EXPLORING MOLECULAR AND CELLULAR MECHANISMS UNDERLYING SEIZURES IN NEUROCYSTICERCOSIS

by

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Abstract

Neurocysticercosis is a disease in which larvae of the tapeworm, *Taenia solium,* infect the central nervous system of humans. Seizures are the most common symptom of NCC, occurring in between 70 % and 90 % of all symptomatic NCC cases. Neurocysticercosis impacts heavily on the quality of life of patients, and further presents a significant drain on the economic resources of endemic countries. Despite its considerable global impact, the molecular and cellular mechanisms underlying seizures in neurocysticercosis remain largely unknown. In this thesis I have explored novel models for neurocysticercosis by combining mouse hippocampal organotypic brain slice cultures with various preparations of a model parasite, *Taenia crassiceps*. Utilising these models, I first explored, using patch clamp and local field potential electrophysiology, how *Taenia* larval extracts directly affect neuronal excitability. I report that extracts of *Taenia crassiceps* resulted in a significant acute excitation of neurons and triggered seizure-like events in brain slices. Further investigation revealed that this excitation was mediated by the activation of glutamate receptors and that, indeed, the larvae of both *Taenia crassiceps* and *Taenia solium* contain and produce levels of glutamate sufficient to explain this effect. Chronic exposure of brain slices to intact, living, larvae did not, however, result in any changes in network excitability. Next, I investigate whether *Taenia* larvae produce acetylcholinesterases, as these enzymes have the potential to affect neuronal signaling by digesting the neurotransmitter acetylcholine. Ellman's assays, *in situ* acetylcholinesterase activity assays, and patch clamp electrophysiology reveal that both *Taenia crassiceps* and *Taenia solium* larvae produce acetylcholinesterases and that the activity of *Taenia* acetylcholinesterases is sufficient to digest acetylcholine at a concentration that alters neuronal signaling. Finally, I explore the effect that *Taenia* larval extracts have on the innate immune cells of the brain, as the responses of these cells can also alter neuronal excitability. Through the measurement of brain slice cytokine release using enzyme-linked immunosorbent assays, I discover that *Taenia crassiceps* extracts have robust anti-inflammatory effects, which involve lipid, protein, and glycan elements. This thesis presents novel findings that reveal ways in which *Taenia* larvae interact with both neuronal and non-neuronal resident brain cells. It further delves into how these interactions could contribute to seizure generation in neurocysticercosis and proposes some potential new therapeutic approaches to treat seizures in neurocysticercosis.

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List of Abbreviations

Symbol	Description
ACh	Acetylcholine
AChE	Acetylcholinesterase
aCSF	Artificial cerebro-spinal fluid
ANOVA	Analysis of variance
AMPA	α-Amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid
APs	Action potentials
ATP	Adenosine triphosphate
BBB	Blood-brain barrier
BSA	Bovine serum albumin
Ca^{2+}	Calcium
$CaCl_2$	Calcium chloride
CsOH	Caesium hydroxide
Cl^-	Chloride
CNQX	Cyanquixaline
CT	Computed tomography
D-AP 5	D-(-)-2-Amino-5-phosphonopentanoic acid
GABA	γ-aminobutyric acid
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
IL-1β	Interleukin-1β
IL-6	Interleukin-6
IQR	Interquartile range
K^+	Potassium
KCl	Potassium chloride
LPS	Lipopolysaccharide
$MgCl_2$	Magnesium chloride
M. corti	*Mesocestoides corti*
Na^+	Sodium
Na_2ATP	Adenosine 5′-triphosphate disodium
NaCl	Sodium chloride
$NaHCO_3$	Sodium bicarbonate
NaH_2PO_4	Sodium phosphate monobasic
NaGTP	Guanosine 5′-triphosphate sodium
NCC	Neurocysticercosis
NMDA	N-methyl-D-aspartate
PBS	Phosphate buffered saline
SEM	Standard error of the mean
SLE	Seizure-like event

T. crassiceps.	*Taenia crassiceps*
TLR	Toll-like receptor
TLR2	Toll-like receptor 2
TLR3	Toll-like receptor 3
TLR4	Toll-like receptor 4
TNF-α	Tumour necrosis factor-α
T. solium	*Taenia solium*

Chapter 1

INTRODUCTION

1.1 NEUROCYSTICERCOSIS: WHY WE NEED TO STOP NEGLECTING THIS TROPICAL DISEASE

Neurocysticercosis (NCC) is a human disease which results when larvae of the tapeworm *Taenia solium (T. solium)* infect the central nervous system (Garcia *et al.*, 2020). It is predominantly a disease of the developing world (Figure 1.1) but with increasing travel to- and migration from- endemic countries, it has steadily becoming a global phenomenon (Burneo and Cavazos, 2014; Carpio and Romo, 2014; White, 2020). Neurocysticercosis has immense public health, social and economic impacts in areas where it is endemic (World Health Organisation, 2016).

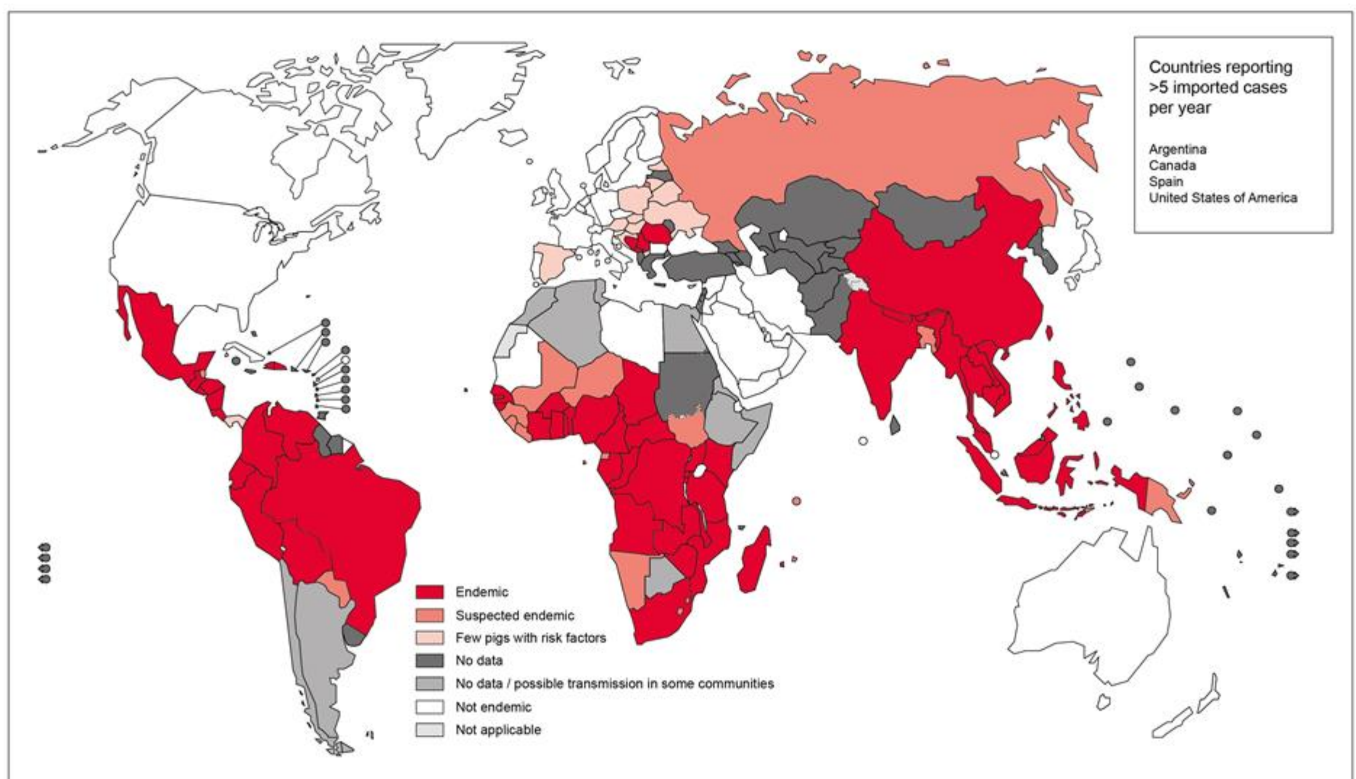
T. solium has been identified as a leading cause of death from food-borne diseases and NCC is estimated to result in approximately 50 000 deaths annually (Roman *et al.*, 2000; World Health Organisation, 2016). Additionally, NCC has been purported to be one of the leading causes of acquired epilepsy, having being associated with almost one third of all epilepsy cases in endemic areas (Gripper and Welburn, 2017; Ndimubanzi *et al.*, 2010; Roman *et al.*, 2000). The causative relationship between NCC and epilepsy has, however, been questioned (Carpio *et al.*, 2021; Gripper and Welburn, 2017). Seizures are the most common symptom of NCC and occur in 70 - 90% of symptomatic NCC cases (Carpio and Romo, 2014). Unfortunately, seizures are heavily stigmatised in many parts of the world, which means that those affected have to deal not only with the health implications but also with immense societal discrimination (World Health Organisation, 2019).

Besides impacting heavily on the quality of life of those infected (Bhattarai *et al.*, 2011), NCC also presents a significant drain on the economic resources due to costs for anticonvulsants, medical resources and lost human work hours (Roman *et al.*, 2000). *T. solium* infection has, for example, been estimated to have had an annual cost in India alone of US$184 million in 2011 (Singh *et al.*, 2017). This gives a clear indication of the extent to which this disease impacts the economies of endemic countries.

In 2010, NCC was added by the World Health Organisation to the list of neglected tropical diseases because of its continued impact on global public health (World Health Organisation, 2016). Neurocysticercosis research, prevention and treatment is “neglected” in part because

it occurs predominantly in poor, rural farming communities in developing countries, which have neither the expertise nor the resources (such as computed tomography (CT) and magnetic resonance imaging (MRI) scanners) required to diagnose and investigate this disease (Mafojane *et al.*, 2003; Ndimubanzi *et al.*, 2010; World Health Organisation, 2016). South Africa is considered an endemic region (Fig. 1.1) and indeed studies suggest that in rural communities of the Eastern Cape Province as many as 20% of the population may have cysticercosis (Mafojane *et al.*, 2003). The South African research community therefore has a responsibility to dedicate attention and resources to the understanding, prevention, control, and treatment of this disease.

Additionally, the study of this disease, and models thereof, present a largely untapped opportunity to elucidate the basic mechanisms of seizures and their transition to chronic epilepsy (Nash *et al.*, 2015b; Sotelo and Rubio-Donndieu, 1989). According to the World Health Organisation (2019), around 50 million people globally suffer from epilepsy, but the cause remains unknown in about 50% of cases. There is therefore a dire necessity for ongoing research into the basic mechanisms of epilepsy. Models of NCC present a potentially powerful tool with which to do said research.



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Data Source: World Health Organization
Map Production: Control of Neglected Tropical Diseases (NTD)
World Health Organization

Figure 1.1: "Endemicity of *Taenia solium*, 2015" (World Health Organisation, 2016). Endemic areas are areas in which the full tapeworm life cycle is prevalent. Areas in which there are "few pigs with risk factors" are areas in which there is lower prevalence of *Taenia solium* infection, as pigs are a carrier of *Taenia solium* larvae and a vector for human disease.

1.2 NEUROCYSTICERCOSIS: AETIOLOGY AND PRESENTATION

1.2.1 The life cycle of *Taenia solium*

In its adult form, *T. solium* is found in the small intestine of *Homo sapiens*(White, Jr., 2000). Adult worms can produce between 200 000 and 400 000 infectious oncospheres per day, which are excreted in human faeces in gravid proglottids (end segments of the worm's body that contain mature eggs) (White, Jr., 2000). If infected faeces are ingested by a pig, the oncospheres become activated/mature in the pig gut due to the presence of bile salts and enzymes, and force their way through the gut wall and into the pig's bloodstream (White, Jr., 2000). The activated oncospheres then lodge in the pig's muscle, nervous, subcutaneous and ocular tissues (White, Jr., 2000). There, each mature oncosphere evolves into a vesicular larvae with an invaginated scolex (also known as a cysticercus) over a period of weeks or months (White, Jr., 2000). These cysticerci have a lifespan of a few years (White, Jr., 2000). If pork meat containing viable cysticerci is ingested by a human, the scolex of the cysticercus evaginates in the small intestine (due to a change in osmotic pressure) and attaches to the intestinal wall. Here the larva develops once more into an adult worm (White, Jr., 2000) (see Fig. 1.2).

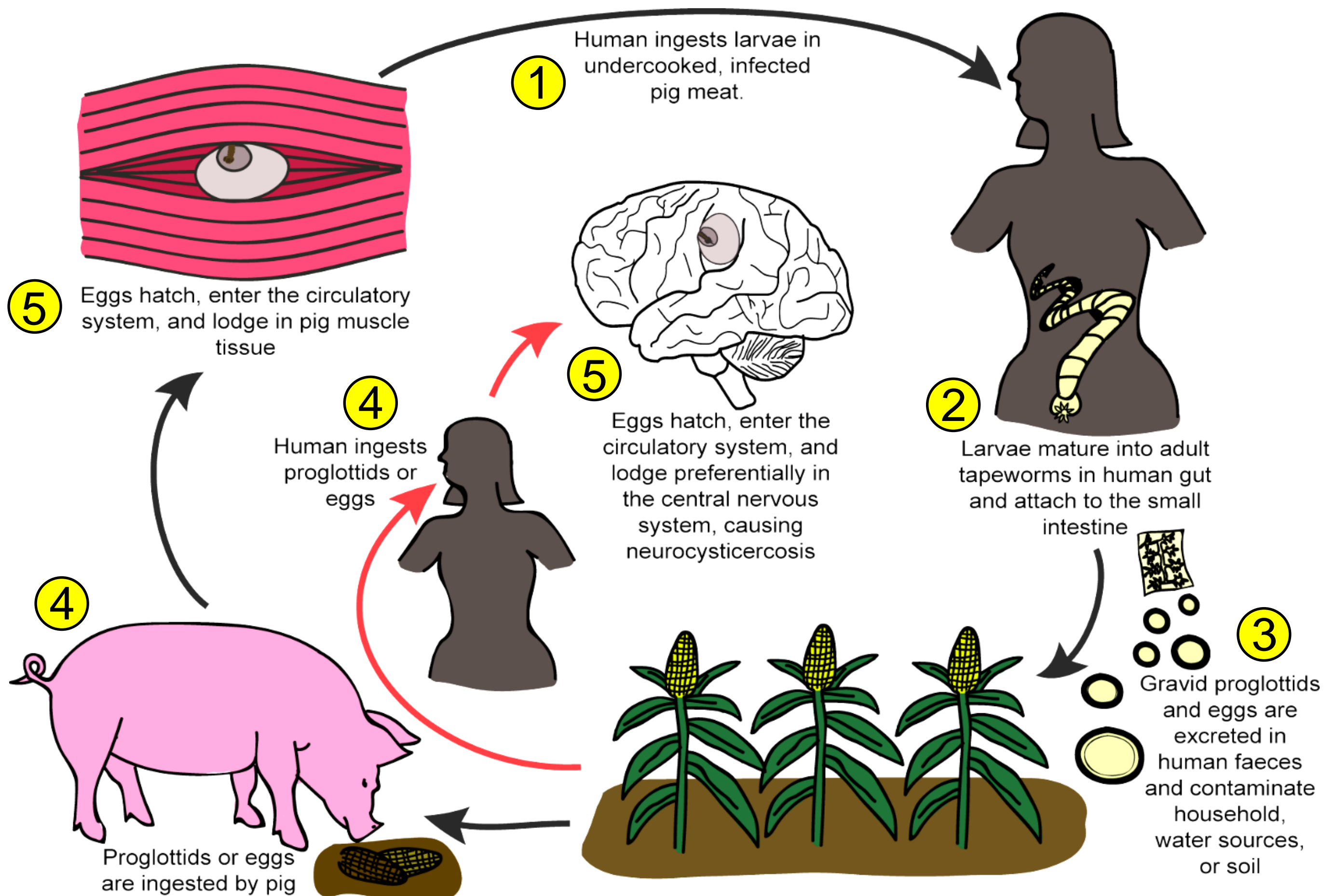


Figure 1.2: Schematic representation of the life cycle of *Taenia solium* and the process by which NCC occurs.

Neurocysticercosis occurs when humans accidentally ingest the oncospheres of *T. solium* (see Fig. 1.2) (de Lange *et al.*, 2018a). This may occur via food or water in areas where water sources are contaminated by human faeces, or via accidental ingestion of tiny amounts of the faeces of a human carrier of the adult tapeworm(Flisser, 1994). Oncospheres are also activated in the human gut as they would be in the pig gut, and are able to penetrate the gut wall and pass into the bloodstream (Carpio, 2002). The activated oncospheres travel to blood vessel terminals and get lodged in capillaries (Escobar, 1983 in Takayanagui, 2004). They then actively cross the capillary wall to lodge in human muscle, ocular, subcutaneous or nervous tissue, with nervous system infection being of the greatest clinical concern (Carpio, 2002; Escobar, 1983 in Takayanagui, 2004).

1.2.2 *Taenia solium* clinical cyst stage progression

Neurocysticercosis is generally described and studied in reference to the stage of the cysticercus/cyst, as first described by Escobar (1983) (see Fig. 1.3). Cyst stages are largely determined using imaging techniques such as CT and MRI scans, or by post-mortem brain dissections (Carpio *et al.*, 1994; Carpio and Romo, 2014).

Viable/vesicular cysts:

Following initial infection and the establishment of cysticerci in the brain parenchyma, sub-arachnoid space or ventricles, there is usually a lengthy period (months to years) in which the host shows little to no immune or inflammatory response to the infection and experiences no clinical symptoms (Garcia and Del Brutto, 2017). This may be because the viable cysticerci (also called vesicular cysts) employ various immune modulatory mechanisms to remain largely unaffected by the host immune system (White, Jr., 2000). Some of these mechanisms include taking up and disposing of host immune proteins (Flores-bautista *et al.*, 2018), directing the host immune response away from the cyst itself by shedding immunogenic molecules, and the excretion/secretion of molecules which can suppress a host inflammatory response (Flores-bautista *et al.*, 2018; White, Jr., 2000). Acetylcholinesterases (AChEs) are a family of enzymes which catalyse the breakdown of acetylcholine, and are one of the agents proposed to be used by helminthic parasite in the modulation of host responses (Darby *et al.*, 2015; Tedla *et al.*, 2019; Vaux *et al.*, 2016). Whether *Taenia* larvae produce AChEs has not yet been established, which I sought to address in this thesis (Aim 3), as, if *Taenia* larvae produce these enzymes, they could potentially disrupt brain acetylcholine signaling, which may, in turn, contribute to seizure generation.

When imaged, viable *T. solium* cysts show no imaging features which are related to an inflammatory host response (*i.e.,* ring-enhancement after contrast administration or visible perilesional oedema) (See Fig. 1.3). Corresponding to this, it has been reported that host

inflammatory responses surrounding viable cysts are minimal, and even that levels of the anti-inflammatory cytokine interleukin -10 (IL-10) are elevated in brain tissue surrounding viable *T. solium* cysts. (Fleury *et al.*, 2016; Singh *et al.*, 2013). In this thesis I investigate the anti-inflammatory potential of extracts of viable *Taenia* larvae (Aim 5), in an attempt to improve our understanding of the mechanisms by which *Taenia* larvae modulate the immune response to favour their survival

Transitional cysts

At some point, the cysts appear to lose their ability to control the host immune response and the cyst wall and fluid become infiltrated by host inflammatory cells. This is termed the colloidal phase. Studies in human brain tissue report that colloidal cysts are surrounded by an intense Th-1 inflammatory host immune response (Restrepo *et al.*, 1998). In the next stage, the cyst cavity collapses, and the host response initiates the process of encompassing the cyst with fibrotic tissue, which is termed the granular-nodular phase. In this stage, the host immune response shifts to a more chronic phenotypic, with mixed Th1 and Th2 immune responses having been observed (Restrepo *et al.*, 2001). Together, cysts in the colloidal or granular-nodular phase are termed as transitional phase cysts. Transitional cysts are also associated with astrocytic gliosis (Alvarez *et al.*, 2002a). Correspondingly, when imaged, *T. solium* cysts in the colloidal/granular-nodular stage are always accompanied by two imaging features which are related to an inflammatory host response: a ring-enhancement after contrast administration and visible perilesional oedema (Fleury *et al.*, 2016) (See Fig. 1.3).

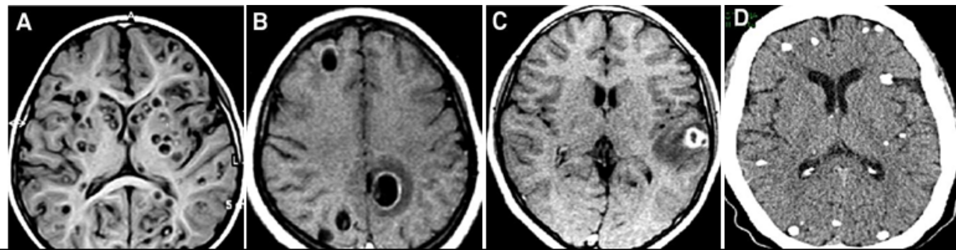
Calcified cysts

Eventually the entire cyst is replaced with connective tissue/calcium by the host's immune system and is then said to be in the calcific stage (White, Jr., 2000). Previously, it has been considered the "healed" stage of the disease and indeed, when imaged, most calcified cysts display no imaging features which are related to an inflammatory host response (Fleury *et al.*, 2016). Recently, however, calcific NCC has increasingly been reported to be associated with ongoing seizures, and alternate imaging techniques and studies in the brains of pigs with calcified NCC have shown that some calcified cysts display perilesional oedema (Nash *et al.*, 2004, 2001; Singh *et al.*, 2013).

1.2.3 Symptoms of neurocysticercosis

A systematic review of the prevalence of different clinical manifestation of NCC reports seizures as the most common symptom, followed by headaches, intracranial hypertension, hydrocephalus and meningitis (Carabin *et al.*, 2011). Seizures appear more commonly in patients with transitional or calcified cysts, whilst intracranial hypertension, hydrocephalus and meningitis seem to be much more common in patients with transitional or active cysts (in these

cases the cysts are often extra-parenchymal) (Carabin *et al.*, 2011; Fleury *et al.*, 2016)(see Fig. 1.3). Additionally, various other less common symptoms secondary to NCC have been reported (Mahanty and Garcia, 2010; Patel *et al.*, 2006; Peon *et al.*, 2016).This diversity in symptoms has been attributed to the diversity in cyst number, antigens, stage and position in the nervous system (Fleury *et al.*, 2004; Yakoleff-Greenhouse *et al.*, 1982). In parenchymal NCC symptom onset also seems to be closely associated with an inflammatory host immune response (Fleury *et al.*, 2016)



Cyst stage: (Escobar, 1983)	Vesicular	Colloidal	Granular-nodular	Calcific
Cyst state: (Escobar, 1983)	Viable.	Dying/transitional.		Dead.
Characterised by: (Carpio *et al.*, 1994)	Little to no inflammatory response.	Cyst is infiltrated by host inflammatory cells.	Cyst cavity collapses, onset of fibrosis.	Cyst fully fibrotic/calcified.
Common clinical manifestations: (Ndimubanzi et al., 2010)	Seizures, intracranial hypertension, hydrocephalus and meningitis.	Seizures.		Seizures.
Suspected main cause of symptoms: (Roman et al., 2000)	Compression of the surrounding brain parenchyma and initial transient inflammatory reaction.	Acute inflammatory changes.	Perilesional gliosis.	Perilesional gliosis/oedema.
Antigens/proteins present:	Excreted/secreted and membrane presenting.	Products from cyst fluid, scolex and membrane.		Some evidence of residual cyst elements in calcific cysts (Gupta et al., 2002).
Predominant immune response: (Peon et al., 2016)	Modulated by Taenia larvae – upon infection there is a transient, inflammatory, T-helper 1 type response, which is shifted to a non-inflammatory T-helper 2 type response by larval excretory/secretory products.	Inflammatory. T-helper 1 type response.		Regulatory. Mixed T-helper 1 & 2 type response.

Figure 1.3: Key differences in clinical, molecular and immune characteristics of *Taenia solium* cysts in NCC [photographs sourced, with permission, from Carpio and Romo, (2014)].

Seizures secondary to NCC

Seizures are by far the most common symptom of NCC, representing between 70 and 90% of all symptomatic NCC cases (Carpio and Romo, 2014). However, the cause of, and risk factors for, seizures secondary to NCC have proven to be difficult to piece apart. Factors such as the size, number, stage, location in the brain, and treatment with anti-helminthic drugs, all seem to affect the likelihood and severity of seizures secondary to neurocysticercosis (Carpio *et al.*, 2019; Montgomery *et al.*, 2019; Takayanagui and Odashima, 2006). Exactly how each of these factors affect seizure likelihood has, however, not necessarily yet been definitively established, with studies sometimes finding contrasting associations (Nash *et al.*, 2004; Prasad *et al.*, 2008).

There do appear to be some commonalities in neurocysticercosis-related seizures. Seizure likelihood seems to relate to the stage of the cyst and seizures are least common while the parasitic cyst remains viable, most prevalent when the cyst is degenerating, and somewhat common in cases where the lesion has become calcified (Herrick *et al.*, 2018). The occurrence of seizures in all cyst stages seems to be closely related to a detectable inflammatory host immune response surrounding the cyst, and as a result, it is widely assumed that seizures result from the host inflammatory response to the cyst (Carpio, 2002; Garcia and Del Brutto, 2017)(See Fig. 1.3). This has not yet, however, been experimentally proven. In this thesis I therefore set out to model the inflammatory host immune response induced by *Taenia* larvae, to enable future investigations into the mechanisms by which the host immune response may result in seizures (Aim 4).

There are also, however, reports of patients with non-inflamed, viable cysts and patients with long-standing, non-inflamed calcified scars having seizures (Garcia and Del Brutto, 2017). This suggests that additional seizurogenic processes may be at play in NCC. Few studies have explored whether larval-derived products may directly affect neuronal signaling. As such, two of my main aims in this thesis involved the investigation of alterations in neuronal excitability after the exposure of brain slices to *Taenia* larvae/larval extracts (see Aims 1 & 2). In addition, a subset of patients experience repeated focal seizures over years or even decades, which suggests that further disruptions in brain structure or circuitry may also be at play (Garcia and Del Brutto, 2017).

The fact that seizures can occur when cysts are at any stage, that they may not occur at all in some NCC patients, and that they may occur together with, or independent of, a host immune response, suggests that the aetiology of seizures in NCC is likely multi-faceted and complex.

1.3 APPROACHES TO STUDYING NEUROCYSTICERCOSIS

Several different models have been utilised to study NCC, including *in vivo* and *in vitro* approaches. Modelling NCC is complex, as there exist a variety of options for model hosts and for model parasites. In this section, I review all the existing models of NCC and their respective strengths and limitations.

1.3.1 Neurocysticercosis is mostly studied in naturally infected human patients or using human cell lines.

Neurocysticercosis is most studied in patients with the disease (*i.e., in vivo* in humans naturally infected with *T. solium* larvae). These studies frequently make use of imaging techniques, such as CT and MRI. Such images have then been used in conjunction with the clinical presentation of the disease to investigate symptom development in NCC. Such studies have provided many valuable insights including: that the presence of vesicular cysts may contribute to a patient remaining asymptomatic, even when other stages of cyst are present (Prasad *et al.*, 2008); that patients with calcified parenchymal cysts appear to be more susceptible to depressive symptoms (Leon *et al.*, 2015); that some cysts which appear to be inactive on an MRI scan are actually surrounded by gliosis, which may contribute to seizure recurrence (Pradhan *et al.*, 2000); and that the best drug regimens vary for different NCC cases (Del Brutto *et al.*, 2006; Garcia *et al.*, 2014; Zhao *et al.*, 2016).

Another popular way of studying disease processes in humans is through the analysis of blood serum or cerebrospinal fluid samples from NCC patients (Arce-Sillas *et al.*, 2016; Chung *et al.*, 1999; Ferrer *et al.*, 2005; Verma *et al.*, 2010). Such studies have discovered certain proteins that appear to be specific to active, symptomatic NCC (Chung *et al.*, 1999; Ferrer *et al.*, 2005); that *T. solium* larvae appear to induce a systemic regulatory T-cell response in the host, likely to create an environment favourable to their survival (Arce-Sillas *et al.*, 2016); and that there appear to be certain polymorphisms in the toll-like receptor 4 (TLR4) gene that are associated with individuals who have symptomatic versus asymptomatic NCC (Verma *et al.*, 2010).

More rarely, researchers may obtain brain tissue samples from NCC patients either post-mortem, or after they have undergone neurosurgical procedures as a part of their standard clinical care (Restrepo *et al.*, 1998; Robinson *et al.*, 2012). Such studies have made huge contributions to our understanding of the host immune responses to cysts at various stages, particularly inflammatory responses to transitional cysts often associated with symptom onset (Alvarez *et al.*, 2002a; Restrepo *et al.*, 2001, 1998). Another notable discovery from one such study is that substance P (a neuropeptide involved in neuropathic inflammation and possibly

seizure induction) is prevalent in cells adjacent to NCC granulomas, but not in areas distant from granulomas, nor in brain tissue from individuals without NCC (Robinson *et al.*, 2012).

Human NCC is also studied *in vitro* utilising normal, healthy cell culture lines of nervous system immune cells, or extracted and cultured immune cells from NCC infected patients. Such cell cultures have been utilised in conjunction with *T. solium* extracts to study immune responses in NCC (Amit *et al.*, 2011; Uddin *et al.*, 2010, 2005). Notable discoveries from *in vitro* human cell culture studies include: that cysts treated with anti-parasitic agents elicit greater chemokine secretion in monocytes than those left untreated (Uddin *et al.*, 2010); that astrocytes play a key role in the inflammatory response to *T. solium* larval extracts (Uddin *et al.*, 2005); that healthy human monocytes respond differently to *T. solium* brain cysts than to *T. solium* muscle cysts from pigs (Uddin *et al.*, 2010); and that the scolex and membrane antigens of *T. solium* stimulate an inflammatory monocyte response, whilst cyst-fluid inhibits this response (Uddin *et al.*, 2010).

A major challenge for the study of NCC in humans is that the disease can only be studied as it occurs naturally. This means that there are large numbers of variables which cannot be controlled in human studies, making it hard to isolate conditions which lead to symptom onset. Additionally, when patients present with symptomatic NCC there exists a moral obligation to start treatment as soon as possible, which also obstructs the understanding of the disease processes underlying symptom development (Cardona *et al.*, 1999). Study of the disease in humans is costly, as the only definitive diagnosis techniques are MRI or CT scans, and these are typically not available in endemic areas with the highest prevalence rates. In addition, the large variability in human pathology and symptom presentation means that large sample sizes are required to obtain statistical significance. Longitudinal studies in humans are also challenging due to the lengthy time course of the disease, both in terms of symptom onset and cyst progression (Cardona *et al.*, 1999).

Studies using human tissue are also limited, as most rely on specimens that are collected using only minimally invasive procedures such as blood sampling. Beyond that, samples of brain tissue or cerebrospinal fluid can only be sourced in cases where these are extracted out of clinical or diagnostic need, a fairly rare occurrence, making it difficult to obtain large sample sizes (Cardona *et al.*, 1999). Taken together, these challenges greatly limit the exploration of the cellular and molecular processes underlying disease progression in NCC in humans.

