *Science, 274*(5284), 99-102.

Hu, W., Chan, H., Lu, L., Wong, K. T., Wong, S. H., Li, M. X., . . . Zhang, L. (2019). Autophagy in intracellular bacterial infection. *Seminars in Cell & Developmental Biology*. doi: https://doi.org/10.1016/j.semcdb.2019.07.014

Huang, J., Yang, J., Shen, Y., Jiang, H., Han, C., Zhang, G., . . . Wang, T. (2017). HMGB1 Mediates Autophagy Dysfunction via Perturbing Beclin1-Vps34 Complex in Dopaminergic Cell Model. *Front Mol Neurosci, 10*, 13. doi: 10.3389/fnmol.2017.00013

Huang, R., Xu, Y., Wan, W., Shou, X., Qian, J., You, Z., . . . Liu, W. (2015). Deacetylation of nuclear LC3 drives autophagy initiation under starvation. *Mol Cell, 57*(3), 456-466. doi: 10.1016/j.molcel.2014.12.013.

Hughes, J.M., Thomasson, D., Craig, P.S., Georgin, S., Pickles, A. and Hide, G. 19 (2008). Neospora caninum: detection in wild rabbits and investigation of co-infection with 20 Toxoplasma gondii by PCR analysis. Experimental Parasitology 120, 255-260.

Hughes, J.M., Williams, R.H., Morley, E.K., Cook, D.A.N., Terry, R.S., Murphy, R.G., 14 Smith, J.E. and Hide, G. (2006). The Prevalence of Neospora caninum and co-

infection 15 with Toxoplasma gondii by PCR analysis in naturally occurring mammal populations. 16 Parasitology 132, 29-36.

Hunt, J. B., Jr., Nash, K. R., Placides, D., Moran, P., Selenica, M. L., Abuqalbeen, F., Ratnasamy, K., Slouha, N., Rodriguez-Ospina, S., Savlia, M., Ranaweera, Y., Reid, P., Dickey, C. A., Uricia, R., Yang, C. G., Sandusky, L. A., Gordon, M. N., Morgan, D. and Lee, D. C. (2015a) Sustained Arginase 1 Expression Modulates Pathological Tau Deposits in a Mouse Model of Tauopathy. *J Neurosci,* 35 (44)**,** 14842-14860.

Hunt, J. B., Nash, K. R., Placides, D., Moran, P., Selenica, M.-L. B., Abuqalbeen, F., Ratnasamy, K., Slouha, N., Rodriguez-Ospina, S., Savlia, M., Ranaweera, Y., Reid, P., Dickey, C. A., Uricia, R., Yang, C. G. Y., Sandusky, L. A., Gordon, M. N., Morgan, D. and Lee, D. C. (2015b) Sustained Arginase 1 Expression Modulates Pathological Tau Deposits in a Mouse Model of Tauopathy. *The Journal of Neuroscience,* 35 (44)**,** 1-12.

Hunt, J., Jr., Nash, K., Placides, D., Moran, P., Rodrigez-Ospina, S., Yang, C. G. Y., Savlia, M., Selenica, M.-L., Gordon, M. N., Morgan, D. and Lee, D. (2013) Arginase-1 overexpression reduces tau pathology in rTg4510 tau transgenic mice. *Alzheimer's & Dementia: The Journal of the Alzheimer's Association,* 9 (4)**,** 307-308.

Hunter, C. A., & Sibley, L. D. (2012). Modulation of innate immunity by Toxoplasma gondii virulence effectors. *Nat Rev Microbiol, 10*(11), 766-778. doi: 10.1038/nrmicro2858

Hunter, C. A., Subauste, C. S., Van Cleave, V. H., & Remington, J. S. (1994). Production of gamma interferon by natural killer cells from Toxoplasma gondii-infected SCID mice: Regulation by interleukin-10, interleukin-12, and tumor necrosis factor alpha. *Infection and Immunity*, *62*(7), 2818–2824. https://doi.org/10.1128/iai.62.7.2818-2824.1994

Hurley, R. A., & Taber, K. H. (2012). Latent Toxoplasmosis gondii: Emerging evidence for influences on neuropsychiatric disorders. *The Journal of neuropsychiatry and clinical neurosciences, 24*(4), 376-383.

Hwang, Y. S., Shin, J.-H., Yang, J.-P., Jung, B.-K., Lee, S. H., & Shin, E.-H. (2018). Characteristics of Infection Immunity Regulated by Toxoplasma gondii to Maintain Chronic Infection in the Brain. *Frontiers in Immunology, 9*(1), 158-168. doi:10.3389/fimmu.2018.00158

Ihara, F., Tanaka, S., Fereig, R. M., Nishimura, M. and Nishikawa, Y. (2019) Involvement of Toll-like receptor 2 in the cerebral immune response and behavioral changes caused by latent Toxoplasma infection in mice. *PloS one,* 14 (8)**,** 1-8. *Infect,* 133, 475-483.

Into, T., Inomata, M., Takayama, E., & Takigawa, T. (2012). Autophagy in regulation of Toll-like receptor signaling. *Cell Signal, 24*(6), 1150-1162. doi: 10.1016/j.cellsig.2012.01.020

Ionita, C., Wasay, M., Balos, L., & Bakshi, R. (2004). MR imaging in toxoplasmosis encephalitis after bone marrow transplantation: paucity of enhancement despite fulminant disease. *AJNR Am J Neuroradiol, 25*(2), 270-273.

J.K.A Beverley (1959).Congenital transmission of toxoplasmosis through successive generations of mice Nature, 183 , pp. 1348-1349.

Remington, J. S., Jacobs, L., & Marjorie, L. (1961). Congenital transmission of toxoplasmosis from mother animals with acute and chronic infections J. Infect. Dis., 108 (1961), pp. 163-173.

Jaeger, P. A., & Wyss-Coray, T. (2010). Beclin 1 complex in autophagy and Alzheimer disease. *Arch Neurol, 67*(10), 1181-1184. doi:10.1001/archneurol.2010.258

Jaeger, P. A., Pickford, F., Sun, C. H., Lucin, K. M., Masliah, E. and Wyss-Coray, T. (2010) Regulation of amyloid precursor protein processing by the Beclin 1 complex. *PLoS One,* 5 (6)**,** 1-10.

Charles A. Janeway, J. and, & Medzhitov, R. (2003). Innate Immune Recognition. *Http://Dx.Doi.Org/10.1146/Annurev.Immunol.20.083001.084359*, 197–216.

Jaso, B. A., Niciu, M. J., Iadarola, N. D., Lally, N., Richards, E. M., Park, M., . . . Zarate, C. A. (2017). Therapeutic Modulation of Glutamate Receptors in Major Depressive Disorder. *Curr Neuropharmacol, 15*(1), 57-70.

Jeon, S.H. and Yong, T.S. (2000). Serological observation of *Toxoplasma gondii* prevalence in *Apodemus agrarius*, a dominant species of field rodents in Korea. Yonsei Medical Journ*al.* 41(4): 491-496.

Jiang, P. D., & Mizushima, N. (2014). Autophagy and human diseases. *Cell Research, 24*(1), 69-79.

Jones, C. D., Okhravi, N., Adamson, P., Tasker, S., & Lightman, S. (2000). Comparison of PCR detection methods for B1, P30, and 18S rDNA genes of *T. gondii* in aqueous humor.*Investigative ophthalmology & visual science*, *41*(3), 634-644.

Jones-Brando, L., Torrey, E. F., & Yolken, R. (2003). Drugs used in the treatment of schizophrenia and bipolar disorder inhibit the replication of Toxoplasma gondii. *Schizophr Res*, *62*(3), 237-244.

Jung, B. K., Pyo, K. H., Shin, K. Y., Hwang, Y. S., Lim, H., Lee, S. J., … Shin, E. H. (2012). Toxoplasma gondii infection in the brain inhibits neuronal degeneration and learning and memory impairments in a murine model of alzheimer's disease. *PLoS ONE*, *7*(3), 1–11. https://doi.org/10.1371/journal.pone.0033312

Kálmán, J., Kitajka, K., Pákáski, M., Zvara, Á., Juhász, A., Vincze, G., … Puskás, L. G. (2005). Gene expression profile analysis of lymphocytes from Alzheimer's patients. *Psychiatric Genetics*, *15*(1), 1–6. https://doi.org/10.1097/00041444-200503000-00001

Kamerkar, S., & Davis, P. H. (2012). Toxoplasma on the brain: Understanding host-pathogen interactions in chronic CNS infection. *Journal of Parasitology Research*, *2012*. https://doi.org/10.1155/2012/589295

Kametani, F. and Hasegawa, M. (2018) Reconsideration of Amyloid Hypothesis and Tau Hypothesis in Alzheimer's Disease. *Frontiers in neuroscience,* 12 (1)**,** 25-35.

Kaňková, Š. and Flegr, J. (2007). Longer pregnancy and slower fetal development in women with latent “asymptomatic” toxoplasmosis. BMC Infection Diseases 7, 114. doi:10.1186/1471-233-7-114

Kannan, G., Moldovan, K., Xiao, J. C., Yolken, R. H., Jones-Brando, L. and Pletnikov, M. V. (2010) Toxoplasma gondii strain-dependent effects on mouse behaviour. *Folia Parasitol,* 57 (2)**,** 151-155.

Kempuraj, D., Thangavel, R., Natteru, P., Selvakumar, G., Saeed, D., Zahoor, H., . . . Zaheer, A. (2016). Neuroinflammation induces neurodegeneration. *Journal of neurology, neurosurgery and spine, 1*(1).

Kershaw, N. J., Murphy, J. M., Lucet, I. S., Nicola, N. A. and Babon, J. J. (2013) Regulation of Janus kinases by SOCS proteins. *Biochem Soc Trans,* 41 (4)**,** 1042-1047.

Kijlstra, A.; Meerburg, B.; Cornelissen, J.; De Craeye, S.; Vereijken, P. and Jongert, E. (2008). The role of rodents and shrews in the transmission of *Toxoplasma gondii* to pigs. Veterinary Parasitology. 156(3-4): 183-190.

Kim, L., & Denkers, E. Y. (2006). Toxoplasma gondii triggers Gi-dependent PI 3-kinase signaling required for inhibition of host cell apoptosis. *J Cell Sci, 119*(10), 2119-2126. doi:10.1242/jcs.02934

Koch, H. J. & Szecsey, A. (2000). A randomized controlled trial of prednisone in Alzheimer's disease. *Neurology*, 55, 1067.

Kokkinopoulos, I., Jordan, W. J., & Ritter, M. A. (2005). Toll-like receptor mRNA expression patterns in human dendritic cells and monocytes. *Molecular Immunology, 42*(8), 957–968. https://doi.org/10.1016/j.molimm.2004.09.037

Komai-Koma, M., Jones, L., Ogg, G. S., Xu, D., & Liew, F. Y. (2004). TLR2 is expressed on activated T cells as a costimulatory receptor. *Proceedings of the National Academy of Sciences, 101*(9), 3029-3034.

Komatsu, M., Waguri, S., Chiba, T., Murata, S., Iwata, J., Tanida, I., . . . Tanaka, K. (2006). Loss of autophagy in the central nervous system causes neurodegeneration in mice. *Nature, 441*(7095), 880-884. doi: 10.1038/nature04723

Komatsu, M., Wang, Q. J., Holstein, G. R., Friedrich, V. L., Jr., Iwata, J., Kominami, E., . . . Yue, Z. (2007). Essential role for autophagy protein Atg7 in the maintenance of axonal homeostasis and the prevention of axonal degeneration. *Proc Natl Acad Sci U S A, 104*(36), 14489-14494. doi: 10.1073/pnas.0701311104

Kompalic-Cristo, A., Frotta, C., Suarez-Mutis, M., Fernandes, O., & Britto, C. (2007). Evaluation of a real-time PCR assay based on the repetitive B1 gene for the detection of Toxoplasma gondii in human peripheral blood. *Parasitol Res, 101*(3), 619-625. doi:10.1007/s00436-007-0524-9

Kong, J. T., Grigg, M. E., Uyetake, L., Parmley, S., & Boothroyd, J. C. (2003). Serotyping of Toxoplasma gondii infections in humans using synthetic peptides. *J Infect Dis, 187*(9), 1484-1495. doi:10.1086/374647

Konradt, C., Ueno, N., Christian, D. A., Delong, J. H., Pritchard, G. H., Herz, J., . . . Hunter, C. A. (2016). Endothelial cells are a replicative niche for entry of Toxoplasma gondii to the central nervous system. *Nat Microbiol, 1*, 16001. doi:10.1038/nmicrobiol.2016.1

Koshy, A. A., Fouts, A. E., Lodoen, M. B., Alkan, O., Helen, M., & Boothroyd, J. C. (2010). Toxoplasma secreting Cre recombinase for analysis of host-Parasite Interactions, *7*(4), 307–309. https://doi.org/10.1038/nmeth.1438.Toxoplasma

Kouno, T., Mizuguchi, M., Tanida, I., Ueno, T., Kanematsu, T., Mori, Y., . . . Kawano, K. (2005). Solution structure of microtubule-associated protein light chain 3 and identification of its functional subdomains. *J Biol Chem, 280*(26), 24610-24617. doi: 10.1074/jbc.M413565200.

Kourenti, C., & Karanis, P. (2006). Evaluation and applicability of a purification method coupled with nested PCR for the detection of Toxoplasma oocysts in water. *Lett Appl Microbiol, 43*(5), 475-481. doi:10.1111/j.1472-765X.2006.02008.x

Krishnamurthy, S., Konstantinou, E. K., Young, L. H., Gold, D. A., & Saeij, J. P. (2017). The human immune response to Toxoplasma: Autophagy versus cell death. *PLoS Pathog, 13*(3), e1006176. doi: 10.1371/journal.ppat.1006176.

Krstic, D., Madhusudan, A., Doehner, J., Vogel, P., Notter, T., Imhof, C., . . . Knuesel, I. (2012). Systemic immune challenges trigger and drive Alzheimer-like neuropathology in mice. *J Neuroinflammation, 9*, 151. doi:10.1186/1742-2094-9-151

Kusbeci, O. Y., Miman, O., Yaman, M., Aktepe, O. C., & Yazar, S. (2011). Could Toxoplasma gondii have any role in Alzheimer disease? *Alzheimer Dis Assoc Disord, 25*(1), 1-3. doi:10.1097/WAD.0b013e3181f73bc2

Lacor, P. N., Buniel, M. C., Furlow, P. W., Clemente, A. S., Velasco, P. T., Wood, M., . . . Klein, W. L. (2007). Abeta oligomer-induced aberrations in synapse composition, shape, and density provide a molecular basis for loss of connectivity in Alzheimer's disease. *J Neurosci, 27*(4), 796-807. doi: 10.1523/JNEUROSCI.3501-06.2007

LaFerla, F. M., Green, K. N., & Oddo, S. (2007). Intracellular amyloid-beta in Alzheimer's disease. *Nat Rev Neurosci, 8*(7), 499-509. doi:10.1038/nrn2168

Lalancette-Hebert, M., Phaneuf, D., Soucy, G., Weng, Y. C. and Kriz, J. (2009) Live imaging of Toll-like receptor 2 response in cerebral ischaemia reveals a role of olfactory bulb microglia as modulators of inflammation. *Brain,* 132 (4)**,** 940-954.

Lambris, J. D., & Sahu, A. (2001). Structure and biology of complement protein C3, a connecting link between innate and acquired immunity. *Immunological Reviews*, *180*(4), 35–48.

Lambert, J. C., Heath, S., Even, G., Campion, D., Sleegers, K., Hiltunen, M., … Pilotto, A. (2009). Genome-wide association study identifies variants at CLU and CR1 associated with Alzheimer’s disease. *Nature Genetics*, *41*(10), 1094–1099. https://doi.org/10.1038/ng.439

Landreth GE, & Reed-Geaghan EG. (2009). Chapter 8. TLRs in Alzheimer’s Disease. *Current Topics in Microbiology and Immunology*, *336*(Gorelick 2004), 1–14. https://doi.org/10.1007/978-3-642-00549-7

Lang, D., Schott, B. H., van Ham, M., Morton, L., Kulikovskaja, L., Herrera-Molina, R., … Dunay, I. R. (2018). Chronic Toxoplasma infection is associated with distinct

alterations in the synaptic protein composition. *Journal of Neuroinflammation*, *15*(1), 1–19. https://doi.org/10.1186/s12974-018-1242-1

Lapierre, J., Rodriguez, M., Ojha, C. R., & El-Hage, N. (2018). Critical Role of Beclin1 in HIV Tat and Morphine-Induced Inflammation and Calcium Release in Glial Cells from Autophagy Deficient Mouse. *J Neuroimmune Pharmacol*. doi: 10.1007/s11481-018-9788-3.

Lau, Y. L., Meganathan, P., Sonaimuthu, P., Thiruvengadam, G., Nissapatorn, V., & Chen, Y. (2010). Specific, sensitive, and rapid diagnosis of active toxoplasmosis by a loop-mediated isothermal amplification method using blood samples from patients. *J Clin Microbiol, 48*(10), 3698-3702. doi:10.1128/JCM.00462-10.

Law, A., Gauthier, S., & Quirion, R. (2001). Say NO to Alzheimer's disease: The putative links between nitric oxide and dementia of the Alzheimer's type. *Brain Research Reviews*, *35*(1), 73–96. https://doi.org/10.1016/S0165-0173(00)00051-5

Lee, S. J., & Lee, S. (2002). Toll-like receptors and inflammation in the CNS. *Current Drug Targets. Inflammation and Allergy*, *1*(2), 181–191.

Lee, Y. J., Song, H. O., Lee, Y. H., Ryu, J. S., & Ahn, M. H. (2013). Proliferation of Toxoplasma gondii suppresses host cell autophagy. *Korean J Parasitol, 51*(3), 279-287. doi: 10.3347/kjp.2013.51.3.279.

Lee, Y., Sasai, M., Ma, J. S., Sakaguchi, N., Ohshima, J., Bando, H., . . . Yamamoto, M. (2015). p62 Plays a Specific Role in Interferon-gamma-Induced Presentation of a Toxoplasma Vacuolar Antigen. *Cell Rep, 13*(2), 223-233. doi: 10.1016/j.celrep.2015.09.005

Lei, P., Ayton, S., Finkelstein, D. I., Adlard, P. A., Masters, C. L., & Bush, A. I. (2010). Tau protein: relevance to Parkinson's disease. *The international journal of biochemistry & cell biology, 42*(11), 1775-1778.

Leonard, B. E., & Myint, A. (2006). Inflammation and depression: Is there a causal connection with dementia? *Neurotoxicity Research*, *10*(2), 149–160. https://doi.org/10.1007/BF03033243

Leonard, B. E. (2007). Inflammation, depression and dementia: Are they connected? *Neurochemical Research*, *32*(10), 1749–1756. https://doi.org/10.1007/s11064-007-9385-y

Li, W., Zhu, J., Dou, J., She, H., Tao, K., Xu, H., . . . Mao, Z. (2017). Phosphorylation of LAMP2A by p38 MAPK couples ER stress to chaperone-mediated autophagy. *Nat Commun, 8*(1), 1763. doi: 10.1038/s41467-017-01609-x

Li, X., Chen, D., Hua, Q., Wan, Y., Zheng, L., Liu, Y., . . . Tan, F. (2016). Induction of Autophagy interferes the tachyzoite to bradyzoite transformation of Toxoplasma gondii. *Parasitology*, *143*(5), 639-645. doi: 10.1017/S0031182015001985

Li, Y., Severance, E. G., Viscidi, R. P., Yolken, R. H. and Xiao, J. (2019) Persistent Toxoplasma Infection of the Brain Induced Neurodegeneration Associated with Activation of Complement and Microglia. *Infection and Immunity,* 87 (8)**,** 139-149.

Li, Z.; Zhao, Z.J.; Zhu, X.Q.; Ren, Q.S.; Nie, F.F.; Gao, J.M.; Gao, X.J.; Yang, T.B.; Zhou, W.L.; Shen, J.L.; Wang, Y.; Lu, F.L.; Chen, X.G.; Hide, G.; Ayala, F.J. and

Lun, Z.R. (2012). Differences in iNOS and arginase expression and activity in the macrophages of rats are responsible for the resistance against *T. gondii* infection. PLoS One 7, e35834.

Liau, N. P. D., Laktyushin, A., Lucet, I. S., Murphy, J. M., Yao, S., Whitlock, E., . . . Babon, J. J. (2018). The molecular basis of JAK/STAT inhibition by SOCS1. *Nat Commun, 9*(1), 1558. doi: 10.1038/s41467-018-04013-1

Lin, C. Y., & Tsai, C. W. (2017). Carnosic Acid Attenuates 6-Hydroxydopamine-Induced Neurotoxicity in SH-SY5Y Cells by Inducing Autophagy Through an Enhanced Interaction of Parkin and Beclin1. *Mol Neurobiol, 54*(4), 2813-2822. doi: 10.1007/s12035-016-9873-7

Ling, Y. M., Shaw, M. H., Ayala, C., Coppens, I., Taylor, G. A., Ferguson, D. J., & Yap, G. S. (2006). Vacuolar and plasma membrane stripping and autophagic elimination of Toxoplasma gondii in primed effector macrophages. *J Exp Med, 203*(9), 2063-2071. doi: 10.1084/jem.20061318.

Lippai, M., & Low, P. (2014). The role of the selective adaptor p62 and ubiquitin-like proteins in autophagy. *Biomed Res Int, 2014*, 832704. doi: 10.1155/2014/832704

Lipton, S. A. (2006). Paradigm shift in neuroprotection by NMDA receptor blockade: memantine and beyond. *Nat Rev Drug Discov, 5*(2), 160-170. doi: 10.1038/nrd1958

Liu CC, Kanekiyo T, Xu H, B. G. (2013). Apolipoprotein E and Alzheimer disease: risk, mechanisms and therapy. Nat Rev Neurol. *Nature Reviews Neuroscience*, *9*(2), 106–118. https://doi.org/10.1038/nrneurol.2012.263.Apolipoprotein

Liu, E., Lopez Corcino, Y., Portillo, J.-A. C., Miao, Y., & Subauste, C. S. (2016). Identification of Signaling Pathways by Which CD40 Stimulates Autophagy and Antimicrobial Activity against Toxoplasma gondii in Macrophages. *Infect Immun, 84*(9), 2616-2626. doi:10.1128/IAI.00101-16

Liu, J., Chang, L., Song, Y., Li, H. and Wu, Y. (2019) The Role of NMDA Receptors in Alzheimer's Disease. *Frontiers in neuroscience,* 13 (1)**,** 43-53.