Animal models of NCC offer the potential to overcome many of these limitations: they allow for the experimental infection of animals in a controlled environment and for the study of disease progression both with and without treatment interventions. They are often much more cost effective, as definitive infection can be confirmed post-mortem without neuroimaging, and

smaller sample sizes can be used due to the controlled experimental environment. They facilitate longitudinal studies, as the time course of disease, especially in smaller mammals, is much shorter and can be accelerated experimentally. Most importantly, animal models allow for access to brain tissue and cerebrospinal fluid, enabling more extensive cellular and molecular exploration. Although animal models have great utility in the study of disease processes in NCC, one should always bear in mind that findings in animals may not necessarily extrapolate to the human condition, as no animal model can fully recapitulate the human disease state.

1.3.2 Parasites utilised in animal model systems of neurocysticercosis.

Model systems for studying NCC typically consist of two components: a cestode species and a host organism. There are three main cestode species that are utilised in animal models of NCC.

Taenia solium

T. solium is the 'gold standard' organism in the study of NCC, as it is the cestode responsible for pathology in humans. Further, the genome for *T. solium* has been sequenced, providing a powerful molecular tool (Tsai *et al.*, 2013). However, *T. solium* has numerous practical limitations for use within the laboratory setting. It is highly infectious to humans and its experimental use requires strict biosafety measures. It is also challenging to obtain *T. solium* larvae, and extremely hard to maintain a steady experimental supply of the larvae. Larvae (or cysticerci) can be obtained in three ways: They can be harvested directly from a naturally or experimentally infected pig; they can be produced experimentally by feeding oncospheres (obtained from gravid proglottids in the stool of infected human patients, or from experimentally infected, immunosuppressed chinchillas or hamsters (Arora *et al.*, 2017)) to a host in which they will become activated and develop naturally into cysticerci (Nguekam *et al.*, 2003); or they can be produced by activating oncospheres *in vitro* and then injecting the activated oncospheres into the brain to develop into cysts (Liu *et al.*, 2002; Verastegui *et al.*, 2015). *T. solium* may also not be infectious to animals utilised in animal models, thus requiring direct intracranial application. However, the larvae of *T. solium* are large and may therefore displace a significant portion of the brain tissue in small mammals.

Taenia crassiceps

Taenia crassiceps (*T. crassiceps)* is the most widely utilised model organism for *T. solium*. The two cestodes are closely related, belonging to the same genus, and have been shown to have significant antigenic similarity (Larralde *et al.*, 1989; Sciutto *et al.*, 1990). *T. crassiceps* very rarely infect humans, making it a reasonably safe laboratory model, not requiring extensive biosafety measures (Willms and Zurabian, 2010). This is particularly the case for

the ORF strain of *T. crassiceps*, which has entirely lost the ability to infect a definitive host and mature into adult worms, meaning that it doesn't even present an infection risk to animals (Willms and Zurabian, 2010). A major advantage of *T. crassiceps* as a model organism is that the larvae are able to asexually divide quite rapidly in the intermediate host (usually mice), providing a simple way to maintain a steady experimental supply of the organism (Stringer *et al.*, 2003; Willms and Zurabian, 2010). The larvae are also able to survive for several weeks *in vitro*.

The use of *T. crassiceps* as a model organism in the study of NCC also has its limitations. The definitive hosts for *T. crassiceps* are carnivores, most often wild canines (Willms and Zurabian, 2010), whilst that of *T. solium* is humans (see Fig. 1.4). *T. crassiceps* larvae are usually hosted by rodents and small moles (Willms and Zurabian, 2010), whilst pigs host larval *T. solium* (see Fig.1.4). As a result, there must exist differences in the antigens of, and the species-specific immune responses induced by, the two organisms (Sciutto *et al.*, 2011). Another concern when utilising *T. crassiceps* is that it has been found that many of the strains undergo morphological and genetic changes when they are maintained via serial intraperitoneal inoculation (for asexual reproduction) in mice, which may affect their immunogenicity and decrease their similarity to *T. solium* larvae (Willms and Zurabian, 2010; Zurabian *et al.*, 2008). Lastly, *T. crassiceps* are difficult to use where intracranial inoculation of small model animals is desired (*e.g.* mice), as the cysticerci displace much of the brain tissue (Alvarez *et al.*, 2010).

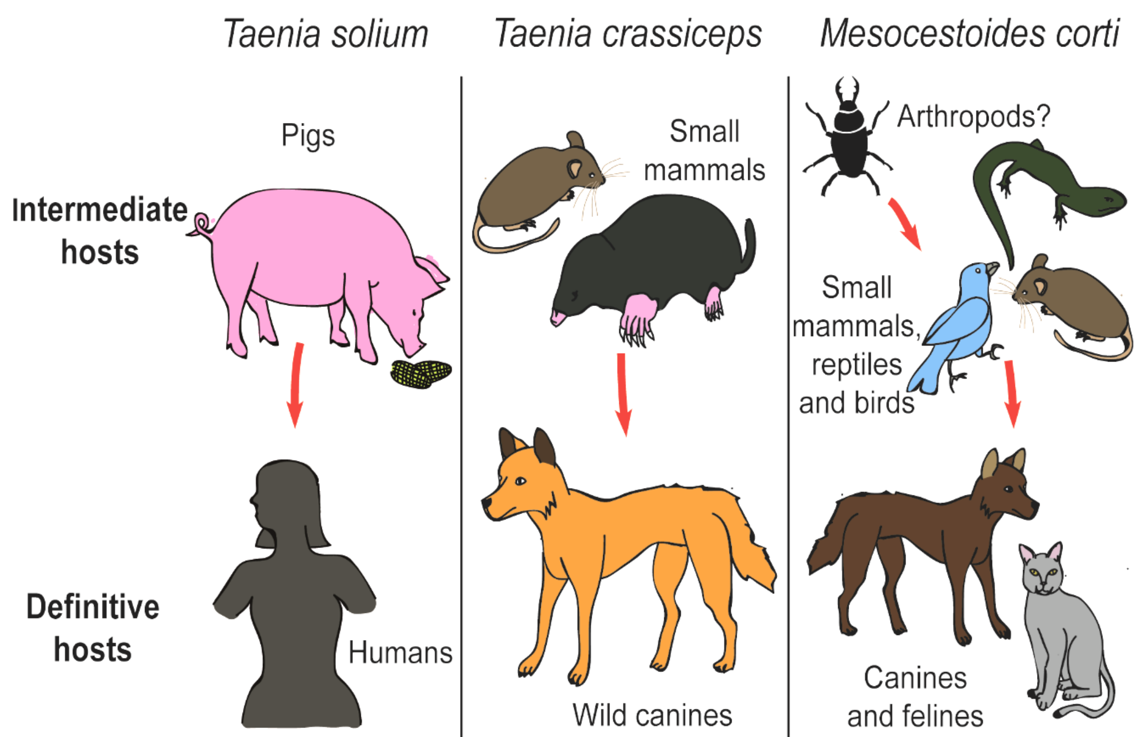


Figure 1.4: The intermediate and definitive hosts of *Taenia solium, Taenia crassiceps* and *Mesocestoides corti.*

Mesocestoides corti

Mesocestoides corti (*M. corti*) is thought to infect arthropods as the initial host, small mammals, birds, reptiles and amphibians in the larval form, and carnivores, such as dogs and cats, as an adult worm (Crosbie *et al.*, 2000) (see Fig. 1.4). *Mesocestoides corti* is not known to infect humans, making it a safe laboratory model not requiring extensive biosafety measures. *M corti* asexually divides both as cysticerci in the intermediate host (Alvarez *et al.*, 2010) and as adult worms in the definitive host (Schmidt and Todd, 1978). The larvae have also been shown to be able to survive and divide under the right *in vitro* conditions (E. Vendelova *et al.*, 2016a; Voge and Coulombe, 1966). A colony of *M. corti* can therefore be experimentally produced and maintained with relative ease (Schmidt and Todd, 1978). *Mesocestoides corti* is closely related to *T. solium*, but is not of the same genus, which means that it may have greater antigenic differences to *T. solium* than would *T. crassiceps* (Alvarez *et al.*, 2010). A major advantage of *M. corti* is that the cysticerci are significantly smaller than those of *T. solium* and *T. crassiceps*, which makes it easier to use for intracranial inoculation of small model animals such as rodents (Alvarez *et al.*, 2010). However, unlike *T. solium* and *T. crassiceps, M. corti* has not been known to infect the central nervous system of any of its hosts during its natural cycle, which means that intracranial injection by the experimenter is the only way in which *M. corti* can enter the central nervous system (Alvarez *et al.*, 2010).

1.3.3 Animal models of neurocysticercosis.

Taenia crassiceps in mice

Mice are attractive model organisms for studying NCC due to their popularity across the life sciences. They are relatively cheap to maintain and have a rapid breeding cycle. Most importantly, the relative ease of modifying the mouse genome means that transgenic strains allowing for exact alterations of immunological and neurological pathways are now widely available. Neurocysticercosis has been modelled in mice by intracranially injecting *T. crassiceps* larval extracts or intact early stage larvae (Leandro *et al.*, 2014; Matos-Silva *et al.*, 2012; Robinson *et al.*, 2012). Different strains of mice show differing susceptibility to *T. crassiceps* intracranial infection (Matos-Silva *et al.*, 2012). There has been one report of *T. crassiceps* in the brain of a wild mouse (Kroeze and Freeman, 1982), normally the intermediate host, which is suggestive that further experimentation on the oral administration of *T. crassiceps* oncospheres in mice (perhaps using immunocompromised individuals) may have potential for the development of a model somewhat more congruent to the condition in humans. Intracranial administration of *T. crassiceps* in mice has provided some valuable insights: one study has shown that early stage granuloma extracts containing substance P may be responsible for seizure activity; another has shown this model results in encephalitis

closely resembling that in human NCC; and a third has shown that *Taenia* larvae in the brain are highly adaptable when faced with adverse conditions (Leandro *et al.*, 2014; Matos-Silva *et al.*, 2012; Robinson *et al.*, 2012).

One strength of this model is that intracranial injection of peritoneal granulomas can produce seizures in the host (Robinson *et al.*, 2012), although it is not known whether these seizures could persist chronically. A significant drawback of this model is that intracranial injection of intact larvae can displace much of the brain tissue as they enlarge.

Mesocestoides corti in mice

Mesocestoides corti can be utilised in conjunction with cultured mouse primary microglia to explore immunomodulation in NCC (Sun *et al.*, 2014). One study utilising this model system elucidated an immunosuppressive mechanism that may help to explain the delay in the onset of neuroinflammation seen in human NCC (Sun *et al.*, 2014). More commonly, however, *M. corti* larvae are administered intracranially in mice (as they do not migrate to the central nervous system if administered orally) to model human NCC (Alvarez *et al.*, 2010; Alvarez and Teale, 2007; Cardona *et al.*, 2003, 1999; Cardona and Teale, 2002). This model presents with an initial relative lack of immune responsiveness, which is thought may be useful as a comparative model for the study of asymptomatic NCC prior to symptomatic NCC (Cardona *et al.*, 1999). Thus far no seizures have been reported in this model system of NCC, but it has provided much insight into potential mechanisms that dictate the severity of inflammation, blood-brain barrier breakdown, parasite burden and neuronal pathology (Alvarez and Teale, 2007, 2006; Cardona *et al.*, 2003, 1999; Cardona and Teale, 2002).

Taenia crassiceps in rats

Elements of *T. crassiceps* larvae have been administered intracranially in rats, but to date no studies have been performed where intact oncospheres/cysts are injected. Studies using this model have shown that the intracranial administration of early stage granuloma extracts can induce seizures in the host (Robinson *et al.*, 2012; Stringer *et al.*, 2003). This is a very promising finding in terms of its potential use in the study of seizures secondary to NCC. It should be noted, however, that these studies are limited by the fact that the granulomas were produced peripherally in mice, and immune responses differ by host and location in the body, so the content of these granulomas may not be reflective of brain cysts. Brain activity was monitored acutely in these studies, but it is not yet known whether recurrent seizures would present in these rats.

Taenia solium in rats

T. solium does not naturally infect rodents, and as such little research has been done using *T. solium* in rats. In one study, however, activated oncospheres were intracranially injected into

rats, and these were found to form cysts in roughly half of the rats after about 4 months (Verastegui *et al.*, 2015). Importantly, infection is much more successful in younger rats. The authors report that this model reflects many NCC characteristics typical of human infection and that 9% of infected rats present with chronic seizures (Verastegui *et al.*, 2015). Although this is not a very efficient model of epilepsy secondary to NCC, the induction of chronic seizures is intriguing, and it may be worth exploring whether this model could be optimised for seizure occurrence. The study further revealed diverse cyst distribution and immunopathology, like that observed in humans. A major advantage of this model over other rodent models is the use of the same parasitic strain responsible for the human form of the disease.

***Taenia solium* in pigs**

The study of *Taenia solium* NCC in pigs often involves the utilisation of pigs reared for agricultural purposes that have naturally acquired the infection. Pigs can also be experimentally infected by oral administration of *T. solium* eggs to induce NCC, with between 20% and 100% of pigs dosed with high numbers of eggs developing NCC (de Aluja *et al.*, 1996; Nguekam *et al.*, 2003; Santamaria *et al.*, 2002). Older pigs appear to be more resistant to infection than younger pigs (Santamaria *et al.*, 2002). Recently, a new model of pig NCC was developed whereby activated *T. solium* oncospheres were surgically implanted into the subarachnoid space (Fleury *et al.*, 2015). All infected pigs developed brain cysts, although at very low infection efficiencies (Fleury *et al.*, 2015). This model surprisingly did not result in any neurological signs (Fleury *et al.*, 2015).

The pig model system of NCC has been extremely useful in characterising immunopathological and proteomic changes in response to *T. solium* in the brain, both in the normal course of disease and after a vaccination or treatment protocol. Significant overlap between human and pig reactions have been found (Christensen *et al.*, 2016; Guerra-Giraldez *et al.*, 2013; Mahanty *et al.*, 2015; Molinari *et al.*, 1983; Navarrete-Perea *et al.*, 2017; Sikasunge *et al.*, 2009; Singh *et al.*, 2013). A recent study reports severe seizures in naturally infected pigs, which could be extremely valuable in furthering understanding of the symptomatic progression in NCC (Trevisan *et al.*, 2016). The pigs that presented with seizures in this study were much older than the others in the sample group, suggesting that a longer infection/experimental period may be necessary for neurological symptoms to present (Trevisan *et al.*, 2016). Neurocysticercosis in pigs also presents with great variation in the infection characteristics and antibody response, which suggests that porcine models may be able to, to some extent, recapitulate the great variation in pathology and disease progression that is observed in human patients (Prasad *et al.*, 2006; Saenz *et al.*, 2008).

Limitations of the pig *T. solium* model system includes that this model can prove very time and resource intensive, with *T. solium* cysts taking as long as 350 days to form in pigs (de Aluja *et al.*, 1996), pig handlers requiring training, and larger animals requiring more resources to feed and maintain (Arora *et al.*, 2017; de Aluja *et al.*, 1996).

Taenia solium in rhesus monkeys

Primate studies of neurological conditions are rare due to the significant ethical (Greene *et al.*, 2005) and legal (Fox, 2009) implications of using primates for research purposes. Many countries have laws in place either preventing primate research or restricting their use only to cases where all other options have been exhausted (Fox, 2009; Greene *et al.*, 2005).

T. solium has, however, been reported to infect several non-human primates in its larval form, although this is considered an "accidental infection" since *T. solium* does not usually rely on the infection of non-human primates to complete its life cycle (Johnston *et al.*, 2016; Kuntz, 1973). Neurocysticercosis can be reliably induced in rhesus monkeys by feeding them large doses of activated *T. solium* oncospheres. The infected monkeys present with seizures and clinical symptoms very similar to those in humans within a matter of days, and if not treated may eventually die from the infection (Saleque *et al.*, 1988). Symptom presentation is delayed and attenuated in monkeys receiving smaller numbers of oncospheres, which may more closely resemble the human condition (Saleque *et al.*, 1988). It is interesting to note that symptom onset could be induced within a matter of days, in contrast to a study reporting a case of naturally acquired NCC in an 8-year-old rhesus monkey, which presented with no symptoms (Johnston *et al.*, 2016). This could potentially be explained by the high dosage of oncospheres used in the experimental study and serves as a reminder of the importance of dose control for eliciting disease phenotypes in models of NCC.

1.3.4 A hippocampal organotypic brain slice culture model as a promising unexplored model for neurocysticercosis.

Due to the remaining uncertainty surrounding disease mechanisms in NCC and the unavoidable limitations of studying the disease in humans, there is a need for continued exploration and improvement of animal models that recapitulate the human disease process. Table 1 summarises the respective utility of currently available model systems used in NCC research and highlights the fact that there still exist many areas that remain unexplored. Amongst others it highlights the paucity of studies which explore seizures in NCC as well as demonstrating that, to date, there have been no studies which have explored brain hyperexcitability/seizure-like events/epileptogenesis in an *in vitro* model. Although *in vitro* models that allow for the study of seizures are limited in their representation of human *in vivo* seizures and ictal activity (Dulla *et al.*, 2018), they represent a valuable resource for NCC

research, particularly for investigations on the molecular, cellular and network brain level, as these would be extremely difficult to study in clinical NCC.

Hippocampal organotypic brain slice cultures have been utilised extensively to study both seizure-like events/epileptogenesis (Holopainen, 2005; Liu *et al.*, 2017; Park *et al.*, 2015; Raimondo *et al.*, 2016; Ziobro *et al.*, 2011) and innate brain immune responses (Bernardino et al., 2008, 2005; Chong et al., 2018; Hailer et al., 2005; Huuskonen et al., 2005; Montpied et al., 2003; Papageorgiou et al., 2016; Yousif et al., 2018) on a molecular, single cell, and cell network level. They have been evaluated to be a powerful brain model system due to the fact that they display well-preserved representation of the various resident cell types and retain excellent structural and functional information processing integrity (Holopainen, 2005). The ways in which clinical seizures can be modelled in in vitro systems such as the hippocampal organotypic brain slice model has also been thoroughly characterised (Dulla *et al.*, 2018).

There are, however, also several limitations to this model that need to be considered. The process of dissecting out, and slicing up, the hippocampus axotomizes many of the neurons in the slice, with the consequence that the network of neurons studied is isolated from other brain areas, and does not fully recapitulate *in vivo* connectivity (Humpel, 2015). Some other considerations include the fact that this model lacks full vasculature, a blood-brain barrier, as well as an adaptive immune response. These may be particularly hindersome in a model of NCC, as blood-brain barrier disruption and the peripheral immune response have both been implicated in pathogenesis of NCC (Alvarez *et al.*, 2002a; Alvarez and Teale, 2007; Carmen *et al.*, 2018; Restrepo *et al.*, 2001, 1998; Sikasunge *et al.*, 2009). Further, hippocampal organotypic cultures can develop spontaneous electrical seizures, which could complicate the study of seizures in an NCC model (Liu *et al.*, 2017).

Based on the abovementioned merits of hippocampal organotypic brain slice cultures and of *T. crassiceps* as a parasite with which to model NCC, I decided to utilise several iterations of a novel model of NCC where mouse hippocampal organotypic brain slice cultures were combined with *T. crassiceps* (intact larvae or larval extracts). Whilst this model lacks key components necessary to fully recapitulate human NCC, it allows for extensive electrical and molecular access to brain tissue, the equivalent of which is difficult or impossible to accomplish *in vivo*.

Table 1: A summary of the characteristics and utilities of existing model systems for the study of neurocysticercosis.

Host	Parasite	In vivo/ *in vitro*	Parasite material administered (if experimental)	Matches human NCC host-parasite stage mismatch?	Enables experimental infection/ manipulation?	Useful to study immune pathology?	Presents with electrographic hyperexcitability/ seizures?	Presents with other clinical symptoms?	Useful in investigating effect of different cyst stages on disease state?	Useful to assess drug treatment? (AEDs, steroids, anthelminthics)	Common experimental techniques
Human	*T. solium*	In vivo	Not applicable	Yes	No	Yes	Yes (Das *et al.*, 2007; Fleury *et al.*, 2004; Garcia *et al.*, 2014; Leon *et al.*, 2015; Pradhan *et al.*, 2003, 2000; Prasad *et al.*, 2008)	Yes (Fleury *et al.*, 2004; Leon *et al.*, 2015)	Yes (Del Brutto *et al.*, 2006; Fleury *et al.*, 2004; Prasad *et al.*, 2008)	Yes (only by repurposing existing human drugs)(Das *et al.*, 2007; Del Brutto *et al.*, 2006; Garcia *et al.*, 2014; Nash *et al.*, 2006; Zhao *et al.*, 2016)	Neuroimaging (Fleury *et al.*, 2004; Leon *et al.*, 2015; Pradhan *et al.*, 2003, 2000; Prasad *et al.*, 2008) Cellular/molecular examination of blood/CSF/tissue (other than histology)(Arce-Sillas *et al.*, 2016; Chung *et al.*, 1999; Ferrer *et al.*, 2005; Fleury *et al.*, 2004; Restrepo *et al.*, 1998; Robinson *et al.*, 2012; Verma *et al.*, 2010)
Human	*T. solium*	*In vitro*	Larval/granuloma extracts.	Yes	Yes (Amit *et al.*, 2011; Uddin *et al.*, 2010, 2005)	Yes (Amit *et al.*, 2011; Uddin *et al.*, 2010, 2005)	Not yet tested	No	Not yet tested	Yes (Uddin *et al.*, 2010)	Cell culture with parasite extract (Amit *et al.*, 2011; Uddin *et al.*, 2010, 2005)
Mouse	*M. corti*	*In vitro*	Larval/granuloma extracts.	No	Yes (Sun *et al.*, 2014)	Yes (Sun *et al.*, 2014)	Not yet tested	No	Not yet tested	Not yet tested	Cell culture with parasite extracts (Sun *et al.*, 2014)
Pig	*T. solium*	In vivo	Oncospheres	No	Yes (de Aluja *et al.*, 1996; Fleury *et al.*, 2015; Nguekam *et al.*, 2003)	Yes (Alvarez *et al.*, 2002b; Cangalaya *et al.*, 2016; Christensen *et al.*, 2016; Ferrer *et al.*, 2005; Guerra-Giraldez *et al.*, 2013; Marzal *et al.*, 2014; Molinari *et al.*, 1983; Nguekam *et al.*, 2003; Sikasunge *et al.*, 2009;	Yes (Trevisan *et al.*, 2016)	Yes (Prasad *et al.*, 2006; Trevisan *et al.*, 2016)	Yes (Alvarez *et al.*, 2002b; Ferrer *et al.*, 2005; Fleury *et al.*, 2015; Saenz *et al.*, 2008; Sikasunge *et al.*, 2009; Singh *et al.*, 2013)	Yes (Cangalaya *et al.*, 2016; de Aluja *et al.*, 1996; Guerra-Giraldez *et al.*, 2013; Marzal *et al.*, 2014)	Neuroimaging (Cangalaya *et al.*, 2016; Prasad *et al.*, 2006; Singh *et al.*, 2013) Cellular/molecular examination of blood/CSF/tissue (other than histology) (Ferrer *et al.*, 2005; Singh *et al.*, 2013) Macroscopic examination of brain (Fleury *et al.*, 2015; Prasad *et al.*, 2006; Saenz *et al.*, 2008; Trevisan *et al.*, 2016) BBB permeability assessment (Cangalaya *et al.*, 2016; Guerra-Giraldez *et al.*, 2013; Marzal *et al.*, 2014) Histological studies (Alvarez *et al.*, 2002b; Cangalaya *et al.*, 2016; Christensen *et al.*, 2016; Fleury *et al.*, 2015; Marzal *et al.*, 2014; Molinari *et al.*, 1983; Prasad *et al.*, 2006; Sikasunge *et al.*, 2009)

						Singh *et al.*, 2013)					
Rat	*T. solium*	In vivo	Activated oncospheres	No	Yes (Verastegui *et al.*, 2015)	Yes (Verastegui *et al.*, 2015)	Yes (Verastegui *et al.*, 2015)	Not yet tested	Not yet tested	Not yet tested	Cellular/molecular examination of blood/CSF/tissue (other than histology) (Verastegui *et al.*, 2015) Macroscopic examination of brain (Verastegui *et al.*, 2015) Histological studies (Verastegui *et al.*, 2015)
Rat	*T. crassiceps*	In vivo	Larval extracts	No	Yes (Robinson *et al.*, 2012; Stringer *et al.*, 2003)	Not yet tested	Yes (Robinson *et al.*, 2012; Stringer *et al.*, 2003)	Not yet tested	Yes (Robinson *et al.*, 2012; Stringer *et al.*, 2003)	Not yet tested	Histological studies (Robinson *et al.*, 2012) Electrophysiological recordings (Robinson *et al.*, 2012; Stringer *et al.*, 2003)
Rhesus monkey	*T. solium*	In vivo	Oncospheres	No	Yes (Saleque *et al.*, 1988)	Yes (Saleque *et al.*, 1988)	Yes (Saleque *et al.*, 1988)	Yes (Saleque *et al.*, 1988)	Not yet tested	Not yet tested	Macroscopic examination of brain (Johnston *et al.*, 2016) Histological studies (Johnston *et al.*, 2016) Behavioural observations (Saleque *et al.*, 1988)
Mouse	*T. crassiceps*	In vivo	Early stage cysticerci	No	Yes (Leandro *et al.*, 2014; Matos-Silva *et al.*, 2012; Robinson *et al.*, 2012)	Yes (Matos-Silva *et al.*, 2012; Robinson *et al.*, 2012)	Yes (Robinson *et al.*, 2012)	Not yet tested	Yes (Robinson *et al.*, 2012)	Yes (Leandro *et al.*, 2014)	Macroscopic examination of brain(Matos-Silva et al., 2012) Histological studies (Matos-Silva *et al.*, 2012) Electrophysiological recordings (Robinson *et al.*, 2012) Gene manipulation(Robinson *et al.*, 2012)
Mouse	*M. corti*	In vivo	Early stage cysticerci	No	Yes (Alvarez and Teale, 2007, 2006; Cardona *et al.*, 2003, 1999; Cardona and Teale, 2002; Gundra *et al.*, 2011; Mishra *et al.*, 2011)	Yes (Alvarez and Teale, 2007, 2006; Cardona *et al.*, 2003, 1999; Cardona and Teale, 2002; Gundra *et al.*, 2011; Mishra *et al.*, 2011)	Not yet tested	Yes (Cardona and Teale, 2002)	Yes (Cardona *et al.*, 1999)	Not yet tested	Cellular/molecular examination of blood/tissue (Cardona *et al.*, 2003; Cardona and Teale, 2002; Gundra *et al.*, 2011; Mishra *et al.*, 2011) BBB permeability (Alvarez and Teale, 2007, 2006; Cardona *et al.*, 1999) Histological studies (Alvarez and Teale, 2007, 2006; Cardona *et al.*, 2003, 1999; Cardona and Teale, 2002; Gundra *et al.*, 2011; Mishra *et al.*, 2011)

1.4 SEIZURES AND EPILEPSY: A CELLULAR UNDERSTANDING

According to the International League Against Epilepsy, an epileptic seizure is defined as: "a transient occurrence of signs and/or symptoms due to abnormal, excessive or synchronous neuronal activity in the brain"(International League Against Epilepsy, 2014). To understand and investigate seizures secondary to NCC, one needs an understanding of the cells (such as neurons) that make up the human nervous system, and how they communicate.

The human nervous system consists of two main cell types: neurons, and glia. Neurons form the most extensive network in the brain and allow for precise and rapid spread of information throughout the human body. Glial cells facilitate and regulate neuronal signaling and are also responsible for immune surveillance of the nervous system (Kandel *et al.*, 2000).

1.4.1 Neuronal signaling: excitatory or inhibitory

Neurons are cells that can respond to both chemical and electrical signals. At rest, there exists an electrical potential difference (voltage) across the membrane of neurons, largely due to differences in the concentrations of ions, mainly sodium (Na^+), potassium (K^+), calcium (Ca^{2+}) and chloride (Cl^-), inside and outside the cells (Kandel *et al.*, 2000). The difference in ion concentration and voltage is created and maintained by several transmembrane ion-transporting proteins in neuronal membranes. The inside surface of the cell membrane is charged negatively relative to the outside (ranging between -55 and -85 mV) (Ackermann and Moshe, 2010).

Neurons form extremely intricate and complex networks, with each neuron making thousands of connections ("synapses") with other neurons (Bear *et al.*, 2016). Upon chemical signaling in the presynaptic cell resulting in the release of neurotransmitters into the synaptic cleft, membrane protein complexes (receptors) on the postsynaptic cell membrane are bound and undergo structural changes that allow particular ions to flow into or out of the neuron, altering the potential difference across the membrane. The movement of ions can have either a "hyperpolarizing" effect, in which the membrane potential is decreased (the inside of the cell becomes more negatively charged) or a "depolarizing" effect, in which the membrane potential difference is increased (the inside of the cell becomes less negatively charged). This depends on the type and charge of the ions that particular receptors allow to pass through the membrane (Ackermann and Moshe, 2010).

If the membrane potential is increased above a cell's "threshold" value (approximately -40mV), usually thanks to a succession of cumulative depolarizing signals, it results in the opening of voltage sensitive ("voltage-gated") ion channels. This results in ionic fluxes that cause a massive depolarization, or increase, of the membrane potential, followed rapidly by a

repolarisation/hyperpolarisation (see Fig. 1.5). This biphasic ionic flux is termed an action potential. It is also said that a neuron “spikes” or “fires” to describe when it generates an action potential. The action potential propagates along the cell membrane (via the opening of adjacent voltage-gated ion channels), resulting in a change in the membrane potential of cells to which the neuron is connected, usually through the release of chemical neurotransmitters into the synaptic space, which bind to receptors on the postsynaptic cell (Ackermann and Moshe, 2010) , repeating the process of de- or hyper-polarization.

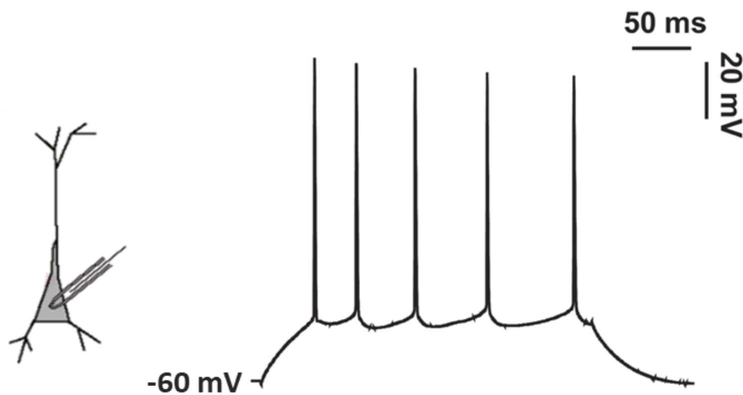


Figure 1.5: A whole-cell recording from a hippocampal pyramidal neuron illustrating a series of five action potentials (McKiernan and Marrone, 2017).

The change in membrane potential induced in the postsynaptic cell can either be depolarising, in which case it is termed “excitatory” as it increases the likelihood of an action potential being generated, or it can be hyperpolarising, in which case it is termed “inhibitory” as it decreases the likelihood of an action potential being generated (Ackermann and Moshe, 2010).

Humans are capable of many varied and nuanced behaviours. Underlying these behaviours are highly specific neuronal firing patterns, which are finely controlled by a dynamic combination of neuronal inhibition and excitation (Bear *et al.*, 2016). Seizures, characterised by “abnormal, excessive or synchronous neuronal activity” (see Fig. 1.6) come about when the balance between excitation and inhibition gets tipped towards excitation (Ackermann and Moshe, 2010).