Lucin, K. M., O'Brien, C. E., Bieri, G., Czirr, E., Mosher, K. I., Abbey, R. J., . . . Wyss-Coray, T. (2013). Microglial beclin 1 regulates retromer trafficking and phagocytosis and is impaired in Alzheimer's disease. *Neuron, 79*(5), 873-886. doi:10.1016/j.neuron.2013.06.046

Luft, B. J., & Chua, A. (2000). Central nervous system toxoplasmosis in HIV pathogenesis, diagnosis, and therapy. *Current Infectious Disease Reports*, *2*(4), 358–362. https://doi.org/10.1007/s11908-000-0016-x

Maes, O. C., Xu, S., Yu, B., Chertkow, H. M., Wang, E., & Schipper, H. M. (2007). Transcriptional profiling of Alzheimer blood mononuclear cells by microarray. *Neurobiology of Aging*, *28*(12), 1795–1809. https://doi.org/10.1016/j.neurobiolaging.2006.08.004

Mahami Oskouei, M., Hamidi, F., Talebi, M., Farhoudi, M., Taheraghdam, A. A., Kazemi, T., . . . Fallah, E. (2016). The correlation between Toxoplasma gondii

infection and Parkinson's disease: a case-control study. *J Parasit Dis, 40*(3), 872-876. doi:10.1007/s12639-014-0595-3

Mahami-Oskouei, M., Hamidi, F., Talebi, M., Farhoudi, M., Taheraghdam, A. A., Kazemi, T., Sadeghi-Bazargani, H. and Fallah, E. (2016) Toxoplasmosis and Alzheimer: can Toxoplasma gondii really be introduced as a risk factor in etiology of Alzheimer? *Parasitol Res,* 115 (8)**,** 3169-3174. doi:10.1007/s00436-016-5075-5

Mahittikorn, A., Wickert, H., & Sukthana, Y. (2005). Comparison of five DNA extraction methods and optimization of a b1 gene nested PCR (nPCR) for detection of Toxoplasma gondii tissue cyst in mouse brain. *Southeast Asian J Trop Med Public Health, 36*(6), 1377-1382.

Mahley, R. W., Weisgraber, K. H., & Huang, Y. (2006). Apolipoprotein E4: a causative factor and therapeutic target in neuropathology, including Alzheimer's disease. *Proc Natl Acad Sci U S A, 103*(15), 5644-5651.

Mammari, N., Vignoles, P., Halabi, M. A., Darde, M. L., & Courtioux, B. (2014). In vitro infection of human nervous cells by two strains of Toxoplasma gondii: A kinetic analysis of immune mediators and parasite multiplication. *PLoS ONE, 9*(6). https://doi.org/10.1371/journal.pone.0098491

Mammari, N., Vignoles, P., Halabi, M. A., Dardé, M. L., & Courtioux, B. (2015). Interferon gamma effect on immune mediator production in human nerve cells infected by two strains of Toxoplasma gondii. *Parasite, 22*. https://doi.org/10.1051/parasite/2015039

Manabe, T. (2017). NMDA receptor-independent long-term potentiation in hippocampal interneurons. *J Physiol, 595*(11), 3263-3264. doi: 10.1113/JP274093

Marshall, P. A., Hughes, J. M., Williams, R. H., Smith, J. E., Murphy, R. G., & Hide, G. (2004). Detection of high levels of congenital transmission of Toxoplasma gondii in natural urban populations of Mus domesticus. *Parasitology, 128*(P1), 39-42. doi:10.1017/s0031182003004189

Martin, H. L., Alsaady, I., Howell, G., Prandovszky, E., Peers, C., Robinson, P., & McConkey, G. A. (2015). Effect of parasitic infection on dopamine biosynthesis in dopaminergic cells. *Neuroscience, 306*, 50-62. doi:10.1016/j.neuroscience.2015.08.005

Mauvezin, C., Orpinell, M., Francis, V. A., Mansilla, F., Duran, J., Ribas, V., . . . Zorzano, A. (2010). The nuclear cofactor DOR regulates autophagy in mammalian and Drosophila cells. *EMBO Rep, 11*(1), 37-44. doi: 10.1038/embor.2009.242

Mazumder, P., Chuang, H. Y., Wentz, M. W., & Wiedbrauk, D. L. (1988). Latex agglutination test for detection of antibodies to Toxoplasma gondii. *J Clin Microbiol, 26*(11), 2444-2446.

McCoy, M. K., Martinez, T. N., Ruhn, K. A., Szymkowski, D. E., Smith, C. G., Botterman, B. R., … Tansey, M. G. (2006). Blocking soluble tumor necrosis factor signaling with dominant-negative tumor necrosis factor inhibitor attenuates loss of dopaminergic neurons in models of Parkinson's disease. *Journal of Neuroscience, 26*(37), 9365–9375. https://doi.org/10.1523/JNEUROSCI.1504-06.2006

Meadowcroft, M. D., Connor, J. R., Smith, M. B., & Yang, Q. X. (2009). MRI and histological analysis of beta-amyloid plaques in both human Alzheimer's disease and APP/PS1 transgenic mice. *Journal of Magnetic Resonance Imaging: An Official Journal of the International Society for Magnetic Resonance in Medicine, 29*(5), 997-1007.

Mehta, P., Henault, J., Kolbeck, R., & Sanjuan, M. A. (2014). Noncanonical autophagy: one small step for LC3, one giant leap for immunity. *Current Opinion in Immunology*, 26, 69-75. doi: https://doi.org/10.1016/j.coi.2013.10.012

Melzer, T., Duffy, A., Weiss, L. M., & Halonen, S. K. (2008). The gamma interferon (IFN-gamma)-inducible GTP-binding protein IGTP is necessary for toxoplasma vacuolar disruption and induces parasite egression in IFN-gamma-stimulated astrocytes. *Infect Immun, 76*(11), 4883-4894. doi: 10.1128/IAI.01288-07

Menati Rashno, M., Fallahi, S., Khreiandish, F. and Birjandi, M. (2016) Seroprevalence of Toxoplasma gondii Infection in Patients with Alzheimer's Disease. *Archives of Clinical Infectious Diseases,* 11 (3)**,** 1-13.

Mendez, O. A., & Koshy, A. A. (2017). Toxoplasma gondii: Entry, association, and physiological influence on the central nervous system. *PLoS Pathog, 13*(7), 1-10. doi:10.1371/journal.ppat.1006351

Miao, D. M., Honda, Y., Tanaka, K., Higashi, A., Nakamura, T., Taguchi, Y., . . . Bagdasarian, M. (1993). A base-paired hairpin structure essential for the functional priming signal for DNA replication of the broad host range plasmid RSF1010. *Nucleic Acids Res, 21*(21), 4900-4903.

Miao, D. M., Honda, Y., Tanaka, K., Higashi, A., Nakamura, T., Taguchi, Y., . . . Bagdasarian, M. (1993). A base-paired hairpin structure essential for the functional priming signal for DNA replication of the broad host range plasmid RSF1010. *Nucleic Acids Res, 21*(21), 4900-4903.

Michiorri, S., Gelmetti, V., Giarda, E., Lombardi, F., Romano, F., Marongiu, R., . . . Casari, G. (2010). The Parkinson-associated protein PINK1 interacts with Beclin1 and promotes autophagy. *Cell Death Differ, 17*(6), 962-974. doi: 10.1038/cdd.2009.200

Miller, M. J., Sunshine, P. J., & Remington, J. S. (1969). Quantitation of cord serum IgM and IgA as a screening procedure to detect congenital infection: results in 5,006 infants. *J Pediatr, 75*(6), 1287-1291.

Miman, O., Kusbeci, O. Y., Aktepe, O. C., & Cetinkaya, Z. (2010). The probable relation between Toxoplasma gondii and Parkinson's disease. *Neurosci Lett, 475*(3), 129-131. doi:10.1016/j.neulet.2010.03.057

Mizushima, N. and Yoshimori, T. (2007) How to interpret LC3 immunoblotting. *Autophagy*, 3 (6)**,** 542-545.

Mizushima, N., & Komatsu, M. (2011). Autophagy: renovation of cells and tissues. *Cell, 147*(4), 728-741. doi: 10.1016/j.cell.2011.10.026

Mizushima, N., Yamamoto, A., Matsui, M., Yoshimori, T., & Ohsumi, Y. (2004). In vivo analysis of autophagy in response to nutrient starvation using transgenic mice expressing a fluorescent autophagosome marker. *Mol Biol Cell, 15*(3), 1101-1111. doi: 10.1091/mbc.E03-09-0704.

Mohle, L., Israel, N., Paarmann, K., Krohn, M., Pietkiewicz, S., Müller, A., . . . Dunay, I. R. (2016). Chronic Toxoplasma gondii infection enhances β-amyloid phagocytosis and clearance by recruited monocytes. *Acta Neuropathologica Communications, 4*(1), 25-32. doi:10.1186/s40478-016-0293-8

Mohle, L., Israel, N., Paarmann, K., Krohn, M., Pietkiewicz, S., Muller, A., . . . Dunay, I. R. (2016). Chronic Toxoplasma gondii infection enhances beta-amyloid phagocytosis and clearance by recruited monocytes. *Acta Neuropathol Commun, 4*, 25. doi:10.1186/s40478-016-0293-8.

Morley, E.K., Williams, R.H., Hughes, J.M., Terry, R.S., Duncanson, P., Smith, J.E. 19 and Hide, G. (2005). Significant familial differences in the frequency of abortion and 20 Toxoplasma gondii infection within a flock of Charollais sheep. Parasitology 131, 181-185.

Mun, H. S., Aosai, F., Norose, K., Chen, M., Piao, L. X., Takeuchi, O., … Yano, A. (2003). TLR2 as an essential molecule for protective immunity against Toxoplasma gondii infection. *International Immunology, 15*(9), 1081–1087. https://doi.org/10.1093/intimm/dxg108

Mun, H. S., Aosai, F., Norose, K., Chen, M., Piao, L. X., Takeuchi, O., . . . Yano, A. (2003). TLR2 as an essential molecule for protective immunity against Toxoplasma gondii infection. *Int Immunol, 15*(9), 1081-1087.

Muniz-Feliciano, L., Van Grol, J., Portillo, J. A., Liew, L., Liu, B., Carlin, C. R., . . . Subauste, C. S. (2013). Toxoplasma gondii-induced activation of EGFR prevents autophagy protein-mediated killing of the parasite. *PLoS Pathog, 9*(12), 1-10. doi:10.1371/journal.ppat.1003809

Murphy RG, Williams RH, Hughes JM, Hide G, Ford NJ, Oldbury DJ. The urban house mouse (Mus domesticus) as a reservoir of infection for the human parasite Toxoplasma gondii: an unrecognised public health issue? International Journal of Environmental Health Research 2008;18:177-185.

Must, K., Hytonen, M. K., Orro, T., Lohi, H., & Jokelainen, P. (2017). Toxoplasma gondii seroprevalence varies by cat breed. *PLoS ONE, 12*(9), 1-12. doi:10.1371/journal.pone.0184659

Nath, A., & Sinai, A. P. (2003). *Cerebral toxoplasmosis. Current Treatment Options in Neurology, 5(1), 3–12.* doi:10.1007/s11940-003-0018-8

Nayeri Chegeni, T., Sarvi, S., Moosazadeh, M., Sharif, M., Aghayan, S. A., Amouei, A., Hosseininejad, Z. and Daryani, A. (2019) Is Toxoplasma gondii a potential risk factor for Alzheimer's disease? A systematic review and meta-analysis. *Microb Pathog*, 137 (1)**,** 1-12.

Ngô, H. M., Zhou, Y., Lorenzi, H., Wang, K., Kim, T. K., Zhou, Y., … McLeod, R. (2017). Toxoplasma Modulates Signature Pathways of Human Epilepsy, Neurodegeneration & Cancer. *Scientific Reports*, *7*(1), 1–32. https://doi.org/10.1038/s41598-017-10675-6

Nguyen, H. M., Berry, L., Sullivan, W. J., Jr., & Besteiro, S. (2017). Autophagy participates in the unfolded protein response in Toxoplasma gondii. *FEMS Microbiol Lett, 364*(15). doi: 10.1093/femsle/fnx153

Niu, H., Xiong, Q., Yamamoto, A., Hayashi-Nishino, M., & Rikihisa, Y. (2012). Autophagosomes induced by a bacterial Beclin 1 binding protein facilitate

obligatory intracellular infection. *Proc Natl Acad Sci U S A, 109*(51), 20800-20807. doi: 10.1073/pnas.1218674109.

Nixon, R. A., & Yang, D. S. (2011). Autophagy failure in Alzheimer's disease--locating the primary defect. *Neurobiol Dis, 43*(1), 38-45. doi: 10.1016/j.nbd.2011.01.021

Nixon, R. A., Cataldo, A. M., & Mathews, P. M. (2000). The endosomal-lysosomal system of neurons in Alzheimer's disease pathogenesis: a review. *Neurochem Res, 25*(9-10), 1161-1172.

Nogareda, F., Le Strat, Y., Villena, I., De Valk, H., & Goulet, V. (2014). Incidence and prevalence of Toxoplasma gondii infection in women in France, 1980-2020: Model-based estimation. *Epidemiology and Infection*, *142*(8), 1661–1670. https://doi.org/10.1017/S0950268813002756

Nourollahpour Shiadeh, M., Rostami, A., Pearce, B.D., Gholipourmalekabadi, M.,

Nowak, R.M. 1991. Walker's Mammals of the World. 5th Ed. Vol II. Johns Hopkins University Press, Baltimore.

O'Brien, R. J. and Wong, P. C. (2011) Amyloid precursor protein processing and Alzheimer's disease. *Annual review of neuroscience,* 34 (1)**,** 185-204.

Oddo, S., Caccamo, A., Shepherd, J. D., Murphy, M. P., Golde, T. E., Kayed, R., . . . LaFerla, F. M. (2003). Triple-transgenic model of Alzheimer's disease with plaques and tangles: intracellular Abeta and synaptic dysfunction. *Neuron, 39*(3), 409-421.

Ohshima, J., Lee, Y., Sasai, M., Saitoh, T., Su Ma, J., Kamiyama, N., . . . Yamamoto, M. (2014). Role of mouse and human autophagy proteins in IFN-gamma-induced cell-autonomous responses against Toxoplasma gondii. *J Immunol, 192*(7), 3328-3335. doi: 10.4049/jimmunol.1302822

Okamoto, T., Takeda, S., Giambarella, U., Murayama, Y., Matsui, T., Katada, T., . . . Nishimoto, I. (1996). Intrinsic signaling function of APP as a novel target of three V642 mutations linked to familial Alzheimer's disease. *EMBO J, 15*(15), 3769-3777.

Oliveira, J., Kazma, R., Le Floch, E., Bennabi, M., Hamdani, N., Bengoufa, D., . . . Tamouza, R. (2016). Toxoplasma gondii exposure may modulate the influence of TLR2 genetic variation on bipolar disorder: a gene-environment interaction study. *Int J Bipolar Disord, 4*(1), 11. doi: 10.1186/s40345-016-0052-6

Osellame, L. D., Rahim, A. A., Hargreaves, I. P., Gegg, M. E., Richard-Londt, A., Brandner, S., . . . Duchen, M. R. (2013). Mitochondria and quality control defects in a mouse model of Gaucher disease--links to Parkinson's disease. *Cell Metab, 17*(6), 941-953. doi: 10.1016/j.cmet.2013.04.014

Owen, M.R. and Trees, A.J. (1998). Vertical transmission of *Toxoplasma gondii* from chronically infected house (*Mus musculus*) and field (*Apodemus sylvaticus*) mice determined by polymerase chain reaction. Parasitology. 116(4): 299-304.

Pajares, M., Rojo, A. I., Arias, E., Diaz-Carretero, A., Cuervo, A. M., & Cuadrado, A. (2018). Transcription factor NFE2L2/NRF2 modulates chaperone-mediated autophagy through the regulation of LAMP2A. *Autophagy*. doi: 10.1080/15548627.2018.1474992.

Pappas, G., Roussos, N., & Falagas, M. E. (2009). Toxoplasmosis snapshots: global status of Toxoplasma gondii seroprevalence and implications for pregnancy and congenital toxoplasmosis. *Int J Parasitol, 39*(12), 1385-1394. doi:10.1016/j.ijpara.2009.04.003

Park, S., Choi, J., Biering, S. B., Dominici, E., Williams, L. E., & Hwang, S. (2016). Targeting by AutophaGy proteins (TAG): targeting of IFNG-inducible GTPases to membranes by the LC3 conjugation system of autophagy. *Autophagy*, 12(7), 1153-1167.

Parker, S.P.; ed. 1990. Grziemek's Encyclopedia. Vol 3. McGraw-Hill Publishers, NY.

Parlog, A., Harsan, L. A., Zagrebelsky, M., Weller, M., Von Elverfeldt, D., Mawrin, C., … Dunay, I. R. (2014). Chronic murine toxoplasmosis is defined by subtle changes in neuronal connectivity. *DMM Disease Models and Mechanisms*, *7*(4), 459–469. https://doi.org/10.1242/dmm.014183

Parlog, A., Schlüter, D., & Dunay, I. R. (2015). Toxoplasma gondii-induced neuronal alterations. *Parasite Immunology*, *37*(3), 159–170. https://doi.org/10.1111/pim.12157

Passeri, E., Jones-Brando, L., Bordon, C., Sengupta, S., Wilson, A. M., Primerano, A., . . . Sawa, A. (2016). Infection and characterization of Toxoplasma gondii in human induced neurons from patients with brain disorders and healthy controls. *Microbes Infect, 18*(2), 153-158. doi:10.1016/j.micinf.2015.09.023

Paula-Lima, A. C., Brito-Moreira, J., & Ferreira, S. T. (2013). Deregulation of excitatory neurotransmission underlying synapse failure in Alzheimer's disease. *J Neurochem, 126*(2), 191-202. doi: 10.1111/jnc.12304

Pedersen, M. G., Mortensen, P. B., Norgaard-Pedersen, B., & Postolache, T. T. (2012). Toxoplasma gondii infection and self-directed violence in mothers. *Arch Gen Psychiatry, 69*(11), 1123-1130. doi:10.1001/archgenpsychiatry.2012.668

Peila, R., & Launer, L. J. (2006). Inflammation and dementia: Epidemiologic evidence. *Acta Neurologica Scandinavica*, *114*(SUPPL. 185), 102–106. https://doi.org/10.1111/j.1600-0404.2006.00693.x

Peng, H., Yang, J., Li, G., You, Q., Han, W., Li, T., . . . Hu, R. (2017). Ubiquitylation of p62/sequestosome1 activates its autophagy receptor function and controls selective autophagy upon ubiquitin stress. *Cell Res, 27*(5), 657-674. doi: 10.1038/cr.2017.40

Peng, H.-J., Tan, F. and Lindsay, D. S. 2015. Pathogenesis of Toxoplasma gondii in Humans. *In:* Singh, S. K. (ed.) *Human Emerging and Re-emerging Infections: Viral and Parasitic Infections, Volume I.* 1st ed. London, UK: Wiley-Blackwell.

Perrin, A. J., Jiang, X., Birmingham, C. L., So, N. S., & Brumell, J. H. (2004). Recognition of bacteria in the cytosol of Mammalian cells by the ubiquitin system. *Curr Biol, 14*(9), 806-811. doi: 10.1016/j.cub.2004.04.033

Perry, C. E., Gale, S. D., Erickson, L., Wilson, E., Nielsen, B., Kauwe, J., & Hedges, D. W. (2016). Seroprevalence and Serointensity of Latent Toxoplasma gondii in a Sample of Elderly Adults With and Without Alzheimer Disease. *Alzheimer Dis Assoc Disord, 30*(2), 123-126. doi:10.1097/WAD.0000000000000108

Persson, C. M., Lambert, H., Vutova, P. P., Dellacasa-Lindberg, I., Nederby, J., Yagita, H., . . . Chambers, B. J. (2009). Transmission of Toxoplasma gondii from infected

dendritic cells to natural killer cells. *Infect Immun, 77*(3), 970-976. doi:10.1128/IAI.00833-08

Phosphoglycerate Kinase 1 Phosphorylates Beclin1 to Induce Autophagy. *Mol Cell, 65*(5), 917-931 e916. doi: 10.1016/j.molcel.2017.01.027

Pickford, F., Masliah, E., Britschgi, M., Lucin, K., Narasimhan, R., Jaeger, P. A., Small, S., Spencer, B., Rockenstein, E., Levine, B. and Wyss-Coray, T. (2008) The autophagy-related protein beclin 1 shows reduced expression in early Alzheimer disease and regulates amyloid beta accumulation in mice. *J Clin Invest,* 118 (6)**,** 2190-2199.

Pickford, F., Masliah, E., Britschgi, M., Lucin, K., Narasimhan, R., Jaeger, P. A., . . . Wyss-Coray, T. (2008). The autophagy-related protein beclin 1 shows reduced expression in early Alzheimer disease and regulates amyloid beta accumulation in mice. *J Clin Invest, 118*(6), 2190-2199. doi:10.1172/jci33585.

Pietkiewicz, H., Hiszczynska-Sawicka, E., Kur, J., Petersen, E., Nielsen, H. V., Paul, M., . . . Myjak, P. (2007). Usefulness of Toxoplasma gondii recombinant antigens (GRA1, GRA7 and SAG1) in an immunoglobulin G avidity test for the serodiagnosis of toxoplasmosis. *Parasitol Res, 100*(2), 333-337. doi:10.1007/s00436-006-0265-1

Pollard, A. M., Knoll, L. J., & Mordue, D. G. (2009). The role of specific Toxoplasma gondii molecules in manipulation of innate immunity. *Trends Parasitol, 25*(11), 491-494. doi: 10.1016/j.pt.2009.07.009

population. J. Affect. Disord. 223, 20–25.

Portillo, J. C., Muniz-Feliciano, L., Lopez Corcino, Y., Lee, S. J., Van Grol, J., Parsons, S. J., . . . Subauste, C. S. (2017). Toxoplasma gondii induces FAK-Src-STAT3 signaling during infection of host cells that prevents parasite targeting by autophagy. *PLoS Pathog, 13*(10), e1006671. doi: 10.1371/journal.ppat.1006671

Porto A.M., Amorim M.M., Coelho I.C., Santos L.C. (2008). Serologic profile of toxoplasmosis in pregnant women attended at a teaching-hospital in Recife. Revista da Associação Médica Brasileira. 54: 242-248.

Portugal, L. R., Fernandes, L. R., Cesar, G. C., Santiago, H. C., Oliveira, D. R., Silva, N. M., . . . Alvarez-Leite, J. I. (2004). Infection with Toxoplasma gondii increases atherosclerotic lesion in ApoE-deficient mice. *Infect Immun, 72*(6), 3571-3576. doi:10.1128/IAI.72.6.3571-3576.2004

Poupel, O., Boleti, H., Axisa, S., Couture-Tosi, E., & Tardieux, I. (2000). Toxofilin, a novel actin-binding protein from Toxoplasma gondii, sequesters actin monomers and caps actin filaments. *Molecular Biology of the Cell*, *11*(1), 355–368. https://doi.org/10.1091/mbc.11.1.355

Prandota, J. (2014). Possible link between Toxoplasma gondii and the anosmia associated with neurodegenerative diseases. *Am J Alzheimers Dis Other Demen, 29*(3), 205-214. doi:10.1177/1533317513517049

Prandovszky, E., Gaskell, E., Martin, H., Dubey, J. P., Webster, J. P., & McConkey, G. A. (2011). The neurotropic parasite Toxoplasma gondii increases dopamine metabolism. *PLoS ONE, 6*(9), e23866. doi:10.1371/journal.pone.0023866

Prestrud, K. W., Åsbakk, K., Mørk, T., Fuglei, E., Tryland, M., & Su, C. (2008). Direct highresolution genotyping of *Toxoplasma gondii* in arctic foxes (*Vulpes lagopus*) in the remote arctic Svalbard archipelago reveals widespread clonal Type II lineage. *Veterinary parasitology*, *158*(1), 121-128.