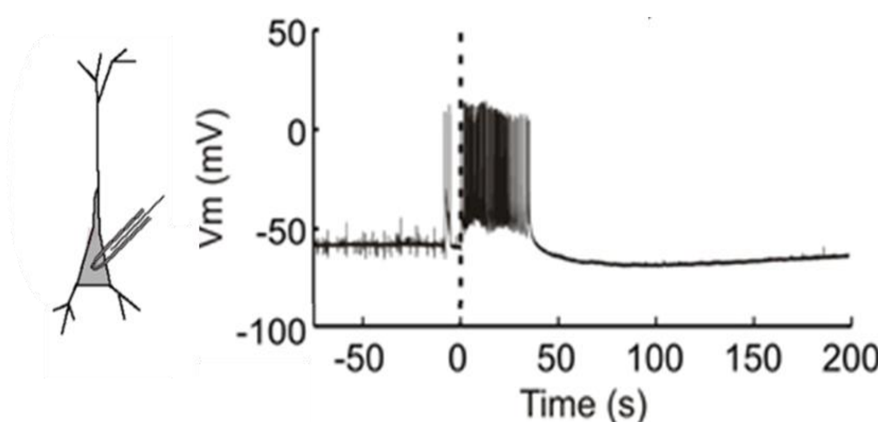


Figure 1.6: A whole cell recording from a hippocampal pyramidal neuron illustrating a series of seizure-like events characterised by rapid, excessive firing of the cell (the start of each event is indicated by a dashed vertical line) (Raimondo *et al.*, 2012).

1.4.2 Neuronal sub-types and the excitation-inhibition balance.

Neurons typically only release one type of neurotransmitter, and as such neurons are often categorised according to the neurotransmitter that they release. There are two cell types that are the primary players in the excitation-inhibition interplay: excitatory pyramidal neurons which release glutamate; and inhibitory interneurons which release γ-aminobutyric acid (GABA) (Bear *et al.*, 2016). Excitatory neurons drive the propagation of signals through neuronal networks, whilst inhibitory neurons counteract the depolarising effects of excitatory neurons, help to coordinate signaling into the necessary patterns, and prevent "runaway" excitation leading to seizures (Ackermann and Moshe, 2010). It is important to note, however, that hundreds of neuronal cell types exist that all contribute to the modulation and control of neuronal signaling (Kandel *et al.*, 2000). Acetylcholinergic neurons, for instance, release acetylcholine, which acts as a neuromodulatory neurotransmitter in the central nervous system. This means that it modifies the response of neuronal networks throughout the brain to internal and external inputs. It does so by altering neuronal excitability, influencing synaptic transmission and plasticity, and even coordinating the firing of groups of neurons. Acetylcholine can induce either excitation or inhibition of neuronal networks, depending on the region of the brain and the cell types it acts upon (Picciotto *et al.*, 2012).

1.4.3 The role of glial cells in neurotransmission

Glial cells, namely astrocytes, microglia, ependymal cells and oligodendrocytes, make up roughly half of all the cells in the human brain (von Bartheld *et al.*, 2016). Glial cells have long been described as cells that primarily "support" the functioning of neurons. While the functions of ependymal cells (lining the fluid-filled ventricles) and oligodendrocytes (myelinating neuronal axons) are well understood in the adult human brain, the roles of astrocytes and microglia appear to be more varied and complex (Bear *et al.*, 2016).

Astrocytes are the most numerous glial cell type, and have been shown to regulate extracellular ion concentration, water homeostasis and the acid-base balance in the brain, all of which are essential to the proper functioning of neurons (Nagelhus *et al.*, 2013). They are further responsible for the majority of the uptake (from synapses), degradation and resupply of glutamate, which is essential to ongoing neurotransmission (Coulter and Eid, 2012). In more recent years it has been described that astrocytic processes regularly associate closely with neuronal synapses and may prevent off-target effects of neurotransmitters (by ensheathing synapses) as well as actively modulate neurotransmission by responding to and releasing (neuro/glio)transmitters (Nagelhus *et al.*, 2013).

Microglia are another major glial cell type, and are considered the resident macrophages of the central nervous system, as they detect and remove cellular debris from dead or dying brain

cells (Bear *et al.*, 2016). They have also been described to be responsible for the removal of excess synapses during brain development and the removal of synapses from damaged neurons in the adult brain (Bar and Barak, 2019). In recent years, research has emerged which further suggests that microglia may be major players in synaptic plasticity, as they appear to periodically contact neuronal synapses with their highly motile, ramified processes during normal function, and this contact seems to reduce/restrain neuronal activity (Wu *et al.*, 2015).

1.4.4 The activation of astrocytes and microglia can disrupt the excitation-inhibition balance

Beyond the functions described above, both astrocytes and microglia play a major role in the innate immune response to infection or injury in the central nervous system (Aronica *et al.*, 2012; Yang *et al.*, 2010). In recent years, it has become increasingly clear that epileptogenesis is associated with strong and persistent neuroinflammation (Rana and Musto, 2018). The "activation" of astrocytes and microglia in response to infection, injury or neuronal degeneration, involves major structural and chemical changes, which can impair their ability to regulate and modulate neurotransmission in the ways mentioned above (Devinsky *et al.*, 2013). Indeed, impaired or altered ion homeostasis (particularly potassium), water homeostasis, glutamate uptake and degradation, and neurotransmitter release by glial cells have all been reported by epilepsy studies (Devinsky *et al.*, 2013).

In addition to a disruption of their normal roles in the facilitation and regulation of neurotransmission, when activated, astrocytes and microglia release large amounts of immune signaling molecules. Amongst these are the pro-inflammatory cytokines interleukin-1β (IL-1β), interleukin-6 (IL-6) and tumour necrosis factor- α (TNF-α), the expression and production of which have been shown to be elevated in clinical and experimental epilepsy studies (Rana and Musto, 2018). Interleukin-1β and TNF-α have both been shown to bind to receptors on excitatory pyramidal neurons and to alter the expression of glutamate and GABA receptors in such a way that makes the neurons more excitable (Rana and Musto, 2018). Further, IL-1β enhances glutamate release and reduces glutamate re-uptake by astrocytes, whilst TNF-α increases glutamate release by microglia (Rana and Musto, 2018). This results in increased glutamate availability in neuronal synapses which makes neurons hyperexcitable. The effect of IL-6 on neuronal excitability seems to be somewhat more complex, with some studies reporting that increased levels of IL-6 have pro-convulsive effects both functionally and molecularly, whilst other studies show IL-6 to be protective against neuronal hyperexcitability on both levels (Rana and Musto, 2018; Vezzani and Viviani, 2015a).

Interleukin-10 has also been found to be elevated in the CSF of patients with epilepsy (de Vries *et al.*, 2016). Interleukin-10 is a regulatory, anti-inflammatory cytokine that can be

produced by both microglia and astrocytes and has been reported to be able to attenuate inflammation-associated tissue damage in response to infection (Couper *et al.*, 2008; Helmut *et al.*, 2011; Wetherington *et al.*, 2008). It has been proposed that IL-10 may be elevated in epilepsy as a mechanism by which to counteract and regulate inflammatory responses (de Vries *et al.*, 2016).

Intriguingly, NCC studies have reported the presence of activated astrocytes and microglia surrounding cysts, as well as the presence of IL-1β, IL-6, TNF-α and IL-10, amongst other cytokines (Alvarez *et al.*, 2002b, 2002a; Fleury *et al.*, 2016; Londoño *et al.*, 2002; Restrepo *et al.*, 2001, 1998; Singh *et al.*, 2013). Together, it seems highly likely that the responses of microglia and astrocytes to *T. solium* larvae play an important role in seizurogenesis in NCC.

1.5 ADDRESSING GAPS IN THE CURRENT UNDERSTANDING OF SEIZURES IN NEUROCYSTICERCOSIS

Despite the extensive existing research on brain and immune responses to *T. solium* larval cysts, some clear gaps in our collective understanding of NCC became apparent upon reviewing the literature. Most strikingly, the aetiology of seizures in NCC remains virtually unknown. I therefore set out to investigate several avenues by which *Taenia* larvae could ostensibly exert an effect on brain excitability. This included exploring the how the larvae may directly affect neurons/neuronal networks (Aims 1 & 2), whether the larvae produce enzymes (AChEs) that could act on a neurotransmitter and thereby indirectly affect neuronal excitability (Aims 3), and, finally, whether larvae could affect neuronal excitability by acting on the immunocompetent cells of the brain and altering their production of inflammatory cytokines which, in turn, alter neuronal excitability (Aims 4 & 5).

1.6 AIMS

Aim 1: Explore the effects of an acute exposure to *Taenia* larval extracts on neuronal excitability.

Aim 2: Investigate the effects of a longer exposure to *Taenia* excretory/secretory extracts on neuronal network excitability.

Aim 3: Investigate and characterise the production of acetylcholinesterases by *Taenia* larvae.

Aim 4: Investigate the potential of *T. crassiceps* larval extracts to induce the release of pro-inflammatory cytokines in brain slices.

Aim 5: Investigate the potential of *T. crassiceps* larval extracts to suppress the release of pro-inflammatory cytokines in brain slices.

1.7 OBJECTIVES

1.7.1 Aim 1: Explore the effects of an acute exposure to *Taenia* larval extracts on neuronal excitability (Chapter 3).

Objective 1: Determine what effect *T. crassiceps* larval extracts have on neuronal excitability.

Objective 2: Establish whether this effect is mediated by glutamate receptors.

Objective 3: Determine the glutamate content in larval extracts of *T. crassiceps* and *T. solium*.

1.7.2 Aim 2: Investigate the effects of a longer exposure to *Taenia* larvae on neuronal network excitability (Chapter 3).

Objective 1: Establish a novel *T. crassiceps* and hippocampal organotypic brain slice co-culture model.

Objective 2: Determine what effect co-culture with *T. crassiceps* larvae has on brain slice neuronal network excitability.

1.7.3 Aim 3: Investigate and characterise the production of acetylcholinesterases by *Taenia* larvae (Chapter 4).

Objective 1: Determine the acetylcholinesterase activity of *T. crassiceps* and *T. solium* larval extracts using Ellman's assays.

Objective 2: Confirm the presence of acetylcholinesterase activity in larval extracts of *T. crassiceps* and *T. solium* using non-denaturing polyacrylamide gel electrophoresis and acetylcholinesterase activity staining.

Objective 3: Characterise the sensitivity of *T. crassiceps* and *T. solium* larval acetylcholinesterases to various cholinesterase inhibitors.

Objective 4: Localise acetylcholinesterases in cross sections and whole mounts of *T. crassiceps* and *T. solium* larvae.

Objective 5: Determine whether the acetylcholinesterase activity of *Taenia* extracts is sufficient to modify neuronal acetylcholine signaling.

1.7.4 Aim 4: Investigate the potential of *T. crassiceps* larval extracts to induce the release of pro-inflammatory cytokines in brain slices (Chapter 5).

Objective 1: Establish baseline release, and the release following application of known immunogenic agents, of pro-inflammatory cytokines by hippocampal organotypic brain slices.

Objective 2: Establish the endotoxin and host cytokine content of *T. crassiceps* larval extracts.

Objective 3: Explore the release of pro-inflammatory cytokine by hippocampal organotypic brain slices in response to larval extracts of viable, dead, and dimethyl sulfoxide solubilised larvae, as well as larval granulomas.

1.7.5 Aim 5: Investigate the potential of *T. crassiceps* larval extracts to suppress the release of pro-inflammatory cytokines by brain slices (Chapter 5).

Objective 1: Explore whether the extracts of viable *T. crassiceps* larvae attenuate cytokine release by hippocampal organotypic brain slices following exposure to several different immunogenic agents.

Objective 2: Determine the involvement of lipid, glycan, and protein components in the anti-inflammatory action of *T. crassiceps* larval extracts.

Chapter 2

MATERIALS AND METHODS

All reagents used were supplied by Sigma-Aldrich, unless stated otherwise.

All animal handling, care and procedures followed the South African National Standard (South African Bureau of Standards, 2008) and were authorised by the University of Cape Town Animal Ethics Committee (Protocols: AEC 015/015, AEC 019/025, AEC 014/035, AEC 018/010). Animal rooms were maintained between 22 - 24 °C, with a lux of 100 - 300 and food and water were available *ad libitum*.

2.1 *TAENIA* MAINTENANCE AND PREPARATION OF LARVAL EXTRACTS

2.1.1 Acquisition and maintenance of *Taenia crassiceps* larvae

Larvae (ORF strain), donated by Dr Siddhartha Mahanty (NIH, Maryland USA), were *in vivo* propagated by serial intraperitoneal infection of 5 - 8-week-old female C57BL/6 mice. Briefly, 20 small, translucent, motile larvae were selected in phosphate buffered saline (PBS; 1X, pH 7.4; Whitehead Scientific) into a 1 ml syringe and injected into a mouse peritoneum through a 20-gauge needle (B&M Scientific). After 12 weeks, during which time the welfare of the mice was monitored daily and their weight was checked weekly, mice were euthanised using an overdose of halothane followed by cervical dislocation and larvae were harvested by peritoneal lavage and washed a further 6 times in PBS (1X, pH 7.4). A small number of these larvae were immediately used to infect a new set of 8 mice, whilst the remaining larvae were processed and used for downstream experiments.

PREPARATION OF LARVAL EXTRACTS

In this thesis, a large variety of *Taenia* extracts were used. For ease of reference and understanding, the preparation and utility of each of these has been summarised in **Table 2.1** and **Table 2.2**, after which follows a detailed description of how each extract was prepared.

Table 2.1: A summary of the preparation and use of the different Taenia somatic cyst extracts utilised in this thesis

EXTRACT	PREPARATION AND USE
Whole cyst homogenate (homogenate)	This extract was prepared from viable larvae by homogenising whole larvae, and it encompassed the cyst vesicular fluid and cyst membrane (and scolex, for *Taenia solium*). This extract mimics the vesicular life stage of *Taenia* cysts. It is used throughout the thesis (chapters 3, 4 & 5).
Dialysed *Taenia crassiceps* whole cyst homogenate	This extract consists of whole cyst homogenate that has been dialysed such that all molecules < 3 kDa are removed. This extract was prepared to help narrow down whether the electrophysiological effects produced by the whole cyst homogenate was mediated by a molecule > 3 kDa or < 3 kDa (chapter 3).
Cyst vesicular fluid	This extract consists of fluid from within viable *Taenia* larvae. This extract was used to assist in the localisation of acetylcholinesterases in *Taenia* larvae (chapter 4) and to assess whether the cyst vesicular fluid or the cyst membrane and scolex (described next) had different effects on the release of cytokines by hippocampal organotypic cultures (chapter 5).
Cyst membrane (and scolex)	This extract was prepared by taking the parts of the cyst that were left after the removal of the cyst vesicular fluid and homogenising them. This extract was used to assist in the localisation of acetylcholinesterases in *Taenia* larvae (chapter 4) and to assess whether the cyst vesicular fluid or the cyst membrane and scolex (described next) had different effects on the release of cytokines by hippocampal organotypic cultures (chapter 5).
Praziquantel treated *T. crassiceps* whole cyst homogenate	This extract was prepared by treating *Taenia* crassiceps larvae in culture with the anthelminthic drug "praziquantel" to kill them, and then preparing a homogenate from the dead larvae. This extract was intended to model transitional stage *Taenia* cysts killed by anthelminthic drugs. The effect of this extract on the release of cytokines by hippocampal organotypic cultures was assessed (chapter 5).
"Starved" *T. crassiceps* whole cyst homogenate	This extract was prepared by leaving *Taenia* larvae in the same medium in culture until they lost motility and shrank, and then preparing a homogenate from the dead larvae. This extract was intended to model transitional stage *Taenia* cysts that had died without anthelminthic treatment. The effect of this extract on the release of cytokines by hippocampal organotypic cultures was assessed (chapter 5).
DMSO *T. crassiceps* whole cyst homogenate	This extract was prepared by adding 2% dimethyl sulfoxide to the pellets that remained after the production of whole cyst homogenate in an attempt to solubilise more elements of the cyst. The effect of this extract on the release of cytokines by hippocampal organotypic cultures was assessed (chapter 5).
Granuloma	This extract was prepared by isolating high density, solid, yellow, cysts from larvae harvested from mouse peritonea and homogenising these. This extract was intended to model calcified *Taenia* cysts. The effect of this extract on the release of cytokines by hippocampal organotypic cultures was assessed (chapter 5).

Periodate treated *T. crassiceps* whole cyst homogenate.	To produce this extract, whole cyst homogenate was treated with sodium metaperiodate, which disrupts glycan moieties. This extract was used to determine whether glycans were involved in the anti-inflammatory effects of *T. crassiceps* whole cyst homogenate observed in a hippocampal organotypic brain slice model of inflammation (chapter 5).
Mock periodate treated *T. crassiceps* whole cyst homogenate.	This extract was subjected to the same protocol as the periodate treated whole cyst homogenate, with the exception that sodium metaperiodate was replaced with acetate buffer for this preparation. This extract served as a control for the periodate treated whole cyst homogenate in experiments performed in a hippocampal organotypic brain slice model of inflammation (chapter 5).
Ammonium sulphate treated *T. crassiceps* whole cyst homogenate	To produce this extract, whole cyst homogenate was subjected to an 80% ammonium sulfate protein precipitation protocol to bulk precipitate proteins and remove lipids. This extract was used to determine whether lipids were involved in the anti-inflammatory effects of *T. crassiceps* whole cyst homogenate observed in a hippocampal organotypic brain slice model of inflammation (chapter 5).
Heat inactivated *T. crassiceps* whole cyst homogenate.	To produce this extract, whole cyst homogenate was heated to 100 °C for 30 min to inactivate proteins. This extract was used to determine whether proteins were involved in the anti-inflammatory effects of *T. crassiceps* whole cyst homogenate observed in a hippocampal organotypic brain slice model of inflammation (chapter 5).

Table 2.2: A summary of the preparation and use of the different Taenia excretory/secretory extracts utilised in this thesis

EXTRACT	PREPARATION AND USE
Total excretory/secretory extracts	This extract was prepared by culturing larvae for up to 28 days and collecting and pooling the media they were cultured in. This extract allows for the study of substances that the larvae produce but that may not be present in the whole cyst homogenate. This extract was used to assess the effect of *Taenia* excretory/secretory proteins on neuronal activity (chapter 3).
Excretory/secretory extracts > 3 kDa and excretory/secretory extracts < 3 kDa.	These extracts consist of the excretory/secretory extracts that have been separated into a fraction containing molecules > 3 kDa and a fraction containing molecules < 3 kDa using a molecular weight cut-off filter. These extracts were prepared to help narrow down whether electrophysiological effects produced by the whole cyst homogenate were mediated by a molecule > 3 kDa or < 3 kDa (chapter 3). The > 3 kDa fraction was also utilised to assess the ability of Taenia acetylcholinesterases to digest acetylcholine and disrupt neuronal signalling (chapter 4)
Daily excretory/secretory extracts	These extracts were prepared by culturing larvae for a period of 6 days and collecting the media every day. These extracts were used to determine the amount of glutamate released by *Taenia* larvae over a 6-day period (chapter 3).
Concentrated excretory/secretory extracts	This extract was prepared by passing total excretory/secretory extracts through a 3 kDa molecular weight cut-off filter to concentrate the solution. It was utilised to investigate the release of acetylcholinesterases by *Taenia* larvae (chapter 4), as well as the effect of *Taenia* excretory/secretory products on cytokine production by hippocampal organotypic brain slices (chapter 5).
Excretory/secretory extracts for cytokine content determination	These extracts are essentially the same as the one described above, with the exception that the medium in which *Taenia* larvae were cultured was collected every 2-3 days for 10 days and each collection was concentrated using a 3 kDa molecular weight cut-off filter. These extracts were made to determine the quantity of cytokines released by *Taenia* larvae in culture over several days (chapter 5).
Praziquantel treated *T. crassiceps* excretory/secretory extracts	This extract was prepared by treating *Taenia* crassiceps larvae in culture with the anthelminthic drug "praziquantel" to kill them, and then collecting the media in which the cysts had died. This extract was intended to model the products released by transitional stage *Taenia* cysts killed by anthelminthic drugs. The effect of this extract on the release of cytokines by hippocampal organotypic cultures was assessed (chapter 5).
"Starved" *T. crassiceps* whole cyst homogenate	This extract was prepared by leaving *Taenia* larvae in the same medium in culture until they lost motility and shrank, and then preparing a homogenate from the dead larvae. This extract was intended to model transitional stage *Taenia* cysts that had died without anthelminthic treatment. The effect of this extract on the release of cytokines by hippocampal organotypic cultures was assessed (chapter 5).

2.1.2 Preparation of *Taenia crassiceps* whole cyst homogenate

Taenia crassiceps whole cyst homogenate. The whole cyst homogenate was often prepared by Hayley Tomes, and only at a later stage by me. Directly after harvest larvae were frozen at -80 °C. Upon thawing, larvae were suspended in a volume of PBS (1X, pH 7.4) threefold that of the larvae. A protease inhibitor cocktail was added to this suspension (1% vol/vol). The larvae were then homogenised on ice using either an electronic tissue homogeniser (Polytron, PT 2500E, Kinematica) or a 40 ml manual glass tissue grinder (Dounce, Sigma-Aldrich). The resulting mixture was centrifuged at 3100 *g* for 20 minutes at 4 °C (Eppendorf, 5810 R). The liquid supernatant (excluding the low density white floating layer) was collected and sterile filtered through a 0.22 µm size filter (Millex-GV syringe filter, Merck). This supernatant was then aliquoted and stored at -80 °C until use.

2.1.3 Preparation of *Taenia crassiceps* cyst membrane and cyst vesicular fluid extracts

After harvesting, washed larvae (+/- 10 ml) were placed onto a piece of filter paper (saturated with PBS, 1X, pH 7.4) in a metal sieve. Cysts were then ruptured using a weighing spatula. The fluid from the ruptured cysts that passed through the filter paper was collected in a beaker and centrifuged at 3100 *g* for 20 min at 4 °C. The supernatant was aliquoted and stored at -80 °C until use. This extract is referred to as "*T. crassiceps* cyst vesicular fluid". The parts of the cysts that remained on the filter paper were scraped off with a weighing spatula and suspended in an equal volume of PBS (1X, pH 7.4) containing a protease inhibitor cocktail (1% vol/vol). This mixture was freeze-thawed once (-80 °C) to lyse the cells and then homogenised on ice using a glass tissue grinder, followed by being centrifuged at 3100 *g* for 20 minutes at 4 °C. The liquid supernatant was collected, aliquoted, and stored at -80 °C until use. This extract is referred to as "*T. crassiceps* cyst membrane".

2.1.4 Preparation of *Taenia crassiceps* excretory/secretory extracts

Total excretory/secretory extracts. After harvesting, washed larvae (+/- 15 ml) were placed in a 50 ml culture flask with 50 ml *T. crassiceps* culture medium (Earle's Balanced Salt Solution with glucose (5.3 g/L), Glutamax (1X), penicillin (50 U/ml), streptomycin (50 µg/ml), gentamicin sulphate (100 µg/ml) and nystatin (11.4 U/ml)). Larvae were maintained at 37 °C in 5 % CO_2. After 24 hours the media was discarded and replaced with fresh media. Thereafter media was collected (and temporarily stored at -20 °C) and replaced every 2-5 days for up to 28 days. At the end of each culture period the media from all collections were thawed and pooled. This pooled solution was utilised as "*T. crassiceps* excretory/secretory extracts" for the electrophysiological experiments.

Excretory/secretory extracts > 3 kDa*, excretory/secretory extracts* < 3 kDa*, and excretory/secretory extracts for cytokine content determination.* To separate and concentrate larger molecules (such as proteins) in the excretory/secretory extracts, a portion of the "Total excretory/secretory extracts" was separated using an Amicon stirred cell with a 3 kDa molecular weight cut-off membrane (Merck). The cell was pressurised with gaseous nitrogen until 99% of the volume had passed through the membrane. The fluid that passed through the membrane was collected and is referred to as "excretory/secretory extracts < 3 KDa". The remaining volume was buffer exchanged with PBS (1X, pH 7.4) in the stirred cell. This was then utilised as "excretory/secretory extracts > 3 kDa". A similar protocol was followed to concentrate excretory/secretory extracts for the assessment of cytokine production by *T. crassiceps* larvae over a 10-day period *in vitro*, with the exception that the collected media was not pooled, but rather each concentrated separately in 2–3-day batches using the Amicon stirred cell. This was done using two separate harvests of *T. crassiceps* larvae.

Daily excretory/secretory extracts (for use in glutamate production determination). After harvesting, washed larvae were placed into 6 well plates (+/- 15 per well, of roughly 5 mm length) with 2 ml *T. crassiceps* culture medium. Every 24 hours 1 ml of culture medium was collected from each well and replaced with fresh culture medium. Media from each well was aliquoted separately and stored at -80 °C.

Concentrated excretory/secretory extracts. After harvesting, washed larvae (+/- 10ml) were placed in a 50 ml culture flask with 10 ml *T. crassiceps* culture medium. Larvae were maintained at 37 °C in 5 % CO_2. After 48 hours the media was discarded and replaced with 10 ml fresh media. No further media was added for the duration of the experiment. At 20 days *in vitro* the culture media was collected, aliquoted, and stored at -80 °C until use.

2.1.5 Preparation of additional *Taenia crassiceps* extracts*.*

Dialysed Taenia crassiceps whole cyst homogenate (Performed by Dr Hayley Tomes). A portion of the whole cyst homogenate was dialysed using a Slide-A-Lyzer™ dialysis cassette (3 kDa MWCO, Separations) in 2l of standard artificial cerebro-spinal fluid (aCSF) at 4° C (see below for composition) to assess the effect of the large versus small molecules on neuronal excitation. The aCSF solution was changed twice over 24 hours. A Cobas 6000 analyser (Roche) was used to determine the ionic composition of the whole cyst homogenate, with ion specific electrodes for K^+ and Na^+. The pH of the homogenate was measured using a Mettler Toledo SevenCompact™ pH meter (S210, Merck).

Praziquantel treated T. crassiceps whole cyst homogenate and excretory/secretory extracts. T. crassiceps larvae (15 ml) were incubated at 37 °C in 5 % CO_2 in 50 ml PBS (1X, pH 7.4) containing 0.2 µg/ml (Palomares *et al.*, 2004) of the anthelminthic drug praziquantel for a

period of 1 week (by which point the larvae had lost all motility). The PBS was then collected, aliquoted and frozen at -80 °C. This was utilised as "Praziquantel excretory/secretory extracts" (PQ excretory/secretory extracts). The larvae were collected and whole cyst homogenate (PQ homogenate) was prepared as described above.

"Starved" T. crassiceps whole cyst homogenate and excretory/secretory extracts. T. crassiceps larvae (15 ml) were incubated at 37 °C in 5 % CO_2 in 50 ml *T. crassiceps* culture medium for a period of 3 weeks (by which point larvae have shrivelled up and lost all motility). The medium was then collected, aliquoted and frozen at -80 °C. This was then utilised as "starved excretory/secretory extracts", whilst the larvae, used to generate whole cyst homogenate, was utilised as "starved whole cyst homogenate" (Starved homogenate).

DMSO T. crassiceps whole cyst homogenate. Viable *T. crassiceps* larvae (7.5 ml) were processed as described for "whole cyst homogenate" above. After the supernatant was collected, 5 ml of PBS (1X, pH 7.4) containing dimethyl sulfoxide (DMSO, 2 % vol/vol) and protease inhibitor cocktail (1 % vol/vol) was added to the remaining solid pellet and the low density white floating layer, and the mixture was vortexed for 2 min. Thereafter, it was re-homogenised in a 40 ml manual glass tissue grinder (Dounce, Sigma-Aldrich) followed by being sonicated at 100 % power for 3 x 20 sec on ice (Soniprep 150, MSE centrifuges). The solution was then centrifuged at 3100 *g* for 20 min at 4 °C (Eppendorf, 5810 R). The liquid supernatant (excluding the, now smaller, solid pellet and low density white floating layer) was collected and sterile filtered through a 0.22 µm filter (Millex-GV syringe filter, Merck). This "DMSO homogenate" (DMSO homogenate) was then aliquoted and stored at -80 °C until use.

Granuloma. The high density, solid, yellowish cysts (late-stage granulomas) were separated from viable larvae upon harvesting of *T. crassiceps*, and were stored at -80 °C. After several harvest rounds, these granulomas were combined and were subjected to the same protocol as described for the "whole cyst homogenate" above. This collected liquid supernatant was aliquoted and frozen is referred to as the "Granuloma" *T. crassiceps* extract.

Periodate treated and mock periodate treated T. crassiceps whole cyst homogenate. Sodium metaperiodate-mediated modification of glycan moieties in homogenate was performed by using a modification of the technique of Tawill *et al.* [2004]. Briefly, 12 ml of a 0.05 M sodium acetate buffer (100 mM, pH 4.5) was added to 12 ml *T. crassiceps* homogenate, which had a concentration of 1.7 mg protein/ml (calculated using a BCA assay from Sigma Aldrich – see **section 2.1.10**). The mixture was gently swirled for 1 min and then allowed to stand for 5 min at room temperature before another 1 min of gentle swirling. This solution was then divided into two equal volumes, one with which to produce periodate treated homogenate (P homogenate), and the other with which to produce a control mock periodate treated

homogenate (MP homogenate). To produce the P homogenate, 240 µl of a 0.5 M stock solution of sodium metaperiodate in acetate buffer was added to 12 ml of the homogenate-acetate buffer mixture to produce a final concentration of 10 mM sodium metaperiodate. For the MP homogenate 240 µl of acetate buffer was substituted for 0.5 M the sodium metaperiodate stock. The solutions were incubated in the dark at room temperature with gentle shaking for 1 h. The reaction was completed by further incubation of each tube with 100 mM sodium borohydride (22.7 µl of 100% sodium borohydride was added to each solution) for 30 min at room temperature. Excess salts were removed, and solutions were buffer exchanged with PBS (1X, pH 7.4) using Millipore Amicon-Ultra 0.5 centrifugal filters (10K MWCO; Merck/Sigma-Aldrich), as per manufacturer instructions. The solutions were sterile filtered through a 0.22 µm filter (Millex-GV syringe filter, Merck/Sigma-Aldrich) and the protein concentrations of each were determined using a BCA assay (Sigma-Aldrich).

Ammonium sulphate treated Taenia crassiceps whole cyst homogenate. To bulk precipitate proteins, 1.7 mg/ml whole cyst homogenate (6 ml) was gradually saturated with ammonium sulfate up to a concentration of 80% on ice, with thorough mixing between additions of ammonium sulfate. The precipitated proteins were obtained by centrifugation (3000 g, 30 min), resuspended in 1000 µl PBS (1X, pH 7.4) and de-salted using Millipore Amicon-Ultra 0.5 centrifugal filters (10K MWCO; Merck/Sigma-Aldrich), as per manufacturer instructions. The solutions were sterile filtered through a 0.22 µm filter (Millex-GV syringe filter, Merck/Sigma-Aldrich) and the final protein concentrations were determined as described in **section 2.1.10**.

Heat inactivated Taenia crassiceps whole cyst homogenate. Heat inactivation of the protein components of the homogenate was achieved by heating 1.7 mg/ml of homogenate (2 ml) to 100 °C using a digital dry bath (Accublock^{TM} digital dry bath, Labnet International), for a 30 min period. The solution was then sterile filtered through a 0.22 µm filter (Millex-GV syringe filter, Merck/Sigma-Aldrich).