Prestrud, K.W.; Dubey, J.P.; Asbakk, K.; Fuglei, E.; Su, C. (2008). First isolate of Toxoplasma gondii from arctic fox (Vulpes lagopus) from Svalbard. Veterinary Parasitology. 151(2-4):110-114.

Priller, C., Bauer, T., Mitteregger, G., Krebs, B., Kretzschmar, H. A., & Herms, J. (2006). Synapse formation and function is modulated by the amyloid precursor protein. *Journal of Neuroscience*, 26(27), 7212-7221.

Prins, N. D., Visser, P. J., & Scheltens, P. (2010). Can novel therapeutics halt the amyloid cascade? *Alzheimers Res Ther, 2*(2), 5. doi:10.1186/alzrt28.

Prokop, S., Miller, K. R., & Heppner, F. L. (2013). Microglia actions in Alzheimer's disease. *Acta Neuropathologica*, *126*(4), 461–477. https://doi.org/10.1007/s00401-013-1182-x

Qian, M., Fang, X., & Wang, X. (2017). Autophagy and inflammation. *Clinical and translational medicine, 6*(1), 24-24. doi: 10.1186/s40169-017-0154-5

Querfurth, H. W., & LaFerla, F. M. (2010). Alzheimer's disease. *N Engl J Med, 362*(4), 329-344. doi: 10.1056/NEJMra0909142

Ramachandran, R., Radhan, P., Anand, R., Subramanian, I., Santosham, R., & Sai, V. (2014). CNS toxoplasmosis in an immunocompetent individual. *Radiology Case Reports*, *9*(1), 908. https://doi.org/10.2484/rcr.v9i1.908

Rashno, M. M., Fallahi, S., Kheirandish, F., Bagheri, S., Kayedi, M., & Birjandi, M. (2016). Seroprevalence of Toxoplasma gondii infection in patients with Alzheimer's disease. *Archives of Clinical Infectious Diseases*.

Reines, S. A., Block, G. A., Morris, J. C., Liu, G., Nessly, M. L., Lines, C. R., … Baranak, C. C. (2004). Rofecoxib: no effect on Alzheimer's disease in a 1-year, randomized, blinded, controlled study. *Neurology,* 62, 66-71.

Reiter-Owona, I., Petersen, E., Joynson, D., Aspock, H., Darde, M. L., Disko, R., . . . Seitz, H. M. (1999). The past and present role of the Sabin-Feldman dye test in the serodiagnosis of toxoplasmosis. *Bull World Health Organ, 77*(11), 929-935.

Reitz, C., & Mayeux, R. (2014). Alzheimer disease: epidemiology, diagnostic criteria, risk factors and biomarkers. *Biochem Pharmacol, 88*(4), 640-651. doi:10.1016/j.bcp.2013.12.024

Reitz, C., Brayne, C., & Mayeux, R. (2011). Epidemiology of Alzheimer disease. *Nat Rev Neurol, 7*(3), 137-152. doi:10.1038/nrneurol.2011.2

Remington, J. S. (1969). The present status of the IgM fluorescent antibody technique in the diagnosis of congenital toxoplasmosis. *J Pediatr, 75*(6), 1116-1124.

Remington, J. S., Miller, M. J., & Brownlee, I. (1968). IgM antibodies in acute toxoplasmosis. II. Prevalence and significance in acquired cases. *J Lab Clin Med, 71*(5), 855-866.

Rickard, E., Costagliola, M., Outen E., Cicero M., Garcia G., Dieguez, N. (1999) Toxoplasmosis antibody prevalence in pregnancy in Buenos Aires Province, Argentina. Clin Microbiol Infec 5: 171–1721.

Robert-Gangneux, F., Commerce, V., Tourte-Schaefer, C., & Dupouy-Camet, J. (1999). Performance of a Western blot assay to compare mother and newborn anti-Toxoplasma antibodies for the early neonatal diagnosis of congenital toxoplasmosis. *Eur J Clin Microbiol Infect Dis, 18*(9), 648-654.

Rodrigues, I. M., Castro, A. M., Gomes, M. B., Amaral, W. N., & Avelino, M. M. (2009). Congenital toxoplasmosis: evaluation of serological methods for the detection of anti-Toxoplasma gondii IgM and IgA antibodies. *Mem Inst Oswaldo Cruz, 104*(3), 434-440.

Rosi, S., Ramirez-Amaya, V., Hauss-Wegrzyniak, B., & Wenk, G. L. (2004). Chronic brain inflammation leads to a decline in hippocampal NMDA-R1 receptors. *Journal of Neuroinflammation, 1*(1), 12. doi: 10.1186/1742-2094-1-12

Rothaug, M., Stroobants, S., Schweizer, M., Peters, J., Zunke, F., Allerding, M., D'Hooge, R., Saftig, P. and Blanz, J. (2015) LAMP-2 deficiency leads to hippocampal dysfunction but normal clearance of neuronal substrates of chaperone-mediated autophagy in a mouse model for Danon disease. *Acta neuropathologica communications,* 3 (1)**,** 6-16.

Rozenfeld, C., Martinez, R., Figueiredo, R. T., Bozza, M. T., Lima, F. R., Pires, A. L., . . . De Souza, W. (2003). Soluble factors released by Toxoplasma gondii-infected astrocytes down-modulate nitric oxide production by gamma interferon-activated microglia and prevent neuronal degeneration. *Infection and immunity*, 71(4), 2047-2057.

Rozenfeld, C., Martinez, R., Seabra, S., Sant'Anna, C., Gonçalves, J. G. R., Bozza, M., … De Souza, W. (2005). Toxoplasma gondii prevents neuron degeneration by interferon-γ- activated microglia in a mechanism involving inhibition of inducible nitric oxide synthase and transforming growth factor-β1 production by infected microglia. *American Journal of Pathology*, *167*(4), 1021–1031. https://doi.org/10.1016/S0002-9440(10)61191-1

Sa, Q., Ochiai, E., Tiwari, A., Perkins, S., Mullins, J., Gehman, M., . . . Suzuki, Y. (2015). Cutting Edge: IFN-gamma Produced by Brain-Resident Cells Is Crucial To Control Cerebral Infection with Toxoplasma gondii. *J Immunol, 195*(3), 796-800. doi:10.4049/jimmunol.1500814

Saeij, J. P., & Frickel, E. M. (2017). Exposing Toxoplasma gondii hiding inside the vacuole: a role for GBPs, autophagy and host cell death. *Curr Opin Microbiol, 40*, 72-80. doi: 10.1016/j.mib.2017.10.021

Saha, T. (2012). LAMP2A overexpression in breast tumors promotes cancer cell survival via chaperone-mediated autophagy. *Autophagy*, *8*(11), 1643-1656. doi: 10.4161/auto.21654

Saha, T. (2012). LAMP2A overexpression in breast tumors promotes cancer cell survival via chaperone-mediated autophagy. *Autophagy*, *8*(11), 1643-1656. doi: 10.4161/auto.21654

Saitoh, T., & Mook-Jung, I. (1999). Is understanding the biological function of APP important in understanding Alzheimer's disease? *J Alzheimers Dis, 1*(4-5), 287-295.

Saitoh, T., & Mook-Jung, I. (1999). Is understanding the biological function of APP important in understanding Alzheimer's disease? *J Alzheimers Dis, 1*(4-5), 287-295

Salloway, S., Sperling, R., Fox, N. C., Blennow, K., Klunk, W., Raskind, M., . . . Clinical Trial, I. (2014). Two phase 3 trials of bapineuzumab in mild-to-moderate Alzheimer's disease. *N Engl J Med, 370*(4), 322-333. doi:10.1056/NEJMoa1304839

Salminen, A., Kaarniranta, K., Haapasalo, A., Hiltunen, M., Soininen, H. and Alafuzoff, I. (2012) Emerging role of p62/sequestosome-1 in the pathogenesis of Alzheimer's disease. *Prog Neurobiol,* 96 (1)**,** 87-95.

Samojłowicz, D., Twarowska-Małczyńska, J., Borowska-Solonynko, A., Poniatowski, Ł. A., Sharma, N. and Olczak, M. (2019) Presence of Toxoplasma gondii infection in brain as a potential cause of risky behavior: a report of 102 autopsy cases. *European journal of clinical microbiology & infectious diseases : official publication of the European Society of Clinical Microbiology,* 38 (2)**,** 305-317.

Sánchez-Martín, P., & Komatsu, M. (2018). p62/SQSTM1–steering the cell through health and disease. *J Cell Sci*, 131(21), jcs222836.

Savva, D., Morris, J. C., Johnson, J. D., & Holliman, R. E. (1990). Polymerase chain reaction for detection of *Toxoplasma gondii*. *Journal of Medical Microbiology*, *32*(1), 25-31.

Scanga, C. A., Aliberti, J., Jankovic, D., Tilloy, F., Bennouna, S., Denkers, E. Y., . . . Sher, A. (2002). Cutting edge: MyD88 is required for resistance to Toxoplasma

gondii infection and regulates parasite-induced IL-12 production by dendritic cells. *The Journal of Immunology*, 168(12), 5997-6001.

Schlüter, D., Deckert, M., Hof, H., & Frei, K. (2001). Toxoplasma gondii infection of neurons induces neuronal cytokine and chemokine production, but gamma interferon- and tumor necrosis factor-stimulated neurons fail to inhibit the invasion and growth of T. gondii. *Infection and Immunity*, *69*(12), 7889–7893. https://doi.org/10.1128/IAI.69.12.7889-7893.2001

Schmitz, F., Mages, J., Heit, A., Lang, R., & Wagner, H. (2004). Transcriptional activation induced in macrophages by Toll-like receptor (TLR) ligands: from expression profiling to a model of TLR signaling. *Eur J Immunol*, *34*(10), 2863-2873. doi: 10.1002/eji.200425228.

Schmued, L. C., & Hopkins, K. J. (2000). Fluoro-Jade B : a high affinity fluorescent marker for the localization of neuronal degeneration, *874*, 123–130.

Schwartz, M., & Deczkowska, A. (2016). Neurological Disease as a Failure of Brain – Immune Crosstalk : The Multiple Faces of Neuroin fl ammation. *Trends in Immunology*, *xx*, 1–12. https://doi.org/10.1016/j.it.2016.08.001

Schwartzman, J. D. (1987). Quantitative comparison of infection of neural cell and fibroblast monolayers by two strains of Toxoplasma gondii. *Proc Soc Exp Biol Med*, *186*(1), 75-78.

Selfridge, A., Hyun, N., Chiang, C. C., Reyna, S. M., Weissmiller, A. M., Shi, L. Z., . . . Berns, M. W. (2015). Rat embryonic hippocampus and induced pluripotent stem cell derived cultured neurons recover from laser-induced subaxotomy. *Neurophotonics*, *2*(1), 015006. doi:10.1117/1.NPh.2.1.015006

Selseleh, M. M., Keshavarz, H., Mohebali, M., Shojaee, S., Modarressi, M., Eshragian, M., & Selseleh, M. M. (2012). Production and Evaluation of Toxoplasma gondii Recombinant Surface Antigen 1 (SAG1) for Serodiagnosis of Acute and Chronic Toxoplasma Infection in Human Sera. *Iran J Parasitol, 7*(3), 1-9.

Selseleh, M., Modarressi, M. H., Mohebali, M., Shojaee, S., Eshragian, M. R., Selseleh, M., . . . Keshavarz, H. (2012). Real-time RT-PCR on SAG1 and BAG1 gene expression during stage conversion in immunosuppressed mice infected with Toxoplasma gondii Tehran strain. *Korean J Parasitol, 50*(3), 199-205. doi:10.3347/kjp.2012.50.3.199

Selseleh, M., Modarressi, M., Shojaee, S., Mohebali, M., Eshraghian, M., Selseleh, M., & Keshavarz, H. (2013). Brain Tissue Cysts in Infected Mice with RH-Strain of Toxoplasma gondii and Evaluation of BAG1 and SAG1 Genes Expression. *Iran J Parasitol, 8*(1), 40-46.

Shaddel M, Mehbod A, Karamy M.(2007) Toxoplasma gondii infection in neonates. Iran J Parasitol; 2: 34-37.

Sher, A., Oswald, I. P., Hieny, S. & Gazzinelli, R. T. (1993). Toxoplasma gondii induces a T-independent IFN-gamma response in natural killer cells that requires both adherent accessory cells and tumor necrosis factor-alpha. *J Immunol,* 150, 3982-9.

Sibley, L. D., & Ajioka, J. W. (2008). Population structure of Toxoplasma gondii: clonal expansion driven by infrequent recombination and selective sweeps. *Annu Rev Microbiol, 62,* 329-351. doi:10.1146/annurev.micro.62.081307.162925

Silva, N. M., Vieira, J. C., Carneiro, C. M. and Tafuri, W. L. (2009) Toxoplasma gondii: the role of IFN-gamma, TNFRp55 and iNOS in inflammatory changes during infection. *Exp Parasitol,* 123 (1)**,** 65-72.

Singh, B., Schwartz, J. A., Sandrock, C., Bellemore, S. M., & Nikoopour, E. (2013). Modulation of autoimmune diseases by interleukin (IL)-17 producing regulatory T helper (Th17) cells. *Indian J Med Res, 138*(5), 591-594.

Skariah, S., Mcintyre, M. K., & Mordue, D. G. (2012). Toxoplasma gondii: determinants of tachyzoite to bradyzoite conversion, *107*(2), 253–260. https://doi.org/10.1007/s00436-010-1899-6.Toxoplasma

Skotnikova, O. I., Sobolev, A. Y., Demkin, V. V., Nikolaeva, N. P., Nosova, E. Y., Isaeva, E. L., . . . Litvinov, V. I. (2000). Application of nested-PCR technique for the diagnosis of tuberculosis. *Bull Exp Biol Med, 129*(6), 612-614.

Small, D. H. (1998). The role of the amyloid protein precursor (APP) in Alzheimer's disease: does the normal function of APP explain the topography of neurodegeneration? *Neurochem Res, 23*(5), 795-806.

Soni, B., & Singh, S. (2019). Cytokine Milieu in Infectious Disease: A Sword or a Boon? *Journal of Interferon & Cytokine Research, 40*(1), 24-32. doi:10.1089/jir.2019.0089

Stan, DM, Heale, R, Barbato, L, Patel, M, Mosses, D, Robinson, A, Davidson, Y, Mann, D, Lace, G (2019); Investigating alterations in autophagy in Alzheimer's Disease. In submission.

Stavoe, A. K. H., & Holzbaur, E. L. F. (2018). Axonal autophagy: Mini-review for autophagy in the CNS. *Neurosci Lett*. doi: 10.1016/j.neulet.2018.03.025

Strobl, J. S., Goodwin, D. G., Rzigalinski, B. A., & Lindsay, D. S. (2012). Dopamine stimulates propagation of Toxoplasma gondii tachyzoites in human fibroblast and primary neonatal rat astrocyte cell cultures. *J Parasitol, 98*(6), 1296-1299. doi:10.1645/GE-2760.1

Stroehle, A., Schmid, K., Heinzer, I., Naguleswaran, A., & Hemphill, A. (2005). Performance of a Western immunoblot assay to detect specific anti-Toxoplasma gondii IgG antibodies in human saliva. *J Parasitol, 91*(3), 561-563. doi:10.1645/GE-423R

Stutz, A., Kessler, H., Kaschel, M. E., Meissner, M., & Dalpke, A. H. (2012). Cell invasion and strain dependent induction of suppressor of cytokine signaling-1 by Toxoplasma gondii. *Immunobiology, 217*(1), 28-36. doi:10.1016/j.imbio.2011.08.008

Su, F., Bai, F., Zhou, H., & Zhang, Z. (2015). Brain , Behavior , and Immunity Microglial toll-like receptors and Alzheimer ' s disease. https://doi.org/10.1016/j.bbi.2015.10.010

Su, H., & Wang, X. (2011). p62 Stages an interplay between the ubiquitin-proteasome system and autophagy in the heart of defense against proteotoxic stress. *Trends Cardiovasc Med, 21*(8), 224-228. doi: 10.1016/j.tcm.2012.05.015

Subauste, C. S. (2019). Interplay Between Toxoplasma gondii, Autophagy, and Autophagy Proteins. *Frontiers in Cellular and Infection Microbiology, 9*(139), 1-15. doi:10.3389/fcimb.2019.00139

Sugden, K., Moffitt, T. E., Pinto, L., Poulton, R., Williams, B. S., & Caspi, A. (2016). Is Toxoplasma Gondii Infection Related to Brain and Behavior Impairments in Humans? Evidence from a Population-Representative Birth Cohort. *PLoS ONE, 11*(2), e0148435. doi:10.1371/journal.pone.0148435

Sutterland, A.L., Fond, G., Kuin, A., Koeter, M.W., Lutter, R., van Gool, T., et al., 2015.Beyond the association. Toxoplasma gondii in schizophrenia, bipolar disorder, and addiction: systematic review and meta-analysis. Acta Psychiatr. Scand. 132 (3),

Suvisaari, J., Torniainen-Holm, M., Lindgren, M., Härkänen, T., Yolken, R.H., 2017.

Suzuki, Y. (2002). Host resistance in the brain against Toxoplasma gondii. *J Infect Dis, 185 Suppl 1*, S58-65. doi:10.1086/337999.

Takahashi, T., & Nakayama, T. (2006). Novel technique of quantitative nested real-time PCR assay for Mycobacterium tuberculosis DNA. *J Clin Microbiol, 44*(3), 1029-1039. doi:10.1128/JCM.44.3.1029-1039.2006.

Tanida, I., Ueno, T., & Kominami, E. (2008). LC3 and Autophagy. *Methods Mol Biol, 445*, 77-88. doi: 10.1007/978-1-59745-157-4_4

Tenner, A. J. (1999). Membrane receptors for soluble defense collagens. *Curr Opin Immunol,* ,34–41.

Tenter, A.M.; Heckeroth, A.R. and Weiss, L.M. (2000). Toxoplasma gondii: from animals to humans. International Journal for Parasitology. 30(12-13): 1217-1258.

Thiermann, E. (1957). Transmission congenita del Toxoplasma gondii en ratas con infeccion leve. Biologica 23: 59.

Thomasson, D., Wright, E. A., Hughes, J. M., Dodd, N. S., Cox, A. P., Boyce, K., . . . Hide, G. (2011). Prevalence and co-infection of Toxoplasma gondii and Neospora caninum in Apodemus sylvaticus in an area relatively free of cats. *Parasitology, 138*(9), 1117-1123. doi:10.1017/s0031182011000904

Torres, L., Robinson, S.-A., Kim, D.-G., Yan, A., Cleland, T. A., & Bynoe, M. S. (2018). Toxoplasma gondii alters NMDAR signaling and induces signs of Alzheimer's disease in wild-type, C57BL/6 mice. *Journal of neuroinflammation, 15*(1), 57-67. doi:10.1186/s12974-018-1086-8

Torrey, E. F. and Yolken, R. H. (2003) Toxoplasma gondii and schizophrenia. *Emerg Infect Dis,* 9 (11)**,** 1375-1380.

Torrey, E. F., Bartko, J. J. and Yolken, R. H. (2012) Toxoplasma gondii and other risk factors for schizophrenia: an update. *Schizophr Bull,* 38 (3)**,** 642-647.

Torrey, E.F., Bartko, J.J., Lun, Z.R., Yolken, R.H., (2007) Antibodies to Toxoplasma gondii in patients with schizophrenia: a meta-analysis. Schizophr. Bull. 33 (3), 729–736.

Suvisaari, J., Torniainen-Holm, M., Lindgren, M., Härkänen, T., Yolken, R.H. (2017) Toxoplasma gondii infection and common mental disorders in the Finnish general population. J. Affect. Disord. 223, 20–25.

Trengove, M. C. and Ward, A. C. (2013) SOCS proteins in development and disease. *Am J Clin Exp Immunol,* 2 (1)**,** 1-29.

Uddin, M. S., Stachowiak, A., Mamun, A. A., Tzvetkov, N. T., Takeda, S., Atanasov, A. G., . . . Stankiewicz, A. M. (2018). Autophagy and Alzheimer's Disease: From

Molecular Mechanisms to Therapeutic Implications. *Frontiers in aging neuroscience, 10*(1), 04-14. doi:10.3389/fnagi.2018.00004

Umeda, K., Tanaka, S., Ihara, F., Yamagishi, J., Suzuki, Y., & Nishikawa, Y. (2017). Transcriptional profiling of Toll-like receptor 2-deficient primary murine brain cells during Toxoplasma gondii infection. *Plos One, 12*(11), e0187703. doi: 10.1371/journal.pone.0187703

Umeda, T., Kimura, T., Yoshida, K., Takao, K., Fujita, Y., Matsuyama, S., . . . Tomiyama, T. (2017). Mutation-induced loss of APP function causes GABAergic depletion in recessive familial Alzheimer's disease: analysis of Osaka mutation-knockin mice. *Acta Neuropathol Commun, 5*(1), 59. doi: 10.1186/s40478-017-0461-5

Vidadala, R. S., Rivas, K. L., Ojo, K. K., Hulverson, M. A., Zambriski, J. A., Bruzual, I., . . . Maly, D. J. (2016). Development of an Orally Available and Central Nervous System (CNS) Penetrant Toxoplasma gondii Calcium-Dependent Protein Kinase 1 (TgCDPK1) Inhibitor with Minimal Human Ether-a-go-go-Related Gene (hERG) Activity for the Treatment of Toxoplasmosis. *J Med Chem, 59*(13), 6531-6546. doi: 10.1021/acs.jmedchem.6b00760

Virreira, S., Niedelman, W., Jensen, K. D., Rosowski, E. E., Julien, L., Spooner, E., … Ploegh, H. L. (2011). Determinants of GBP Recruitment to Toxoplasma gondii Vacuoles and the Parasitic Factors That Control It, *6*(9). https://doi.org/10.1371/journal.pone.0024434

Vyas, A., Kim, S. K., Giacomini, N., Boothroyd, J. C., & Sapolsky, R. M. (2007). Behavioral changes induced by Toxoplasma infection of rodents are highly

specific to aversion of cat odors. *Proc Natl Acad Sci U S A, 104*(15), 6442-6447. doi:10.1073/pnas.0608310104.

Walker, D. G., Whetzel, A. M. and Lue, L. F. (2015) Expression of suppressor of cytokine signaling genes in human elderly and Alzheimer's disease brains and human microglia. *Neuroscience,* 302 (1)**,** 121-137.

Wang, H., Lei, C., Li, J., Wu, Z., Shen, G., & Yu, R. (2004). A piezoelectric immunoagglutination assay for Toxoplasma gondii antibodies using gold nanoparticles. *Biosens Bioelectron, 19*(7), 701-709.

Wang, L. L., & Law, H. K. W. (2015). The Role of Autophagy in Lupus Nephritis. *International Journal of Molecular Sciences, 16*(10), 25154-25167.

Wang, S.; Lan, C.; Zhang, L.; Zhang, H.; Yao, Z.; Wang, D.; Ma, J.; Deng, J.; Liu, S. (2015). Seroprevalence of *Toxoplasma gondii* infection among patients with hand, foot and mouth disease in Henan, China: a hospital-based study. Infectious Diseases of Poverty. 4: 53.

Wang, T., Tang, Z. H., Li, J. F., Li, X. N., Wang, X., & Zhao, Z. J. (2013). A potential association between Toxoplasma gondii infection and schizophrenia in mouse models. *Exp Parasitol, 135*(3), 497-502. doi:10.1016/j.exppara.2013.08.012

Wang, W., Fan, H., Zhou, Y., Duan, P., Zhao, G., & Wu, G. (2013). Knockdown of autophagy-related gene BECLIN1 promotes cell growth and inhibits apoptosis in the A549 human lung cancer cell line. *Mol Med Rep, 7*(5), 1501-1505. doi: 10.3892/mmr.2013.1379

Wang, Y. H., Li, X. R., Wang, G. X., Yin, H., Cai, X. P., Fu, B. Q., & Zhang, D. L. (2011). Development of an immunochromatographic strip for the rapid detection

of Toxoplasma gondii circulating antigens. *Parasitol Int, 60*(1), 105-107. doi:10.1016/j.parint.2010.11.002

Wang, Y., & Yin, H. (2014). Research progress on surface antigen 1 (SAG1) of Toxoplasma gondii. *Parasit Vectors, 7*, 180. doi:10.1186/1756-3305-7-180

Wang, Y., Weiss, L. M., & Orlofsky, A. (2009). Host cell autophagy is induced by Toxoplasma gondii and contributes to parasite growth. *J Biol Chem, 284*(3), 1694-1701. doi: 10.1074/jbc.M807890200

Warner, T. D., Mitchell, J. A., & Cyclooxygenases, J. A. (2016). Cyclooxygenases: new forms , new inhibitors , and lessons from the clinic, *18*(7), 790–804. https://doi.org/10.1096/fj.03-0645rev

Webster, J. P. (2001) Rats, cats, people and parasites: the impact of latent toxoplasmosis on behaviour. *Microbes Infect,* 3 (12)**,** 1037-1045.

Weidberg, H., Shpilka, T., Shvets, E., Abada, A., Shimron, F., & Elazar, Z. (2011). LC3 and GATE-16 N termini mediate membrane fusion processes required for autophagosome biogenesis. *Developmental cell,* 20(4), 444-454.

Welikovitch, L. A., Do Carmo, S., Maglóczky, Z., Szocsics, P., Lőke, J., Freund, T., & Cuello, A. C. (2018). Evidence of intraneuronal Aβ accumulation preceding tau pathology in the entorhinal cortex. *Acta Neuropathologica*, *136*(6), 901–917. https://doi.org/10.1007/s00401-018-1922-z

Whyte, C. S., Bishop, E. T., Ruckerl, D., Gaspar-Pereira, S., Barker, R. N., Allen, J. E., . . . Wilson, H. M. (2011). Suppressor of cytokine signaling (SOCS)1 is a key

determinant of differential macrophage activation and function. *J Leukoc Biol, 90*(5), 845-854. doi: 10.1189/jlb.1110644

Wolfe, D. M., Lee, J. H., Kumar, A., Lee, S., Orenstein, S. J., & Nixon, R. A. (2013). Autophagy failure in Alzheimer's disease and the role of defective lysosomal acidification. *Eur J Neurosci, 37*(12), 1949-1961. doi: 10.1111/ejn.12169.

Wyss-coray, T., & Rogers, J. (2012). Inflammation in Alzheimer Disease — A Brief Review of the Basic Science and Clinical Literature, 1–24.

Wyss-Coray, T. (2006). Inflammation in Alzheimer disease: driving force, bystander or beneficial response? *Nat Med, 12*(9), 1005-1015. doi:10.1038/nm1484.

Xuan, F., Huang, M., Liu, W., Ding, H., Yang, L., & Cui, H. (2016). Homeobox C9 suppresses Beclin1-mediated autophagy in glioblastoma by directly inhibiting the transcription of death-associated protein kinase 1. *Neuro Oncol, 18*(6), 819-829. doi: 10.1093/neuonc/nov281

Xue, Z., Zhang, S., Huang, L., He, Y., Fang, R., & Fang, Y. (2013). Increased expression of Beclin-1-dependent autophagy protects against beta-amyloid-induced cell injury in PC12 cells [corrected]. *J Mol Neurosci, 51*(1), 180-186. doi:10.1007/s12031-013-9974

Yamazaki, Y., & Kanekiyo, T. (2017). Blood-brain barrier dysfunction and the pathogenesis of alzheimer's disease. *International Journal of Molecular Sciences, 18*(9). https://doi.org/10.3390/ijms18091965

Yang, X., Yao, C., Tian, T., Li, X., Yan, H., Wu, J., . . . Lu, Y. (2018). A novel mechanism of memory loss in Alzheimer's disease mice via the degeneration of

entorhinal-CA1 synapses. *Mol Psychiatry, 23*(2), 199-210. doi: 10.1038/mp.2016.151

Yang, Z., & Ming, X.-F. (2014). Functions of arginase isoforms in macrophage inflammatory responses: impact on cardiovascular diseases and metabolic disorders. *Frontiers in immunology, 5*, 533-533. doi: 10.3389/fimmu.2014.00533

Yarovinsky, F. (2014). Innate immunity to Toxoplasma gondii infection. *Nat Rev Immunol, 14*(2), 109-121. doi: 10.1038/nri3598

Yazar, S., & Arman, F. (2003). Investigation of probable relationship between Toxoplasma gondii and cryptogenic epilepsy, *1311*(02), 107–109. https://doi.org/10.1016/S1059

Zannis, V. I., Cole, F. S., Jackson, C. L., Kurnit, D. M., & Karathanasis, S. K. (1985). Distribution of apolipoprotein A-I, C-II, C-III, and E mRNA in fetal human tissues. Time-dependent induction of apolipoprotein E mRNA by cultures of human monocyte-macrophages. *Biochemistry, 24*(16), 4450-4455.

Zare-Shahabadi, A., Masliah, E., Johnson, G. V. W., & Rezaei, N. (2015). Autophagy in Alzheimer's disease. *Reviews in the neurosciences, 26*(4), 385-395. doi:10.1515/revneuro-2014-0076

Zhang, Y., Li, P., Feng, J., & Wu, M. (2016). Dysfunction of NMDA receptors in Alzheimer's disease. *Neurol Sci, 37*(7), 1039-1047. doi:10.1007/s10072-016-2546-5

Zhao, Z.J.; Zhang, J.; Wei, J.; Li, Z.; Wang, T.; Yi, S.Q.; Shen, J.L.; Yang, T.B.; Hide, G. and Lun Z.R. (2013). Lower expression of inducible nitric oxide synthase and

higher expression of arginase in rat alveolar macrophages are linked to their susceptibility to *Toxoplasma gondii* infection. PLoS One 8, e63650.

Zheng, H. and Koo, E. H. (2011) Biology and pathophysiology of the amyloid precursor protein. *Molecular Neurodegeneration,* 6 (1)**,** 27-37.

Zhong, Y., Wang, Q. J., Li, X., Yan, Y., Backer, J. M., Chait, B. T., . . . Yue, Z. (2009). Distinct regulation of autophagic activity by Atg14L and Rubicon associated with Beclin 1–phosphatidylinositol-3-kinase complex. *Nature Cell Biology, 11*(4), 468-476. doi: 10.1038/ncb1854.

Appendix 1

Table 1. A) APP DNA sequence (variant 1, exon 16) from *Mus musculus*, B) APP DNA sequence (variant 1, exon 16) from *Rattus norvegicus* C) Clustal alignment of variants of the APP gene from *Mus musculus* and *Rattus norvegicus*.

A

GTCGACGCCGCCGTGACCCCAGAGGAGCGCCATCTCTCCAAGATGCAGCAGAACGGATATGAGAATCCAACTTACAAGTTCTTTGAG
CAAATGCAGAACTAAGCCCCACCCGCGCCACAGCAGCGGCCTCTGAACTTGGACAGCGAAACCATTGCTTCACTACCCATCGGTGTT
CATTTATAAAATAACGTGGAAAGAAACAAACCCTTCCGTTTATTTACTCACCCTCGGCTTTTGACAGCTGTGCTGTAACACAAGTAG
ATGCCTGAACTTGAATTAATATACAAATCAGTAATGTATTCTCGCTTTCTCTCTTTACATTCTGGTCTCTACATTACATGATTCATG
GGTTTTGTGTACTGTAAAAAAAAAAATTAGCTGTATCAAACTAGTGCATGAATAGATTCTCTCCTAATTATTTATCACATACATAGC
CCCTTAGCCCGTTGTATATTATTCTTGTGGTTTGTGGCCCGGAAAAAACTCCTACTTGAAATATGCTTTAAAAATCGATGGGGGATG
CTTCTTGTGAACGTGGGCGTCTAGCTGCTTCTCCTACGTATTCTTTTCCTGATCACTATGCATTTTGAACATTTTTTTAAGTATTCC
AAATGACTTAGAAAATTCTTTTTCCATGACTGCATCTTACTGTACAGATTGCTGCTTCTGCTCTCTTTGTGATATAGGAATAAGAGG
ATACACATTGATTTCTTTGTGCCTGTTTTATGTGCACACATTAGGCATTGAGAATTTGAACATTTTTTTTGTCCATGTATCTTTGGA
TCTTTGATAAAAAAAAAATTAAAAAAAAATTATCCCTGTTCATCATAAGCACTTTTACGGGTGGGGGGAGGGAGTGTTCTGCTGGTCT
CCAATTACCAAGAATTCTCCAAAAATTAATTTTCTGCAGGATGATTGTACAGAATCATTGCTTATGCCATGATAGCTTTCTACACTG
TATTACATAAATAAATTAAATAAAATAACTCCAGGCAAGACGTTTCTTTGAAGGGTGATTGTGTGGGAGGGAAAAGGCAAGATCCCT
GCATTTATCAACCCAAGGCGCTTCGTTTAATAATACTGACAGAGAATGAAAAGCCAGGAAGAGAGTAAGAGGGAAGAGAGGGCACCC
TGAGACACCCCTGAGGGTTTGTATAAAATTGTGTTTTCATAGGAATACCTTAGACTGGTATGATTGTATGGTGAGAGTTTATTGCTT
AGTCATCTAGACCCGTGAGAACACCTCATCTGGTCACTCACACCTAACCTGTGAGTCTGATGAGGAAATTGTGGGCGCACACAGCTC
TTAGTGCTGGTAAACTAACTGGTGGAGTTTGTTGTTTTTAAAAGGCTGTGCTTCCCTGGCTTGGTTCTTCCTTTCTGTCTTAGTCAG
GGTTTGTATTCCTGCACAAAACATCATGGCCAAGAAGCAAGTTGGGGAGGAAAGGGTTTATTCAGCTTACACTTCCACATTGCTCTT
TTGTTTATCACCAAAGGAAGTCAGGACAGGAACTCACACAGGGCAGGAACTTGGAGGCAGGAGCTGATGCAGAGGCCATGGAGGATG
CTGCTTACTGGCTTGCTTCCCCTGGCTTGCTCAGTTTGCTTTCTTATAGAACCCAAGACTACCAGTCCAGGGATGGCACCACCCACA
ATGGGCCCTCCCCCCTTGATCACAATAATTGAGAAAATGCCTTACAGCTAGATCGCATGGAGGCATTTCCTTGAGGGAGGCTCCTTT
CTCTATGATAACTCCAGCTTGTGTCAAGTTGACACACAAAAACTAACAGGTTCCAAATGTGAAACACACACACACACACACACAC
ACAAACAAAACAAAAACAGAGAAAAGCTGTAGAGTTTTGATTTGGGGGTTTTGTTTTTTGTTTTGTTTTGTTTTGTTTTTCG
CTTTCAAGCTAGGGTCTCAAGTATCCCAATCTAACCTTTAACTCATGTAGCCAAAGATGGCCTTATCCTCCTGATGGTCCTGCCTCT
ACCTTGCAAATATTGGGACTGCAGCATGCGCCATCGTGCCCTGTGTATATGAAGTGCTGGGATTTGACTTCAAGGCTCCCTGTATGC
TAAGTAAGTTCTCTACCAAGTGAGCTACATCCCCAGGCCCTGGAAATGATGGGTAGAGCAGCTGATAGTCTGCAGCAAGCTCTGGAC
CTGGGCACTCCCCACATTTTTGGGGGAGTCACTCTTATTCGTGGGGATACGAACGTTCTAGGATATTTAACCGCATCCGCCTGATTC
TAACCAGAAATGTCTCCCGAGCTGTATGATGTCTCACACGCCTGTGACTCCAGTGCTTGGGAGGGAAGCAAGAGACCAGGCCTGCCT
GGAAAACAGGGTGAGATCCTGTCTCAAAGAGTGAGAAGCAGAAGTGTTTCCAGAAGACGTTCTCCAACACCAGAAGACGGATCAAGT
GTCCAGATCCTGCCAAACATCTCTTACGGAAGCGAGTTACAAACCACTGATCTATAAACAGTATTTTGACCTTTATATTATGTGGCA
TGTGTTGTATATGATACAATGAACGTGCGTTTGAGTTTCAAAGGACTACTGGCTGGGAAGTGTGTGCCTTCCACTCTGTGTGGTCTG
GAATCAGACTCAAGTCATCAGGCTTGGCAGCAAGTACTTTTACCCACTGAGCCTTCCTACCATCCTTCATTTTAGCTTAATGGCCTG
TGCACACAACCTTTTATTGAGGATGCTTGTATTAATTCCAGCTGCTATAAGAATAAACAATAGACAAAGA

B

AGTCCTCGGTGTATTTTAAACCCTAGTCTCTATACTTGCTTTCTAGGTTGACGCTGCTGTGACCCCGGAGGAGCGCCACCTCTCCAA
GATGCAGCAGAATGGATATGAGAATCCAACATACAAGTTCTTTGAGCAGATGCAGAACTAAACCCCGCCCCCCGCCACAGCAGCGGC
CTCTGAACTTGGACAGCAAAACCATTGCTTCACTACCCATCGGTGTTCATTTATAAAATAACGTGGAAAGAAACAAACCCGTCTGTT
TATTTACTCACCCTCGGCTTTTGACAGCTGTGCTGTAACACAAGTAGATGCCTGAACTCGAATTAATATACAAATCAATAATGTATT
CTCTCTTTCTCTCTTTACATTCTGGTCTCTACACTACATTATTAATGGGTTTTGTGTACTGTAAAAAAAAAATTTAGCTGTATCAAA
CTAGTGCATGAATAGATTCTCTCCTGAGTATCTGTCACATACATAGCCCCTTAGCCCGTTGTATATTATTCTTGTGGTTTGTGGCCC
GAAAAACTCCTACTTGAAATACGCTTTAAAAAAATCGATGGGGGATGCTTCGTGTGAACGTGGGCGTTTAGCTGCTTCTCCTCTCTT
AAGTATTCTTTTCCTGATCACTATGCATTTTGGACATTTTTGAGTATTCCAAATGATTTAGAAAAGTTCGTTTTCCATGATTGCATC

TTACTGTACAGATTGCTGCTTCTGCTCTCTTTGCGATATAGGAATACGAGGATACACATTGATTTCTTTGTGCCTGTTTTATGTGCA
CACATTAGGCATTGAGGATTTGAACTTTTTTTTGTCCATGTATCTTTGGATCTTTGATAAAAAAAAAATTTAAAAAAAAAAATCCCT
GTTCATCGTAAGCACTTTTACGGGTGGGGGGAGGGAGTGTTCTGCTGGTCTCCAATTACCAAGAATTCTCCAAAAATAATTTTCTGC
AGGATGATTGTACAGAATCATTGCTTATGCCATGATAGCTTTCTACACTGTATTACATAAATAAATTAAATTAAATAACTCCA

C

CLUSTAL O(1.2.4) multiple sequence alignment

```
APP      ---------------------------------------------GTCGACGCCGCCGT       14
Rat      AGTCCTCGGTGTATTTTAAACCCTAGTCTCTATACTTGCTTTCTAGGTTGACGCTGCTGT       60
                                                       ** ***** ** **

APP      GACCCCAGAGGAGCGCCATCTCTCCAAGATGCAGCAGAACGGATATGAGAATCCAACTTA       74
Rat      GACCCCGGAGGAGCGCCACCTCTCCAAGATGCAGCAGAATGGATATGAGAATCCAACATA       120
         ****** *********** ****************** ***************** **

APP      CAAGTTCTTTGAGCAAATGCAGAACTAAGCCCCA-CCCGCGCCACAGCAGCGGCCTCTGA       133
Rat      CAAGTTCTTTGAGCAGATGCAGAACTAAACCCCGCCCCCCGCCACAGCAGCGGCCTCTGA       180
         *************** ************ ****  *** **********************

APP      ACTTGGACAGCGAAACCATTGCTTCACTACCCATCGGTGTTCATTTATAAAATAACGTGG       193
Rat      ACTTGGACAGCAAAACCATTGCTTCACTACCCATCGGTGTTCATTTATAAAATAACGTGG       240
         *********** **************************************************

APP      AAAGAAACAAACCCTTCCGTTTATTTACTCACCCTCGGCTTTTGACAGCTGTGCTGTAAC       253
Rat      AAAGAAACAAACCCGTCTGTTTATTTACTCACCCTCGGCTTTTGACAGCTGTGCTGTAAC       300
         ************** ** ******************************************

APP      ACAAGTAGATGCCTGAACTTGAATTAATATACAAATCAGTAATGTATTCTCGCTTTCTCT       313
Rat      ACAAGTAGATGCCTGAACTCGAATTAATATACAAATCAATAATGTATTCTCTCTTTCTCT       360
         ****************** ***************** ************ ********

APP      CTTTACATTCTGGTCTCTACATTACATGATTCATGGGTTTTGTGTACTGTAAAAAAAAAA       373
Rat      CTTTACATTCTGGTCTCTACACTACATTATTAATGGGTTTTGTGTACTGTAAAAAAAAAA       420
         ******************** ***** *** ****************************

APP      ATTAGCTGTATCAAACTAGTGCATGAATAGATTCTCTCCTAATTATTTATCACATACATA       433
Rat      TTTAGCTGTATCAAACTAGTGCATGAATAGATTCTCTCCTGAGTATCTGTCACATACATA       480
          ************************************ * *** * ***********

APP      GCCCCTTAGCCCGTTGTATATTATTCTTGTGGTTTGTGGCCCGGAAAAAACTCCTACTTG       493
Rat      GCCCCTTAGCCCGTTGTATATTATTCTTGTGGTTTGTGGCCCGAAAAACTCCTACTTGAA       540
         ****************************************** ****  *

APP      AAATATGCTTTAAAAATCGATGGGGGATGCTTCTTGTGAACGTGGGCGTCTAGCTGCTTC       553
Rat      ATACGCTTTAAAAAAATCGATGGGGGATGCTTCGTGTGAACGTGGGCGTTTAGCTGCTTC       600
         * *     *  ********************** *************** **********

APP      TCCTA-----CGTATTCTTTTCCTGATCACTATGCATTTTGAACATTTTTTTAAGTATTC       608
Rat      TCCTCTCTTAAGTATTCTTTTCCTGATCACTATGCATTTTGGACATTTTTG--AGTATTC       658
         ****       ****************************** ********   *******

APP      CAAA-TGACTTAGAAAATTCTTTTTCCATGACTGCATCTTACTGTACAGATTGCTGCTTC       667
Rat      CAAATGATTTAGAAAAGTTCGTTTTCCATGATTGCATCTTACTGTACAGATTGCTGCTTC       718
         ****     *   *** *** ********** ****************************

APP      TGCTCTCTTTGTGATATAGGAATAAGAGGATACACATTGATTTCTTTGTGCCTGTTTTAT       727
Rat      TGCTCTCTTTGCGATATAGGAATACGAGGATACACATTGATTTCTTTGTGCCTGTTTTAT       778
         *********** ************ ***********************************

APP      GTGCACACATTAGGCATTGAGAATTTGAACATTTTTTTTGTCCATGTATCTTTGGATCTT       787
Rat      GTGCACACATTAGGCATTGAGGATTTGAACTTTTTTTTG-TCCATGTATCTTTGGATCTT       837
         ******************** ******** *******  ********************

APP      TGAT-AAAAAAAAATTAAAAAAAAATTATCCCTGTTCATCATAAGCACTTTTACGGGTGG       846
```

```
Rat      TGATAAAAAAAAAATTTAAAAAAAAAAATCCCTGTTCATCGTAAGCACTTTTACGGGTGG      897
         **** *********** ********  ************* ******************

APP      GGGGAGGGAGTGTTCTGCTGGTCTCCAATTACCAAGAATTCTCCAAAAATTAATTTTCTG      906
Rat      GGGGAGGGAGTGTTCTGCTGGTCTCCAATTACCAAGAATTCTCCAAAA-ATAATTTTCTG      956
         **********************************************  **********

APP      CAGGATGATTGTACAGAATCATTGCTTATGCCATGATAGCTTTCTACACTGTATTACATA      966
Rat      CAGGATGATTGTACAGAATCATTGCTTATGCCATGATAGCTTTCTACACTGTATTACATA     1016
         ************************************************************

APP      AATAAATTAAATAAAATAACTCCAGGCAAGACGTTTCTTTGAAGGGTGATTGTGTGGGAG     1026
Rat      AATAAATTAAATTAAATAACTCCA-----------------------------------     1040
         ************ ***********

APP      GGAAAAGGCAAGATCCCTGCATTTATCAACCCAAGGCGCTTCGTTTAATAATACTGACAG     1086
Rat      ------------------------------------------------------------     1040


APP      AGAATGAAAAGCCAGGAAGAGAGTAAGAGGGAAGAGAGGGCACCCTGAGACACCCCTGAG     1146
Rat      ------------------------------------------------------------     1040


APP      GGTTTGTATAAAATTGTGTTTTCATAGGAATACCTTAGACTGGTATGATTGTATGGTGAG     1206
Rat      ------------------------------------------------------------     1040


APP      AGTTTATTGCTTAGTCATCTAGACCCGTGAGAACACCTCATCTGGTCACTCACACCTAAC     1266
Rat      ------------------------------------------------------------     1040


APP      CTGTGAGTCTGATGAGGAAATTGTGGGCGCACACAGCTCTTAGTGCTGGTAAACTAACTG     1326
Rat      ------------------------------------------------------------     1040


APP      GTGGAGTTTGTTGTTTTTAAAAGGCTGTGCTTCCCTGGCTTGGTTCTTCCTTTCTGTCTT     1386
Rat      ------------------------------------------------------------     1040


APP      AGTCAGGGTTTGTATTCCTGCACAAAACATCATGGCCAAGAAGCAAGTTGGGGAGGAAAG     1446
Rat      ------------------------------------------------------------     1040


APP      GGTTTATTCAGCTTACACTTCCACATTGCTCTTTTGTTTATCACCAAAGGAAGTCAGGAC     1506
Rat      ------------------------------------------------------------     1040


APP      AGGAACTCACACAGGGCAGGAACTTGGAGGCAGGAGCTGATGCAGAGGCCATGGAGGATG     1566
Rat      ------------------------------------------------------------     1040


APP      CTGCTTACTGGCTTGCTTCCCCTGGCTTGCTCAGTTTGCTTTCTTATAGAACCCAAGACT     1626
Rat      ------------------------------------------------------------     1040


APP      ACCAGTCCAGGGATGGCACCACCCACAATGGGCCCTCCCCCCTTGATCACAATAATTGAG     1686
Rat      ------------------------------------------------------------     1040


APP      AAAATGCCTTACAGCTAGATCGCATGGAGGCATTTCCTTGAGGGAGGCTCCTTTCTCTAT     1746
Rat      ------------------------------------------------------------     1040


APP      GATAACTCCAGCTTGTGTCAAGTTGACACACAAAAACTAACAGGTTCCAAATGTGAAACA     1806
Rat      ------------------------------------------------------------     1040


APP      CACACACACACACACACACACACAAACAAAACAAAAACAGAGAAAAGCTGTAGAGTTTTG     1866
Rat      ------------------------------------------------------------     1040
```

```
APP     ATTTGGGGGTTTTGTTTTTTGTTTTGTTTTGTTTTGTTTTGTTTTTCGCTTTCAAGCTAG     1926
Rat     ------------------------------------------------------------     1040


APP     GGTCTCAAGTATCCCAATCTAACCTTTAACTCATGTAGCCAAAGATGGCCTTATCCTCCT     1986
Rat     ------------------------------------------------------------     1040


APP     GATGGTCCTGCCTCTACCTTGCAAATATTGGGACTGCAGCATGCGCCATCGTGCCCTGTG     2046
Rat     ------------------------------------------------------------     1040


APP     TATATGAAGTGCTGGGATTTGACTTCAAGGCTCCCTGTATGCTAAGTAAGTTCTCTACCA     2106
Rat     ------------------------------------------------------------     1040


APP     AGTGAGCTACATCCCCAGGCCCTGGAAATGATGGGTAGAGCAGCTGATAGTCTGCAGCAA     2166
Rat     ------------------------------------------------------------     1040


APP     GCTCTGGACCTGGGCACTCCCCACATTTTTGGGGGAGTCACTCTTATTCGTGGGGATACG     2226
Rat     ------------------------------------------------------------     1040


APP     AACGTTCTAGGATATTTAACCGCATCCGCCTGATTCTAACCAGAAATGTCTCCCGAGCTG     2286
Rat     ------------------------------------------------------------     1040


APP     TATGATGTCTCACACGCCTGTGACTCCAGTGCTTGGGAGGGAAGCAAGAGACCAGGCCTG     2346
Rat     ------------------------------------------------------------     1040


APP     CCTGGAAAACAGGGTGAGATCCTGTCTCAAAGAGTGAGAAGCAGAAGTGTTTCCAGAAGA     2406
Rat     ------------------------------------------------------------     1040


APP     CGTTCTCCAACACCAGAAGACGGATCAAGTGTCCAGATCCTGCCAAACATCTCTTACGGA     2466
Rat     ------------------------------------------------------------     1040


APP     AGCGAGTTACAAACCACTGATCTATAAACAGTATTTTGACCTTTATATTATGTGGCATGT     2526
Rat     ------------------------------------------------------------     1040


APP     GTTGTATATGATACAATGAACGTGCGTTTGAGTTTCAAAGGACTACTGGCTGGGAAGTGT     2586
Rat     ------------------------------------------------------------     1040


APP     GTGCCTTCCACTCTGTGTGGTCTGGAATCAGACTCAAGTCATCAGGCTTGGCAGCAAGTA     2646
Rat     ------------------------------------------------------------     1040


APP     CTTTTACCCACTGAGCCTTCCTACCATCCTTCATTTTAGCTTAATGGCCTGTGCACACAA     2706
Rat     ------------------------------------------------------------     1040


APP     CCTTTTATTGAGGATGCTTGTATTAATTCCAGCTGCTATAAGAATAAACAATAGACAAAG     2766
Rat     ------------------------------------------------------------     1040


APP     A         2767
Rat     -         1040
```