2.1.6 Acquisition of *Taenia solium* larvae

Larvae of *T. solium* were harvested from the muscles of a heavily infected, freshly slaughtered pig in Lusaka, Zambia, by Fabien Prodjinotho and Clarissa Prazeres da Costa (Technical University of Munich (TUM), Munich, Germany) in collaboration with the research group of Chummy Sikasunge (University of Zambia, Lusaka, Zambia). Larvae were obtained from pig muscles by vigorous shaking of pieces of skeletal muscle until cysts fell out into a collecting tray. Cysts were then transferred to petri dishes containing sterile PBS (1X, pH 7.4). Preparation of all *T. solium* extracts was performed by Fabien Prodjinotho at the University of Zambia and stored in a liquid nitrogen tank. Larvae and larval extracts were transported to

The Technical University of Munich in a dry shipper and stored either in liquid Nitrogen or at -80°C.

2.1.7 Preparation of *Taenia solium* whole cyst homogenate

After extensive washing with sterile PBS (1X, pH 7.4), fresh larvae were suspended in a volume of PBS threefold that of the larvae, containing phenylmethyl-sulphonyl fluoride (5 mM) and leupeptin (2.5 µM). Larvae were then homogenised using a sterile handheld homogenizer (T10 Basic Ultra-Turrax, IKA-Werke) at 4 °C. The resulting homogenate was sonicated (4 x 60 s at 20 kHz, 1 mA, with 30 s intervals; Sonopuls HD 2070.2, Bandelin Electronic), gently stirred with a magnetic stirrer (2 h at 4 °C; Corning PC-420D, Corning), and centrifuged (15 000 *g* for 60 min at 4 °C; Sigma 3-16KL Refrigerated centrifuge, SciQuip Ltd). The liquid supernatant (excluding the low density white floating layer) was collected and sterile filtered through 0.45 µm filters (Millex-GV syringe filter, Merck). This supernatant was then aliquoted and stored at -80 °C until use.

2.1.8 Preparation of *Taenia solium* cyst vesicular fluid, cyst membrane and scolex extracts

Cyst vesicular fluid. After extensive washing with sterile PBS (1X, pH 7.4) larvae were placed in a petri dish and individually ruptured with a sterile needle. The resulting fluid in the petri dish was collected and centrifuged (15 000 *g* for 60 min at 4 °C). The supernatant was then sonicated (4 x 60 s at 20 kHz, 1 mA, with 30 s intervals), phenylmethyl-sulphonyl fluoride (5 mM) and leupeptin (2.5 µM) were added, and the solution was centrifuged a second time (15 000 *g* for 60 min at 4 °C). The supernatant was aliquoted and stored at -80 °C until use.

Cyst membrane and scolex. The remaining parts of the larvae were again extensively washed with PBS (1X, pH 7.4) and then suspended in an equal volume of PBS, containing phenylmethyl-sulphonyl fluoride (5 mM) and leupeptin (2.5 µM). This suspension was then homogenised using a sterile handheld homogenizer at 4 °C. The resulting homogenate was sonicated (4 x 60 s at 20 kHz, 1 mA, with 30 s intervals), gently stirred with a magnetic stirrer (2 h at 4 °C), and centrifuged (15,000 g for 60 min at 4 °C). The liquid supernatant (excluding the low density white floating layer) was collected and sterile filtered through a 0.45 µm filter (Millex-GV syringe filter, Merck). This supernatant was then aliquoted and stored at -80 °C until use.

2.1.9 Preparation of *Taenia solium* excretory/secretory extracts

After harvesting, washed larvae were placed into 6 well plates (+/- 15 per well, of roughly 5 mm length) with 2 ml *T. solium* culture medium (RPMI 1640 with 10 mM HEPES buffer, 100 U/ml penicillin, 100 µg/ml streptomycin, 0.25 µg/ml amphotericin B and 2 mM L-glutamine). Every

24 hours 1 ml of culture medium was collected from each well and replaced with fresh culture medium. Media from all wells was pooled each day, aliquoted and stored at -80 °C. Media collected on day 1, day 2, and day 3 *in vitro* were pooled, and this mixture is what is generally referred to as "*T. solium* excretory/secretory extracts".

2.1.10 Protein concentration determination

All *T. crassiceps* and *T. solium* larval extracts were assessed for protein concentration using a BCA- or Bradford's- protein assay kit, respectively. The concentration of glutamate in the various larval extracts was measured using a glutamate assay kit according to the supplier's instructions (Sigma-Aldrich).

2.2 HIPPOCAMPAL BRAIN SLICE PREPARATION AND TREATMENT

2.2.1 Brain slice preparation

Organotypic hippocampal brain slices were prepared using 6-8-day old male and female Wistar rats or C57BL/6 mice, as first described by Stoppini *et al.* [1991]. Rat brain slices were used for almost all experiments in chapters 3 & 4 (except for the calcium imaging experiments in chapter 3), whilst mouse slices were utilised for all experiments in chapter 5. The use of the two species for different experiments was motivated by accessibility at the time, and the suitability of antibodies for performing ELISAs in the respective species. Briefly – the brain was removed and immediately placed in cooled (4 °C) dissection media (EBSS with 6.1 g/L HEPES, 6.6 g/L D-glucose and 5 μM saturated sodium hydroxide). The brain was then dissected into its two hemispheres and the hippocampus dissected out of each. Hippocampi were then cut into 350 μm slices using a McIlwain tissue chopper (Brinkmann, Mickle) and slices were separated from one another by gentle swirling in cooled dissection media. Slices with the greatest structural integrity were selected and placed onto 6-well-plate Millicell-CM cell culture inserts (Merck) in warmed (37 °C) culture medium (1.2 ml per well in 6-well plates) consisting of (vol / vol): 50 % MEM with Glutamax, 23 % EBSS, 25 % heat-inactivated horse serum (Biochrom), 6.5 g/L glucose and 2 % B27. Slices were incubated at 37 °C and 5 % CO_2 in a humidified incubator. Culture medium was replaced every 2-3 days.

2.2.2 *Taenia crassiceps* and hippocampal brain slice co-culture

Hippocampal brain slices and *T. crassiceps* larvae were cultured together (with the brain slices on the membranes of Millicell-CM cell culture inserts, and the larvae in the culture medium) in 6-well plates for a period of 7-9 days in conditions as described above. Three to five *T. crassiceps* larvae of 3-5 mm in length were added to each well. A set of control brain slices were exposed to identical culture conditions, but in the absence of *T. crassiceps* larvae.

2.2.3 Hippocampal organotypic brain slice preparation for the determination of daily baseline cytokine release

Hippocampal brain slices were prepared as described above, with the exception that the culture medium was collected and replaced daily, for later cytokine determination. Six brain slices were placed on each insert with 1.2 ml of culture medium.

2.2.4 Treatment of hippocampal organotypic brain slice cultures with different doses of immunogenic substances.

On day 6 *in vitro* brain slice cultures were exposed for 24 hrs to various doses of known immunogenic antigens in culture medium. We waited six days before exposure to antigens as this allows time for slices to recover from slicing induced changes and for the elimination of dead cells and tissue debris (Lein *et al.*, 2011). A 24 hr exposure time to antigens was chosen as previous studies have demonstrated that inflammatory cytokine release tends to peak 24 hrs after antigen exposure (Papageorgiou *et al.*, 2016; Yousif *et al.*, 2018). After 24 hrs of antigen exposure the medium was collected from each well and stored at -80 °C until use for cytokine determination.

I utilised three immunogenic agents, each of which act on a different toll-like receptor. The first was lipopolysaccharides (LPS) from the bacteria *Escherichia coli* (O127:B8, Sigma-Aldrich). Lipopolysaccharides are molecules that are found in the membrane of some bacteria and have been described to activate neuroinflammatory pathways by binding to TLR4 (Vargas-Caraveo *et al.*, 2020). LPS was utilised at concentrations of 10^1 ng/ml, 10^2 ng/ml, 10^3 ng/ml, and 10^4 ng/ml. These concentrations were chosen as previous publications in similar models have used each of these concentrations and I wanted to establish which concentration would be optimal for my model (Papageorgiou *et al.*, 2016; Terrazas *et al.*, 2011; Emilia Vendelova *et al.*, 2016; Yousif *et al.*, 2018).

The second immunogenic antigen was zymosan A from *Saccharomyces cerevisiae* (Sigma-Aldrich).Zymosan A is a molecule found on the surface of fungi, such as yeast, and cause an inflammatory host immune response by activating TLR2 and dectin-1 (Emilia Vendelova *et al.*, 2016). Zymosan A was tested at concentrations of 10^1 ng/ml, 10^2 ng/ml, 10^3 ng/ml, and 10^4 ng/ml. These concentrations were chosen as an existing study using microglial cultures utilised a concentration of zymosan A of 10^4 ng/ml to stimulate a potent inflammatory response, so I chose to utilise this concentration as well as several lower concentrations (Facci *et al.*, 2014).

The third immunogenic antigen utilised was polyinosinic–polycytidylic acid (poly(I:C)) (poly(I:C) acid salt, Sigma-Aldrich). Poly(I:C) is a double stranded RNA analogue that

stimulates TLR3, resulting in an inflammatory host immune response. Poly(I:C) was tested at concentrations of 10^2 ng/ml, 10^3 ng/ml, 10^4 ng/ml, and 10^5 ng/ml. These concentrations were chosen as I found a previous study in which a poly(I:C) concentration of 5^4 ng/ml had been utilised and I decided to try a few concentrations spanning this concentration (Facci *et al.*, 2014).

2.2.5 Treatment of hippocampal organotypic brain slice cultures with various *Taenia crassiceps* extracts, alone or together with lipopolysaccharides.

For all cases where hippocampal organotypic brain slice cultures were exposed to *T. crassiceps* extracts, alone or together with lipopolysaccharides, the extracts were added to the medium on day 6 *in vitro*, allowed to incubate for 24 hrs, and then collected, aliquoted and stored at -80°C for later cytokine determination. The excretory/secretory extracts utilised for these experiments were the concentrated excretory/secretory extracts, except where it is specified that excretory/secretory extracts > 3 kDa was utilised. In all experiments where homogenate, excretory/secretory extracts, cyst vesicular fluid, or cyst membrane were added, they made up 150 µg/ml of the final concentration in the brain slice culture medium. However, the final concentrations of the PQ homogenate (50 µg/ml), PQ excretory/secretory extracts (100 µg/ml), Starved homogenate (65 µg/ml), Starved excretory/secretory extracts (100 µg/ml), DMSO homogenate (500 µg/ml) and Granuloma (100 µg/ml) varied.

The LPS concentration utilised for all experiments was 10 ng/ml, except for experiments performed specifically to determine the percentage suppression of cytokine production in response to different doses of LPS by *T. crassiceps* homogenate. For these experiments LPS concentrations of 10^1 ng/ml, 10^2 ng/ml, 10^3 ng/ml, and 10^4 ng/ml were utilised both on their own, and together with 150 µg/l *T. crassiceps* homogenate. Percentage suppression was calculated for each dose of LPS as follows: $\%\,suppresion = 1 - \left(\frac{Median\ LPS+Hom\ cytokine\ release}{Median\ LPS\ cytokine\ release}\right) * 100.$

2.2.6 Treatment of hippocampal organotypic brain slice cultures with zymosan A or poly(I:C) alone, and together with *Taenia crassiceps* homogenate.

For experiments exploring whether the *T. crassiceps* homogenate can attenuate cytokine production induced by zymosan A or poly(I:C), a concentration of 5 µg/ml of zymosan A or poly(I:C) was utilised. Experiments were done both with these toxins on their own, and with 150 µg/ml *T. crassiceps* homogenate.

2.3 ELECTROPHYSIOLOGY

2.3.1 *In vitro* patch-clamp recordings

Brain slices were removed from the Millicell-CM membrane by cutting the membrane around each slice with a scalpel (no 10 blade, B&M Scientific). Each slice was then placed in the submerged recording chamber on the patch-clamp rig and continuously superfused (using 102R peristaltic pumps, Watson-Marlow Fluid Technology Group) with artificial cerebrospinal fluid (aCSF; deionised water with 120 mM sodium chloride (NaCl), 23 mM sodium bicarbonate ($NaHCO_3$), 11 mM D-glucose, 3 mM potassium chloride (KCl), 2 mM calcium chloride ($CaCl_2$), 2 mM magnesium chloride ($MgCl_2$), 1.2 mM sodium phosphate monobasic (NaH_2PO_4); pH between 7.35-7.40) which was bubbled with carbogen gas (95 % O_2: 5 % CO_2) and kept at a temperature of 28-32 °C. An upright microscope (Zeiss Axioskop or Olympus BX51WI) with a 20x or 40x fluid-immersion objective lens was used to identify pyramidal neurons in the CA3 region of the hippocampus suitable for patch-clamp recordings. Surface cells with a typical pyramidal cell body morphology were selected for whole-cell patching

Micropipettes, for patching and for puffing of solutions (tip resistance between 3 and 7 MΩ), were made from borosilicate glass capillaries (outer diameter 1.2 mm, inner diameter 0.69 mm, Harvard Apparatus Ltd) using a horizontal puller (Sutter). Recordings were made in current clamp mode using Axopatch 200B amplifiers (Axon Instruments) and data was acquired using WinWCP (University of Strathclyde) or Igor (Markram Laboratory, Ecole polytechnique fédérale de Lausanne). Data were analysed using custom Matlab scripts (R2015b, MathWorks). Experimental substances were puffed onto neurons in a controlled manner using an OpenSpritzer (Forman *et al.*, 2017).

Patch-clamp recordings to determine the acute effects of T. crassiceps extracts on neurons. Two internal solutions were used: a “standard” internal solution (126 mM potassium gluconate, 4 mM KCl, 10 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), 4 mM adenosine 5′-triphosphate disodium (Na_2ATP), 0.3 mM guanosine 5′-triphosphate sodium (NaGTP), 10 mM phosphocreatine disodium) and a “caesium” internal solution (120 mM caesium hydroxide (CsOH), 120 mM gluconic acid, 40 mM HEPES, 2 mM Na_2ATP, 0.3 mM NaGTP, 10 mM NaCl). Caesium, a potassium channel blocker, was used in the internal solution for most whole-cell patch experiments that involved the puffing of *T. crassiceps* homogenate or excretory/secretory extracts to establish their acute effect on neurons, since Hayley Tomes had previously established that a small component of the *T. crassiceps* depolarizing response resulted from potassium in the homogenate. We thus wanted to exclude the contribution of potassium in subsequent experiments. Additionally, most of these recordings were performed in the presence of of 2 µM tetrodotoxin (TTX). TTX blocks voltage-

gated sodium channels and thereby prevents the firing of action potentials. We utilised TTX to largely reduce the inputs from connected cells, enabling us to better establish/isolate the direct effect of the *T. crassiceps* extracts on the patched cell. Current was injected, if required, to maintain a neuronal resting membrane potential within 2 mV of -60 mV. Each data point represents the mean peak puff-induced change in membrane potential from 10 sweeps. Pharmacological blockade of glutamate receptors was achieved by adding the following drugs to the aCSF superfusing the slice: 10 µM cyanquixaline (CNQX) disodium, 50 µM D-(-)-2-Amino-5-phosphonopentanoic acid (D-AP5) (Tocris, WhiteSci) and 2 mM kynurenic acid.

Patch-clamp recordings to demonstrate the effect of larval acetylcholinesterases on neuronal acetylcholine signaling. The standard internal solution was utilised. Two puffer pipettes were lowered down (in addition to the pipette containing the recording electrode), one containing a solution of 200 µm acetylcholine with 1.3 mg/ml *T. crassiceps* excretory/secretory extracts > 3 kDa, and the other a solution of 200 µm acetylcholine with 1.3 mg/ml *T. crassiceps* excretory/secretory extracts > 3 kDa that had been heated to 56 °C for 30 min. Neurons were subjected to the following protocol: current was injected in order to hold the membrane potential of cells close to their action potential firing threshold. The cell was then subjected to cycles of the following: after 4 s a train of 5 x 30 ms puffs of one of the experimental solutions to the cell body; the neuron's response to this puff was then recorded for 26 s; a 90 s "recovery" period was allowed; an identical puff train of the other experimental solution was applied; the neuron's response was again recorded for 26 sec; a 90 s recovery period was allowed, and the cycle was then repeated. Post-recording analysis consisted of manually counting the number of action potentials induced by the application of each solution within a 5 sec period of the onset of the puff train. Only traces with a baseline membrane potential prior to the puff protocols of between -60 mV and -45 mV were included. Each data point in the puffing experiments represents the average of between 2 and 5 repeats of the puff cycle.

2.3.2 Calcium imaging

For calcium imaging, organotypic hippocampal mouse brain slices were virally transfected with a genetically encoded Ca^{2+} reporter (GCAMP6s under the synapsin promoter, AAV1.Syn.GCaMP6s.WPRE.SV40) on day 2 *in vitro* using the OpenSpritzer, and imaged 5 days later using an Olympus BX51WI microscope, 20x water-immersion objective, CCD camera (CCE-B013-U, Mightex) and 470 nm LED (Thorlabs). Images were collected using µManager (Edelstein *et al.*, 2010) and analysed using Caltracer3 beta scripts in Matlab.

2.3.3 Interface local field potential recordings

Brain slices were extracted from the Millicell-CM membrane and transferred to an interface recording chamber where they were continuously superfused with 5 mM K^+ aCSF (to make

the slices more excitable) which was bubbled with carbogen gas and kept at 28-32 °C. Local field potential (LFP) recordings were performed in the CA3 region using an AC-coupled amplifier (A-M Systems). Data were acquired using LabChart Pro (AD Instruments) with recordings processed using a 140 Hz low-pass filter. The LFP of each slice was recorded for a 60 min period. Seizure-like events (SLEs) were defined as events where there were large amplitude deviations from the resting potential with a characteristic "rapidly spiking" appearance lasting at least 5 s and having a defined start and end (Dulla *et al.*, 2018). Seizure-like events were only defined as separate events if there was a 20 s return to baseline in between events. If a slice was undergoing an SLE at the start of the recording this was counted as the first SLE. If a slice was part-way through a SLE at the end of the recording period, then this SLE was excluded from analysis. For the purposes of analysing time to first SLE, slices that did not experience any SLEs during the 60 min recording were assigned a maximum set value of 3600 s. LFP traces were analysed manually in LabChart Pro.

2.4 ACETYLCHOLINESTERASE PRESENCE AND ACTIVITY

2.4.1 Acetylcholinesterase activity and inhibitor sensitivity

Acetylcholinesterase activity was determined by the method of Ellman *et al.* (1961). *T. crassiceps* whole cyst homogenate concentrated excretory/secretory extracts, cyst vesicular fluid, and cyst membrane extracts were exposed to 1 mM acetylthiocholine iodide (ATI) substrate in 100 mM sodium phosphate buffer (pH 7.0). 1 mM 5,5'-dithiobis (2-nitrobenzoic acid) (DTNB) was then added to create a coloured product in direct proportion to the amount of ATI hydrolysed by larval/larval-element AChEs. The reaction was monitored by measuring the absorbance at 412 nm and the hydrolysis of ATI was calculated from the extinction coefficient of DTNB (1.36 x 10^{-4}, Ellman *et al.* 1961). Activity was expressed as nanomoles of acetylthiocholine hydrolysed per minute per milligram of total protein (ng.min^{-1}.mg^{-1}). To test the sensitivity of *Taenia* AChEs to different inhibitors, extracts were preincubated with different concentrations of 1,5-bis(4-allyldimethylammoniumphenyl) pentan-3-one dibromide (BW 284c51), tetraisopropyl pyrophosphoramide (iso-OMPA) or eserine salicylate for 20 min at room temperature in Ellman buffer, prior to the addition of the other reagents and subsequent enzyme activity determination. Each reaction was assayed a minimum of three times.

2.4.2 Non-denaturing polyacrylamide gel electrophoresis (PAGE)

Extracts were electrophoresed in Tris-glycine buffer, pH 8.3, through 7.5 % polyacrylamide gels in the absence of denaturing and reducing agents. Electrophoresis was performed at 150 V for 3 hrs on ice. Protein staining was performed on one set of PAGE gels using EZBlue™ Gel Staining Reagent (Sigma-Aldrich) according to the manufacturer's instructions, and

specific staining for AChE activity was performed overnight on another set of gels (Selkirk and Hussein (2000), adapted from the method of Karnovsky and Roots (1964)). Briefly, each gel was placed in 65 ml $NaPO_4$ buffer (pH 6) with 50 mg ATI for 10 min. Thereafter, the following solutions were added sequentially with gentle mixing in between each addition: 5 ml of 0.1 M sodium citrate, 10 ml of 30 mM copper sulphate, 10 ml distilled water and 10 ml of 5 mM potassium ferricyanide. Gels were left to stain overnight. To control for non-specific staining another set of gels was exposed to the same staining treatment, but with the omission of ATI. The maximum volume of each protein extract was loaded (20ul), to ensure maximal staining. Protein concentrations of the different extracts varied (*Taenia crassiceps*: homogenate = 1.9 mg/ml, cyst membrane = 3.4 mg/ml, cyst vesicular fluid = 3.0 mg/ml and excretory/secretory extracts = 1.32 mg/ml; *Taenia solium*: All extracts = 1.5 mg/ml). Each experiment was performed three times to confirm the results.

2.4.3 *In situ* localisation of *Taenia* acetylcholinesterases

To localise *Taenia* AChEs, fresh *Taenia* larvae were fixed in 10 % formalin for 60 min. Some of the larvae were then stained overnight for AChE activity (as described above for the PAGE gels) and mounted onto slides as whole mounts. A few of the fixed larvae were embedded in Optimal Cutting Temperature reagent (Jung), frozen overnight at -80 °C, and then cryo-sectioned at -20 °C on a Leica CM 1850 cryostat microtome into 50 um sections. The sections were then similarly stained overnight for AChE activity, placed on positively charged slides and dehydrated in graded alcohols before mounting. To assess non-specific staining ATI was omitted in a subset of the whole mount and cryo-section specimens. Specimens were imaged using an upright light microscope (Zeiss Axioskop 2 MOT, Zeiss).

2.5 Cytokine detection

To determine the concentration of various cytokines in the culture medium of experimental hippocampal organotypic brain slice cultures, and in the extracts of *T. crassiceps*, enzyme linked immunosorbent assays for IL-6, IL-10, IL-1β and TNF-α were prepared in-house. A detailed protocol is available online at protocols.io (dx.doi.org/10.17504/protocols.io.bh2fj8bn).

2.6 Endotoxin detection

Endotoxin concentrations in single samples of *T. crassiceps* whole cyst homogenate, concentrated excretory/secretory extracts, cyst vesicular fluid and cyst membrane, were detected using a Pierce™ LAL Chromogenic Endotoxin Quantitation Kit (Thermo Fisher Scientific) as according to the manufacturer’s instructions.

2.7 DATA ANALYSIS AND STATISTICS

Data was graphed and analysed using Matlab R2015b, Microsoft excel v14, GraphPad Prism v5 and LabChart Pro. Adobe Illustrator vCS6 was used to create all figures.

All data were subjected to a Shapiro-Wilk test for normality. This test for normality was chosen based on the recommendation of Ghasemi and Zahediasl (2012). Normally distributed populations were subjected to parametric statistical analyses, whilst data sets with skewed distributions were assessed using non-parametric statistical analyses. Parametric tests included: unpaired student's t-test; paired student's t-test; one-way analysis of variance (ANOVA) with Tukey's Multiple Comparisons post hoc test and repeated measures ANOVA with Tukey's post-hoc test. Non-parametric tests included: Mann-Whitney U test; Wilcoxin ranked pairs test; Kruskal Wallis one-way ANOVA with post hoc Dunn's Multiple Comparison test; and Friedman test with post-hoc Dunn's Multiple Comparison test. For parametric data sets mean and standard error of the mean (SEM) are reported, whilst median and interquartile range (IQR) are reported for non-parametric data sets with $N > 3$, and median and range are reported for non-parametric data sets with $N \leq 3$, as in this case IQR cannot be reported. The confidence interval for all statistical analyses was set at 95%, meaning that differences were only considered significant if $p \leq 0.05$.

Chapter 3

THE EFFECTS OF *TAENIA CRASSICEPS* LARVAL EXTRACTS ON NEURONS AND NEURONAL NETWORKS *IN VITRO*.

3.1 INTRODUCTION

Seizures have been reported to occur in 70-90 % of symptomatic NCC cases (Carpio and Romo, 2014). Despite this, knowledge of the mechanisms underlying seizures secondary to NCC is still incredibly limited.

It is widely assumed that seizures result from the host response to the parasitic material as the occurrence of seizures seems to be closely related to a detectable host immune response surrounding the cyst (Garcia and Del Brutto, 2017; Robinson *et al.*, 2012). Seizures secondary to NCC are less common while the parasitic cyst remains viable, most prevalent when the cyst is degenerating, and somewhat common in cases where the lesion has become calcified (Garcia and Del Brutto, 2017). The possibility that parasite-derived mediators may themselves induce or contribute to seizures in NCC has been largely overlooked. We were interested in the possibility that *Taenia* excreted/secreted extracts (which would be present while cysts are viable) and/or *Taenia* somatic extracts (which would become exposed to the brain parenchyma when the cyst degenerates and may linger in calcific cysts) could act on neurons and neuronal networks in a way that makes them more excitable. Together with collaborators, I investigated the acute electrophysiological effects of *Taenia* excreted/secreted and somatic extracts (in the form of whole cyst homogenate) directly on neurons utilising a whole-cell patch-clamp set-up. Remarkably, we found that both extracts elicit a large depolarising shift in the neuronal membrane potential. Dialysis of the extracts revealed that a small molecule (< 3 kDa) was the active agent in depolarisation and the blockade of glutamate receptors revealed that the effect was mediated by glutamate receptors. Glutamate assays of these extracts revealed a concentration of glutamate great enough to explain the observed depolarisation, leading to the conclusion that glutamate was responsible for the effects observed. In addition, I confirmed that the human pathogen, *Taenia solium*, also contains and consistently produces significant amounts of glutamate.

I further investigated whether chronic exposure to the viable parasite (and thereby its excreted/secreted extracts) could affect brain excitability. To do so I created a novel co-cultured model of NCC in which hippocampal brain slices were cultured together with *T. crassiceps* larvae for several days. Thereafter, the excitability of the neuronal networks of brain slices co-cultured with *Taenia* larvae were compared to that of brain slices that were not, utilising local field potential recordings. Interestingly, prolonged brain exposure to viable parasites did not result in any statistically significant changes in brain excitability.

3.2 *TAENIA CRASSICEPS* EXTRACTS EXCITE NEURONS AND CAN ELICIT EPILEPTIFORM ACTIVITY

To explore the potential acute effects of *T. crassiceps* larval extracts on neurons, *T. crassiceps* larvae were harvested from the peritonea of mice, after which they were either freeze-thawed and homogenised (to produce *T. crassiceps* whole cyst homogenate) or cultured *in vitro* for several days (to produce *T. crassiceps* excretory/secretory extracts). Whole-cell patch-clamp recordings were made from CA3 pyramidal neurons in rat hippocampal organotypic brain slice cultures and pico-litre volumes of *T. crassiceps* extracts were applied onto the soma of neurons using a glass micropipette attached to a custom built pressure ejection system (**Fig 3.1 A**) (Forman *et al.*, 2017). A 20 ms puff of *T. crassiceps* homogenate resulted in a large depolarisation in the membrane potential and consequently the firing of a train of action potentials (**Fig. 3.1 B** – performed by Hayley Tomes). To exclude the possibility that this depolarisation resulted from a mechanical disruption of the membrane by the puff application itself I examined the effects of a 20 ms puff of artificial cerebrospinal fluid, which did not elicit a similar depolarising response (**Fig. 3.1 B**). A 20 ms puff of *T. crassiceps* excretory/secretory extracts elicited a remarkably similar response to that induced by the *T. crassiceps* homogenate (**Fig. 3.1 C**). As an additional control, a 20 ms puff of the culture medium in which the excretory/secretory extracts were collected resulted in no obvious membrane potential depolarisation, thereby excluding the possibility that a component of the medium may be responsible for the effect observed (**Fig. 3.1 C**).

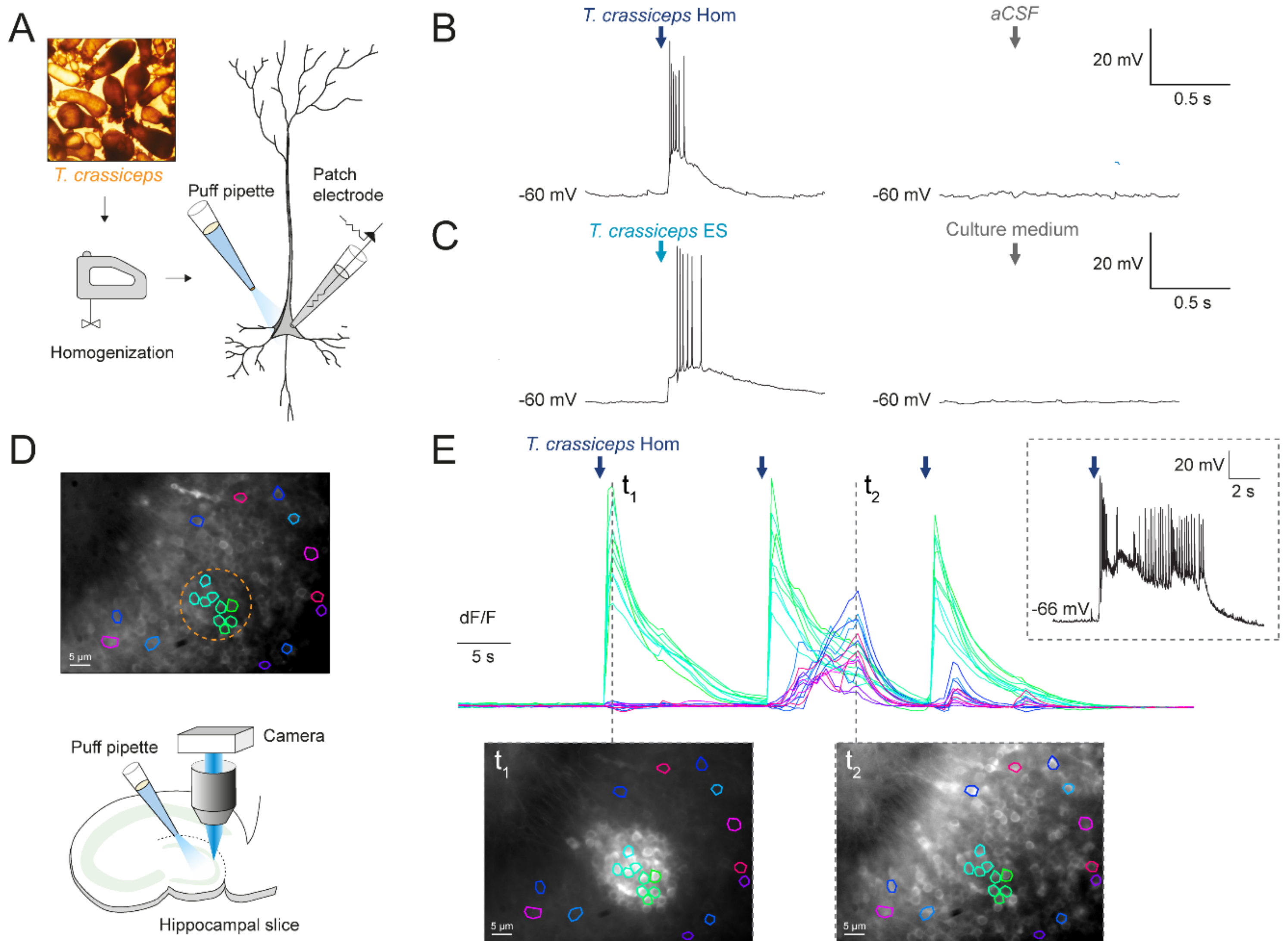


Figure 3.1: *Taenia crassiceps* extracts excite neurons and can elicit epileptiform activity. A) Schematic diagram of experimental set up. Whole-cell patch-clamp recordings were made from CA3 hippocampal pyramidal neurons in rodent organotypic slice cultures while a puffer pipette applied pico-litre volumes of either *Taenia crassiceps (T. crassiceps)* larval homogenate (Hom) or *Taenia crassiceps* larval excretory/secretory extracts (ES) to the soma. B) Current clamp recording from a rat pyramidal neuron while a 20 ms puff of *T. crassiceps* homogenate was applied via the puff pipette (dark blue arrow indicates puff). Application of *T. crassiceps* homogenate resulted in depolarization and the firing of multiple action potentials (left). A similar puff of aCSF had no effect on the neuronal membrane potential (right, grey arrow), showing that potential mechanical disruption of the cell by a puff is not sufficient to depolarise it. C) As in 'B', remarkably similar effects to those of *T. crassiceps* homogenate could be elicited in current clamp recordings from a CA3 hippocampal pyramidal rat neuron by *T. crassiceps* E/S extracts (left). A similar puff of the culture medium in which *T. crassiceps* larvae were cultured did not elicit a similar response (right). D) Top: widefield fluorescence image of neurons in the dentate gyrus of a mouse hippocampal organotypic brain slice culture expressing the genetically encoded Ca^{2+} reporter, GCAMP6s, under the synapsin promoter. A subset of neurons used to generate the Ca^{2+} traces in 'E' are indicated by different colours. The orange dotted circle indicates where *T. crassiceps* homogenate was delivered using the puff pipette. Bottom: schematic of the experimental setup including puffer pipette and CCD camera for Ca^{2+} imaging using a 470 nm LED. E) Top: dF/F traces representing Ca^{2+} dynamics from the GCAMP6s expressing neurons labelled in 'D' when 3 puffs of *T. crassiceps* homogenate were applied (30 ms duration, 15 s apart). The slice was maintained in aCSF with 0.5 mM Mg^{2+} for this recording. Inset, top-right: whole-cell current-clamp recording from an individual neuron in the slice demonstrates that *T. crassiceps* homogenate application evoked a seizure-like event. Bottom: two images of raw Ca^{2+} fluorescence at two time points; t_1 and t_2. Note how at time point t_2 neurons distant to the site of *puff* are also activated, indicating spread of neuronal activity.