Table 2. A) TLR2 DNA sequence (variant 1, exon 2) from *Mus musculus*, B) TLR2 DNA sequence (variant 1, exon 2) from *Rattus norvegicus* C) Clustal alignment of variants of the Tlr2 gene from *Mus musculus* and *Rattus norvegicus*.

A

TCACATGAGCGTCATTTGTTTTAAGGTCAAATCTCAGAGGATGCTACGAGCTCTTTGGCTCTTCTGGATCTTGGTGGCCATAACAGT
CCTCTTCAGCAAACGCTGTTCTGCTCAGGAGTCTCTGTCATGTGATGCTTCTGGGGTGTGTGATGGCCGCTCCAGGTCTTTCACCTC
TATTCCCTCCGGACTCACAGCAGCCATGAAAAGCCTTGACCTGTCTTTCAACAAGATCACCTACATTGGCCATGGTGACCTCCGAGC
GTGTGCGAACCTCCAGGTTCTGATGTTGAAGTCCAGCAGAATCAATACAATAGAGGGAGACGCCTTTTATTCTCTGGGCAGTCTTGA
ACATTTGGATTTGTCTGATAATCACCTATCTAGTTTATCTTCCTCCTGGTTCGGGCCCCTTTCCTCTTTGAAATACTTAAACTTAAT
GGGAAATCCTTACCAGACACTGGGGGTAACATCGCTTTTTCCCAATCTCACAAATTTACAAACCCTCAGGATAGGAAATGTAGAGAC
TTTCAGTGAGATAAGGAGAATAGATTTTGCTGGGCTGACTTCTCTCAATGAACTTGAAATTAAGGCATTAAGTCTCCGGAATTATCA
GTCCCAAAGTCTAAAGTCGATCCGCGACATCCATCACCTGACTCTTCACTTAAGCGAGTCTGCTTTCCTGCTGGAGATTTTTGCAGA
TATTCTGAGTTCTGTGAGATATTTAGAACTAAGAGATACTAACTTGGCCAGGTTCCAGTTTTCACCACTGCCCGTAGATGAAGTCAG
CTCACCGATGAAGAAGCTGGCATTCCGAGGCTCGGTTCTCACTGATGAAAGCTTTAACGAGCTCCTGAAGCTGTTGCGTTACATCTT
GGAACTGTCGGAGGTAGAGTTCGACGACTGTACCCTCAATGGGCTCGGCGATTTCAACCCCTCGGAGTCAGACGTAGTGAGCGAGCT
GGGTAAAGTAGAAACAGTCACTATCCGGAGGTTGCATATCCCCCAGTTCTATTTGTTTTATGACCTGAGTACTGTCTATTCCCTCCT
GGAGAAGGTGAAGCGAATCACAGTAGAGAACAGCAAGGTCTTCCTGGTTCCCTGCTCGTTCTCCCAGCATTTAAAATCATTAGAATT
CTTAGACCTCAGCGAAAATCTGATGGTTGAAGAATATTTGAAGAACTCAGCCTGTAAGGGAGCCTGGCCTTCTCTACAAACCTTAGT
TTTGAGCCAGAATCATTTGAGATCAATGCAAAAAACAGGAGAGATTTTGCTGACTCTGAAAAACCTGACCTCTCTTGACATCAGCAG
GAACACTTTTCATCCGATGCCCGACAGCTGTCAGTGGCCAGAAAAGATGCGCTTCCTGAATTTGTCCAGTACAGGGATCCGGGTGGT
AAAAACGTGCATTCCTCAGACGCTGGAGGTGTTGGATGTTAGTAACAACAATCTTGACTCATTTTCTTTGTTCTTGCCTCGGCTGCA
AGAGCTCTATATTTCCAGAAATAAGCTGAAAACACTCCCAGATGCTTCGTTGTTCCCTGTGTTGCTGGTCATGAAAATCAGAGAGAA
TGCAGTAAGTACTTTCTCTAAAGACCAACTTGGTTCTTTTCCCAAACTGGAGACTCTGGAAGCAGGCGACAACCACTTTGTTTGCTC
CTGCGAACTCCTATCCTTTACTATGGAGACGCCAGCTCTGGCTCAAATCCTGGTTGACTGGCCAGACAGCTACCTGTGTGACTCTCC
GCCTCGCCTGCACGGCCACAGGCTTCAGGATGCCCGGCCCTCCGTCTTGGAATGTCACCAGGCTGCACTGGTGTCTGGAGTCTGCTG
TGCCCTTCTCCTGTTGATCTTGCTCGTAGGTGCCCTGTGCCACCATTTCCACGGACTGTGGTACCTGAGAATGATGTGGGCGTGGCT
CCAGGCCAAGAGGAAGCCCAAGAAAGCTCCCTGCAGGGACGTTTGCTATGATGCCTTTGTTTCCTACAGTGAGCAGGATTCCCATTG
GGTGGAGAACCTCATGGTCCAGCAGCTGGAGAACTCTGACCCGCCCTTTAAGCTGTGTCTCCACAAGCGGGACTTCGTTCCGGGCAA
ATGGATCATTGACAACATCATCGATTCCATCGAAAAGAGCCACAAAACTGTGTTCGTGCTTTCTGAGAACTTCGTACGGAGCGAGTG
GTGCAAGTACGAACTGGACTTCTCCCACTTCAGGCTCTTTGACGAGAACAACGACGCGGCCATCCTTGTTTTGCTGGAGCCCATTGA
GAGGAAAGCCATTCCCCAGCGCTTCTGCAAACTGCGCAAGATAATGAACACCAAGACCTACCTGGAGTGGCCCTTGGATGAAGGCCA
GCAGGAAGTGTTTTGGGTAAATCTGAGAACTGCAATAAAGTCCTAGGTTCTCCACCCAGTTCCTGACTTCCTTAACTAAGGTCTTTG
TGACACAAACTGTAACAAAGTTTATAAGTAACATAGAATTGTATTATTGAGGATATTAACTATGGGTTTTGTCTTGAATACTGTTAT
ATAAATATGTGACATCAGGA

B

TGCTTCGTTTGTTTTCAGGTCAAATCTCAGAGGATGCTGCAAGCTCTTTGGCTCTTCTGGATCTTGATGGCTGTGATAGGCCTCTCA
AGGGAAGGCCATTCTGCCCAGGCATCTCTGTCATGTGATGCTGCTGGTGTGTGTGATGGCAGCTCCAGGTCTTTCACCTCTATTCCC
TCGGGACTCACAGCAAACACAAAGAAGCTTGACCTGTCTTTCAACAAGATCACCTACATTGGCCATGGTGACTTGCGAGCCTGTGTG
AACCTCCGGGTTCTGACATTGGAGTCCAGCGGAATCAACACAATAGAGGGAGATGCCTTTTATTCTCTGGGCAGTCTTGAACACTTG
GACTTGTCTAATAATCACCTATCTAGTTTATCTTCCTCCTGGTTCAGGCCCCTTTCCTCTTTGAAATACTTAAACTTAATGGGAAAT
CCTTACAGGACACTGGGGGAAACATCACTGTTCTCCAATCTCACAAATTTACAAAACCTTAGGGTAGGAAATGTTGACACTTTCA
GTGAGATAAGGAGAATAGATTTTGCTGGGCTGACCTCTCTCAACGAACTTGAAATTCAGGTATTAAGTCTCGGCAACTATGAGTCCC
GAAGTCTACAGTCAATTAGAGACATCTATCACCTGACCCTGCACTTGAGCGAGTCTGCTTTCCTGCTGGGGATTTTTGCAGATATTC
TGAGTTCCGTGAGATATTTAGAACTAAGAGATACTAACTTGGCTAGGTTCCAATTTTCTGAACTGTCTGTAGACGAAATCAATTCGC
CAATGAAGAAGCTGGCATTCCGGAATGCAGATCTCACCGATAAAAGTTTTAATGAACTCCTGAAGCTGTTGCGTTACATCTTGGAAC
TGATGGAGGTGGAGTTTGATCACTGCACCCTCAATGGGGTTGGTAATTTCAACCCCTCAGAGTCAGACGTAGTGAGGGAGCTAGGTA
AAGTAGAAACGGTAACAATACGGAGCCTGCACATCCCCCAGTTCTATTTGTTCTATGATCTGAGCACTGTGTATTCCCTCCTGGAGA
AAGTGAAGCGAATCACAGTAGAGAACAGTAAGGTCTTTCTGGTTCCCTGCTCTTTCTCACAGCATTTAAAATCATTAGAGTTCTTAG
ACCTCAGCGAAAATCTGATGGTTGAAGAGTATTTGAAAAACTCAGCCTGTGAGGGTGGCTGGCCTTCTCTACAAAGCTTGGTTTTGA
GTCAGAATCATTTGAGATCAATACGAAAAACTGCTGAGATTTTGCTGACTCTGAAAAACCTGACAGCCCTTGACATCAGCAAGAACA

GTTTTCAGCCTATGCCCGACAGTTGTCAGTGGCCAGGAAAGATGCGCTTCCTGAACTTGTCCAGTACAGGGATACAGGCCGTCAAGA
CATGCATTCCTCAGACCCTGGAGGTGTTGGATGTTAGTAACAACAATCTCGATTCATTTTCTTTGTTTTTGCCTCGGCTCCAAGAGC
TGTATATTTCCAGAAATAAACTGAAGACACTCCCAGAAGCTTCATTGTTCCCTGTGTTACAGGTCATGAAGATCAGAGAGAACGCAA
TAAGTACTTTCTCTAAAGACCAGCTTGGTTCTTTTCCCAAACTGGAGACTCTGGAAGCAGGTGACAACCATTTCATCTGCTCCTGTG
AACTCCTGTCCTTCATTCTGGAGAGGCCAGCCCTGGTCCATGTCCTGGTTGACTGGCCAGACAGCTACCTGTGTGATTCTCCGCCTC
GCCTGCATGGCCAGAGGCTTCAGGATGCCCGGCCCTCAGTCTTGGAGTGTCACCAGGCCGCACTGGTCTCTGGAGTCTGCTGTGCCC
TTCTCCTGTTGATCCTGCTCTTAGGCGCCCTGTGTTACCATTTCCATGGGCTGTGGTATCTGAGAATGATGTGGGCGTGGCTCCGGG
CCAAGAGGAAGCCCAAGAAAGCTCCTTGCAGGGACCTTTGCTATGATGCCTTTGTTTCCTACAGCGAGCAGGATTCCTATTGGGTGG
AGAACCTCATGGTCCAGCAGCTGGAGAACTCTGACCCACCCTTCAAGCTGTGTCTCCACAAGCGGGACTTTGTTCCGGGCAAATGGA
TCATTGACAACATCATTGATTCCATCGAAAAGAGCCACAAAACAGTGTTCGTGCTTTCTGAGAACTTCGTACGCAGTGAGTGGTGCA
AGTATGAACTGGACTTCTCCCACTTCAGGCTCTTTGACGAGAACAATGACGCGGCCATCCTTGTTTTGCTGGAACCCATTGAGAAGA
AAGCCATTCCCCAGCGCTTCTGCAAACTGCGCAAGATAATGAACACTAAGACATACCTGGAGTGGCCCTTGGATGAAGGCCAGCGGG
AAGTGTTTTGGGCAAATCTGAGAACTGCAATAAAATCCTAGGTTCTCCACCCTGTTTCTGTCTTGCCTTAACTAAAGGTCTTTGTGA
CAGGAACTGTAACAAAGTTTATTCTGACATAATTATATAAGTATCATAGAATTGTACTACTGAGGATTTGGTAAGTATAACTAGTAA
CTATGAGCTTTATCTTGAATACTGTTATATAAATATTTGACATCAGTTTCTCTGTTTTTCTTTATCTTTCTCCATTATTGCTATCTG
AATAACCTGTTCACTTAGAGCATCTGGAAATGGTACATGTAGAAAGATGGTTTAATGCCTATTATTTAATGCCCAGGCAGTGTGACA
TAGGGTCACAGAAAGTATTTCCTAATCTACTCACCTGCCATCCCTCCGTGCAGCTGATATCAAGAGTAAGGAGTCACGATATTTGGG
GCATCTAGGCAAATCTCTTGCTATTCCTAGAGAGGGGGTTGTATCCCACCATCCCCTCAGACCTTCCAGTACAAATGACACAAGCTG
CAAAACTAGGCTTGTTTGTCTGAGTATTTGATAGTTTTCTCTAAGTATGAATTAAATGTTGATTTGTAAGGATCTGGTGCAGATTCC
CACTGGACTTTAGCTCTGAATTTCCAGTGACTGGTTGCTGTGGCTCCTGATCCCCTTGGATGTTCTGTTGCCTTTTTTCCTGTGCAG
GGTCTCATTTGTAATTCTATAAAGCTTTTCTTTGCCTGTATGGAAAAAATACATGTGTGCAAAAGATTTTTAAAGTAA

C

CLUSTAL O(1.2.4) multiple sequence alignment

```
TLR      TCACATGAGCGTCATTTGTTTTAAGGTCAAATCTCAGAGGATGCTACGAGCTCTTTGGCT       60
Rat      -------TGCTTCGTTTGTTTTCAGGTCAAATCTCAGAGGATGCTGCAAGCTCTTTGGCT       53
                ** ** ******** ********************** * ************

TLR      CTTCTGGATCTTGGTGGCCATAACAGTCCTCTTCAGCAAACGCTGTTCTGCTCAGGAGTC       120
Rat      CTTCTGGATCTTGATGGCTGTGATAGGCCTCTCAAGGGAAGGCCATTCTGCCCAGGCATC       113
         ************* ****  * * ** *****  **  ** **  ****** ****  **

TLR      TCTGTCATGTGATGCTTCTGGGGTGTGTGATGGCCGCTCCAGGTCTTTCACCTCTATTCC       180
Rat      TCTGTCATGTGATGCTGCTGGTGTGTGTGATGGCAGCTCCAGGTCTTTCACCTCTATTCC       173
         **************** **** ************ ************************

TLR      CTCCGGACTCACAGCAGCCATGAAAAGCCTT GACCTGTCTTTCAACAAGATCACC TACAT       240
Rat      CTCGGGACTCACAGCAAACACAAAGAAGCTTGACCTGTCTTTCAACAAGATCACCTACAT       233
         *** *************  **  ** *  ********************************

TLR      TGGCCATGGTGACCTCCGAGCGTGTGCGAACCTCCAGGTTCTGATGTTGAAGTCCAGCAG       300
Rat      TGGCCATGGTGACTTGCGAGCCTGTGTGAACCTCCGGGTTCTGACATTGGAGTCCAGCGG       293
         ************* * ***** **** ******** ********  *** ******** *

TLR      AATCAATACAATAGAGGGAGACGCCTTTTATTCTCTGGGCAGTCTTGAACATTTGGATTT       360
Rat      AATCAACACAATAGAGGGAGATGCCTTTTATTCTCTGGGCAGTCTTGAACACTTGGACTT       353
         ****** ************** ***************************** ***** **

TLR      GTCTGATAATCACCTATCTAGTTTATCTTCCTCCTGGTTCGGGCCCCTTTCCTCTTTGAA       420
Rat      GTCTAATAATCACCTATCTAGTTTATCTTCCTCCTGGTTCAGGCCCCTTTCCTCTTTGAA       413
         **** ********************************** ******************

TLR      ATACTTAAACTTAATGGGAAATCCTTACCAGACACTGGGGGTAACATCGCTTTTTCCCAA       480
Rat      ATACTTAAACTTAATGGGAAATCCTTACAGGACACTGGGGGAAACATCACTGTTCTCCAA       473
         ***************************  *********** ****** ** **  ****
```

```
TLR     TCTCACAAATTTACAAACCCTCAGGATAGGAAATGTAGAGACTTTCAGTGAGATAAGGAG    540
Rat     TCTCACAAATTTACAAAACCTTAGGGTAGGAAATGTTGACACTTTCAGTGAGATAAGGAG    533
        ***************** *** *** ********** ** ********************

TLR     AATAGATTTTGCTGGGCTGACTTCTCTCAATGAACTTGAAATTAAGGCATTAAGTCTCCG    600
Rat     AATAGATTTTGCTGGGCTGACCTCTCTCAACGAACTTGAAATTCAGGTATTAAGTCTCGG    593
        ********************** ******** ************ *** ********** *

TLR     GAATTATCAGTCCCAAAGTCTAAAGTCGATCCGCGACATCCATCACCTGACTCTTCACTT    660
Rat     CAACTATGAGTCCCGAAGTCTACAGTCAATTAGAGACATCTATCACCTGACCCTGCACTT    653
         ** *** ****** ******* **** **  * ****** ********** ** *****

TLR     AAGCGAGTCTGCTTTCCTGCTGGAGATTTTTGCAGATATTCTGAGTTCTGTGAGATATTT    720
Rat     GAGCGAGTCTGCTTTCCTGCTGGGGATTTTTGCAGATATTCTGAGTTCCGTGAGATATTT    713
         ********************** ********************** ***********

TLR     AGAACTAAGAGATACTAACTTGGCCAGGTTCCAGTTTTCACCACTGCCCGTAGATGAAGT    780
Rat     AGAACTAAGAGATACTAACTTGGCTAGGTTCCAATTTTCTGAACTGTCTGTAGACGAAAT    773
        ********************** ******** *****   **** * ***** *** *

TLR     CAGCTCACCGATGAAGAAGCTGGCATTCCGAGGCTCGGTTCTCACTGATGAAAGCTTTAA    840
Rat     CAATTCGCCAATGAAGAAGCTGGCATTCCGGAATGCAGATCTCACCGATAAAAGTTTTAA    833
        **  ** ** ********************     * * ****** *** **** *****

TLR     CGAGCTCCTGAAGCTGTTGCGTTACATCTTGGAACTGTCGGAGGTAGAGTTCGACGACTG    900
Rat     TGAACTCCTGAAGCTGTTGCGTTACATCTTGGAACTGATGGAGGTGGAGTTTGATCACTG    893
         ** ********************************  ****** ***** **  ****

TLR     TACCCTCAATGGGCTCGGCGATTTCAACCCCTCGGAGTCAGACGTAGTGAGCGAGCTGGG    960
Rat     CACCCTCAATGGGGTTGGTAATTTCAACCCCTCAGAGTCAGACGTAGTGAGGGAGCTAGG    953
         ************ * **  ************* ***************** ***** **

TLR     TAAAGTAGAAACAGTCACTATCCGGAGGTTGCATATCCCCCAGTTCTATTTGTTTTATGA   1020
Rat     TAAAGTAGAAACGGTAACAATACGGAGCCTGCACATCCCCCAGTTCTATTTGTTCTATGA   1013
        ************ ** ** ** *****  **** ******************** *****

TLR     CCTGAGTACTGTCTATTCCCTCCTGGAGAAGGTGAAGCGAATCACAGTAGAGAACAGCAA   1080
Rat     TCTGAGCACTGTGTATTCCCTCCTGGAGAAAGTGAAGCGAATCACAGTAGAGAACAGTAA   1073
         ***** ***** ***************** ************************** **

TLR     GGTCTTCCTGGTTCCCTGCTCGTTCTCCCAGCATTTAAAATCATTAGAATTCTTAGACCT   1140
Rat     GGTCTTTCTGGTTCCCTGCTCTTTCTCACAGCATTTAAAATCATTAGAGTTCTTAGACCT   1133
        ****** ************** ***** ******************** ***********

TLR     CAGCGAAAATCTGATGGTTGAAGAATATTTGAAGAACTCAGCCTGTAAGGGAGCCTGGCC   1200
Rat     CAGCGAAAATCTGATGGTTGAAGAGTATTTGAAAAACTCAGCCTGTGAGGGTGGCTGGCC   1193
        ************************ ******** ************ **** * ******

TLR     TTCTCTACAAACCTTAGTTTTGAGCCAGAATCATTTGAGATCAATGCAAAAAACAGGAGA   1260
Rat     TTCTCTACAAAGCTTGGTTTTGAGTCAGAATCATTTGAGATCAATACGAAAAACTGCTGA   1253
        *********** *** ******** ******************** * ****** *  **

TLR     GATTTTGCTGACTCTGAAAAACCTGACCTCTCTTGACATCAGCAGGAACACTTTTCATCC   1320
Rat     GATTTTGCTGACTCTGAAAAACCTGACAGCCCTTGACATCAGCAAGAACAGTTTTCAGCC   1313
        **************************  * ************* ***** ****** **

TLR     GATGCCCGACAGCTGTCAGTGGCCAGAAAAGATGCGCTTCCTGAATTTGTCCAGTACAGG   1380
Rat     TATGCCCGACAGTTGTCAGTGGCCAGGAAAGATGCGCTTCCTGAACTTGTCCAGTACAGG   1373
         *********** ************* ****************** **************

TLR     GATCCGGGTGGTAAAAACGTGCATTCCTCAGACGCTGGAGGTGTTGGATGTTAGTAACAA   1440
Rat     GATACAGGCCGTCAAGACATGCATTCCTCAGACCCTGGAGGTGTTGGATGTTAGTAACAA   1433
        *** * **  ** ** ** ************** *************************

TLR     CAATCTTGACTCATTTTCTTTGTTCTTGCCTCGGCTGCAAGAGCTCTATATTTCCAGAAA   1500
Rat     CAATCTCGATTCATTTTCTTTGTTTTTGCCTCGGCTCCAAGAGCTGTATATTTCCAGAAA   1493
        ****** ** ************** ************ ******** **************

TLR     TAAGCTGAAAACACTCCCAGATGCTTCGTTGTTCCCTGTGTTGCTGGTCATGAAAATCAG   1560
Rat     TAAACTGAAGACACTCCCAGAAGCTTCATTGTTCCCTGTGTTACAGGTCATGAAGATCAG   1553
        *** ***** ************ ***** ************** * ********* *****
```

```
TLR      AGAGAATGCAGTAAGTACTTTCTCTAAAGACCAACTTGGTTCTTTTCCCAAACTGGAGAC       1620
Rat      AGAGAACGCAATAAGTACTTTCTCTAAAGACCAGCTTGGTTCTTTTCCCAAACTGGAGAC       1613
         ****** *** ********************** *************************

TLR      TCTGGAAGCAGGCGACAACCACTTTGTTTGCTCCTGCGAACTCCTATCCTTTACTATGGA       1680
Rat      TCTGGAAGCAGGTGACAACCATTTCATCTGCTCCTGTGAACTCCTGTCCTTCATTCTGGA       1673
         ************ ******** **  * ******** ******** ***** * * ****

TLR      GACGCCAGCTCTGGCTCAAATCCTGGTTGACTGGCCAGACAGCTACCTGTGTGACTCTCC       1740
Rat      GAGGCCAGCCCTGGTCCATGTCCTGGTTGACTGGCCAGACAGCTACCTGTGTGATTCTCC       1733
         ** ****** ****  **  ********************************** *****

TLR      GCCTCGCCTGCACGGCCACAGGCTTCAGGATGCCCGGCCCTCCGTCTTGGAATGTCACCA       1800
Rat      GCCTCGCCTGCATGGCCAGAGGCTTCAGGATGCCCGGCCCTCAGTCTTGGAGTGTCACCA       1793
         ************ ***** ********************** ******** ********

TLR      GGCTGCACTGGTGTCTGGAGTCTGCTGTGCCCTTCTCCTGTTGATCTTGCTCGTAGGTGC       1860
Rat      GGCCGCACTGGTCTCTGGAGTCTGCTGTGCCCTTCTCCTGTTGATCCTGCTCTTAGGCGC       1853
         *** ******** ********************************** ***** **** **

TLR      CCTGTGCCACCATTTCCACGGACTGTGGTAC**CTGAGAATGATGTGGGCGTG**GCTCCAGGC       1920
Rat      CCTGTGTTACCATTTCCATGGGCTGTGGTATCTGAGAATGATGTGGGCGTGGCTCCGGGC       1913
         ******  ********** ** ******** ************************ ***

TLR      CAAGAGGAAGCCCAAGAAAGCTCCCTGCAGGGACGTTTGCTATGATGCCTTTGTTTCCTA       1980
Rat      CAAGAGGAAGCCCAAGAAAGCTCCTTGCAGGGACCTTTGCTATGATGCCTTTGTTTCCTA       1973
         ************************ ********* *************************

TLR      CAGTGAGCAGGATTCCCATTGGGTGGAGAACCTCATGGTCCAGCAGCTGGAGAACTCTGA       2040
Rat      CAGCGAGCAGGATTCCTATTGGGTGGAGAACCTCATGGTCCAGCAGCTGGAGAACTCTGA       2033
         *** ************ ******************************************

TLR      CCCGCCCTTTAAGCTGTGTCTCCACAAGCGGGACTTCGTTCCGGGCAAATGGATCATTGA       2100
Rat      CCCACCCTTCAAGCTGTGTCTCCACAAGCGGGACTTTGTTCCGGGCAAATGGATCATTGA       2093
         *** ***** ************************** ***********************

TLR      CAACATCATCGATTCCATCGAAAAGAGCCACAAAACTGTGTTCGTGCTTTCTGAGAACTT       2160
Rat      CAACATCATTGATTCCATCGAAAAGAGCCACAAAACAGTGTTCGTGCTTTCTGAGAACTT       2153
         ********* ************************** ***********************

TLR      CGTACGGAGCGAGTGGTGCAAGTACGAACTGGACTTCTCCCACTTCAGGCTCTTTGACGA       2220
Rat      CGTACGCAGTGAGTGGTGCAAGTATGAACTGGACTTCTCCCACTTCAGGCTCTTTGACGA       2213
         ****** ** ************** ***********************************

TLR      GAACAACGACGCGGCCATCCTTGTTTTGCTGGAGCCCATTGAGAGGAAAGCCATTCCCCA       2280
Rat      GAACAATGACGCGGCCATCCTTGTTTTGCTGGAACCCATTGAGAAGAAAGCCATTCCCCA       2273
         ****** ************************** ********** ***************

TLR      GCGCTTCTGCAAACTGCGCAAGATAATGAACACCAAGACCTACCTGGAGTGGCCCTTGGA       2340
Rat      GCGCTTCTGCAAACTGCGCAAGATAATGAACACTAAGACATACCTGGAGTGGCCCTTGGA       2333
         ********************************** ***** ********************

TLR      TGAAGGCCAGCAGGAAGTGTTTTGGGTAAATCTGAGAACTGCAATAAAGTCCTAGGTTCT       2400
Rat      TGAAGGCCAGCGGGAAGTGTTTTGGGCAAATCTGAGAACTGCAATAAAATCCTAGGTTCT       2393
         *********** ************** ********************** ***********

TLR      CCACCCAGTTCCTGACTTCCTTAACTAA--GGTCTTTGTGACACAAACTGTAACAAAGTT       2458
Rat      CCACCCTGTTTCTGTCTTGCCTTAACTAAAGGTCTTTGTGACAGGAACTGTAACAAAGTT       2453
         ****** *** *** *** * * *   *  *************  ***************

TLR      TATAAGTAACAT----------------AGAATTGTATTATTG---------------AG       2487
Rat      TATTCTGACATAATTATATAAGTATCATAGAATTGTACTACTGAGGATTTGGTAAGTATA       2513
         ***    *                    ********* ** **

TLR      GATATTAACTATGGGTTTTGTCTTGAATACTGTTATATAAATATGTGACATCAGGA----       2543
Rat      ACTAGTAACTATGAGCTTTATCTTGAATACTGTTATATAAATATTTGACATCAGTTTCTC       2573
           ** ******** * *** ********************** *********

TLR      ------------------------------------------------------------       2543
Rat      TGTTTTTCTTTATCTTTCTCCATTATTGCTATCTGAATAACCTGTTCACTTAGAGCATCT       2633
```

```
TLR      ------------------------------------------------------------        2543
Rat      GGAAATGGTACATGTAGAAAGATGGTTTAATGCCTATTATTTAATGCCCAGGCAGTGTGA        2693

TLR      ------------------------------------------------------------        2543
Rat      CATAGGGTCACAGAAAGTATTTCCTAATCTACTCACCTGCCATCCCTCCGTGCAGCTGAT        2753

TLR      ------------------------------------------------------------        2543
Rat      ATCAAGAGTAAGGAGTCACGATATTTGGGGCATCTAGGCAAATCTCTTGCTATTCCTAGA        2813

TLR      ------------------------------------------------------------        2543
Rat      GAGGGGGTTGTATCCCACCATCCCCTCAGACCTTCCAGTACAAATGACACAAGCTGCAAA        2873

TLR      ------------------------------------------------------------        2543
Rat      ACTAGGCTTGTTTGTCTGAGTATTTGATAGTTTTCTCTAAGTATGAATTAAATGTTGATT        2933

TLR      ------------------------------------------------------------        2543
Rat      TGTAAGGATCTGGTGCAGATTCCCACTGGACTTTAGCTCTGAATTTCCAGTGACTGGTTG        2993

TLR      ------------------------------------------------------------        2543
Rat      CTGTGGCTCCTGATCCCCTTGGATGTTCTGTTGCCTTTTTTCCTGTGCAGGGTCTCATTT        3053

TLR      ------------------------------------------------------------        2543
Rat      GTAATTCTATAAAGCTTTTCTTTGCCTGTATGGAAAAAATACATGTGTGCAAAAGATTTT        3113

TLR      --------  2543
Rat      TAAAGTAA  3121
```

Table 3. A) Beclin1 DNA sequence (variant 1, exon 12) from *Mus musculus*, B) Beclin1 DNA sequence (variant 1, exon 12) from Rattus norvegicus, C)Clustal alignment of variants of the beclin1 gene from *Mus musculus* and *Rattus norvegicus*.

A

```
GACTTAGGTTGGCTCTGTTACTAATGAGCTCTGCTATGTCCACCTGCAGGATGGACGTGGAGAAAGGCAAGATTGAAGACACTGGAG
GCAGTGGCGGCTCCTATTCCATCAAAACCCAGTTTAACTCGGAGGAGCAGTGGACAAAAGCGCTCAAGTTCATGCTGACCAATCTCA
AGTGGGGTCTTGCCTGGGTGTCCTCACAGTTCTATAACAAGTGACTTGCTCCTTAGGGGATGTTTGCCTTTAAGGTTTTATACTTTG
TTTGGTTTGGAAAGATGCTTTAAATTAAATTTGGGTAATATTAAACCACATGTTTACAATACCAAAATCCACAAAAGCTACTTTATT
TTCAAATATGACAGATAGTTTCCAGAGTACGCCATGTATAGCAAAGAACCCTGCCATAGTTTTGACTCAGCCCCATGCATCCTTTCC
CTCTTTCCTGAAAACAACTAATTTAAATTTGCTTTGTTTTCTTTTTTAAGTTGAATTGACGTTAATGTGTTTTCACTGGATTTTATC
TCTCTCAACTTCCTGCACTTAAAATTTGAAACAGCAAAGGTTTGAGATGAGATGCTTGTGGCACACAGTTGGGTGATGTGGGGAAAG
GACACCGGGTCAGGAGTTGCAAGTTTAACTCCGTCCTCACTTGTAGCATTGAATGCCTCCTGTGCTGTCTAGTGGGACTACAGAATG
CTGTTTGATACTGTGTGCGACGTGGAGAGATTTAATTATTTGTAATAAAGGATTTGCTATGGTCTATTAATTA
```

B

```
ACCTGCAGGATGGACGTGGAGAAAGGCAAGATTGAAGACACTGGAGGCAGTGGCGGCTCCTATTCCATCAAAACCCAGTTTAACTCT
GAGGAGCAGTGGACAAAGGCGCTCAAGTTCATGCTGACGAATCTCAAGTGGGGTCTTGCTTGGGTGTCCTCACAGTTCTATAACAAG
```

TGACTTGCTCCTTAGGGGATATTTGCCTTTAAGGTTTTACATTTTGTTTGGTTTGGAAAGATGCTTTAAATTAAATTTGGGTAATAT
TAAACCACATGTTTACAATACCAGAATCCACAAAAGCTACTTTATTTTACAATATGACAGTTTTCAGAGCACAGCACGCCATGTATA
GCAAAGAGCCCTGCCGTAGTTTGGCTCAACCCCATGCTGTCCTTTCCCTCTTCCCTGGAAACAACTAATTTAAATTTGCTTTGTTTT
CTTTTTTAAGTTGAATTGACATTAATGTGTTTTCACTGGATTTTATCTCTCTCAACTTCCTGTACTTGAAACAGAAAAGTTTTGAGA
TGAGATGCTTGTGGCACACAGTTGGGTAATGTGGGGAAGGGACAAGAGGTAAAGGTTTAACTCTGTCCTCACTTCTAGTATTGAACG
CTTACTGTGCTAACTATTGGAATTACAAAATGCTGTTTGATACTGTTTGAGATGTGGAAGGATTTAATTATTTGTAATAAAGGATTT
GCTATGGTCTATTAATTA

C

CLUSTAL O(1.2.4) multiple sequence alignment

```
BECN1      GACTTAGGTTGGCTCTGTTACTAATGAGCTCTGCTATGTCCACCTGCAGGATGGACGTGG      60
Rat        ----------------------------------------ACCTGCAGGATGGACGTGG      19
                                                    ********************

BECN1      AGAAAGGCAAGATTGAAGACACTGGAGGCAGTGGCGGCTCCTATTCCATCAAAACCCAGT      120
Rat        AGAAAGGCAAGATTGAAGACACTGGAGGCAGTGGCGGCTCCTATTCCATCAAAACCCAGT      79
           ************************************************************

BECN1      TTAACTCGGAGGAGCAGTGGACAAAAGCGCTCAAGTTCATGCTGACCAATCTCAAGTGGG      180
Rat        TTAACTCTGAGGAGCAGTGGACAAAGGCGCTCAAGTTCATGCTGACGAATCTCAAGTGGG      139
           ******* ***************** ******************* *************

BECN1      GTCTTGCCTGGGTGTCCTCACAGTTCTATAACAAGTGACTTGCTCCTTAGGGGATGTTTG      240
Rat        GTCTTGCTTGGGTGTCCTCACAGTTCTATAACAAGTGACTTGCTCCTTAGGGGATATTTG      199
           ******* ********************************************** ****

BECN1      CCTTTAAGGTTTTATACTTTGTTTGGTTTGGAAAGATGCTTTAAATTAAATTTGGGTAAT      300
Rat        CCTTTAAGGTTTTACATTTTGTTTGGTTTGGAAAGATGCTTTAAATTAAATTTGGGTAAT      259
           ************** * ******************************************

BECN1      ATTAAACCACATGTTTACAATACCAAAATCCACAAAAGCTACTTTATTTTCAAATATGAC      360
Rat        ATTAAACCACATGTTTACAATACCAGAATCCACAAAAGCTACTTTATTTTACAATATGAC      319
           ************************ ************************  ********

BECN1      AGATAG-TTTCCAGAGTACGCCATGTATAGCAAAGAACCCTGCCATAGTTTTGACTCAGC      419
Rat        AGTTTTCAGAGCACAGCACGCCATGTATAGCAAAGAGCCCTGCCGTAGTTTGGCTCAACC      379
           ** *       ** ** ****************** ******* ****** *    * *

BECN1      CCCATGCATCCTTTCCCTCTTTCCTGAAAACAACTAATTTAAATTTGCTTTGTTTTCTTT      479
Rat        CCATGCTGTCCTTTCCCTCTTCCCTGGAAACAACTAATTTAAATTTGCTTTGTTTTCTTT      439
           **      ************* **** **********************************

BECN1      TTTAAGTTGAATTGACGTTAATGTGTTTTCACTGGATTTTATCTCTCTCAACTTCCTGCA      539
Rat        TTTAAGTTGAATTGACATTAATGTGTTTTCACTGGATTTTATCTCTCTCAACTTCCTGTA      499
           ***************** ****************************************** *

BECN1      CTTAAAATTTGAAACAGCAAAGGTTTGAGATGAGATGCTTGTGGCACACAGTTGGGTGAT      599
Rat        CTTGA-------AACAGAAAAGTTTTGAGATGAGATGCTTGTGGCACACAGTTGGGTAAT      552
           *** *       ***** **** ********************************** **

BECN1      GTGGGGAAAGGACACCGGGTCAGGAGTTGCAAGTTTAACTCCGTCCTCACTTGTAGCATT      659
Rat        GTGGGGAAGGGACAAG--------AGGTAAAGGTTTAACTCTGTCCTCACTTCTAGTATT      604
           ******** *****          ** *  * ********* ********** *** ***

BECN1      GAATGCCTCCTGTGCTGTCTAGTGGGACTACAGAATGCTGTTTGATACTGTGTGCGACGT      719
Rat        GAACGCTTACTGTGCTAACTATTGGAATTACAAAATGCTGTTTGATACTGTTTGAGATGT      664
           *** ** * *******  *** *** * **** ****************** ** ** **

BECN1      GGAGAGATTTAATTATTTGTAATAAAGGATTTGCTATGGTCTATTAATTA      769
Rat        GGAAGGATTTAATTATTTGTAATAAAGGATTTGCTATGGTCTATTAATTA      714
           ***  ****************************************
```

Table 4. A) Tau DNA sequence (variant 1, exon 12) from *Mus musculus*, B) Tau DNA sequence (variant 1, exon 12) from *Rattus norvegicus* C) Clustal alignment of variants of the Tau gene from *Mus musculus* and *Rattus norvegicus*.

A

ATTGAAACCCACAAGCTGACCTTCAGGGAGAATGCCAAAGCCAAGACAGACCATGGAGCAGAAATTGTGTATAAGTCACCCGTGGTG
TCTGGGGACACATCTCCACGGCACCTCAGCAATGTGTCTTCCACGGGCAGCATCGACATGGTGGACTCACCACAGCTTGCCACACTA
GCCGATGAAGTGTCTGCTTCCTTGGCCAAGCAGGGTTTGTGATCAGGCTCCCAGGGCAGTCAATAATCATGGAGAGAAGAGAGAGTG
AGAGTGTGGAAAAAAAAAAAAAAAAAAAGAATGATCTGGCCCCTTGCCCTCTGCCCTCCCCGCTGCTCCTCATAGACAGGCTGACCTGC
TTGTCACCTAACCTGCTTTTGTGGCTCGGATTTGGCTCGGGACTTCAAAATCAGTGATGGGAAAAGTACATTTCATCTTTCCAAATT
GATTTGTGGGCTAAAAATAAAACATATTTAAGGGAAAAAAAAACATGTAAAAACATGGCCAAAAAATTTCCTTGGGCAATTGCTAAT
TGATTTCCCCCCCCTGACCCCGCCCTCCCTCTCTGAGTATTAGAGGGTGAAGAAGGCTCTGGAGGCTGCTTCTGGGGAGTGGCTGAG
GGACTAGGGCAGCTAATTGCCCATAGCCCCATCCTAGGGGCTTCAGGGACAGTGGCAGCAATGAGAGATTTGAGACTTGGTGTGTTC
GTGGGGCCGTAGGCAGGTGCTGTTAACTTGTGTGGGTGTGAGTGGGGACTGAAACAGCGACAGCGAAGGCTGAGAGATGGATGGGTG
GACTGAGTTAGAGGACAGAGGTGAGGAAGGCAGGTTGGGAGAGGGGACACTGGCTCCTTGCCAAGTAGCTTGGGGAGGACAGGGTGC
TGCAGCTGCCTGCAGCAGTCCTAGCTAGCTCAGATGCCTGCTTGATAAAGCACTGTGGGGGTAACGTGGGTGTGTGTGCCCCTTCTG
CAGGGCAGCCTGTGGGAGAAGGGGTATTGGGCAGAAGGAAGGTAAGCCAGCAGGTGGTACCTTGTAGATTGGTTCTCTTGAAGGCTG
CTCTTGACATCCCAGGGCACTGGCTTCTTCCTCCCTCCCCGCAAGGTGGGAGGTCCTGAGCGAGGTGTTTCCCTTCGCTCCCACAGG
AAAAGCTGCTTTACTGAGTTCTCAAGTTTGGAACTACAGCCATGATTTGGCCACCATTACAGACCTGGGACTTTAGGGCTAACCAGA
TCTTTGTAAGGACTTGTGCCTCTTGGGGGACCTCTGCCTGTTCTCATGCTTGGCCCTCTGGCACTTCTGTAGTGGGAGGGATGGGGG
GTGGTATTCTGGGATGTGGGTCCCAGGCCTCCCATCCCTCACACAGCCACTGTATCCCCTCTCTCTGTCCTATCATGCCCACGTCTG
CCACGAGAGCTAGTCACTGCCGTCCGTACATCACGTCTCACTGTCCTGAGTGCCATGCCTCTCCCAGCCCCCATCCCTGGCCCCTGG
GTAGATATGGGCAATATCTGCTCTACACTAGGGGTTGGAGTCCAGGGAAGGCAAAGATTTGGGCCTCAGTCTCTAGTCCTACGTTCC
ACGAATCCAACCAGTGTGCCTCCCACAAGGAACCTTACGACCTTGTTTGGTTCACTCCATTACTTCCTATCCTGGATGGGAACTGGT
GTGTGCCTGCCTGGGGATGACCTTGGACCTCTGCCTTTTCTTTTATCTAAGTGGATGCCTCCTAGGCCTGACTCCTTGTGTTGAGCT
GGAGGCAGCCAAGTCAGGTGCCAATGTCTTGGCATCAGTAAGAACAGTCAAGAGTCCCAGGGCAGGGCCACACTTCTCCCATCTTTC
GCTTCCACCCCAGCTTGTGATCGCTAGCCTCCCAGAGCTCAGCTGCCATTAAGTCCCCATGCACGTAATCAGTCTCCACACCCCAGT
TTGGGGAACATACCCCCTTGATTGAAGTGTTTTTTTCCTCCGGTCCCATGGAAACCATGCTGCCTGCCCTGCTGGAGCAGACGGCCA
CCTCCATAGATGCAGCCCTTTCTTTCCCGTCTTCGCCCTGTTACGTTGTAGTTGGATTTGTCTGTTTGTCTGGGTTCACCAGAGTGA
CTATGATAGTGAAAAGAAAAGAAAGAAAAAGAAAAAAAAAAAAAAAAAAAGAAAAAGAAAAAGGAAAAAAAAAAGGACGCATGTATCTT
GAAATATTTGTCAAAAGGTTCTAGCCCACCACGTGATGGAGAGTCTGGATATCTCCTTCCTGACGTGGCTCCAGGCCAGTGCAGTGC
TAACCTGCTGGGACATCCCATGTTTTGAAGGGTTTCTTCTGCATCTGGGACCTCACAGACACTGGATTGTGACATTGGAGGTCTGTG
ACATTGGAGGTCAATGGCATTGGCCAAGGCCTGAAGCACAGGACCAGCTAGAGGCAGCAGGCTCCGAGTGCCAGGGAGAGCTTGTGG
CTGGCCTGTTTTGTATGAAGATGGTCCTTTCTGATCACGACTTCAAATCCCACAGTAGCCCTGAAAGACATCTAAGAACTCCTGCAT
CACAAGAGAAAAGGACACCAGTACCAGCAGGGAGAGCTGTGACCCTAGAAATTCCATGACGACCCAGTAGATATCCTTGGGCCCTCT
CCAAGCCTGGGCCTTTTCACCATAGAGTTTGGGATGGACTGTCCCACTGATGAAGGGGACATCTTAGGAGACTCCCTTGGTTTCCAA
GCTGTCAGCCCCCTGAACTTGCACGACCTCCTACAGCTTCAGGGACTAGGCCTTTGAAGATTAGGAACCTCAGGCCCACATCAGCCA
CTTCTGATGTACAGTTAAGGACAATGTGGAGACTAGGAGGAAGCAGCCAGCCTTTCCCATTAAAGAACTCTTGAGTGCCCAGGGCTA
CCTATTGTGAGCTTCCCCACTGATAAGACTTTAGCTGTCCATAGAAGTGAGTCCGAGGGAGGAAAAGTGTGGTTTCTTCATCATGGT
TACCTGTCGTGGTTCTCTCTCTTACACCCATTTACCCATCCCGCAGTTCCTGTCCTTGAATGGGGGGTGGGGTGCTCTGCCTATCTC
TTGTGGGGTGATCAGCCCAAAAATCATGATTTGGAGTGATCTGATCAGTGCTGATAGGCAGTTTACAAAGGGATTCTGGCTTGTGAC
TTCAGTGAGGACAATCCCCCAGGGCCCTTTCTTTCCATGCCTCTCCAACTCAGAGCCAATGTCTTTGGGTGGGCTAGATAGATAGGG
CATACAATTGGCCTGGTTCCTCCAAGCTCTTAATTCACTTTATCAATAGTTCCATTTAAATTGACTTCAATGATAAGAGTGTATCCC
ATTTGAGATTGCTTGCGTTGTGGGGGAGGGGGAGGAGGAACACATTAAGATAATTCACATGGGCAAAGGGAGGTCTTGGAGTGTAGC
CGTTAAGCCATCTTGTAACCCCATTCATGATTTTGACCACCTGCTAGAGAGAAGAGGTGCCAAGAGACTAGAACTTGGAGGCTTGGC
TGTCCCACTAATAGGCTTTCGCAAGGCAGAGGTAGCCAGCTAGGTCCCTGCCTTCCCAGCCAGGTACAGCTCTCAGGTTTGTGGAGG
TAATCTGTGAACTTCTCTTCCTGCTGCCTTCTTGTGATGTCCAGAGCCCACAGTCAAATACCTCCTAAGAACCCTGGCTTCCTTCCC
TCTAATCCACTGGCACATGACTATCACCTCTGGATTGACCTCAGATCCATAGCCTACACACTGCTAGCAGTGGCCAAGATCACTTCC
TTTATCTCCATCTGTTCTGTTCTCCAGGAAAGTAAGTGGGGATGAGGGTGGAGGTGGTAATCAACTGTAGATCTGTGGCTTTATGAG
CCTTCAGACTTCTCTCTGGCTTCTTCTGGAAGGGTTACTATTGGCAGTATTGCAATCTCACCCTCCTGATGAACTGTAGCCTGTGCC
GTTACTGTGCTGGGCATGATCTCCAGTGCTTGCAAGTCCCATGATTTCTTTGGTGATTTTGAGGGTGGGGGGAGGGACACAAATCAG
CTTAGCTTAGCTTCCTGTCTGTGAATGTCCATATAGTGTATTGTGTTTTAACAAATGATCTACACTGACTGTTGCTGTAAAAGTGAA
TTTGGAAATAAA