I was curious as to whether the effects of the *T. crassiceps* extracts on individual neurons may propagate to other parts of the slice. To explore this, Dr Raimondo performed fluorescence Ca^{2+} imaging in mouse hippocampal organotypic brain slice cultures. Neurons were virally transfected with the genetically encoded Ca^{2+} reporter, GCAMP6s, under the synapsin promoter and imaged using widefield epifluorescence microscopy (**Fig 3.1 D**). To encourage excitatory responses a low Mg^{2+} aCSF was used (0.5 mM Mg^{2+}). Neurons in the dentate gyrus were imaged whilst being exposed to 30 ms, spatially restricted, puffs of *T. crassiceps* homogenate every 15 ms (**Fig 3.1 D**). There was a sharp increase in the fluorescence of cells near the puffer pipette after each puff, which is indicative of Ca^{2+} entry following membrane depolarization and action potential generation (**Fig 3.1 E**, green and light blue traces). Notably, cells less proximal to the puffer pipette also showed increases in fluorescence following some puffs, although these responses lagged behind those of the more proximal cells (**Fig 3.1 E**, purple and dark blue traces). This indicates seizure-like propagation following the application of *T. crassiceps* homogenate. This is supported by a current clamp recording from a neuron in the same slice that displays a prolonged, regenerative, seizure-like response to a 30 ms puff of *T. crassiceps* homogenate (**Fig. 3.1 E** - inset). Thus *T. crassiceps* homogenate can induce prolonged seizure-like events that propagate through the brain slice under suitable conditions.

3.3 *TAENIA CRASSICEPS* HOMOGENATE INDUCED NEURONAL DEPOLARIZATION IS NOT A RESULT OF MECHANICAL MEMBRANE DISPLACEMENT, A GENERIC PROTEIN COMPONENT, OR A MOLECULE LARGER THAN 3 KDA

Through the utilisation of whole-cell patch-clamping, Dr Hayley Tomes, and I set out to discriminate which property of the *T. crassiceps* homogenate puff is responsible for the depolarisation in membrane potential. First, *T. crassiceps* homogenate was puffed onto neurons (in the presence of 2 μM TTX) to quantify the magnitude of the membrane depolarisation caused by the homogenate. The median size of the depolarisation induced by the homogenate was determined to be 13.76 mV (IQR 10.87 – 17.24 mV, N = 49, **Fig. 3.2 A**, population data in **Fig. 3.2 E** – These experiments were performed by Hayley Tomes). Then, to confirm that the mechanical displacement of the cell membrane by the puff application was not responsible for the depolarisation seen in a population of cells, I puffed aCSF onto neuronal cell bodies at a proximity that resulted in visual disruption of the cell membrane. The aCSF puffs resulted in only an exceedingly small shift in neuronal membrane potential, which was significantly smaller than that of the homogenate (median 0.05 mV, IQR -0.09 – 1.04 mV, N = 5, $P \leq 0.01$, Kruskal Wallis test with Dunn's multiple comparison post-hoc test, **Fig. 3.2 B**, population data in **Fig. 3.2 E**).

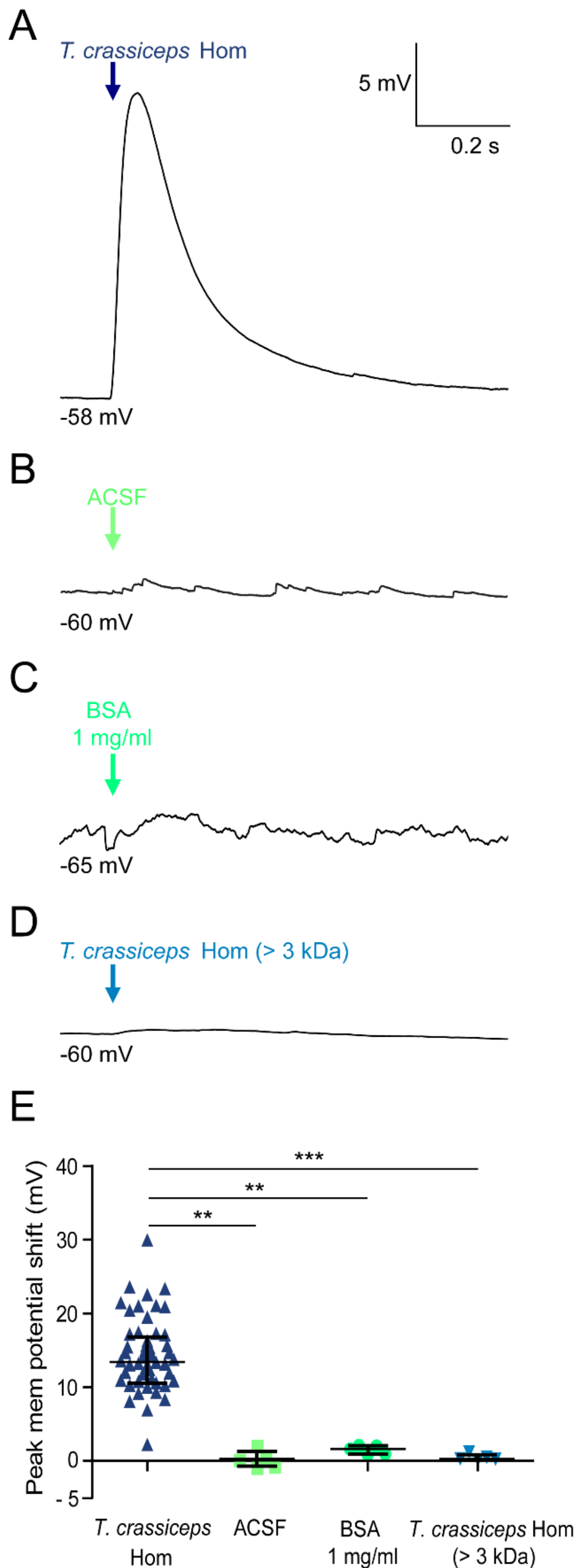


Figure 3.2: *Taenia crassiceps* homogenate induced neuronal depolarization is not a result of mechanical membrane displacement, a generic protein component, or a molecule larger than 3 kDa. Whole-cell patch recordings in current clamp were made from CA3 pyramidal neurons in rat organotypic hippocampal slice cultures using a caesium based internal in the presence of 2 µM TTX. A) 20 ms puff of *Taenia crassiceps* (*T. crassiceps)* homogenate to the soma resulted in a large depolarizing shift in membrane potential. B) 20 ms puff of aCSF to the soma did not produce a similar depolarizing response. C) Puff application of a generic protein (bovine serum albumin - BSA) at a similar concentration to the protein concentration of *T. crassiceps* homogenate, also failed to produce a large depolarising shift in membrane potential. D) The depolarizing response to *T. crassiceps* homogenate was largely abolished by dialysing out all molecules smaller than 3 kDa, which suggests that the excitatory agent is a molecule smaller than 3 kDa. E) Population data (with means ± SEM) demonstrating that the depolarising shift in neuronal membrane potential induced by *T. crassiceps* homogenate (N = 49) is significantly larger than that induced by aCSF (N = 5), 1 mg/ml BSA (N = 5) or dialysed *T. crassiceps* homogenate (N = 5). The membrane potential shift induced by aCSF, 1 mg/ml BSA and by dialysed *T. crassiceps* homogenate did not differ significantly from one another. Values with medians ± IQR; ** $p \leq 0.01$; *** $p \leq 0.001$; each data point represents a cell.

Next, to exclude the possibility that a generic protein component may be responsible for the depolarising effect, I puffed a 1 mg/ml solution of bovine serum albumin (BSA) onto neuronal somas. This resulted in a much smaller depolarising membrane response to that elicited by *T. crassiceps* homogenate (median 0.57 mV, IQR 0.86 – 2.03 mV, N = 5, $P \leq 0.01$, Kruskal Wallis test with Dunn's multiple comparison post-hoc test, **Fig. 3.2 C**, population data in **Fig. 3.2 E**). This established that a generic protein component is not responsible for the observed effect. Finally, Dr Hayley Tomes removed the small molecules from the *T. crassiceps* homogenate (using a 3 kDa molecular weight cut-off dialysis cassette in a large volume of aCSF overnight) before applying it to neurons, to establish whether a small molecule (< 3 kDa) was responsible for the effect (**Fig. 3.2**). The dialysed *T. crassiceps* homogenate failed to induce a large membrane potential shift such as that induced by non-dialysed *T. crassiceps* homogenate (median 0.27 mV, IQR 0.20 – 0.85, N = 5, $P \leq 0.01$, Kruskal Wallis test with Dunn's multiple comparison post-hoc test, **Fig. 3.2 D**, population data in **Fig. 3.2 E**), indicating that a small molecule (< 3 kDa) is responsible for the large depolarising membrane potential shift induced by non-dialysed *T. crassiceps* homogenate. The small shifts in membrane potential induced by aCSF, 1 mg/ml BSA and dialysed homogenate were not significantly different to one another (Kruskal Wallis test with Dunn's multiple comparison post-hoc test, $P > 0.05$, **Fig. 3.2 E**).

3.4 NEURONAL DEPOLARIZATION INDUCED BY *TAENIA CRASSICEPS* EXCRETORY/SECRETORY EXTRACTS IS MEDIATED BY A SMALL MOLECULE

In **Figure 3.1 C** I demonstrated that *T. crassiceps* excretory/secretory extracts appear to induce a large depolarising shift in neuronal membrane potential. To establish whether (as shown above for *T. crassiceps* homogenate) a small molecule (< 3 kDa) was responsible for this effect, and to exclude the possibility that the depolarising effects of *T. crassiceps* excretory/secretory extracts could be attributed to their solvents, I performed another set of patch-clamp recordings in rat CA3 hippocampal pyramidal neurons. First, I puffed *T. crassiceps* excretory/secretory extracts onto neurons in the presence of 2 μM TTX to confirm and quantify its depolarising effect. These experiments revealed that *T. crassiceps* excretory/secretory extracts result in a mean depolarising shift of 12.29 mV (SEM 1.74 mV, N = 7, **Fig. 3.3 A**, population data in **Fig. 3.3 F**). Puff application of the *T. crassiceps* culture medium onto neurons established that the effect induced by *T. crassiceps* excretory/secretory extracts was not because of its solvent, as the culture medium induced a negligible membrane potential shift that was significantly smaller than that induced by the *T. crassiceps* excretory/secretory extracts (mean -0.48 mV, SEM 0.5 mV, N = 7, $p \leq 0.001$, 1-way ANOVA with Tukey's Multiple Comparison test, **Fig. 3.3 D**, population data in **Fig. 3.3 F**).

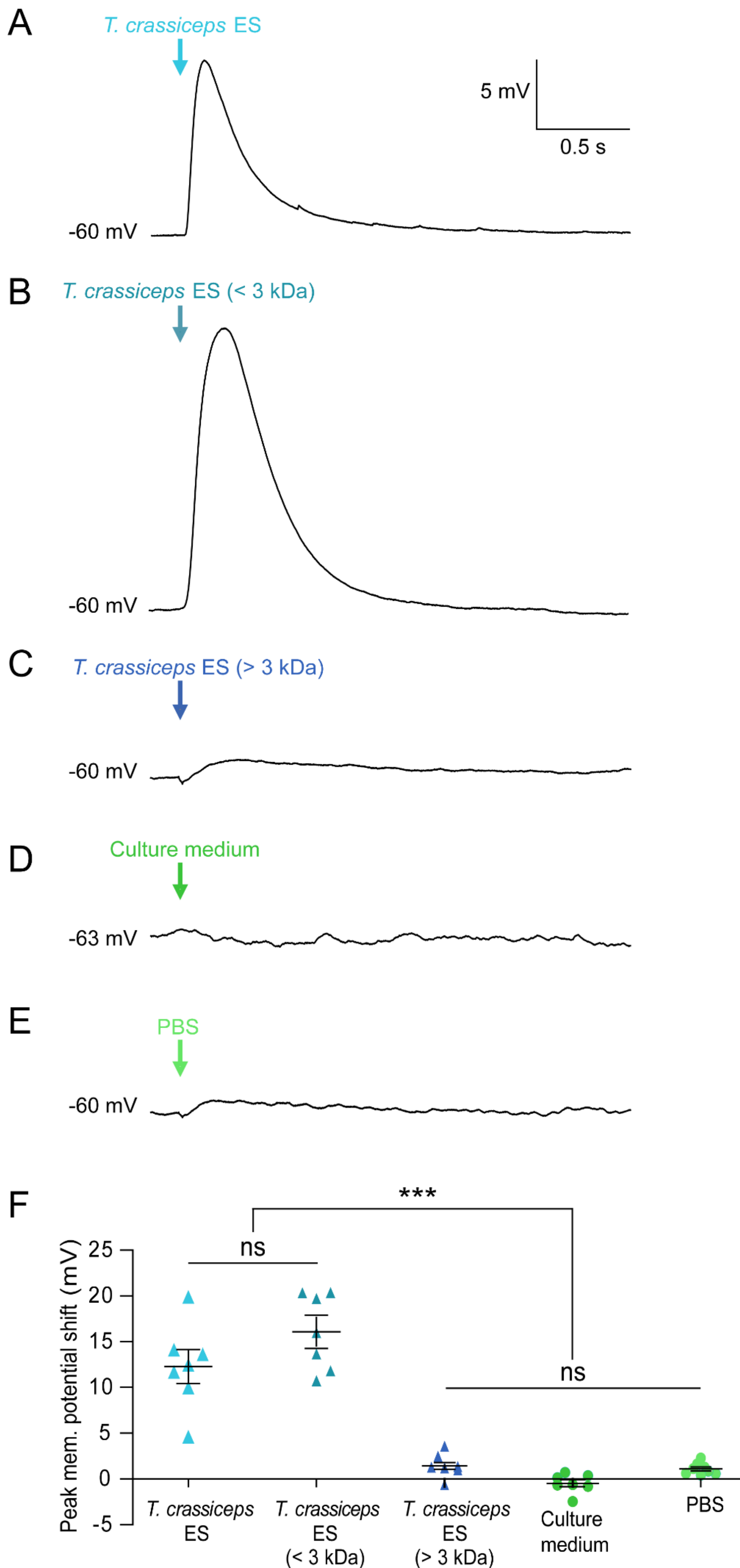


Figure 3.3: Neuronal depolarization induced by *Taenia crassiceps* excretory/secretory extracts are mediated by a small molecule. Whole-cell patch recordings in current clamp were made from CA3 pyramidal neurons in rat organotypic hippocampal slice cultures using a caesium based internal in the presence of 2 μM TTX. A) 20 ms puff of *T. crassiceps* excretory/secreted extracts (ES) to the soma resulted in a large depolarizing shift in membrane potential. B) Application of *T. crassiceps* ES from which molecules larger than 3 kDa had been removed also resulted in a large depolarizing shift in membrane potential. C) In contrast, a puff of *T. crassiceps* ES larger than 3 kDa resulted in a negligible shift in membrane potential. D) No change in membrane potential occurred in response to a puff of growth medium, a control for the 'ES < 3 kDa'. E) A negligible depolarization of the membrane potential occurred in response to a puff of phosphate-buffered saline (PBS), a control for the 'E/S extracts > 3kDa'. F) Population data demonstrating that complete *T. crassiceps* ES (N = 7) and ES < 3kDa (N = 7) cause a significant depolarization of neuronal membrane potential when compared to ES > 3kDa (N = 8), culture medium (N = 7) or PBS (N = 8). Values with means ± SEM; *** $p \leq 0.001$; ns = not significant; $p > 0.05$; each data point represents one cell.

I then separated the *T. crassiceps* excretory/secretory extracts into two fractions using an Amicon stirred cell – one containing molecules smaller than 3 kDa in the original *T. crassiceps* culture medium, and one containing components larger than 3 kDa in PBS (1X, pH 7.4). Application of the < 3 KDa fraction resulted in a large mean membrane depolarisation shift (16.09 mV, SEM 1.56 mV, N = 7) that did not differ significantly from that induced by the whole *T. crassiceps* excretory/secretory extracts ($p > 0.05$, 1-way ANOVA with Tukey's Multiple Comparison test, **Fig. 3.3 B**, population data in **Fig. 3.3 F**). In contrast, the application of the > 3kDa fraction resulted in a significantly smaller depolarising shift in membrane potential (mean 1.43 mV, SEM 0.43 mV, N = 8, $p \leq 0.001$, 1-way ANOVA with Tukey's Multiple Comparison test, **Fig. 3.3 C**, population data in **Fig. 3.3 F**). Puff application of PBS (1X, pH 7.4) on its own also resulted in only a very small depolarisation in membrane potential, which was significantly smaller than that of the whole excretory/secretory extracts and the excretory/secretory extracts < 3 kDa (mean 1.10 mV, SEM 0.22 mV, N = 8, $p \leq 0.001$, 1-way ANOVA with Tukey's Multiple Comparison test, **Fig. 3.3 E**, population data in **Fig. 3.3 F**) but did not differ significantly from the membrane potential responses induces by puffs of the culture medium or the excretory/secretory extracts > 3 kDa. Together, this set of experiments demonstrate that *T. crassiceps* excretory/secretory extracts induces a large depolarising membrane effect, and that this effect is largely mediated by a small molecule (< 3 kDa).

3.5 THE EXCITATORY EFFECTS OF *T. CRASSICEPS* EXTRACTS ARE MEDIATED BY GLUTAMATE

Most excitatory signaling in the human nervous system is mediated by the glutamatergic system (Bear et al., 2016). We therefore wanted to establish whether *T. crassiceps* extracts induce their effect by acting on glutamate receptors. To achieve this, Dr Hayley Tomes and I exposed CA3 pyramidal neurons to sweeps of puffs of *T. crassiceps* extracts and recorded the response to these puffs prior to, during, and after the wash in of a glutamate receptor blocker cocktail (containing 50 μM D-AP5, 10 μM CNQX disodium and 2 mM Kynurenic acid). When *T. crassiceps* homogenate was applied prior to the wash in of the glutamate receptor blockers, it predictably induced a large membrane potential depolarization (median 14.72 mV, IQR 13.39 - 15.68 mV). After the glutamate receptor blocker cocktail, however, this response was significantly reduced (median 1.90 mV, IQR 1.09 - 2.21 mV, N = 9, $p \leq 0.01$, Friedman test with Dunn's multiple comparison test, **Fig. 3.4 A & B),** but was restored when the blockade was washed out (median 14.37 mV, IQR 7.32 - 16.38 mV, N = 9, $p \leq 0.01$, Friedman test with Dunn's multiple comparison test, **Fig. 3.4 A & B**)(All experiments for **Fig. 3.4 A & B** were performed by Dr Hayley Tomes).

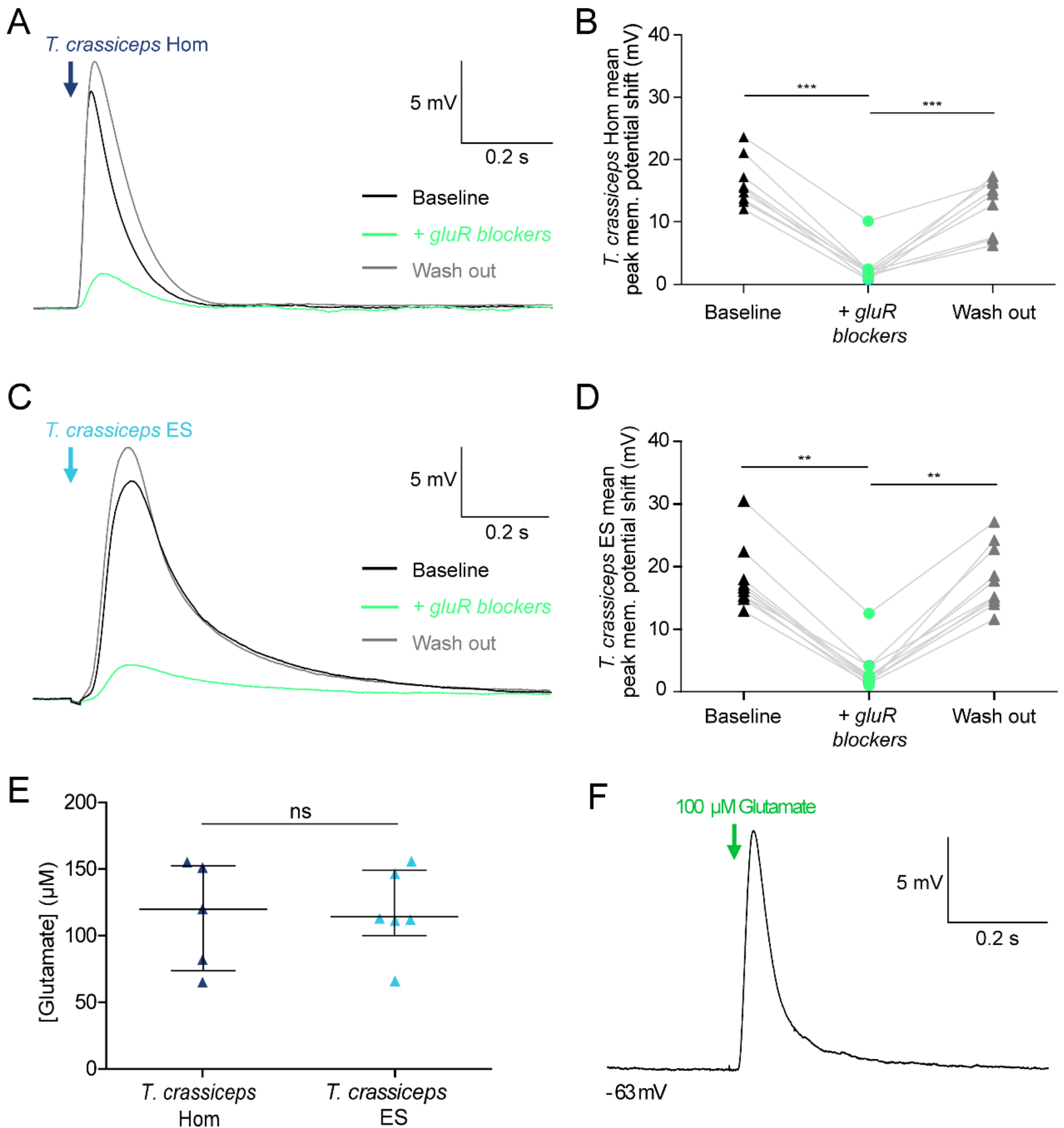


Figure 3.4: The depolarizing effects of *Taenia crassiceps* are mediated by glutamate. A) Whole-cell current-clamp recordings were obtained from rat CA3 pyramidal neurons in hippocampal organotypic slice cultures. Neuronal membrane potential in response to a 20 ms puff of *Taenia crassiceps* (*T. crassiceps)* homogenate (dark blue arrow) before (black trace), during (green trace) and following wash out (grey trace) of a pharmacological cocktail to block glutamate receptors (10 μM cyanquixaline, 50 μM D-AP5, 2 mM kynurenic acid). B) Population data shows that the depolarization response to *T. crassiceps* homogenate is significantly reduced in the presence of glutamate receptor blockers and returns upon wash out (N = 9). C) Neuronal membrane depolarization response to a 20 ms puff of *T. crassiceps* E/S extracts is also markedly reduced during glutamate receptor blockade. D) Population data for glutamate receptor blockade during *T. crassiceps* E/S extracts application shows that the reduction in membrane depolarization resulting from glutamate receptor blockers is statistically significant (N = 10).

E) *T. crassiceps* homogenate (N = 5) and E/S extracts (N = 6) were assayed for glutamate and both extracts were found to have considerable glutamate concentrations. F) Whole-cell current-clamp recording trace (mean response of 10 sweeps) showing that a 20 ms application of a 100 μM glutamate solution induces a neuronal membrane depolarization of similar amplitude to that induced by *T. crassiceps* extracts (N = 1). This implies that the glutamate concentration found in the *T. crassiceps* extracts is sufficient to explain the depolarizing responses that these extracts induce. Values with medians ± IQR; ***$p \le 0.001$; **$p \le 0.01$; ns = not significant, $p > 0.05$; each data point represents a cell.

When *T. crassiceps* excretory/secretory extracts were utilised, a remarkably similar reduction was observed – the median membrane response of 16.35 mV prior to glutamate receptor blockade (IQR 15.17 - 19.03 mV), reduced significantly to 2.19 mV during blockade (IQR 1.67 - 4.16 mV, N = 10, $p \le 0.01$, Friedman test with Dunn's multiple comparison test, **Fig. 3.4 C & D**), and recovered to a median value of 16.43 mV (IQR 14.41 - 23.14 mV) following blockade washout (N = 10, $p \le 0.01$, Friedman test with Dunn's multiple comparison test, **Fig. 3.4 C & D**). This demonstrates that the *T. crassiceps* extracts act on glutamate receptors to induce their large depolarising membrane shifts.

Having established that *T. crassiceps* extracts exert their effect by acting on glutamate receptors, I used a glutamate assay kit to probe whether the *T. crassiceps* extracts contain glutamate, or whether the effect was due to some glutamate agonist. The glutamate assay revealed that, indeed, the *T. crassiceps* homogenate solutions utilised for the puffing experiments had a median glutamate concentration of 120.0 μM (IQR 73.50 – 153.00, N = 5, **Fig. 3.4 E**) and the *T. crassiceps* solutions had a median glutamate concentration of 115.50 μM (IQR 101.3 – 150.8, N = 6, **Fig. 3.4 E**). Statistical analysis found that there was no statistically significant difference between the distributions of the two *T. crassiceps* product's glutamate concentrations (Mann-Whitney test, $p = 1.00$, **Fig. 3.4 E**). We then wanted to assess this concentration of glutamate was sufficient to explain membrane potential shifts induce by the *T. crassiceps* extracts. To do this Dr Hayley Tomes puffed a 100 μM solution of glutamate onto a CA3 pyramidal rat neuron and observed the membrane potential response (10 puffs of 30 ms duration). We observed a depolarising shift of similar magnitude to that induced by the *T. crassiceps* extracts (**Fig. 3.4 F**). This indicated that the membrane potential shifts induced by *T. crassiceps* extracts can largely, perhaps even entirely, be attributed to their glutamate content.

Having established that *T. crassiceps* homogenate contains glutamate, I set out to determine whether the whole cyst homogenate of *Taenia solium* (the species of worm that causes pathology in humans) also contains glutamate, as this could have potential clinical relevance. I was further curious as to which part of the cyst the glutamate was present in; the cyst vesicular fluid or the cyst membrane (for *T. solium* the cyst membrane fraction also includes the larvae's scolex). Glutamate content was expressed relative to the protein concentration of

each solution (as determined using a BCA assay kit), to make the glutamate concentrations comparable between the two species. The glutamate assays revealed *T. crassiceps* homogenate solutions to have a mean glutamate concentration of 6.47 µg/mg total protein (SEM 0.97 µg/mg total protein, N = 5, **Fig. 3.5 A**), whilst the glutamate concentration in *T. solium* homogenate was 4.559 µg/mg total protein (N = 1, **Fig. 3.5 A**). Additionally, *T. crassiceps* cyst vesicular fluid glutamate concentration (1.02 µg/mg total protein, N=1, **Fig. 3.5 A**) and *T. crassiceps* cyst membrane glutamate concentration (1.13 µg/mg total protein, N =1, **Fig. 3.5 A**) were very similar, however, the glutamate concentration of *T. solium* cyst membrane and scolex (10.64 µg/mg total protein, N =1, **Fig. 3.5 A**) was considerably higher than that of the *T. solium* cyst vesicular fluid (3.90 µg/mg total protein, N =1, **Fig. 3.5 A**). \

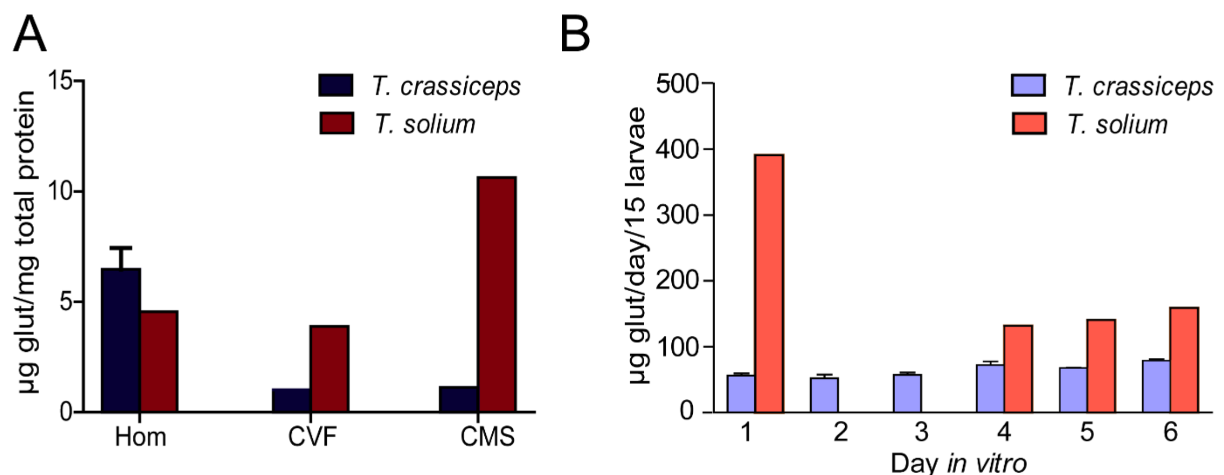


Figure 3.5: *Taenia crassiceps* and *Taenia solium* larvae contain and produce glutamate. A) Glutamate content of *T. crassiceps* whole cyst homogenate (Hom) (N = 5), cyst vesicular fluid (CVF) (N = 1) and cyst membrane (CM) (N = 1) versus *T. solium* Hom (N = 1), CVF (N = 1) and cyst membrane & scolex (CMS) (N = 1). B) *De novo* glutamate production per day per 15 larvae for 6 days of *in vitro* culture of *T. crassiceps* (N = 3) and *T. solium* larvae (N = 1). Values with means ± SEM.