B

ATTGAAACCCACAAGCTGACCTTCAGGGAGAATGCCAAAGCCAAGACAGACCATGGAGCAGAAATCGTGTACAAGTCACCTGTGGTG
TCTGGGGACACATCTCCACGGCACCTCAGCAACGTCTCCTCCACGGGCAGCATCGACATGGTGGACTCTCCACAGCTTGCCACGTTA
GCCGATGAAGTGTCCGCCTCTTTGGCCAAGCAGGGTTTGTGATCAGGCCCCTGGGGCCGTCACTGATCATGGAGAGAAGAGAGAGTG
AGAGTGTGGAAAAAAAAAAAAAAAAAAAGAATGACCTGGCCCCTCACCCTCTGCCCTCCCCGCTGCTCCTCATAGACAGGCTGACCAG
CTTGTCACCTAACCTGCTTTTGTGGCTCGGGTTTGGCTCGGGACTTCAAAATCAGTGATGGGAAAAAGTAAATTTCATCTTTCCAAA
TTGATTTGTGGGCTAGTAATAAAATATTTTTAAGGAAGGAAAAAAAAAAAAAACACGTAAAACCATGGCCAAACAAAACCCAACATTT
CCTTGGCAATTGTTATTGACCCCGCCCCCCCCCTCTGAGTTTTAGAGGGTGAAGGAGGCTTTGGATGGAGGCTGCTTCTGGGGATTG
GCTGAGGGACTAGGGCAACTAATTGCCCACAGCCCCATCTTAGGGGCATCAGGGACAGCGGCAGCAATGAAAGACTTGGGACTTGGT
GTGTTTGTGGAGCCGTAGGCAGGTGATGTTAACTTTGTGTGGGTTTGAGGGAGGACTGTGATAGTGAAGGCTGAGAGATGGGTGGGC
TGGGAGTCAGAGGAGAGAGGTGAGGAAGACAGGTTGGGAGAGGGGACATTGGCTCCTTGCCAAGGAGCTTGGGAAGCACAGGTAGCC
CTGGCTGCCTGCAGCAGTCTTAGCTAGCACAGATGCCTGCCTGAGAAAGCACAGTGGGGTACAGTGGGTGTGTGTGCCCCTTCTGAA
GGGCAGCCCATGGGAGAAGGGGTATTGGGCAGAAGGAAGGTAGGCCAGAAGGTGGCACCTTGTAGATTGGTTCTCTGAAGGCTGACC
TTGCCATCCCAGGGCACTGGCTCCCACCCTCCAGGGAGGGAGGTCCTGAGCTGAGGAGCTTCCCTTTGCTCTCACAGGAAAACCTGT
GTTACTGAGTTCTGAAGTTTGGAACTACAGCCATGATTTTGGCCACCATACAGACCTGGGACTTTAGGGCTAACCAGTTCTTTGTAA
GGACTTGTGCCTCTTGCGGGAACATCTGCCTGTTCTCAAGCCTGGTCCTCTGGCACTTCTGCAGTGTGAGGGATGGGGGTGGTAATT

```
CTGGGATGTGGGTCCCAGGCCTCCCATCCTCGCACAGCCACTGTATCCCCTCTACCTGTCCTATCATGCCCACGTCTGCCATGAGAG
CCAGTCACTGCCGTCCGTACATCACGTCTCACCGTCCTGAGTGCCCAGCCTCCCCAAGCCCCATCCCTGGCCCCTGGGTAGTTATGG
CCAATATCTGCTCTACACTAGGGGTTGGAGTCCAGGGAAGGCAAAGATTTGGGCCTTGGTCTCTAGTCCTACGTTGCACGAATCCAA
CCAGTGTGCCTCCCACAAGGAACCTTACAACCTTGTTTGGTTTGCTCCATCATTTCCCATCGTGGATGGGAGTCCGTGTGTGCCTGG
AGATTACCCTGGACACCTCTGCTTTTTTTTTTTTACTTTAGCGGTTGCCTCCTAGGCCTGACTCCTTCCCATGTTGAACTGGAGGCA
GCCACGTTAGGTGTCAATGTCCTGGCATCAGTATGAACAGTCAGTAGTCCCAGGGCAGGGCCACACTTCTCCCATCTTCTGCTTCCA
CCCCAGCTTGTGATTGCTAGCCTCCCAGAGCTCAGCCGCCATTAAGTCCCCATGCACGTAATCAGCCCTTCATACCCCAATTTGGGG
AACATACCCCTTGATTGAAATGTTTTCCCTCCAGTCCTATGGAAGCGGTGCTGCCTGCCCTGCTGGAGCAGCCAGCCATCTCCAGAG
ACGCAGCCCTTTCTCTCCTGTCCGCACCCTGTTGCGCTGTAGTCGGATTCGTCTGTTTGTCTGGGTTCACCAGAGTGACTATGATAG
TGAAAAGAAAAAGAAAAAGAAAAAAGAAAAAAGAAAAAAAAAAAAGGACGCATGTTATCTTGAAATATTTGTCAAAAGGTTGTAGCC
CACCGCAGGGATTGGAGGGCCTGGATATTCCTTGTCTTCTTCGTGACTTAGGTCCAGGCCGGTGCAGTGCTACCCTGCTGGGACATC
CCATGTTTTGAAGGGTTTCTTCTTCATCTGGGACCCTGCAGACACTGGATTGTGACATTGGAGGTCTATGACATTGGCCAAGGCCTG
AAGCACAGGACCCGTTAGAGGCAGCAGGCTCCGACTGTCAGGGAGAGCTTGTGGCTGGCCTGTTTCTCTGAGTGAAGATGGTCCTCT
CTAATCACAACTTCAAGTCCCACAGCAGCCCTGGCAGACATCTAAGAACTCCTGCATCACAAGAGAAAAGGACACTAGTACCAGCAG
GGAGAGCTGTGGCCCTAGAAATTCCATGACTCTCCACTACATATCCGTGGGTCCTTTCCAAGCCTTGGCCTCGTCACCAAGGGCTTG
GGATGGACTGCCCCACTGATGAAAGGGACATCTTTGGAGACCCCCTTGGTTTCCAAGGCGTCAGCCCCCTGACCTTGCATGACCTCC
TACAGCTGTAAGGATGAGGCCTTTAAAGATTAGGAACCTCAGGCCCAGGTCGGCCACTTTGGGCTTGGGTACAGTTAGGGACGATGC
GGTAGAAGGAGGTGGCCAACCTTTCCCATATAAGAGTTCTGTGTGCCCAGAGCTACCCTATTGTGAGCTCCCCACTGCTGATGGACT
TTAGCTGTCCTTAGAAGTGAAGAGTCCAACGGAGGAAAAGGAAGTGTGGTTTGATGGTCTGTGGTCCCTTCATCATGGTTACCTGTT
GTGGTTTTCTCTCGTATACCCATTTACCCATCCTGCAGTTCCTGTCCTTGAATAGGGGTGGGGGTACTCTGCCATATCTCTTGTAGG
GCAGTCAGCCCCCAAGTCATAGTTTGGAGTGATCTGGTCAGTGCTAATAGGCAGTTTACAAAGGAATTCTGGCTTGTTACTTCAGTG
AGGACAATCCCCCAAGGGCCCTGGCACCTGTCCTGTCTTTCCATGGCTCTCCACTGCAGAGCCAATGTCTTTGGGTGGGCTAGATAG
GGTGTACAATTTGCCTGGTTCCTCCAAGCTCTTAATCCACTTTATCAATAGTTCCATTTAAATTGACTTCAATGATAAGAGTGTATC
CCATTTGAGATTGCTTGTGTTGTGGGGTAAAGGGGGGAGGAGGAACATGTTAAGATAATTGACATGGGCAAGGGGAAGTCTTGAAGT
GTAGCAGTTAAACCATCTTGTAGCCCCATTCATGATGTTGACCACTTGCTAGAGAGAAGAGGTGCCATAAGGCTAGAACCTAGAGGC
TTGGCTGTCCCACCAACAGGCAGGCTTTTGCAAGGCAGAGGCAGCCAGCTAGGTCCCTGACTTCCCAGCCAGGTGCAGCTCTAAGAA
CTGCTCTTGCCTGCTGCCTTCTTGTGGTGTCCAGAGCCCACAGCCAATGCCTCCTCAAAACCCTGGCTTCCTTCCTTCTAATCCACT
GGCACATCAGCATCACCTCCGGATTGACTTCAGATCCACAGCCTACACTACTAGCAGTGGGTAAGACCACTTCCTTTGTCCTTGTCT
GTTCTCCAGAAAAGTGGGCATGGAGGCGGTGTTAATAACTATAGGTCTGTGGCTTTATGAGCCTTCAAACTTCTCTCTAGCTTCTGA
AAGGGTTACTTTTGGGCAGTATTGCAGTCTCACCCTCCCGATGGGCTGTAGCCTGTGCAGTTGCTGTACTGGGCATGATCTCCAGTG
CTTGCAAGTCCCATGATTTCTTTGGTGATTTTGAGGGTGGGGGGAGGGACATGAATCATCTTAGCTTAGCTTCCTGTCTGTGAATGT
CCATATAGTGTACTGTGTTTTAACAAACGATTTACACTGACTGTTGCTGTACAAGTGAATTTGGAAATAAAGTTA
```

C

CLUSTAL O(1.2.4) multiple sequence alignment

```
Rat        ATTGAAACCCACAAGCTGACCTTCAGGGAGAATGCCAAAGCCAAGACAGACCATGGAGCA      60
Mouse      ATTGAAACCCACAAGCTGACCTTCAGGGAGAATGCCAAAGCCAAGACAGACCATGGAGCA      60
           ************************************************************

Rat        GAAATCGTGTACAAGTCACCTGTGGTGTCTGGGGACACATCTCCACGGCACCTCAGCAAC      120
Mouse      GAAATTGTGTATAAGTCACCCGTGGTGTCTGGGGACACATCTCCACGGCACCTCAGCAAT      120
           ***** ***** ******** ************************************

Rat        GTCTCCTCCACGGGCAGCATCGACATGGTGGACTCTCCACAGCTTGCCACGTTAGCCGAT      180
Mouse      GTGTCTTCCACGGGCAGCATCGACATGGTGGACTCACCACAGCTTGCCACACTAGCCGAT      180
           ** ** ***************************** **************  ********

Rat        GAAGTGTCCGCCTCTTTGGCCAAGCAGGGTTTGTGATCAGGCCCCTGGGGCCGTCACTGA      240
Mouse      GAAGTGTCTGCTTCCTTGGCCAAGCAGGGTTTGTGATCAGGCTCCCAGGGCAGTCAATAA      240
           ******** ** ** ************************** **  **** **** * *

Rat        TCATGGAGAGAAGAGAGAGTGAGAGTGTGGAAAAAAAAAAAAAAAAAAAGAATGACCTGGC      300
Mouse      TCATGGAGAGAAGAGAGAGTGAGAGTGTGGAAAAAAAAAA-AAAAAAAGAATGATCTGGC      299
           ************************************** ************* *****

Rat        CCCTCACCCTCTGCCCTCCCCGCTGCTCCTCATAGACAGGCTGACCAGCTTGTCACCTAA      360
Mouse      CCCTTGCCCTCTGCCCTCCCCGCTGCTCCTCATAGACAGGCTGACCTGCTTGTCACCTAA      359
           ****  ****************************************** *************

Rat        CCTGCTTTTGTGGCTCGGGTTTGGCTCGGGACTTCAAAATCAGTGATGGGAAAAAGTAAA      420
Mouse      CCTGCTTTTGTGGCTCGGATTTGGCTCGGGACTTCAAAATCAGTGATGGGAAAA-GTACA      418
           ****************** ********************************** *** *

Rat        TTTCATCTTTCCAAATTGATTTGTGGGCTAGTAATAAAATATTTTTAAGGAAGGAAAAAA      480
Mouse      TTTCATCTTTCCAAATTGATTTGTGGGCTAAAAATAAAACATATTTAAGGGAAAAAAAAA      478
           ******************************  ******* ** ******* *  ******
```

```
Rat        AAAAAAACACGTAAAACCATGGCCAAACAAAACCCAAC-------ATTTCCTTGGCAATT      533
Mouse      CATGTAA-------AAACATGGCCAAAAAATTTCCTTGGGCAATTGCTAATTGATTTCCC      531
            *   **       ** ********** **   **           *   *

Rat        GTTATTGACCCCGCCCCCCCCCTCTGAGTTTTAGAGGGTGAAGGAGGCTTTGGATGGAGG      593
Mouse      CCCCCTGACCCCGCCCTCCCTCTCTGAGTATTAGAGGGTGAAGAA----GGCTCTGGAGG      587
                ********** *** ******** ************ *         ******

Rat        CTGCTTCTGGGGATTGGCTGAGGGACTAGGGCAACTAATTGCCCACAGCCCCATCTTAGG      653
Mouse      CTGCTTCTGGGGAGTGGCTGAGGGACTAGGGCAGCTAATTGCCCATAGCCCCATCCTAGG      647
           ************* ****************** *********** ********* ****

Rat        GGCATCAGGGACAGCGGCAGCAATGAAAGACTTGGGACTTGGTGTGTTTGTGGAGCCGTA      713
Mouse      GGCTTCAGGGACAGTGGCAGCAATGAGAGATTTGAGACTTGGTGTGTTCGTGGGGCCGTA      707
           *** ********** *********** *** *** ************* **** ******

Rat        GGCAGGTGATGTTAACTTTGTGTGGGTTTGAGGGAGGAC------TGTGATAGTGAAGGC      767
Mouse      GGCAGGTGCTGTTAACTTGTGTGGGTGTG-AGTGGGGACTGAAACAGCGACAGCGAAGGC      766
           ******** *********     **  *  ** * ****       * ** ** ******

Rat        TGAGAGATGGG--TGGGCTGGGAGTCAGAGGAGAGAGGTGAGGAAGACAGGTTGGGAGAG      825
Mouse      TGAGAGATGGATGGGTGGACTGAGTTAGAGGACAGAGGTGAGGAAGGCAGGTTGGGAGAG      826
           **********    * *    **** ****** ************* *************

Rat        GGGACATTGGCTCCTTGCCAAGGAGCTTGGGAAGCACAGGTAGCCCTGGCTGCCTGCAGC      885
Mouse      GGGACACTGGCTCCTTGCCAAGTAGCTTGGGGAGGACAGGGTGCTGCAGCTGCCTGCAGC      886
           ****** *************** ******** ** *****  **    ************

Rat        AGTCTTAGCTAGCACAGATGCCTGCCTGAGAAAGCACAGTGG-GGTACAGTGGGTGTGTG      944
Mouse      AGTCCTAGCTAGCTCAGATGCCTGCTTGATAAAGCACTGTGGGGGTAACGTGGGTGTGTG      946
           **** ******** *********** *** ******* **** ****  ***********

Rat        TGCCCCTTCTGAAGGGCAGCCCATGGGAGAAGGGGTATTGGGCAGAAGGAAGGTAGGCCA     1004
Mouse      TGCCCCTTCTGCAGGGCAGCCTGTGGGAGAAGGGGTATTGGGCAGAAGGAAGGTAAGCCA     1006
           *********** *********  ******************************** ****

Rat        GAAGGTGGCACCTTGTAGATTGGTTCTCTGAAGGC-TGACCTTGCCATCCCAGGGCACTG     1063
Mouse      GCAGGTGGTACCTTGTAGATTGGTTCTCTTGAAGGCTGCTCTTGACATCCCAGGGCACTG     1066
           * ****** ********************  * *  **  **** ***************

Rat        GCTCCCACCCTCC-------AGGGAGGGAGGTCCTGAGCTGAGGAGCTTCCCTTTGCTCT     1116
Mouse      GCTTCTTCCTCCCTCCCCGCAAGGTGGG-AGGTCCTGAGCGAGGTGTTTCCCTTCGCTCC     1125
           *** *  **  **       * ** ***  *  *      **** * ******* ****

Rat        CACAGGAAAACCTGTGTTACTGAGTTCTGAAGTTTGGAACTACAGCCATGATTTTGGCCA     1176
Mouse      CACAGGAAAAGCTGCTTTACTGAGTTCTCAAGTTTGGAACTACAGCCATGATTTGGCCAC     1185
           ********** ***  ************ ************************ * *

Rat        CCATACAGACCTGGGACTTTAGGGCTAACCAGTTCTTTGTAAGGACTTGTGCCTCTTGCG     1236
Mouse      CATTACAGACCTGGGACTTTAGGGCTAACCAGATCTTTGTAAGGACTTGTGCCTCTTGGG     1245
           *  *************************** ************************ *

Rat        GGAACATCTGCCTGTTCTCAAGCCTGGTCCTCTGGCACTTCTGCAGTGTGAGGGATGGGG     1296
Mouse      GG-ACCTCTGCCTGTTCTCATGCTTGGCCCTCTGGCACTTCTGTAGTGGGAGGGATGGGG     1304
           ** ** ************** ** *** *************** **** ************

Rat        GTGGTAATTCTGGGATGTGGGTCCCAGGCCTCCCAT-CCTCGCACAGCCACTGTATCCCC     1355
Mouse      GGTGGTATTCTGGGATGTGGGTCCCAGGCCTCCCATCCCTCACACAGCCACTGTATCCCC     1364
           *  *  ***************************** **** ******************

Rat        TCTACCTGTCCTATCATGCCCACGTCTGCCATGAGAGCCAGTCACTGCCGTCCGTACATC     1415
Mouse      TCTCTCTGTCCTATCATGCCCACGTCTGCCACGAGAGCTAGTCACTGCCGTCCGTACATC     1424
           ***  ************************* ****** *********************

Rat        ACGTCTCACCGTCCTGAGTGCCCAGCCTCCCCAA-GCCCCATCCCTGGCCCCTGGGTAGT     1474
Mouse      ACGTCTCACTGTCCTGAGTGCCATGCCTCTCCCAGCCCCCATCCCTGGCCCCTGGGTAGA     1484
           ********* ************  ***** ** *  ***********************

Rat        TATGGCCAATATCTGCTCTACACTAGGGGTTGGAGTCCAGGGAAGGCAAAGATTTGGGCC     1534
Mouse      TATGGGCAATATCTGCTCTACACTAGGGGTTGGAGTCCAGGGAAGGCAAAGATTTGGGCC     1544
```

```
           ***** ****************************************************

Rat        TTGGTCTCTAGTCCTACGTTGCACGAATCCAACCAGTGTGCCTCCCACAAGGAACCTTAC     1594
Mouse      TCAGTCTCTAGTCCTACGTTCCACGAATCCAACCAGTGTGCCTCCCACAAGGAACCTTAC     1604
           *  ***************** ****************************************

Rat        AACCTTGTTTGGTTTGCTCCATCATTTCCCATCGTGGATGGGAGTCCGTGTGTGCCTGGA     1654
Mouse      GACCTTGTTTGGTTCACTCCATTACTTCCTATCCTGGATGGGAACTGGTGTGTGCCTGCC     1664
            *************  ****** * **** *** *********    ***********

Rat        GATTACCCTGGACACCTCTGCTTTTTTTTTTTTACTTTAGCGGTTGCCTCCTAGGCCTGA     1714
Mouse      TGGGGATGACCTTGGACCTCTGCCTTTTCTTTTATCTAAGTGGATGCCTCCTAGGCCTGA     1724
                         **     **** *****  * ** ** ****************

Rat        CTCCTTCCCATGTTGAACTGGAGGCAGCCACGTTAGGTGTCAATGTCCTGGCATCAGTAT     1774
Mouse      CTC---CTTGTGTTGAGCTGGAGGCAGCCAAGTCAGGTGCCAATGTCTTGGCATCAGTAA     1781
           ***   *   ****** ************* ** ***** ******* ***********

Rat        GAACAGTCAGTAGTCCCAGGGCAGGGCCACACTTCTCCCATCTTCTGCTTCCACCCCAGC     1834
Mouse      GAACAGTCAAGAGTCCCAGGGCAGGGCCACACTTCTCCCATCTTTCGCTTCCACCCCAGC     1841
           *********  *********************************  **************

Rat        TTGTGATTGCTAGCCTCCCAGAGCTCAGCCGCCATTAAGTCCCCATGCACGTAATCAGCC     1894
Mouse      TTGTGATCGCTAGCCTCCCAGAGCTCAGCTGCCATTAAGTCCCCATGCACGTAATCAGTC     1901
           ******* ********************* ***************************** *

Rat        CTTCATACCCCAATTTGGGGAACATACCCCTTGATTGAAATG--TTTTCCCTCCAGTCCT     1952
Mouse      TCCACACCCCAGTTTGGGGAACATACCCCCTTGATTGAAGTGTTTTTTTCCTCCGGTCCC     1961
                  ***   ** *** *     ************* **  **** ***** ****

Rat        ATGGAAGCGGTGCTGCCTGCCCTGCTGGAGCAGCCAGCCATCTCCAGAGACGCAGCCCTT     2012
Mouse      ATGGAAACCATGCTGCCTGCCCTGCTGGAGCAGACGGCCACCTCCATAGATGCAGCCCTT     2021
           ****** *  ********************** * **** ***** *** *********

Rat        TCTCTCCTGTCCGCACCCTGTTGCGCTGTAGTCGGATTCGTCTGTTTGTCTGGGTTCACC     2072
Mouse      TCTTTCCCGTCTTCGCCCTGTTACGTTGTAGTTGGATTTGTCTGTTTGTCTGGGTTCACC     2081
           *** *** ***  * ******* ** ****** ***** *********************

Rat        AGAGTGACTATGATAGTGAAAAGAAAAAGAAAAAGAAAAAAGAAAAAAGAAAAAA-----     2127
Mouse      AGAGTGACTATGATAGTGAAAAGAAAAGAAAGAAAAAAGAAAAAAAAAAAAAAAAGAAAA     2141
           ***************************  ** ** ** *** ****** ******

Rat        ------------AAAAAAGGACGCATGTTATCTTGAAATATTTGTCAAAAGGTTGTAGCC     2175
Mouse      AGAAAAAGGAAAAAAAAAAGGACGCATGTATCTTGAAATATTTGTCAAAAGGTTCTAGCC     2201
                     ****** *        ************************* *****

Rat        CACCGCAGGGATTGGAGGGCCTGGATATTCCTTGTCTTCTTCGTGACTTAGGTCCAGGCC     2235
Mouse      CACCACGTGATG---------GAGAGTCTGGATATCTCCTTCCTGACGTGGCTCCAGGCC     2252
           **** *  *              **   *   * *** **** **** * * ********

Rat        GGTGCAGTGCTACCCTGCTGGGACATCCCATGTTTTGAAGGGTTTCTTCTTCATCTGGGA     2295
Mouse      AGTGCAGTGCTAACCTGCTGGGACATCCCATGTTTTGAAGGGTTTCTTCTGCATCTGGGA     2312
            *********** ************************************ *********

Rat        CCCTGCAGACACTGGATTGTGACATTGGAGGTCT-----------------ATGACATTGG     2339
Mouse      CCTCACAGACACTGGATTGTGACATTGGAGGTCTGTGACATTGGAGGTCAATGGCATTGG     2372
           **   *****************************                *** ******

Rat        CCAAGGCCTGAAGCACAGGACCCGTTAGAGGCAGCAGGCTCCGACTGTCAGGGAGAGCTT     2399
Mouse      CCAAGGCCTGAAGCACAGGACCAGCTAGAGGCAGCAGGCTCCGAGTGCCAGGGAGAGCTT     2432
           ********************** * ******************** ** ************

Rat        GTGGCTGGCCTGTTTCTCTGAGTGAAGATGGTCCTCTCTAATCACAACTTCAAGTCCCAC     2459
Mouse      GTGGCTGGCCTGTTT---TGTATGAAGATGGTCCTTTCTGATCACGACTTCAAATCCCAC     2489
           ***************   **  ************* *** ***** ******* ******

Rat        AGCAGCCCTGGCAGACATCTAAGAACTCCTGCATCACAAGAGAAAAGGACACTAGTACCA     2519
Mouse      AGTAGCCCTGAAAGACATCTAAGAACTCCTGCATCACAAGAGAAAAGGACACCAGTACCA     2549
           ** *******  ************************************** *******

Rat        GCAGGGAGAGCTGTGGCCCTAGAAATTCCATGACTCTCCACTACATATCCGTGGGTCCTT     2579
```

```
Mouse       GCAGGGAGAGCTGTGACCCTAGAAATTCCATGACGACCCAGTAGATATCCTTGGGCCCTC      2609
            *************** *****************   *** ** ****** **** ***

Rat         TCCAAGCCTTGGCCTCGTCACCAA-GGGCTTGGGATGGACTGCCCCACTGATGAAAGGGA      2638
Mouse       TCCAAGCCTGGGCCTTTTCACCATAGAGTTTGGGATGGACTGTCCCACTGATGAAGGGGA      2669
            ********* *****  ******  * * ************* ************ ****

Rat         CATCTTTGGAGACCCCCTTGGTTTCCAAGGCGTCAGCCCCCTGACCTTGCATGACCTCCT      2698
Mouse       CATCTTAGGAGACTCCCTTGGTTTCCAAGCTGTCAGCCCCCTGAACTTGCACGACCTCCT      2729
            ****** ****** ***************  ************* ****** ********

Rat         ACAGCTGTAAGGATGAGGCCTTTAAAGATTAGGAACCTCAGGCCCAGGTCGGCCACTTTG      2758
Mouse       ACAGCTTCAGGGACTAGGCCTTTGAAGATTAGGAACCTCAGGCCCACATCAGCCACTTCT      2789
            ******  * ***  ******** ***********************  ** *******

Rat         GGCTTGGGTACAGTTAGGGACGATGCGGTAGAAGGAGGTGGCCAACCTTTCCCATATAAG      2818
Mouse       GATGTACAGTTAAGGACAATGTGGAGACTAGGAGGAAGCAGCCAGCCTTTCCCATTAAAG      2849
            *   *      *   *            *** **** *  **** **********  ***

Rat         AGTTCTGTGTGCCCAGAGCTACCCTATTGTGAGCTCCCCACTGCTGATGGACTTTAGCTG      2878
Mouse       AACTCTTGAGTGCCCAGGGCTACCTATTGTGAGCTTCCCCACTGA-TAAGACTTTAGCTG      2908
            *  ***      **   *    ************* ***          ***********

Rat         TCCTTAGAAGTGAAGAGTCCAACGGAGGAAAAGGAAGTGTGGTTTGATGGTCTGTGGTCC      2938
Mouse       TCCATAGAAGTG----------------------AGTCCGAGGGAGGAAAAGTGTGGTTT      2946
            *** ********                      *      *   *      ******

Rat         CTTCATCATGGTTACCTGTTGTGGTTTTCTCTCGTATACCCATTTACCCATCCTGCAGTT      2998
Mouse       CTTCATCATGGTTACCTGTCGTGGTTCTCTCTCTTACACCCATTTACCCATCCCGCAGTT      3006
            ****************** ****** ****** ** ***************** ******

Rat         CCTGTCCTTGAATAGGGGTGGGGGTACTCTGCCATATCTCTTGTAGGGCAGTCAGCCCCC      3058
Mouse       CCTGTCCTTGAATGGGGGGTGGGGTGCTCTG-CCTATCTCTTGTGGGGTGATCAGCCCAA      3065
            ************* ****  ***** ***** * ********** ***   *******

Rat         AAGTCATAGTTTGGAGTGATCTGGTCAGTGCTAATAGGCAGTTTACAAAGGAATTCTGGC      3118
Mouse       AAATCATGATTTGGAGTGATCTGATCAGTGCTGATAGGCAGTTTACAAAGGGATTCTGGC      3125
            ** ****  ************** ******** ****************** ********

Rat         TTGTTACTTCAGTGAGGACAATCCCCCAAGGGCCCTGGCACCTGTCCTGTCTTTCCATGG      3178
Mouse       TTGTGACTTCAGTGAGGACAATCCCCCAGGGCCCTT-------------TCTTTCCATGC      3172
            **** ********************** ** ** *             **********

Rat         CTCTCCACTGCAGAGCCAATGTCTTTGGGTGG----GCTAGATAGGGTGTACAATTTGCC      3234
Mouse       CTCTCCAACTCAGAGCCAATGTCTTTGGGTGGGCTAGATAGATAGGGCATACAATTGGCC      3232
            *******   **********************    * *********  ******* ***

Rat         TGGTTCCTCCAAGCTCTTAATCCACTTTATCAATAGTTCCATTTAAATTGACTTCAATGA      3294
Mouse       TGGTTCCTCCAAGCTCTTAATTCACTTTATCAATAGTTCCATTTAAATTGACTTCAATGA      3292
            ********************* ****************************************

Rat         TAAGAGTGTATCCCATTTGAGATTGCTTGTGTTGTGGGGTAAAGGGGGGAGGAGGAACAT      3354
Mouse       TAAGAGTGTATCCCATTTGAGATTGCTTGCGTTGTGGGGGAGGG---GGAGGAGGAACAC      3349
            ***************************** ********* *  *   ************

Rat         GTTAAGATAATTGACATGGGCAAGGGGAAGTCTTGAAGTGTAGCAGTTAAACCATCTTGT      3414
Mouse       ATTAAGATAATTCACATGGGCAAAGGGAGGTCTTGGAGTGTAGCCGTTAAGCCATCTTGT      3409
             *********** ********** **** ****** ******** ***** *********

Rat         AGCCCCATTCATGATGTTGACCACTTGCTAGAGAGAAGAGGTGCCATAAGGCTAGAACCT      3474
Mouse       AACCCCATTCATGATTTTGACCACCTGCTAGAGAGAAGAGGTGCCAAGAGACTAGAACTT      3469
            * ************* ******** *********************  ** ******* *

Rat         AGAGGCTTGGCTGTCCCACCAACAGGCAGGCTTTTGCAAGGCAGAGGCAGCCAGCTAGGT      3534
Mouse       GGAGGCTTGGCTGTCCCACTAATAGG----CTTTCGCAAGGCAGAGGTAGCCAGCTAGGT      3525
             ****************** ** ***    **** ************ ************

Rat         CCCTGACTTCCCAGCCAGGTGCAGCTCTAAGAACTGCTCT-------------------T      3575
Mouse       CCCTGCCTTCCCAGCCAGGTACAGCTCTCAGGTTTGTGGAGGTAATCTGTGAACTTCTCT      3585
            ***** ************** ******* **   **                       *
```

```
Rat        GCCTGCTGCCTTCTTGTGGTGTCCAGAGCCCACA-GCCAATGCCTCCTCAAAACCCTGGC      3634
Mouse      TCCTGCTGCCTTCTTGTGATGTCCAGAGCCCACAGTCAAATACCTCCTAAGAACCCTGGC      3645
            ***************** ***************  * *** ****** * *********

Rat        TTCCTTCCTTCTAATCCACTGGCACATCAGCATCACCTCCGGATTGACTTCAGATCCACA      3694
Mouse      TTCCTTCCCTCTAATCCACTGGCACATGACTATCACCTCTGGATTGACCTCAGATCCATA      3705
           ******** ****************** *  ******** ******** ********* *

Rat        GCCTACACTA--CTAGCAGTGGGTAAGACCACTTCCTTTGTCCTTGTCTGTTCT-----C      3747
Mouse      GCCTACACACTGCTAGCAGTGGCCAAGATCACTTCCTTTATCTCCATCTGTTCTGTTCTC      3765
           ********    **********  **** ********** **    ********     *

Rat        CAGAAAAGTGGGC--------ATGGAGGCGGTGTTAATAACTATAGGTCTGTGGCTTTAT      3799
Mouse      CAGGAAAGTAAGTGGGGATGAGGGTGGAGGTGGTAATCAACTGTAGATCTGTGGCTTTAT      3825
           *** *****  *           *  *  *  ** *  **** *** *************

Rat        GAGCCTTCAAACTTCTCTCTAGCTTCTGAA--AGGGTTACTTTTGGGCAGTATTGCAGTC      3857
Mouse      GAGCCTTCAGACTTCTCTCTGGCTTCTTCTGGAAGGGTTACTATTGGCAGTATTGCAATC      3885
           ********* ********** ******     * ** *   * * ************ **

Rat        TCACCCTCCCGATGGGCTGTAGCCTGTGCAGTTGCTGTACTGGGCATGATCTCCAGTGCT      3917
Mouse      TCACCCTCCTGATGAACTGTAGCCTGTGCCGTTACTGTGCTGGGCATGATCTCCAGTGCT      3945
           ********* ****  ************* *** **** *********************

Rat        TGCAAGTCCCATGATTTCTTTGGTGATTTTGAGGGTGGGGGGAGGGACATGAATCATCTT      3977
Mouse      TGCAAGTCCCATGATTTCTTTGGTGATTTTGAGGGTGGGGGGAGGGACACAAATCAGCTT      4005
           ***********************************************  ***** ***

Rat        AGCTTAGCTTCCTGTCTGTGAATGTCCATATAGTGTACTGTGTTTTAACAAACGATTTAC      4037
Mouse      AGCTTAGCTTCCTGTCTGTGAATGTCCATATAGTGTATTGTGTTTTAACAAATGATCTAC      4065
           ************************************ ************** *** ***

Rat        ACTGACTGTTGCTGTACAAGTGAATTTGGAAATAAAGTTA       4077
Mouse      ACTGACTGTTGCTGTAAAAGTGAATTTGGAAATAAA----       4101
           *************** ******************
```

Table 5. A) Lamp2a DNA sequence (variant 1, exon 9) from *Mus musculus*, B) Lamp2a DNA sequence (variant 1, exon 9) from *Rattus norvegicus* C) Clustal alignment of variants of the Lamp2a gene from *Mus musculus* and *Rattus norvegicus*.

A

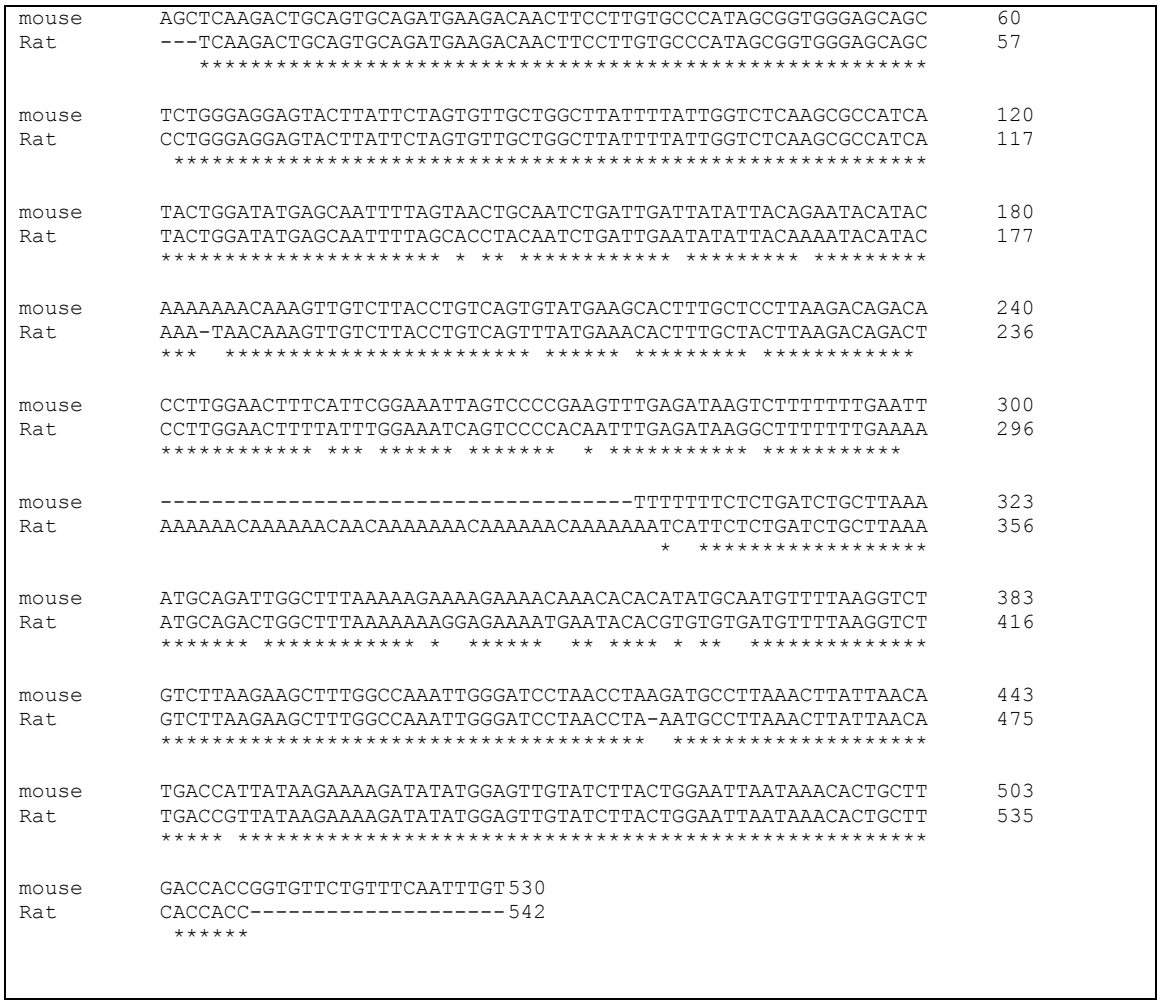
```
AGCTCAAGACTGCAGTGCAGATGAAGACAACTTCCTTGTGCCCATAGCGGTGGGAGCAGCTCTGGGAGGAGTACTTATTCTAGTGTT
GCTGGCTTATTTTATTGGTCTCAAGCGCCATCATACTGGATATGAGCAATTTTAGTAACTGCAATCTGATTGATTATATTACAGAAT
ACATACAAAAAAACAAAGTTGTCTTACCTGTCAGTGTATGAAGCACTTTGCTCCTTAAGACAGACACCTTGGAACTTTCATTCGGAA
ATTAGTCCCCGAAGTTTGAGATAAGTCTTTTTTTGAATTTTTTTTTCTCTGATCTGCTTAAAATGCAGATTGGCTTTAAAAAGAAAA
GAAAACAAACACACATATGCAATGTTTTAAGGTCTGTCTTAAGAAGCTTTGGCCAAATTGGGATCCTAACCTAAGATGCCTTAAACT
TATTAACATGACCATTATAAGAAAAGATATATGGAGTTGTATCTTACTGGAATTAATAAACACTGCTTGACCACCGGTGTTCTGTTT
CAATTTGT
```

B

```
TCAAGACTGCAGTGCAGATGAAGACAACTTCCTTGTGCCCATAGCGGTGGGAGCAGCCCTGGGAGGAGTACTTATTCTAGTGTTGCT
GGCTTATTTTATTGGTCTCAAGCGCCATCATACTGGATATGAGCAATTTTAGCACCTACAATCTGATTGAATATATTACAAAATACA
TACAAATAACAAAGTTGTCTTACCTGTCAGTTTATGAAACACTTTGCTACTTAAGACAGACTCCTTGGAACTTTTATTTGGAAATCA
GTCCCCACAATTTGAGATAAGGCTTTTTTTGAAAAAAAAAACAAAAAACAACAAAAAAACAAAAAACAAAAAAATCATTCTCTGATC
TGCTTAAAATGCAGACTGGCTTTAAAAAAAGGAGAAAATGAATACACGTGTGTGATGTTTTAAGGTCTGTCTTAAGAAGCTTTGGCC
AAATTGGGATCCTAACCTAAATGCCTTAAACTTATTAACATGACCGTTATAAGAAAAGATATATGGAGTTGTATCTTACTGGAATTA
ATAAACACTGCTTCACCACC
```

C

```
CLUSTAL O(1.2.4) multiple sequence alignment
```


mouse AGCTCAAGACTGCAGTGCAGATGAAGACAACTTCCTTGTGCCCATAGCGGTGGGAGCAGC 60
Rat ---TCAAGACTGCAGTGCAGATGAAGACAACTTCCTTGTGCCCATAGCGGTGGGAGCAGC 57
mouse TCTGGGAGGAGTACTTATTCTAGTGTTGCTGGCTTATTTTATTGGTCTCAAGCGCCATCA 120
Rat CCTGGGAGGAGTACTTATTCTAGTGTTGCTGGCTTATTTTATTGGTCTCAAGCGCCATCA 117
mouse TACTGGATATGAGCAATTTTAGTAACTGCAATCTGATTGATTATATTACAGAATACATAC 180
Rat TACTGGATATGAGCAATTTTAGCACCTACAATCTGATTGAATATATTACAAAATACATAC 177
mouse AAAAAAACAAAGTTGTCTTACCTGTCAGTGTATGAAGCACTTTGCTCCTTAAGACAGACA 240
Rat AAA-TAACAAAGTTGTCTTACCTGTCAGTTTATGAAACACTTTGCTACTTAAGACAGACT 236
mouse CCTTGGAACTTTCATTCGGAAATTAGTCCCCGAAGTTTGAGATAAGTCTTTTTTTGAATT 300
Rat CCTTGGAACTTTTATTTGGAAATCAGTCCCCACAATTTGAGATAAGGCTTTTTTTGAAAA 296
mouse ----------TTTTTTTCTCTGATCTGCTTAAA 323
Rat AAAAAACAAAAAACAACAAAAAAACAAAAAACAAAAAAATCATTCTCTGATCTGCTTAAA 356
mouse ATGCAGATTGGCTTTAAAAAGAAAAGAAAACAAACACACATATGCAATGTTTTAAGGTCT 383
Rat ATGCAGACTGGCTTTAAAAAAAGGAGAAAATGAATACACGTGTGTGATGTTTTAAGGTCT 416
mouse GTCTTAAGAAGCTTTGGCCAAATTGGGATCCTAACCTAAGATGCCTTAAACTTATTAACA 443
Rat GTCTTAAGAAGCTTTGGCCAAATTGGGATCCTAACCTA-AATGCCTTAAACTTATTAACA 475
mouse TGACCATTATAAGAAAAGATATATGGAGTTGTATCTTACTGGAATTAATAAACACTGCTT 503
Rat TGACCGTTATAAGAAAAGATATATGGAGTTGTATCTTACTGGAATTAATAAACACTGCTT 535
mouse GACCACCGGTGTTCTGTTTCAATTTGT530
Rat CACCACC----------542