Having previously established that *T. crassiceps* excretory/secretory extracts have a significant glutamate concentration (**Fig. 3.4 E**), I wanted to investigate how glutamate is released from the cyst *in vitro* over several days. I also wanted to establish whether *T. solium* larvae excrete/secrete glutamate and decided to do so over the same period. To achieve this, we cultured *Taenia* larvae in 6 well plates, with 15 larvae in 2 ml of culture medium in each well (I performed the culturing of *T. crassiceps* larvae, whilst Fabien Prodjinotho performed the culturing of *T. solium* larvae). One millilitre of medium was collected and replaced every 24 hours, and the collected medium was utilized for glutamate assessment (using a glutamate assay kit). *De novo* daily glutamate production was estimated by calculating the total amount of glutamate in a well (µg/15 larvae) and subtracting half of the previous day's glutamate content (µg/15 larvae), as this accounts for the glutamate that remained from the previous day's remaining 1 ml of medium.

Glutamate assays revealed that *T. crassiceps* larvae release a consistent quantity of glutamate daily, across a 6-day period **(Fig. 3.5 B**, values in **Table 3**). They further revealed that *T. solium* also release significant quantities of glutamate into the culture medium, but that the pattern of release differs somewhat from that of *T. crassiceps*, with a spike occurring on day 1 *in vitro*, no apparent glutamate being released on days 2 and 3, and then a lower but consistent quantity of glutamate being released over days 4-6 (**Fig. 3.5 B**, values in **Table 3**). This set of results shows that both *T. crassiceps* and *T. solium* larvae contain glutamate, and that they continually release glutamate into their environment over a 6-day period.

Table 3: *De novo* daily glutamate release by *T. solium* and *T. crassiceps* larvae *in vitro.*

Day *in vitro*		**1**	**2**	**3**	**4**	**5**	**6**
		***De novo* glutamate production (µg glut/day/15 larvae)**					
***T. crassiceps* (N = 3)**	**Mean**	55.97	52.13	57.22	72.50	67.71	78.86
	SEM	3.49	5.58	3.14	5.25	0.95	2.26
***T. solium* (N = 1)**	**Mean**	391.11	0.00	0.00	131.87	140.57	158.81

It may also be of interest to note that the concentration of glutamate in the excretory/secretory extracts (in a sample where equal volumes of the excretory/secretory extracts from D1-D3 were combined and then assayed for glutamate and protein concentration) of both *T. crassiceps* and *T. solium* is much higher relative to the total protein content of these solutions (*T. crassiceps* excretory/secretory extracts = 173.0 µg/mg total protein, N =1, and T Solium excretory/secretory extracts = 65.4 µg/mg total protein, N =1) compared to the somatic extracts of these larvae (see **Fig. 3.5A**).

3.6 CHRONIC EXPOSURE TO *TAENIA CRASSICEPS* LARVAE DOES NOT ALTER BRAIN SLICE NETWORK EXCITABILITY

Having established that *Taenia* larvae produce and continually release glutamate, I wanted to explore whether network excitability of hippocampal organotypic brain slices would be altered if chronically exposed to *Taenia* larvae. To do this, I set up a novel model, in which I cultured hippocampal brain slices and *T. crassiceps* larvae together in 6-well plates for a period of 7-9 days. After this period, the network excitability of *T. crassiceps* co-cultured brain slices was assessed by placing them in an interface recording chamber, perfused with aCSF containing 5 mM K^+ to make the slices more excitable, and recording local field potentials from the CA3 region. The excitability of *T. crassiceps* co-cultured slices was compared to a control group of brain slices that were cultured under identical conditions but without *T. crassiceps* larvae. (see **Fig. 3.6 A** for a schematic diagram of experimental set-up).

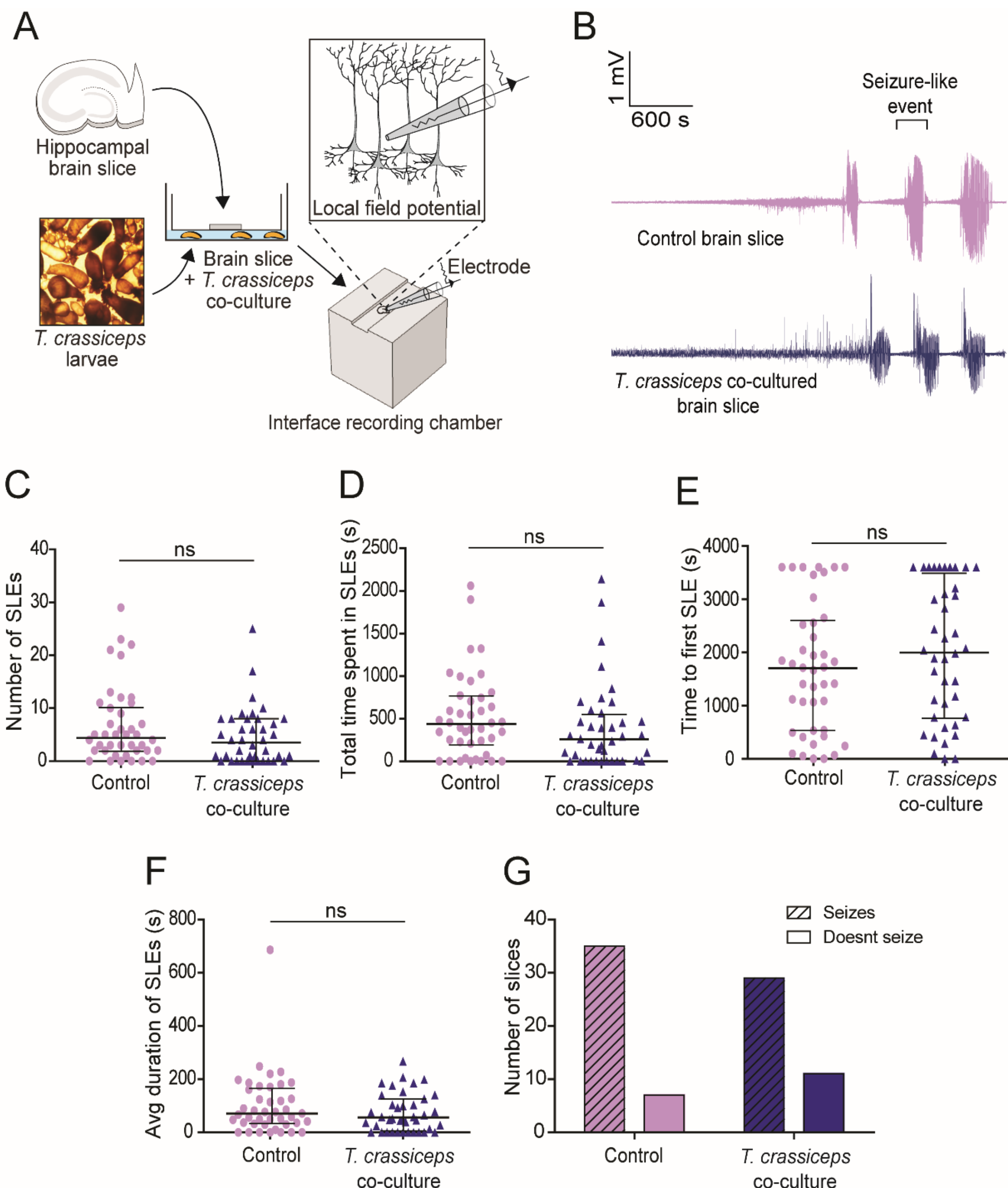


Figure 3.6: Chronic exposure to *Taenia crassiceps* larvae does not alter network excitability. A) Schematic diagram of experimental set up. *Taenia crassiceps (T. crassiceps)* larvae were co-cultured with hippocampal brain slices for 7-9 days and the excitability of the brain slices was then assessed by maintaining them in an interface chamber with 5 mM K^+ aCSF whilst recording local field potentials in the CA3 region using an extracellular microelectrode. B) Traces depicting a typical hour-long recording for a control brain slice (top) and a *T. crassiceps* co-cultured brain slice (bottom) also showing seizure-like events (SLEs). C) Population data depicting that the number of SLEs in control slices (N = 42) and *T. crassiceps* co-cultured brain slices (N = 40) did not differ significantly. D) Population data depicting that the total time that slices spent in a seizure-like state did not differ

significantly between control slices and *T. crassiceps* co-cultured slices. E) Population data depicting that the time to first SLE did not differ significantly between control slices and *T. crassiceps* co-cultured slices. F) Population data depicting that the average duration of SLEs did not differ significantly between control slices and *T. crassiceps* co-cultured slices. G) Graph depicting the number of slices in both the control group and in the *T. crassiceps* co-cultured group that displayed SLEs, versus the number of slices that displayed no SLEs. Values with medians ± IQR; ns = not significant, $p > 0.05$; each data point represents a cell.

The majority of both the *T. crassiceps* co-cultured slices and the control slices underwent multiple seizure-like events (SLEs) during a 60 min recording period, although a slightly larger proportion of the *T. crassiceps* co-cultured slices did not experience any SLEs (7/42 control slices did not seize, 11/40 *T. crassiceps* co-cultured slices did not seize, **Fig. 3.6 B & G**). The median number of SLEs occurring in control slices was 4.50 (IQR 2.00 – 10.25, N = 42), whilst *T. crassiceps* co-cultured slices had a slightly lower median of 3.50 SLEs (IQR 0.00 – 8.00, N = 40), although this did not prove to be statistically significant ($p = 0.19$, Mann Whitney test, **Fig. 3.6 C**). To assess whether the SLEs of one group were of a significantly different length to that of the other, the total time that the slices spent in a seizure-like state, as well as the average seizure duration, were calculated. Control slices spent a median total time of 422.90 s in a seizure-like state (IQR 175.20 – 751.70 s, N = 42), whilst *T. crassiceps* co-cultured slices spent a lower median total time of 250.10 s in a seizure-like state (IQR 0.00 – 540.60 s, N = 40), although this difference was not statistically significant ($p = 0.09$, Mann Whitney test, **Fig. 3.6 D**). The median average duration of SLEs in control slices was 69.47 s (IQR 31.59 – 163.4 s, N = 42) with *T. crassiceps* co-cultured slices having a median average SLE duration that was not significantly lower at 50.84 s (IQR 0.00 – 120.70 s, N = 40, $p = 0.28$, Mann Whitney test, **Fig. 3.6 F**). Finally, I calculated whether there existed a difference between the two groups in the amount of time spent in the 5 mM K^+ aCSF before the occurrence of the first SLE. I found that control slices had a median lag time of 1688.00 s until the occurrence of the first SLE (IQR 522.9 – 2582 s, N = 42), whilst *T. crassiceps* co-cultured brain slices had a slightly longer, but not significantly different, median lag time of 2013.00 s (IQR 779.50 – 3502.00 s, N = 40, $p = 0.21$, Mann Whitney test, **Fig. 3.6 E**). Collectively, these results show that, although there appears to be a trend towards *T. crassiceps* co-cultured slices being slightly less excitable, differences between the *T. crassiceps* co-cultured and control groups did not prove to be of statistical significance. Chronic exposure to *T. crassiceps* larvae therefore does not appear to alter brain slice network excitability.

3.7 **DISCUSSION**

Seizures are the most common symptom of NCC, occurring in up to 90% of symptomatic cases (Carpio and Romo, 2015). The bulk of research in the field of NCC centres around the role of the host immune response in symptom presentation, and little regard has been given to the possibility that parasite-derived extracts may act directly on brain cells. Here, using

patch-clamp electrophysiology, my collaborators and I show that *Taenia* larval extracts cause large depolarising shifts in neuronal membrane potential in hippocampal pyramidal neurons via glutamatergic pathways. In addition, calcium imaging revealed that, given the right conditions, *T. crassiceps* homogenate can induce SLEs in rodent hippocampal organotypic brain slices. These results were unexpected, as, to my knowledge, this is the first time that a direct effect of *Taenia* larval extracts on neurons has been shown. In addition, it challenges the idea that the host inflammatory immune response is solely responsible for seizures secondary to NCC.

Our initial results, depicted in **Figure 3.1**, show that *T. crassiceps* extracts can induce the firing of action potential trains in neurons (**Fig. 3.1 B&C**), and furthermore, that given the correct conditions *T. crassiceps* extracts can result in action potential generation and epileptiform activity in hippocampal brain slices (**Fig. 3.1 E**). This is significant, as it suggests that the larval-derived extracts themselves could, in fact, contribute to the causation of seizures in NCC. This experimental protocol has obvious limitations, however, in that it involves an *in vitro* model as opposed to an *in vivo* one, and in that the application of the larval extracts was not done in a manner that closely resembles the clinical scenario, where whole larvae have extended contact directly with brain tissue.

We next set out to quantify the magnitude to which *T. crassiceps* extracts could affect neurons. We found that both *T. crassiceps* homogenate and *T. crassiceps* excretory/secretory extracts induce large depolarising shifts in neuronal membrane potential. Interestingly, these responses occur within tens of milliseconds of the application of the *T. crassiceps* extracts. In light of this, the later discovery that the depolarising effect of *T. crassiceps* extracts effects are mediated by a small molecule (< 3kDa) (**Fig. 3.2 & Fig. 3.3**) makes sense, as molecules that mediate fast synaptic transmission are typically small ions (Smart and Paoletti, 2012)The lack of large depolarising effects caused by puffing aCSF (**Fig. 3.2 B&E**), culture medium (**Fig. 3.3 D&F**), and PBS (**Fig. 3.3 E&F**) helped to establish that the active component of the *T. crassiceps* extracts originated from the larvae themselves, as opposed to from the solvents utilised. The further lack of a large depolarising effect from BSA (**Fig. 3.2 C&E),** the fraction of *T. crassiceps* homogenate larger than 3 kDa (Fig. 3.2 D&E) and the fraction *T. crassiceps* excretory/secretory extracts larger than 3 kDa (**Fig. 3.3 C&**F) confirm that the effect is predominantly mediated by a small molecule.

The *T. crassiceps* homogenate response, and the *T. crassiceps* excretory/secretory extracts response, show large between-cell variability (**Fig. 3.2 E & Fig. 3.3 F**). This may partly be because whole cyst homogenate from different batches of *T. crassiceps* was utilised, and

these data sets contain recordings from many different neurons in different slices from different animals, all of which may help explain the observed inter-recording variabilities.

Once we established that small molecules drive the depolarising neuronal membrane potential responses to *T. crassiceps* extracts, we investigated whether this small molecule acted on glutamate receptors to exert its effect (**Fig. 3.4**). The reduction in neuronal membrane depolarisation in the presence of a glutamate receptor blockade was dramatic for both *T. crassiceps* homogenate and *T. crassiceps* excretory/secretory extracts, providing convincing evidence that the depolarising effect is exerted via glutamate receptors. The small residual depolarising effect observed in the presence of the glutamate receptor blockade was similar in magnitude to that which is observed when PBS or a 1 mg/ml solution of BSA is applied, and these could perhaps be explained by a disruption of the normal ion/pH homeostasis in the neuronal environment. In both the *T. crassiceps* homogenate (**Fig. 3.4 B**) and the *T. crassiceps* excretory/secretory extracts (**Fig. 3.4 D**) datasets there is one datapoint in which the depolarising effect of the larval extracts was not as effectively blocked by the glutamate receptor blockade as the rest of the neurons in the experimental population. In both datasets the outlying datapoint was one of the strongest responders to the *T. crassiceps* extracts. Perhaps the concentration of glutamate receptor blockade utilised was not sufficient to fully block all the glutamate receptors on these particular cells, or perhaps in these recordings the time given for the blockade solution to wash in and take effect was not sufficient to allow complete permeation of the blockers. Further, it is possible that these cells were slightly deeper within the tissue than the other cells, and that the glutamate receptor blockade solution could not as easily affect these cells. The possibility also exists that these cells responded to an additional excitatory substance in the larval extracts, that did not act via the glutamate receptors, but this seems unlikely, considering that the rest of the population of neurons in these datasets responded very uniformly to glutamate receptor blockade. Had there been an additional excitatory pathway one would expect a larger portion of the population to show a significant residual depolarisation in the presence of the glutamate receptor blockade, and certainly more variability within the responses.

I then went on to quantify the glutamate concentration in *T. crassiceps* extracts and found that *T. crassiceps* homogenate and *T. crassiceps* excretory/secretory extracts contain significant amounts of glutamate (**Fig. 3.4 E**), and that the concentration of glutamate appeared to be sufficient to explain the depolarisation induced by the *T. crassiceps* extracts (**Fig. 3.4 F**). Notably, the glutamate content in different batches of *T. crassiceps* extracts show large variability (**Fig. 3.4 E**). This could be attributed to different batches of the larvae containing/releasing different amounts of glutamate but is more likely a result of experimental variability in the precise larvae: PBS or larvae: medium ratios utilised. In **Figure 3.4 F** we

demonstrate that a 100 μM solution of glutamate is sufficient to induce a large membrane potential depolarisation, a finding corroborated by existing studies (Lu *et al.*, 2019; Swartz and Bean, 1992). The magnitude of the glutamate-induced depolarisation was very similar to that seen in response to *T. crassiceps* extracts, but it should be noted that this finding is limited, as it was only demonstrated in a single neuron, and population data would be required to corroborate this.

Having confirmed the presence of significant amounts of glutamate in *T. crassiceps* extracts, I proceeded to confirm whether the primary human pathogen, *T. solium*, also contains and produces glutamate. In **Figure 3.5 A** I demonstrate that the whole cyst homogenate of *T. solium* contains a similar concentration of glutamate to that of *T. crassiceps*. It should be kept in mind when considering these data that I were able to assay the glutamate concentration in a small population of different homogenates of *T. crassiceps* larvae (N = 5) whilst I only had access to a single batch of *T. solium* homogenate (N = 1). I also explored whether the somatic glutamate of these larvae is located primarily in the cystic fluid or in the cyst membrane (& scolex) (**Fig. 3.5 A**). For *T. crassiceps* it seems that the cyst vesicular fluid and the cyst membrane both contain similar concentrations of glutamate when these are normalised to the total protein content. Strangely, the glutamate content in these two fractions of the larvae is lower than what I report for the *T. crassiceps* homogenate. This can potentially be because I only assayed the cyst vesicular fluid and cyst membrane extracts from one batch of *T. crassiceps* larvae, whilst the reported glutamate concentration of the homogenate is the median measured in 5 batches. In the *T. solium* extracts, I observed a much greater concentration of glutamate (relative to total protein) in the cyst membrane and scolex fraction than in the cyst vesicular fluid fraction. I hypothesise that the difference seen between *T. crassiceps* and *T. solium* somatic glutamate distribution may be due to a strong presence of glutamate in the scolex of *T. solium ((*not present in the *T. crassiceps* samples), perhaps as a part of the parasite's own nervous system. Again, the results reported for the cyst vesicular fluid and cyst membrane of both parasites are from a single sample for each extract and should thus be interpreted with caution.

I next set out to determine whether *T. crassiceps* and *T. solium* larvae continually produce and release glutamate, or whether the glutamate concentration I had found in the excretory/secretory extracts merely represented a sudden release of the somatic glutamate due to a change in environment. I found that the larvae of both species do seem to actively produce glutamate *in vitro*, even though in the case of *T. solium* one does see a sudden release of glutamate within the first day *in vitro*, followed by two days without glutamate release, before the resumption of glutamate release on day 4 (**Fig. 3.5 B**). It should be noted that the actual values of glutamate production in these experiments cannot be reliably

compared between the two species, as the collection of the excretory/secretory extracts from the two species was performed at different times, on different continents and by different researchers, and as such it is difficult to be certain that the experimental protocols were enacted identically. Furthermore, the *T. solium* readings represent a pooled collection of medium from several wells, whilst for the *T. crassiceps* experiment the medium collected from each well was collected and assayed separately.

Having discovered that the *Taenia* larvae release significant amounts of glutamate, I explored whether chronic exposure to the larvae may have any effect on network excitability of hippocampal organotypic slices. To achieve this, I used a novel larvae-brain slice co-culture model (pictured in **Fig. 3.6 A**). My results revealed no statistically significant differences between *Taenia* co-cultured and control brain slices in the number, duration, or time to onset of SLEs (**Fig. 3.6**). There may, however, be a subtle trend toward decreased excitability in the *Taenia* co-cultured slices, with the median number of SLEs, the total time spent in a seizure-like state, and the average duration of SLEs all being slightly lower in the *T. crassiceps* co-cultured slice populations than in the control population. Additionally, the delay to the onset of SLEs and the total number of slices that had no seizure like events are slightly higher.

After some consideration, I identified several limitations in the experimental set-up which could potentially explain why I saw no significant differences between the two groups. The first is that I only added between 3 and 5 larvae to 1.2 ml of culture medium. This larvae:medium ratio is much lower than that which I used to create the excretory/secretory extracts in which I measured glutamate concentration, and as such the glutamate concentration in the brain slice culture medium was likely significantly lower, and perhaps not sufficient to exert any obvious effects. In the clinical setting the larval cysts are typically lodged in the brain parenchyma, meaning that they are in direct contact with brain tissue, and thereby much more likely to exert an effect on neuronal networks than they are in my co-culture model. Additionally, clinical NCC frequently presents with an extended asymptomatic period before symptom/seizure onset, which implies that the brain can co-exist with the viable larvae for an extended period without displaying obvious network hyperexcitability. My experimental model may therefore be flawed in its use of viable *T. crassiceps* larvae and could perhaps benefit from adding dead or degrading larvae.

Another confounding factor in this experimental set-up is that the brain slices are exposed to all extracts excreted/secreted by viable larvae, making it difficult to isolate the effects of individual molecules. Viable *Taenia* larvae are, for example, known to employ strategies which shift the host immune system to a permissive, anti-inflammatory state (Flores-bautista *et al.*, 2018; White, Jr., 2000). Neuroinflammation is increasingly being linked with seizures and

epilepsy, and as such this shift toward an anti-inflammatory environment might explain the subtle trend toward reduced brain slice excitability in *T. crassiceps* co-cultured brain slices noted above. It is only when the larval cysts lose viability that symptoms/seizure onset commonly occurs, and as such it is perhaps not entirely surprising that brain slices show no shift towards greater neuronal network excitability in the presence of viable larvae. The release of AChE enzymes is one strategy thought to be used by helminthic parasites to modulate the host immune response to the parasite, but it has not yet been determined whether *Taenia* larvae utilise this strategy (Darby *et al.*, 2015; Tedla *et al.*, 2019; Vaux *et al.*, 2016). I therefore set out in Chapter 4 to determine the production, localisation, and activity of AChEs in *T. crassiceps* and *T. solium* larvae.

Chapter 4

TAENIA CRASSICEPS AND *TAENIA SOLIUM* LARVAE HAVE ACETYLCHOLINESTERASE ACTIVITY WHICH MAY DISRUPT HOST CHOLINERGIC SIGNALING.

4.1 INTRODUCTION

In the previous chapter I established, with the assistance of collaborators, that *Taenia* larval extracts contain molecules that act directly on neurons in a way that is likely to promote seizures. However, the complex nature of seizures in neurocysticercosis (NCC) suggests that there are likely multiple contributing factors. As such, I next decided to explore the possibility that *Taenia* larvae may produce substances that act indirectly on neurons by affecting the availability of an endogenous neurotransmitter. I came to be interested in the possibility that *Taenia* larvae may release enzymes called acetylcholinesterases (AChEs).

Acetylcholinesterases are a family of enzymes which catalyse the breakdown of acetylcholine, and are thought to be used by many helminthic parasites in the modulation of host responses (Darby *et al.* 2015; Tedla *et al.* 2019; Vaux *et al.* 2016). Helminths widely express membrane-bound forms of AChEs, which are classically associated with the facilitation of rapid acetylcholine signaling to parasite muscle, as well as sensory and neural structures (Leflore and Smith, 1976; M E Selkirk *et al.*, 2005). Some helminths also produce surface-presenting membrane-bound AChEs (Schwabe, Koussa, and Acra 1961; Espinoza *et al.* 1991; Gimenez-Pardo *et al.* 2004; Camacho *et al.* 1994). Some helminths can actively excrete/secrete AChEs, which may modulate acetylcholine dependent components of the host immune response, play a role in the detoxification of ingested cholinesterase inhibitors, or inhibit smooth muscle contraction and mucus and fluid secretion associated with clearance of intestinal parasites (Vaux *et al.* 2016; M E Selkirk, Lazari, and Matthews 2005; Tedla *et al.* 2019; Darby *et al.* 2015).

Acetylcholine is also a major neurotransmitter in the human brain, with powerful effects on the excitability of cortical circuits (Colangelo *et al.*, 2019; Friedman *et al.*, 2007). It is a critical component of multiple brain systems that are responsible for functions such as attention, learning, memory, sleep and motor activity (Gotti *et al.*, 2006; Picciotto *et al.*, 2012). Disruption of cholinergic signaling is well known to lead to seizures; for instance mutations of the nicotinic acetylcholine receptor underlies a heritable form of epilepsy (Raggenbass and Bertrand,

2002), and pilocarpine (an acetylcholine muscarinic receptor agonist) is a well described proconvulsant (Curia *et al.*, 2008). Furthermore, blockade of endogenous brain AChEs by organophosphate pesticides or poisons can also lead to seizures (Cordner *et al.*, 1986; Tattersall, 2009).

Since *T. solium* larvae invade the central nervous system in NCC, it is important to determine potential AChE activity expressed by these larvae, as such activity could conceivably interfere with endogenous cholinergic signaling in the brain, by breaking acetylcholine down into neurologically inactive products (Soreq, 2001). *Taenia crassiceps* (*T. crassiceps*) is widely utilised as a model parasite for *T. solium* in animal models of cysticercosis and NCC, and has also been known on rare occasions to invade the human nervous system. (de Lange *et al.*, 2018b; Ntoukas *et al.*, 2013). It is therefore also important to ascertain how AChE activity might compare between the larvae of these two *Taenia* species.

The presence of AChEs has been reported in several members of the broader *Taeniidae* family, both in the adult forms (Eranko *et al.*, 1968; Lee *et al.*, 1963; Shield, 1969) and in larval stages (Cumino *et al.*, 2012; Gimenez-Pardo *et al.*, 2004, 2000; Leflore and Smith, 1976; Schwabe *et al.*, 1961). The presence of AChEs in metacestodes of *Echinococcus granulosus* is particularly noteworthy, as these are known to infect the nervous system of children (Ciurea *et al.*, 2006). The AChEs are often associated with the neural structures and tegument of Taeniid*s*, and there is also the suggestion that some larvae may release AChEs into the host environment (Cumino *et al.*, 2012). Studies describing cholinesterases in *T. crassiceps* larvae are scarce, with only one report of AChEs in the bladder wall of *T. crassiceps* (Koziol *et al.*, 2013), and one other study which refers to the presence of “unidentified esterases” in the cystic fluid of *T. crassiceps* (Trejo-Chávez *et al.*, 2011).

The genome of *T. solium* has recently been sequenced (Tsai *et al.*, 2013) and bioinformatics have revealed three *T. solium* AChE homologs (Tedla *et al.*, 2019). Whether the AChEs are expressed during the larval stage of *T. solium* is not yet clear, nor where they may be located or what the activity of these enzymes may be. A histological study by Vasantha *et al.* (Vasantha *et al.*, 1992) performed on *T. solium* larvae demonstrated AChE staining in neural structures, but no obvious staining of AChEs on the surface of the larvae, apart from positive staining of a few surface nerve endings. One other report of cholinesterase activity in *T. solium* larvae found cholinesterase activity to be predominantly present in the isolated cyst bladder (Martínez-Zedillo and González-Barranco, D González-Angulo, 1983; cited in Parija and Ar, 2011).

Considering the scarcity of knowledge of how AChEs function in *T. crassiceps* and *T. solium* larvae, I set out to perform a detailed characterization of AChE activity in the larvae of these

species. To do so, I employed Ellman's activity assays as well as AChE activity stains. I found that both the larvae of *T. crassiceps* and *T. solium* contain significant AChE activity, but it is broadly lower in *T. solium* as compared to *T. crassiceps* larvae. In addition, whilst AChEs were present in all fractions of *T. crassiceps* larvae, including the membrane surface and excreted/secreted extracts, I could not identify AChEs on the membrane surface or within the excreted/secreted extracts of *T. solium* larvae. I further wanted to investigate whether larval derived AChEs could conceivably disrupt host neuronal cholinergic signaling. Utilising whole-cell patch clamp recordings in rodent hippocampal brain slice cultures I demonstrate that *Taenia* larval derived AChEs can break down acetylcholine at a concentration which induces changes in neuronal signaling.

4.2 *TAENIA CRASSICEPS* AND *TAENIA SOLIUM LARVAL* EXTRACTS HAVE ACETYLCHOLINESTERASE ACTIVITY.

In order to quantify AChE activity in *T. crassiceps and T. solium* larvae, Ellman's assays were employed, using acetylthiocholine as a substrate (Ellman *et al.*, 1961). These assays revealed that all *T. crassiceps and T. solium* larval extracts had significant AChE activity, with the exception of *T. solium* excretory/secretory extracts (**Fig. 4.1 A&C, Table 4.1**) Kruskal Wallis one-way ANOVAs with post hoc Dunn's multiple comparison post-hoc test revealed that the only statistically significant difference between the median activities of the different *T. crassiceps* larval extracts was between that of the cyst vesicular fluid and the excretory/secretory extracts ($p \leq 0.01$, **Fig. 4.1 A, Table 4.1**), and amongst the *T. solium* extracts the cyst membrane and scolex had a statistically significantly higher activity than that of the whole cyst homogenate and the cyst vesicular fluid ($p \leq 0.01$, **Fig. 4.1 C, Table 4.1**)., whilst the median activity of two latter extracts did not differ significantly from one another ($p > 0.05$, **Fig. 4.1 C, Table 4.1**).

Table 4.1: Acetylcholinesterase (AChE) activity of different larval extracts of *Taenia crassiceps* and *Taenia solium.*

Larval species	Larval extract	# of assays	Median AChE activity (nmol min^{-1} mg^{-1})
	Whole cyst homogenate	10	31.7 (IQR 24.0 – 58.3)
Taenia crassiceps	Cyst membrane	10	39.7 (IQR 36.1 – 46.3)
	Cyst vesicular fluid	10	51.5 (IQR 50.9 – 54.1)
	Excretory/secretory extracts	10	31.8 (IQR 50.9 – 54.1)
	Whole cyst homogenate	5	4.1 (IQR 4.1 – 5.1)
Taenia solium	Cyst membrane & scolex	5	14.0 (IQR 13.8 – 15.3)
	Cyst vesicular fluid	5	4.7 (IQR 3.37 – 5.03)
	Excretory/secretory extracts	4	Undetectable

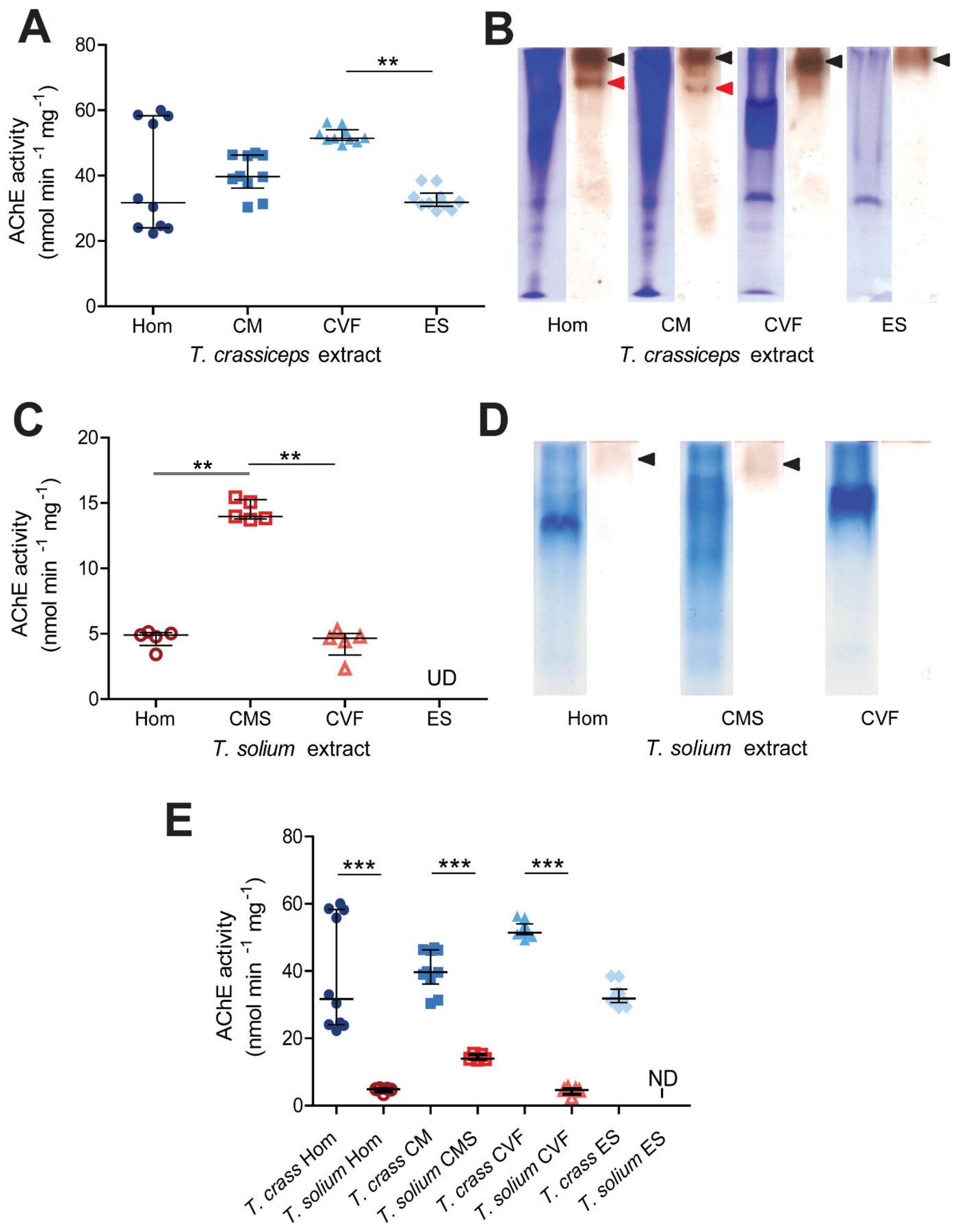


Figure 4.1: Acetylcholinesterase activity in extracts of *Taenia crassiceps* and *Taenia solium* larvae. A) Quantification of acetylcholinesterase (AChE) activity in different *Taenia crassiceps* larval extracts. Acetylcholinesterase activity was quantified using the method of Ellman *et al.* (Ellman *et al.*, 1961) with 1 mM acetylthiocholine iodide as substrate in the presence of 1 mM 5,5'-dithiobis(2-nitrobenzoic acid) in 100 mM sodium phosphate, pH 7.0, at room temperature. The extracts assessed were: whole cyst homogenate (Hom); cyst membrane (CM); cyst vesicular fluid (CVF); and larval excretory/secretory extracts (ES). Values with median ± IQR, N = 10 for all extracts assayed, $^{**}p \leq 0.01$, Kruskal-Wallis test with Dunn's multiple comparison post-hoc test

B) Non-denaturing polyacrylamide gel electrophoresis of *Taenia crassiceps* extracts. Extracts were electrophoresed in Tris-glycine buffer, pH 8.3, through 7.5% polyacrylamide gels in the absence of denaturing and reducing agents. Coomassie staining was performed on one set of gels (left tracks), and staining for AChE activity (Karnovsky and Roots, 1964) was performed on another set of gels for 16 hours following addition of the substrate (right tracks). The maximum volume of each extract was loaded (20 µl), to ensure maximal staining. Protein concentrations of the different extracts varied: Hom = 1.9 mg ml^{-1}, CM = 3.4 mg ml^{-1}, CVF = 3.0 mg ml^{-1} and ES = 1.32 mg ml^{-1}**.** C) Quantification of AChE activity in different *Taenia solium* extracts. Activity assays were performed as described for "A". The extracts assessed were: Hom; cyst membrane and scolex (CMS); CVF; and ES. Values with median ± IQR, N = 5 for all extracts assayed, $^{**}p \leq 0.01$, Kruskal-Wallis test with Dunn's multiple comparison post-hoc test D) Non-denaturing polyacrylamide gel electrophoresis of different *Taenia solium* extracts, as described for *Taenia crassiceps* in "B". The maximum volume of each extract was loaded (20 µl), to ensure maximal staining. Protein concentrations of all extracts was 1.5 mg/ml. E) Statistical comparison of AChE activity in larval extracts of *Taenia crassiceps* (A) versus *Taenia solium* (C). $^{***}p \leq 0.001$, Mann-Whitney U tests.

To visually confirm *T. crassiceps* and *T. solium* AChE activity, and to assess whether there may be more than one AChE isoform in these larval extracts, non-denaturing PAGE gels were run, and stained for AChE. A second set of non-denaturing PAGE gels were run simultaneously and Coomassie stained. The Coomassie stains demonstrated that the different larval extracts contained different protein compositions (left-hand tracks in **Fig. 4.1 B&D**). The AChE-stained gels (right-hand track for each larval extract in **Fig. 4.1 B&D**) showed distinct dark bands (indicated by black arrowheads) in the tracks of all the *T. crassiceps* larval extracts (**Fig. 4.1 B)**, thereby confirming that all the *T. crassiceps* larval extracts show AChE activity. In the whole cyst homogenate and the cyst membrane tracks of the *T. crassiceps* AChE-stained gels, there was an additional smaller band (indicated by the red arrowheads in **Fig. 4.1 B**). These results suggest that *T. crassiceps* larvae express more than one isoform of AChE. The AChE stained gels on which *T. solium* extracts were run revealed bands in the whole cyst homogenate and in the cyst membrane and scolex preparations (indicated by black arrowheads in **Fig. 4.1 D**), but no apparent band in the cyst vesicular fluid track, The absence of bands in the cyst vesicular fluid tract, and the relatively faint bands in the tracks of the other extracts can be attributed to the fact that the amount and concentrations of the extracts loaded contain a drastically lower total AChE activity than is usually recommended for this technique (Selkirk and Hussein, 2000). In future investigations this could be solved by concentration or purification of the AChEs in *T. solium* extracts.

Comparison of the AChE activity of *Taenia crassiceps* extracts with the corresponding *Taenia solium* extracts using Mann-Whitney U tests, revealed that *T. crassiceps* larval extracts uniformly have greater AChE activity than the corresponding *T. solium* larval extract ($p \leq 0.001$) (**Fig. 4.1 E, Table 4.1**). It further highlights that *T. crassiceps* larvae excrete/secrete AChEs, whilst *T. solium* do not appear to (**Fig. 4.1 E, Table 4.1**). Additionally, the comparison of AChE activity in *T. crassiceps* versus *T. solium* larval extracts also reveals a different pattern of AChE distribution within the cyst – *T. solium* AChEs appear to be predominantly located in the cyst membrane and scolex, whilst *T. crassiceps* AChEs appear abundant in both the cyst membrane and the cyst vesicular fluid and are additionally excreted/secreted (**Fig. 4.1 E**).

4.3 Inhibitor sensitivities of *Taenia crassiceps* and *Taenia solium* larval extracts.

The sensitivity of AChE activity in *T. crassiceps* and *T. solium* larval extracts to different inhibitors was tested by preincubating them for 20 min with different concentrations of BW 284c51 (a selective AChE inhibitor), iso-OMPA (a selective butyryl cholinesterase inhibitor) or eserine salicylate (a nonselective cholinesterase inhibitor) before assaying AChE activity (Ellman *et al.*, 1961). All *T. crassiceps* extracts showed a similar dose-dependent inhibitory response to BW 284c51, with AChE activity in all extracts being almost completely inhibited by the presence of 1000 µM BW 284c51 (**Fig. 4.2 A, Table 4.2**). Conversely, the AChE activity of *T. crassiceps* extracts was not greatly inhibited by iso-OMPA, with only small reductions in activity being observed even at high (1 mM) inhibitor concentration (**Fig 4.2 A, Table 4.2**). Acetylcholinesterase activity in *T. crassiceps* cyst membrane, cyst vesicular fluid and excretory/secretory extracts was highly sensitive to eserine inhibition, with strong inhibition apparent at low (1 µM) eserine concentration (**Fig. 4.2 A, Table 4.2**). The AChE activity of *T. crassiceps* whole cyst homogenate was less sensitive to eserine inhibition, only displaying strong inhibition at a much higher eserine concentration (100 µM) (**Fig. 42 A, Table 4.2**). These inhibition patterns suggest that the enzymes produced by *T. crassiceps* larvae can be classified as true AChEs, as opposed to pseudocholinesterases (Austin and Berry, 1953).

The sensitivity of AChE activity in *T. solium* larval extracts differed somewhat from those of *T. crassiceps*. All *T. solium* extracts showed a similar dose-dependent inhibitory response to BW 284c51, although the *T. solium* whole cyst homogenate appeared somewhat less sensitive to inhibition when compared to the cyst membrane and scolex and cyst vesicular fluid (**Fig. 4.2 B, Table 4.3**). *T. solium* extracts showed low sensitivity to inhibition by iso-OMPA (**Fig. 4.2 B, Table 4.3**). *T. solium* whole cyst homogenate, cyst membrane and scolex, and cyst vesicular fluid showed very variable sensitivities to inhibition by increasing concentrations of eserine but were ultimately all strongly inhibited at an eserine concentration of 1 mM or less (**Fig. 4.2 B, Table 4.3**). These inhibition patterns suggest that the cholinesterases produced by *T. solium* larvae can be classified as true AChEs, although the fact that some inhibition is displayed at high iso-OMPA concentrations may suggest a small pseudocholinesterase component (Austin and Berry, 1953).

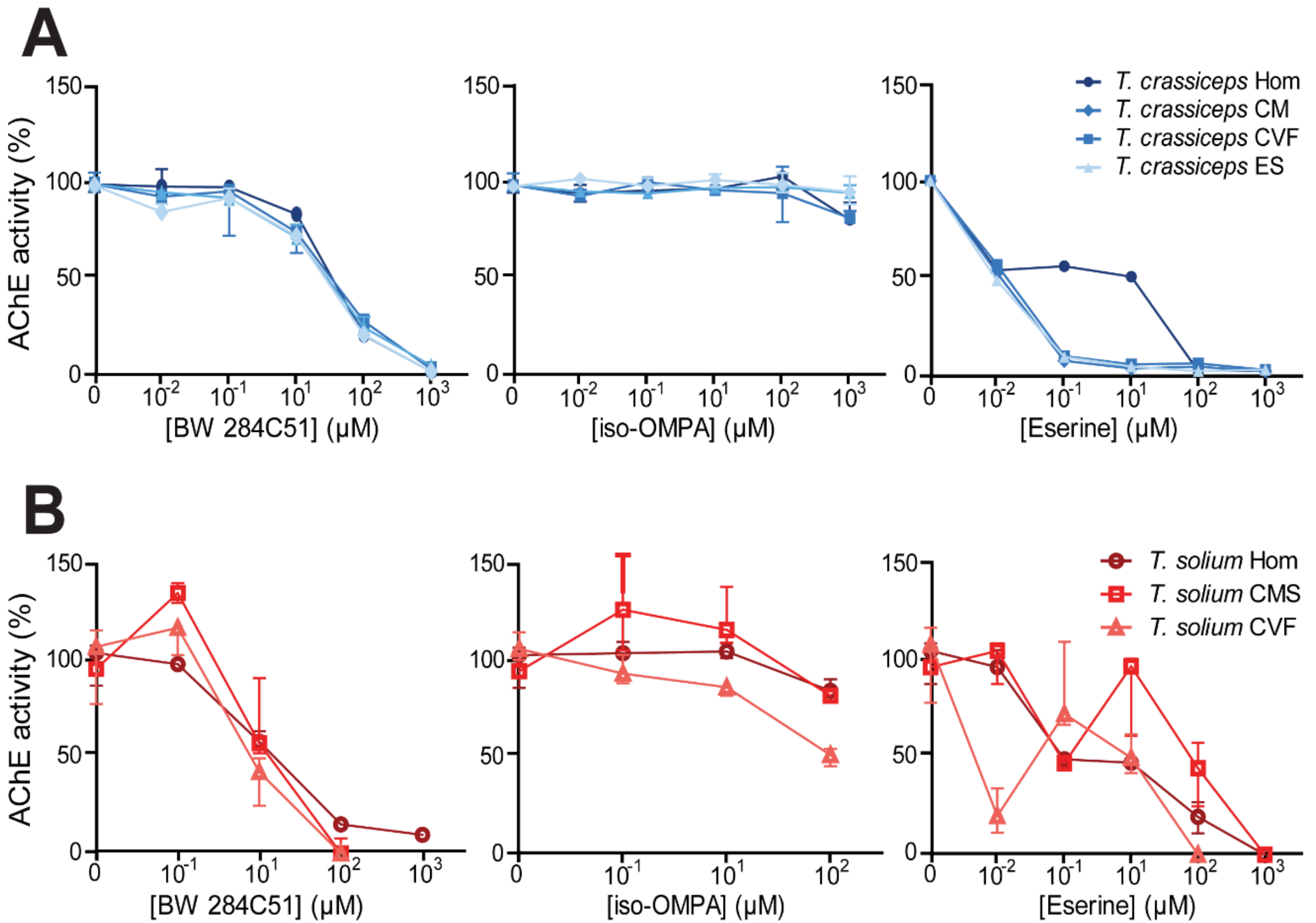


Figure 4.2: Inhibitor sensitivity of *Taenia crassiceps* and *Taenia solium* acetylcholinesterases. A) Inhibitor sensitivity of *Taenia crassiceps* larval acetylcholinesterase (AChE) activity. Extracts were preincubated with BW 284C51, iso-OMPA or eserine salicylate for 20 min at room temperature in Ellman buffer, prior to the addition of 1 mM acetylthiocholine iodide and enzyme activity determination. Median ± Range, N = 10 for all extracts in the absence of inhibitors, N = 3 for all extracts at all inhibitor concentrations. B) Inhibitor sensitivity of *Taenia solium* larval AChE activity. Inhibitor sensitivity was determined as described for *Taenia crassiceps* in A. Median ± Range, N = 5 for all *Taenia solium* extracts in absence of inhibitors and at 10 μM inhibitor concentration, N = 3 for all extracts at all other inhibitor concentrations.

Table 4.2: Sensitivity of *T. crassiceps* cholinesterase activity in various extracts to different inhibitors.

Inhibitor	Extract		Inhibitor concentration (µM)					
			Uninhibited	10^{-2}	10^{-1}	10^{1}	10^{2}	10^{3}
			Cholinesterase activity (% of mean uninhibited)					
BW284C51	Whole cyst homogenate	Median	100.4	99.4	98.9	84.8	21.3	2.2
		Range	96.0 – 104.0	95.1 – 108.3	98.8 – 101.7	83.9 – 85.6	18.8 – 22.8	1.3 – 4.1
		# of assays	10	3	3	3	3	3
	Cyst membrane	Median	100.5	94.0	96.8	75.3	28.7	3.6
		Range	81.6 – 109.2	93.2 – 95.3	73.4 – 97.0	64.5 – 78.9	28.4 – 30.1	2.9 – 4.3
		# of assays	10	3	3	3	3	3
	Cyst vesicular fluid	Median	100.5	96.3	93.2	72.6	25.5	5.2
		Range	97.0 – 101.4	94.3 – 96.8	92.4 – 93.4	72.5 – 79.2	25.2 – 30.8	5.1 – 5.3
		# of assays	10	3	3	3	3	3
	Excretory/ secretory extracts	Median	99.7	85.9	93.3	73.2	21.5	2.2
		Range	97.6 – 105.1	85.6 – 90.1	93.1 – 98.3	70.6 – 75.7	19.5 – 21.8	UD – 4.4
		# of assays	10	3	3	3	3	3
Iso-OMPA	Whole cyst homogenate	Median	100.4	96.2	97.5	98.4	105.0	82.3
		Range	96.0 – 104.0	91.7 – 100.4	91.2 – 101.0	96.6 – 101.2	94.1 – 113.9	19.1 – 100.8
		# of assays	10	3	5	3	5	5
	Cyst membrane	Median	100.5	94.6	101.9	97.8	96.2	83.2
		Range	81.6 – 109.2	94.1 – 98.0	92.5 – 107.2	97.1 – 100.2	75.0 – 116.9	80.2 – 91.5
		# of assays	10	3	5	3	5	5
	Cyst vesicular fluid	Median	100.5	97.0	96.1	98.9	99.3	96.3
		Range	97.0 – 101.4	96.4 – 97.4	95.0 – 97.5	98.0 – 99.1	97.3 – 101.3	89.4 – 103.0
		# of assays	10	3	5	3	5	5
	Excretory/ secretory extracts	Median	99.7	103.7	99.9	103.1	100.6	96.8
		Range	97.5 – 105.1	102.9 – 105.7	96.7 – 104.3	99.5 – 106.0	95.0 – 111.2	87.3 – 106.6
		# of assays	10	3	5	3	5	5
Eserine	Whole cyst homogenate	Median	100.4	52.5	55.4	49.6	UD	UD
		Range	96.0 – 104.0	52.1 – 54.4	53.9 – 56.4	49.1 – 50.2	UD	UD
		# of assays	10	3	3	3	3	3
	Cyst membrane	Median	100.5	52.1	5.8	1.8	2.1	UD
		Range	81.6 – 109.2	49.3 – 53.7	4.7 – 6.2	0.8 – 2.1	1.8 – 3.2	UD
		# of assays	10	3	3	3	3	3
	Cyst vesicular fluid	Median	100.5	56.4	8.1	3.8	4.1	0.7
		Range	97.0 – 101.4	54.8 – 56.4	6.8 – 9.3	2.4 – 4.9	3.8 – 4.6	0.5 – 1.3
		# of assays	10	3	3	3	3	3
	Excretory/ secretory extracts	Median	99.7	47.3	8.5	2.8	UD	0.4
		Range	97.5 – 105.1	46.3 – 49.5	5.3 – 8.8	0.7 – 4.2	UD	0.3 – 2.1
		# of assays	10	3	3	3	3	3

UD = undetectable, where activity was so low as to be undetectable.

Table 4.3: Sensitivity of *T. solium* cholinesterase activity in various extracts to different inhibitors.

			Inhibitor concentration (μM)					
			Uninhibited	**10^{-2}**	**10^{-1}**	**10^{1}**	**10^{2}**	**10^{3}**
			Cholinesterase activity (% of mean uninhibited)					
BW284C51	Whole cyst homogenate	Median	105.3	-	99.5	58.7	15.3	9.9
		Range	73.7 – 110.5	-	97.8 – 102.0	55.3 – 66.3	12.8 – 17.9	8.2 – 9.9
		# of assays	5	-	3	5	3	3
	Cyst membrane & scolex	Median	96.9	-	136.8	57.9	0.0	-
		Range	95.2 – 107.1	-	131.6 – 142.1	52.6 – 97.4	0.0 – 7.9	-
		# of assays	5	-	3	5	3	-
	Cyst vesicular fluid	Median	108.6	-	118.6	42.9	0.0	-
		Range	54.3 – 122.9	-	104.3 – 121.4	21.4 – 50.0	0.0 – 2.9	-
		# of assays	5	-	3	5	3	-
Iso-OMPA	Whole cyst homogenate	Median	105.3	-	106.3	107.1	86.7	-
		Range	73.7 – 110.5	-	103.7 – 112.2	100.3 – 113.1	85.8 – 92.7	-
		# of assays	5	-	3	5	3	-
	Cyst membrane & scolex	Median	96.9	-	128.9	118.4	84.2	-
		Range	95.2 – 107.1	-	107.9 – 152.6	107.9 – 152.6	84.2 – 86.8	-
		# of assays	5	-	3	5	3	-
	Cyst vesicular fluid	Median	108.6	-	108.6	95.7	88.6	-
		Range	54.3 – 122.9	-	102.9 – 122.9	85.7 – 112.9	82.9 – 90.0	-
		# of assays	5	-	3	5	3	-
Eserine	Whole cyst homogenate	Median	105.3	96.9	49.3	47.6	19.6	UD
		Range	73.7 – 110.5	95.2 – 107.1	49.3 – 51.0	46.8 – 50.2	9.4 – 28.1	UD
		# of assays	5	5	3	3	5	3
	Cyst membrane & scolex	Median	96.9	105.3	47.4	97.4	44.7	UD
		Range	95.2 – 107.1	73.7 – 110.5	44.7 – 47.4	60.0 – 102.6	7.9 – 63.2	UD
		# of assays	5	5	3	3	5	3
	Cyst vesicular fluid	Median	108.6	20.0	72.8	50.0	UD	-
		Range	54.3 – 122.9	11.4 – 34.3	67.1 – 110.0	38.6 – 64.3	UD	-
		# of assays	5	5	3	3	5	-

UD = undetectable, where activity was so low as to be undetectable.

4.4 LOCALISATION OF *TAENIA CRASSICEPS* AND *TAENIA SOLIUM* ACETYLCHOLINESTERASES IN THE LARVAE.

To spatially localise AChEs within *T. crassiceps* and *T. solium* larvae, both cross-sections of larvae and whole larvae were subjected to AChE activity staining (Karnovsky and Roots, 1964), (**Fig. 4.3**). To evaluate non-specific staining, a second set of larval cross-sections and whole larvae were subjected to the same staining procedure, with the exception that the substrate (acetylthiocholine iodide) was omitted. In the case of *T. crassiceps*, samples where the substrate was omitted (**top row, Fig. 4.3 A**) showed minimal staining. In contrast, cross sections stained for AChE activity displayed dense, uniform staining, indicating that AChEs are localised ubiquitously throughout the tegument membrane (**bottom-left panel, Fig. 4.3 A**). *T. crassiceps* whole larvae stained for AChE showed light surface staining at low magnification (**bottom-center panel, Fig. 4.3 A**), and high magnification revealed that the staining was localised to numerous small protrusions on the surface of the cyst tegument membrane (**bottom-right panel, Fig. 4.3 A**). Cross-sections of *T. solium* larvae where the substrate was omitted, to evaluate non-specific staining (**top row, Fig. 4.3 B**), showed some patchy black background staining. Acetylcholinesterase-stained cross-sections and whole mounts showed light, uniform, brown AChE staining throughout the tegument membrane (in addition to the black background staining) (**bottom-left and bottom-center panel, Fig. 4.3 B**). High magnification images of the surface of the tegument membrane in whole-mounted AChE-stained larvae revealed that, unlike in *T. crassiceps*, AChEs in the tegument membrane of *T. solium* are not surface-presenting (**bottom-right panel, Fig, 4.3 B**).

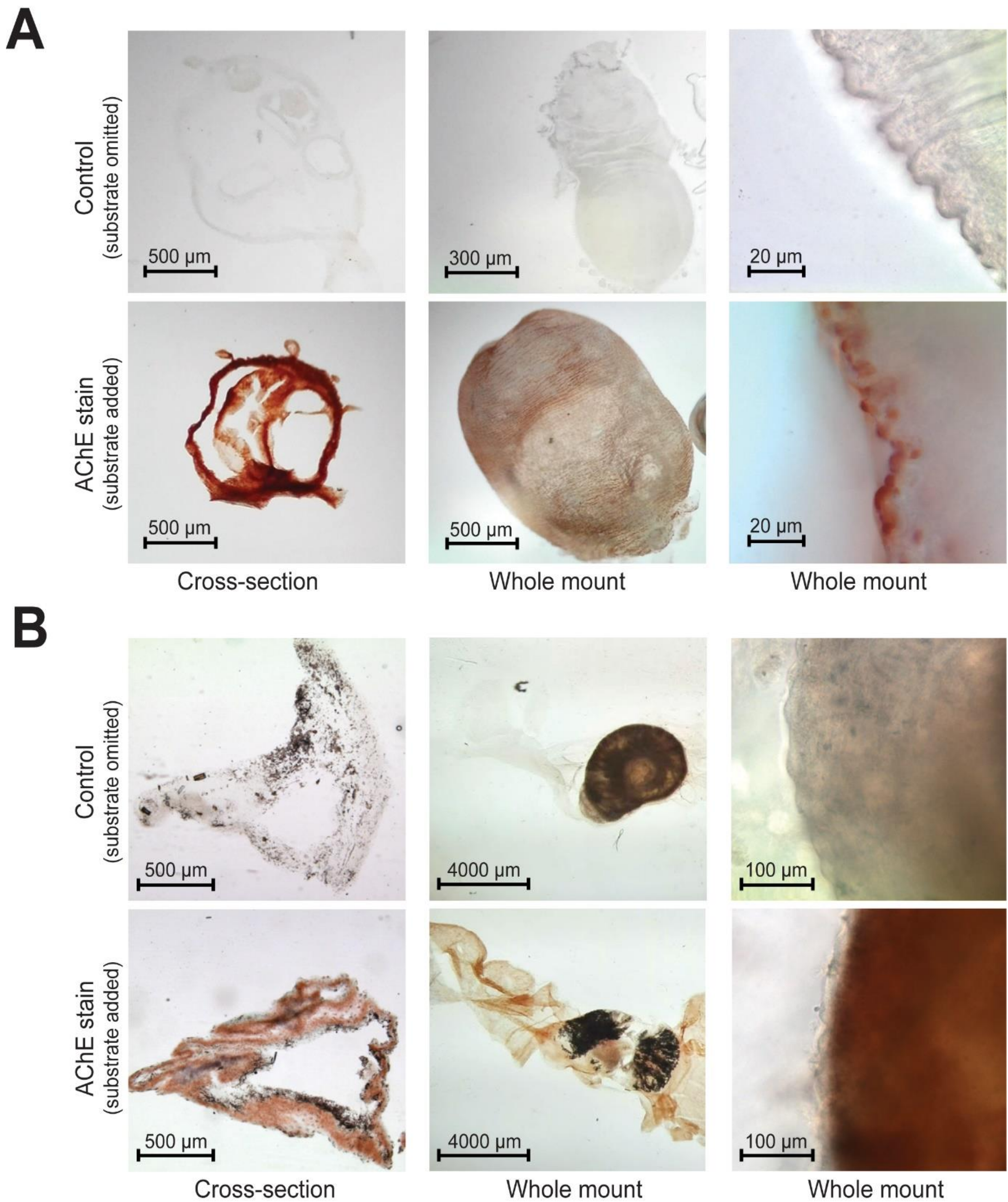


Figure 4.3: Localisation of *Taenia crassiceps* and *Taenia solium* larval acetylcholinesterases. A) Localisation of *Taenia crassiceps* larval AChEs. Cryo-sections (N = 19 for control and N = 19 for AChE stain) and whole mounts (N = 15 for control and N = 15 for AChE stain) of *Taenia crassiceps* larvae were subjected to AChE activity staining (Karnovsky and Roots, 1964) for 16 hrs prior to dehydration and mounting. Images in the top row show time-matched controls for non-specific staining, where acetylthiocholine iodide was omitted from the staining solution. Cross sections were imaged at 5X magnification and whole mounts were imaged at 100X magnification. B) Localisation of *Taenia solium* larval AChEs. Cryo-sections (N = 20 for control and N = 20 for AChE stain) and whole mounts (N = 4 for control and N = 4 for AChE stain) of *Taenia solium* larvae were stained as described for *Taenia crassiceps* in "A". Cross sections were imaged at 5X magnification and whole mounts were imaged at 40X magnification.

4.5 *TAENIA* LARVAL ACETYLCHOLINESTERASES HAVE SUFFICIENT ACTIVITY TO BREAK DOWN ACETYLCHOLINE AT A CONCENTRATION WHICH INDUCES CHANGES IN NEURONAL SIGNALING IN AN *IN VITRO* BRAIN SLICE MODEL.

In order to investigate what implications the presence of *Taenia* larval AChEs may have in the context of NCC, 200 µM acetylcholine (known to induce depolarisation in hippocampal pyramidal neurons (Cole and Nicoll, 1984) was applied to neurons in hippocampal organotypic cultures, either on its own or together with either heat-inactivated *T. crassiceps* excretory/secretory extracts, or active *T. crassiceps* excretory/secretory extracts. For this investigation I elected to use *T. crassiceps* excretory/secretory extracts > 3 kDa, because I had previously established that this filtered version of the excretory/secretory extracts does not induce action potentials when applied on its own (see Chapter 1). The response of the membrane potential of the neurons was measured using whole-cell patch-clamp recordings (**Fig. 4.4 A, B**). When neurons were held at a voltage close to their action potential threshold and a picolitre volume of 200 µM acetylcholine with heat-inactivated excretory/secretory extracts was puffed onto the soma of each neuron, they depolarised and fired action potentials (APs) (median = 3.6 APs, IQR = 1.0 – 11.2 APs, N = 16, **Fig. 4.4 B, C**). However, when 200 µM of acetylcholine with active excretory/secretory extracts were applied to the same neurons just 2 min prior to/after this, the neurons did not show the same response, often firing no action potentials despite still being held at a voltage close to their action potential threshold (median = 0.2 APs, IQR = 0.0 – 1.7 APs, N = 16, $P \leq 0.05$, Kruskal Wallis one-way ANOVAs with Dunn's Multiple Comparison post hoc test, **Fig. 4.4 C**). As a positive control, 200 µM acetylcholine on its own was applied to a different set of neurons but using the same experimental protocol. The application of acetylcholine on its own resulted in a median number of APs (45.4 APs, IQR = 21.0 – 54.5 APs, N = 7) that was significantly higher than that produced by the application of 200 µM acetylcholine with active excretory/secretory extracts ($p \leq 0.001$, Kruskal Wallis test with Dunn's Multiple Comparison post hoc test) but did not differ significantly from that produced by the application of 200 µM acetylcholine with heat-inactivated excretory/secretory extracts ($p > 0.05$, Kruskal Wallis test with Dunn's Multiple Comparison post hoc test). This demonstrates that the AChEs in *T. crassiceps* larval excretory/secretory extracts have sufficient activity to break down acetylcholine at a concentration which induces changes in neuronal signaling *in vitro*. This would hold true for *T. solium* cyst membrane and scolex AChEs, given twice the amount of time, or the use of an extract of double the concentration, as these break down acetylcholine at roughly half the rate of *T. crassiceps* larval excretory/secretory extracts (**Table 4.1**).

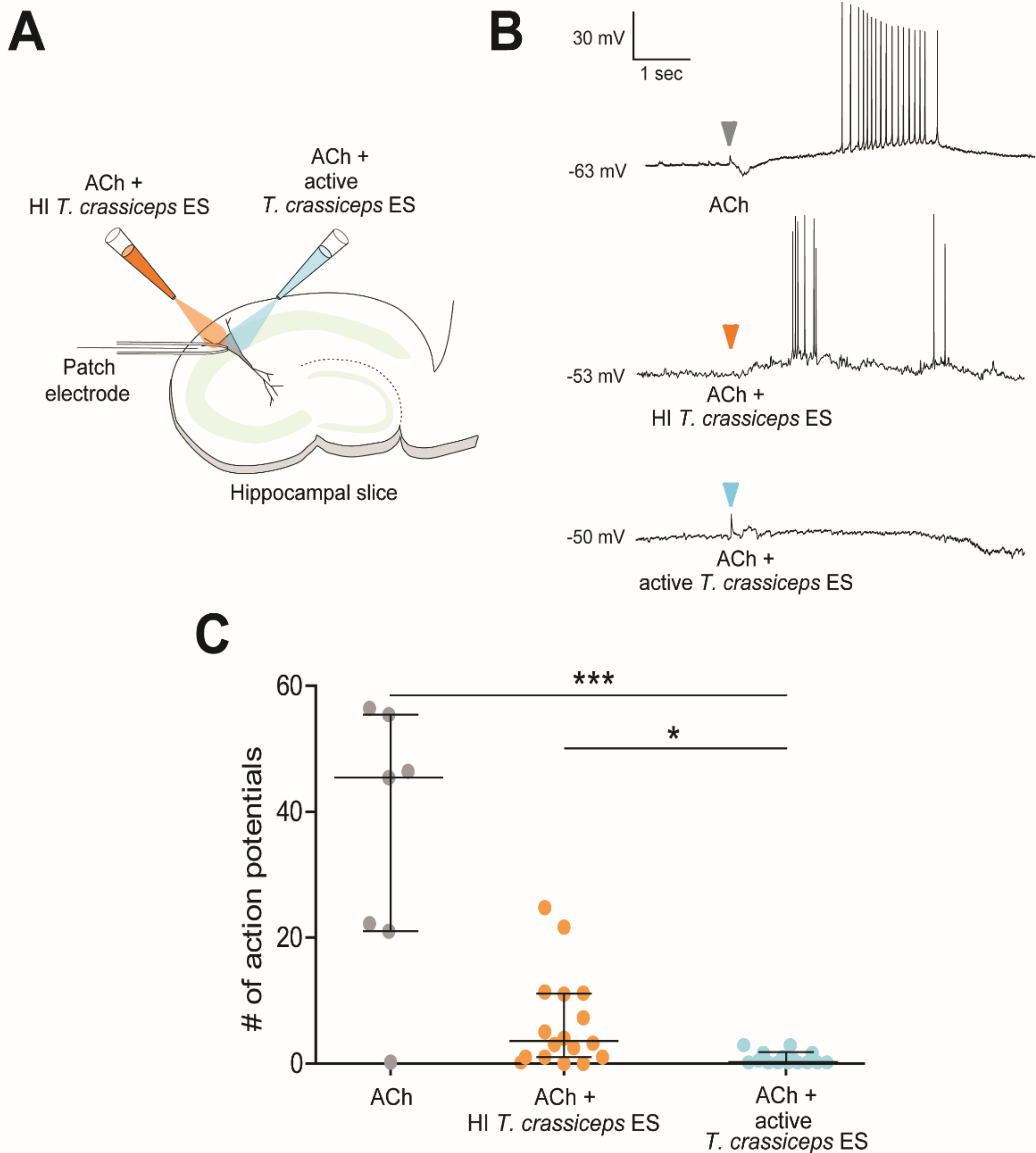


Figure 4.4: The functional effect of *Taenia* acetylcholinesterases on neuronal acetylcholine signaling. A) Schematic depicting the experimental setup. Whole-cell patch-clamp recordings were made from rat CA3 pyramidal neurons in organotypic hippocampal brain slice cultures. Whilst recording the electrical activity from neurons, two glass pipettes delivered picolitre volumes of either acetylcholine (ACh) (200 μM) with heat-inactivated (HI) *Taenia crassiceps* (*T. crassiceps)* excretory/secretory extracts (ES) (1.3 mg ml^{-1}) (orange pipette), or ACh with active *T. crassiceps* ES (1.3 mg ml^{-1} (blue pipette). As a positive control, ACh (200 μM) on its own was applied to a separate cell population using the same experimental protocol. B) Recordings of the membrane potential responses of individual pyramidal neurons to the application of either a solution of ACh alone (top trace), ACh with HI *T. crassiceps* ES (middle trace) or ACh with active *T. crassiceps* ES (bottom trace) onto the cell body (arrowheads indicate moment of application). C) Population data (median ± IQR) where each point represents the mean number (#) of action potentials evoked in 5 s after neurons were exposed to 5 x 30 ms puffs (2 – 5 cycles) of either a solution of ACh alone (N = 7), ACh with HI *T. crassiceps* ES (N = 13), or ACh with active *T. crassiceps* ES (N = 13). *$p \leq 0.05$, ***$p \leq 0.001$, Kruskal-Wallis test with Dunn's multiple comparison post-hoc test.

4.6 DISCUSSION

Helminths have been widely reported to contain and produce AChEs, but despite this knowledge of these enzymes in *T. crassiceps* and *T. solium* larvae to date has been scarce. Considering that parasitic AChEs could ostensibly disrupt host cholinergic brain signaling in the context of NCC, I felt that it would be valuable to investigate the activity and localization of AChEs in *T. crassiceps* and *T. solium* larvae. Here, using Ellman's assays and AChE activity stains, I show that the larvae of both species produce AChEs, but that AChE activity is broadly lower in *T. solium* extracts as compared to the corresponding *T. crassiceps* extracts. Additionally, I show that *T. crassiceps* larvae excrete/secrete AChEs, and have surface-presenting AChEs, whilst *T. solium* larvae do not. I further show that the activity of the *Taenia* larval AChEs is sufficient to alter acetylcholine signaling in an *in vitro* brain model.

Ellman's assays of AChE activity in various extracts of larvae of *T. crassiceps* (**Fig. 4.1 A**) and *T. solium* (**Fig. 4.1 C**) revealed that the larvae of both species contain AChEs in the whole cyst homogenate, as well as in the cyst vesicular fluid and in the cyst membrane (& scolex) extracts, but that *T. solium* larvae do not excrete/secrete AChEs, whilst *T. crassiceps* larvae do. The finding that *T. solium* larvae do not excrete/secrete AChEs is interesting, as many helminth species have been observed to secrete these enzymes in substantial amounts, with proposed benefits to parasite survival, such as protection against ingested AChE inhibitors and modulation of the host immune response (Mcsorley and Maizels, 2012; M E Selkirk *et al.*, 2005; Tedla *et al.*, 2019). Recently, a study by Vaux *et al.* (2016) demonstrated that *in vivo* exposure to secreted AChE from *Nippostrongylus brasiliensis*, a gastro-intestinal helminth, promoted classical activation of macrophages (as opposed to alternative activation), a state which is permissive to the survival of parasitic nematodes. In contrast, classically activated macrophages appear to be deleterious to the survival of Taeniid larvae, occurring in the resistant Th1 acute phase of infection, whilst their phenotype is shifted to an alternatively activated state during chronic Taeniid infection (Peon *et al.*, 2016; Prodjinotho *et al.*, 2020). This could potentially explain why it may not be beneficial for Taeniids to secrete large amounts of AChE.

Ellman's assays performed using *T. crassiceps* homogenate showed two "clusters" of AChE activity, whilst in the other *T. crassiceps* extracts the variability in AChEs activity is relatively small (**Fig. 4.1 A**). There is a noticeably large variability in the AChE activity of the whole cyst homogenate, which may be explained by the fact that the homogenate utilized in these assays was a "master batch" made using a great many larvae from two separate harvests of *T. crassiceps*, whilst the other extracts were each made using a smaller number of larvae from a single harvest. An interesting observation from the Ellman's assays on *T. solium* extracts (**Fig. 4.1 C**) is that the AChE activity of the whole cyst homogenate was closer to that of the

cyst vesicular fluid than the cyst membrane and scolex, which suggests that the whole cyst homogenate may be more representative of the contents of the cyst vesicular fluid, rather than being representative of all the cyst extracts. It is also interesting to note that *T. crassiceps*, which shows AChE activity in its excretory/secretory extracts has higher AChE activity in the cyst vesicular fluid than in the cyst membrane extract, whilst *T. solium*, which does not show AChE activity in excretory/secretory extracts has much higher AChE activity in its cyst membrane and scolex extract than in its cyst vesicular fluid. This may imply that the excretory/secretory extracts are made in, or released into, the cyst vesicular fluid before being released by the larvae.

To my knowledge these data represent the first definitive measurements of AChE activity from larvae of *T. crassiceps*. Previous studies have identified AChE activities in the larvae of multiple species of the broader Taeniidae family including *Echinococcus granulosus* (dog tapeworm) and *Taenia pisiformis* (rabbit tapeworm). The amount of AChE activity I found in *T. crassiceps* whole cyst homogenate is similar to that previously reported for the larval homogenate of *T. pisiformis* (*T. crassiceps* whole cyst homogenate mean activity = 39.1 nmol min^{-1} mg^{-1}, *T.* pisiformis homogenate mean activity = 24.8 nmol min^{-1} mg^{-1})(Gimenez-Pardo *et al.*, 2004).

Next, I ran non-denaturing PAGE gels of *T. crassiceps* and *T. solium* extracts and either Coomassie stained these or stained them for AChE activity using the Karnovsky & Roots (1964) method (**Fig. 4.1 B&D**). The Coomassie stained gels of *T. crassiceps* extracts revealed similar protein staining between the whole cyst homogenate and the cyst membrane extract, whilst the cyst membrane showed a slightly different staining profile, and the excretory/secretory extracts contained one dominant protein band (**Fig. 4.1 B**). Interestingly, the AChE activity staining of the *T. crassiceps* gels revealed more than one band in the whole cyst homogenate and the cyst membrane extract (**Fig. 4.1 B**). This implies that *T. crassiceps* larvae produce more than one isoform of AChE, perhaps one that is membrane bound and another that is soluble, as has been described in some nematodes (Murray E Selkirk *et al.*, 2005). AChE activity staining of the *T. solium* gels showed only faint staining in the whole cyst homogenate and cyst membrane and scolex lanes. This is not surprising, as, according to Selkirk and Hussein (2000), approximately 0.005 units of activity (i.e.. 5 nmoles of substrate hydrolysed per minute) is required to discern clear bands using this method, and I was only able to load far less than this (0.12 nmol/min for the whole cyst homogenate, 0.14 nmol/min for the cyst vesicular fluid and 0.42 nmol/min for the cyst membrane and scolex) (**Fig. 4.1 D**). This result could potentially be improved by performing protein concentration of the *T. solium* extracts and then repeating the described protocol.

For all three *T. solium* extracts some staining was visible at the interface between the stacking gel and the running gel, which raises the possibility that there were AChEs in the solutions which did not run into the running gel. This, together with the fact that all the AChE-stained bands appeared close to the top of gels, implies that the enzymes are quite large and perhaps are not strongly negatively charged. I did try to get the AChE bands to resolve more clearly from one another and run further into the gel by altering the acrylamide concentration (to 5% or 10%) and by running the gel for several hours longer, and at different voltages (100 mV or 150 mV), but none of these attempts lead to a different result. As such, running a denaturing PAGE gel (such as an SDS-PAGE gel), and utilizing a different, non-activity-based AChE stain (perhaps antibody based), might yield better resolution and identification of AChE isoforms. That said, it is unclear whether existing AChE antibodies would bind to the specific AChEs of *T. solium*, so specific antibodies would likely need to be raised to perform an antibody-based *T. solium* AChE stain.

I also decided to explore whether the AChE activity detected by the Ellman's assays was due to true acetylcholinesterases or other "pseudocholinesterases" – enzymes that are also able to hydrolyse acetylcholine but primarily function to digest other substrates, such as buterylcholine. To do this I incubated *Taenia* larval extracts with various concentrations of BW 284C51 (a specific AChE inhibitor), iso-OMPA (a specific buterylcholinesterase inhibitor) and eserine (a nonselective cholinesterase inhibitor) prior to assessing AChE activity using Ellman's assays. *T. crassiceps* larval extracts all showed remarkably similar inhibition patterns to all three inhibitors, apart from the whole cyst homogenate at concentrations of 10^{-1} and 10^{1} µM eserine, it which it was less strongly inhibited than the other extracts. This is extremely unexpected, as the AChEs present in the whole cyst homogenate should be the same as/a combination of those in the other cyst extracts. Again, because the homogenate was made from a different, more varied batch of *T. crassiceps* larvae than the other extracts, it is possible that it has a different AChE composition, which has resulted in this aberrant eserine inhibition curve. It seems more likely, however, that some experimental error may be responsible for this outcome. The fact that the *T. crassiceps* AChE activities could be completely inhibited by BW 284C51 but appeared unaffected by iso-OMPA means that the AChEs from *T. crassiceps* larvae can be classified true AChEs. It is interesting, and somewhat curious, that eserine seems to inhibit the *T. crassiceps* AChEs much more efficiently than BW 284C51.

T. solium larval extracts showed slightly different, but broadly similar patterns of inhibition to those of *T. crassiceps* extracts. There generally exists more variability within and between the activity readings of the different *T. solium* extracts than in the *T. crassiceps* extracts, which I believe may be attributable to the fact that the *T. solium* extracts generally have lower AChE activity, and the assay method may not have as much sensitivity in this lower activity range.

This may potentially also explain why, at low inhibitor concentrations, the AChE activity seems to be enhanced, as opposed to inhibited by the inhibitors, although it is still a possibility that this is a true phenomenon.

Next, I explored the localization of AChEs in the larvae by utilizing the Karnovsky & Roots (1984) AChE activity stain on cross-sections and whole mounts of *T. crassiceps* and *T. solium* larvae. These stains revealed dense, ubiquitous staining of AChEs in the membrane of *T. crassiceps* larvae (**Fig. 4.3 A**) and more diffuse AChE activity staining in the membrane of *T. solium* (**Fig. 4.3 B**), which corroborates the findings of the Ellman's assays. Interestingly, the staining technique resulted in some black background staining in *T. solium* larvae, but not in *T. crassiceps* larval samples. These outcomes, in addition to the conspicuous absence of a scolex in the *T. crassiceps* whole mounted larvae, serve as a pertinent reminder that although *T. crassiceps* is utilized as a model parasite for *T. solium* (Leandro *et al.*, 2014; Matos-Silva *et al.*, 2012; Robinson *et al.*, 2012; Stringer *et al.*, 2003), the two species may differ considerably, and findings made using *T. crassiceps* in NCC research may not necessarily translate to *T. solium* or clinical human NCC. The *T. solium* larvae available for staining had been frozen, and as such had ruptured, so intact specimens were not available for staining. Careful examination of the available specimens did allow me to establish which side of the membrane would have been outward facing in the intact larvae, however, and upon h examination under high magnification it became clear that *T. solium*, unlike *T. crassiceps*, does not have surface presenting AChEs. The lack of surface staining in *T. solium* larvae is in accordance with a previous study by Vasantha *et al.* (1992), which reports AChEs in *T. solium* larvae to be predominantly associated with a sub-tegmental network of nerves in the strobila and bladder wall.

Finally, I wanted to explore whether the AChE activity of the *Taenia* larvae was sufficient to affect brain AChE activity. I utilized patch-clamp electrophysiology to confirm that acetylcholine results in the firing of action potentials when puffed onto the cell surface of hippocampal pyramidal CA3 neurons (**Fig. 4.4 B & C**). I then set out to see whether the AChEs in *T. crassiceps* excretory/secretory extracts could abrogate this effect In order to ensure that any change in acetylcholine-mediated signaling was a result of the degradation of acetylcholine by the larval AChEs rather than an additional action by the larval extracts, I utilized an experimental set-up in which I puffed cycles of both acetylcholine with active excretory/secretory extract, and acetylcholine with excretory/secretory extract in which the proteins had been heat-inactivated (with the intent of inactivating the larval AChEs), onto the same neurons (see **Fig. 4.4 A**).

As I had hoped, the active excretory/secretory extracts altered the neuron's response to the application of acetylcholine, significantly reducing the number of action potentials the neuron

fired (**Fig. 4.4 C**). Unexpectedly, however, the heat inactivated excretory/secretory extracts also seemed to reduce the number of action potentials induced by acetylcholine application, although the number of action potentials the neuron fired in response to the application of acetylcholine with heat inactivated excretory/secretory extracts did not prove to be significantly lower than that induced by acetylcholine, but was significantly higher than that induced by acetylcholine with active excretory/secretory extracts (**Fig. 4.4 C**). Even though not statistically significant, the acetylcholine with heat inactivated excretory/secretory extracts may have induced a weaker excitatory response in neurons than acetylcholine alone due to incomplete inactivation of the larval AChEs by the heating protocol. Alternatively, it is possible that the excretory/secretory extracts contain a substance other than the AChEs that opposes the action of acetylcholine. In Chapter 1, however, I showed that the application of *T. crassiceps* excretory/secretory extracts do not result in an inhibitory effect on neurons, so if an acetylcholine-opposing substance does exist in the excretory/secretory extracts, it would have to act by some other mechanism, such as by competitively binding to acetylcholine receptors.

In summary, in this chapter I have described distinct profiles of AChE activity in *T. crassiceps* and *T. solium* larvae and have demonstrated that *Taenia* larval AChEs have sufficient activity to alter neuronal acetylcholine signalling. Considering, however, that a distinct correlation between the host immune response and seizure onset in NCC has been established (Carpio, 2002; Garcia and Del Brutto, 2017), I further wanted to explore the release of host inflammatory cytokines by hippocampal brain slices in response to extracts of *T. crassiceps*, which I set out to do in Chapter 5.

Chapter 5

THE EFFECTS OF *TAENIA CRASSICEPS* LARVAL EXTRACTS ON BRAIN IMMUNE CELL CYTOKINE PRODUCTION.

5.1 INTRODUCTION

In the previous two chapters I demonstrated that *Taenia* larval extracts may disrupt normal neuronal signaling by acting directly on neurons via glutamate, or by acting on the neurotransmitter acetylcholine through the production of AChEs. For this third and final chapter, I decided to "zoom out" yet further, and explore whether *Taenia* larval extracts may additionally act on immunocompetent brain cells and alter their production of cytokines, which could have implications for brain excitability.

My interest in the potential of *Taenia* extracts to alter cytokine release by innate immune cells was twofold: Firstly, it has been well documented that a strong inflammatory host immune response occurs when NCC cysts begin to degenerate in the brain, and it has been suggested that this inflammatory host immune response likely underlies the occurrence of seizures in NCC (Nash *et al.*, 2015b). Secondly, whilst it has been well established that *Taenia* larvae modulate the immune response to one that is more permissive to their survival whilst they remain viable (Peon *et al.*, 2016), the mechanisms involved in the modulation and induction of host immune responses have not yet been fully elucidated. More in-depth knowledge of the inflammatory immune response in NCC and how this contributes to seizurogenesis will aid in the development of more effective treatments for NCC and related seizures, and potentially even for other seizure disorders. A better understanding of the immune-modulatory mechanisms employed by viable *Taenia* larvae is also essential for a comprehensive understanding of NCC symptom onset and progression.

The release of the cytokines IL-1β, TNF-α and IL-6 by astrocytes and microglia have been reported to be elevated in subjects with epilepsy and have been shown to increase brain excitability via multiple mechanisms (Rana and Musto, 2018; Vezzani and Viviani, 2015b). The regulatory/anti-inflammatory cytokine IL-10 has also been reported to be elevated in the CSF of epilepsy patients (de Vries *et al.*, 2016). Interestingly, TNF-α, IL-6 and IL-10 have also been reported to be involved in the immune response to *T. solium* brain cysts (Restrepo *et al.*, 2001, 1998; Singh *et al.*, 2013). As such, I decided to explore how *Taenia crassiceps* larval

extracts affect the release of IL-1β, TNF-α, IL-6 and IL-10 by innate brain immune cells in a hippocampal organotypic brain slice model. I chose this model particularly because it would also allow for later interrogation (by other members of our research group) of the electrophysiological consequences of immune activation or modulation by *Taenia* larval extracts.

To explore the potential of *Taenia* extracts to induce cytokine release, I treated *Taenia* larvae in various ways – some extracts were prepared from viable larvae, some from larvae that had been treated with a fatal dose of an anthelminthic, praziquantel, others from larvae that were "starved" to death in culture and yet others from cysts that had been calcified by the host immune response. The rationale behind the use of each of these extracts is laid out in **Table 2.1** and **Table 2.2** in Chapter 2 – Materials and Methods. These extracts were applied to hippocampal brain slices for 24 hrs, after which time the culture medium was collected and assessed for presence of the cytokines IL-1β, TNF-α, IL-6 and IL-10. Surprisingly, none of the larval extracts produced large, significant changes in the release of these cytokines, when compared to control brain slices.

When I explored the ability of *Taenia* extracts to inhibit cytokine release, however, I found that whole cyst homogenate prepared using viable larvae was able to robustly and significantly attenuate the release of IL-6 and TNF-α induced by the exposure of brain slices to known inflammatory agents - lipopolysaccharides and zymosan A. Further investigation revealed that the effectivity of the *Taenia* whole cyst homogenate was reduced when glycan, protein or lipid components were manipulated, suggesting that multiple types of molecules may contribute to the ability of *Taenia* extracts to inhibit cytokine release by brain immunocompetent cells.

The description of the robust anti-inflammatory potential of *Taenia crassiceps* whole cyst homogenate is exciting not only in the context of contributing to a better understanding of NCC disease progression, but also as an avenue by which to identify new anti-inflammatory agents, with potential application in the treatment of neuroinflammation.

5.2 HIPPOCAMPAL ORGANOTYPIC BRAIN SLICE CULTURES HAVE LOW BASELINE RELEASE OF PRO-INFLAMMATORY CYTOKINES, BUT PRODUCTION OF PRO-INFLAMMATORY CYTOKINES CAN BE INDUCED BY LIPOPOLYSACCHARIDES, ZYMOSAN A AND POLY(I:C).

I wanted to utilise the hippocampal organotypic brain slice culture model to explore some of the ways in which *Taenia* larvae may modulate host immunity. To this end, I needed first to establish what the baseline levels of the release of pro-inflammatory cytokines by hippocampal organotypic brain slice cultures were. ELISAs for IL-6 and TNF-α revealed that, over the first

6 days in culture, the slices release relatively low levels of these cytokines (N = 10 wells, **Fig. 5.1A**). A Kruskal-Wallis test with Dunn's multiple comparison post-hoc test revealed that the release of IL-6 on day 1 *in vitro* was significantly higher than that on days 3 – 6 ($p \leq 0.001$). Note that most of the samples for days 2-6 had an IL-6 concentration below the 3.91 pg/ml detection limit of the assay. Similarly, the release of TNF-α on day 1 *in vitro* was significantly higher than that on days 3 – 5 ($p \leq 0.001$) (**Suppl Table 5.1A; Fig. 5.1A**). ELISAs run for IL-1β revealed all the samples (all wells over all days) to have a concentration of IL-1β that was below the assay detection limit (7.8 pg/ml).

Now that the baseline levels were established, I wanted to explore whether a release of pro-inflammatory cytokines could be induced by stimulation of the hippocampal organotypic brain slices with known immunogenic antigens. First, I exposed slices to increasing doses of lipopolysaccharides (LPS) from *Escherichia coli*. Lipopolysaccharides are molecules that are found in the membrane of some bacteria and have been described to activate neuroinflammatory pathways by binding to TLR4 (Vargas-Caraveo *et al.*, 2020). ELISAs for IL-6 and TNF-α confirmed that LPS induces the release of pro-inflammatory cytokines by hippocampal brain slices in a broadly dose-dependent manner (**Fig. 5.1B**). This increase was significant for both cytokines at LPS doses of 10^1 ng/ml, 10^3 ng/ml, and 10^4 ng/ml (Kruskal-Wallis test with Dunn's multiple comparison post hoc test, $p \leq 0.05$, **Suppl table 5.1B; Fig. 5.1B**). ELISAs for IL-1β (using samples that had been treated with either 10^1 ng/ml or 10^4 ng/ml LPS) showed no significant increase in IL-1β production in the presence of LPS (Kruskal-Wallis test with Dunn's multiple comparison post hoc test, $p = 0.09$, **Suppl table 5.1B; Fig. 5.1B**). ELISAs were also run for IL-10 on a subset of control samples (N = 5) and samples of slices exposed to the different doses of LPS (N = 3 for all doses). These revealed all the samples (control and different LPS doses) to consistently have a concentration of IL-10 that was below the assay detection limit (31 pg/ml).

I further explored the release of IL-6 and TNF-α by hippocampal brain slices in response to treatment with zymosan A and (poly(I:C). Zymosan A is a molecule found on the surface of fungi, such as yeast, and activated inflammatory host immune pathways by activating TLR2 and dectin-1 (Emilia Vendelova *et al.*, 2016). Poly(I:C) is a double stranded RNA analogue that stimulates TLR3 (Yousif *et al.*, 2018). The sample size for these experiments was N = 1 for each dose, as these were performed as pilot experiments to establish a dose of zymosan A and poly(I:C) that induced the release of a considerable amount of IL-6 and TNF- α, so that the appropriate dose could be used for subsequent experiments investigating whether the *T. crassiceps* homogenate could prevent the release of cytokines by agents that bind to TLRs other than TLR4. ELISAs for IL-6 and TNF-α revealed that brain slices exposed to zymosan A or poly(I:C) also show a trend towards a dose-dependent increase in the release of these

cytokines (**Fig. 5.1C & D**). Importantly, the two highest doses of poly(I:C) exceeded the maximum detection limit of the IL-6 ELISA (4 ng/ml) and are thus simply reported as the maximum detection limit value.

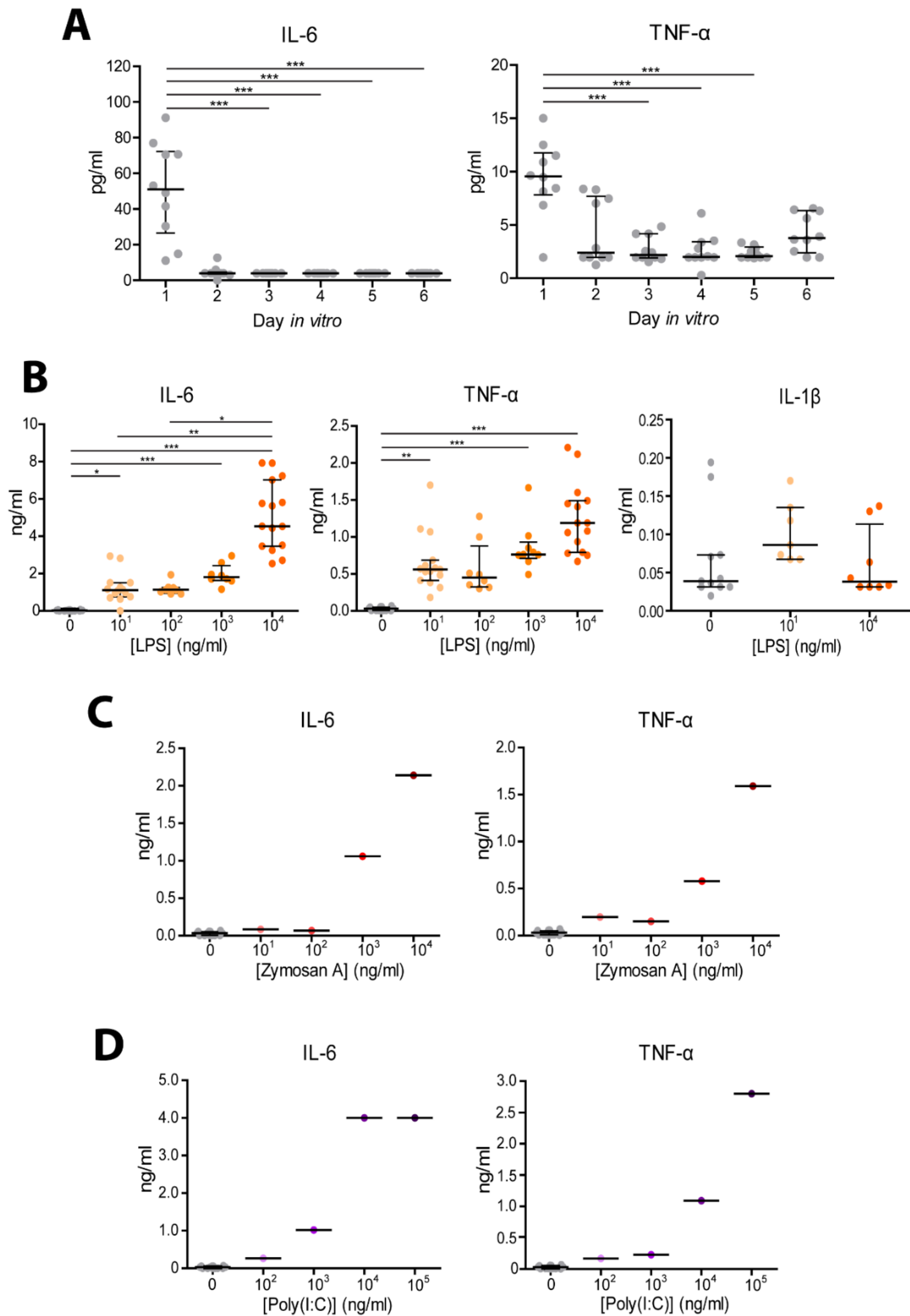


Figure 5.1: Characterising pro-inflammatory cytokine release and induction in an *in vitro* hippocampal organotypic brain slice culture model. A) Release of pro-inflammatory cytokines by hippocampal organotypic brain slice cultures over the first 6 days *in vitro*. Hippocampal organotypic cultures were prepared from 7 - 9-day old mice and cultured on Millicell cell culture inserts for 6-well plates (6 slices per insert with 1.2 ml of culture medium) for a 6-day period. Each day the culture medium was collected from each well (N = 10) and replaced, and later the concentrations of IL-6 and TNF-α in the culture media were determined using enzyme-linked immunosorbent assays (ELISAs). Values with median ± IQR, ***$p \leq 0.001$, Kruskal-Wallis test with Dunn's multiple comparison post-hoc test. B) Release of pro-inflammatory cytokines by hippocampal organotypic brain slice cultures in the presence of increasing doses of lipopolysaccharides (LPS). On day 6 *in vitro*, hippocampal organotypic brain slice cultures were exposed to various concentrations of LPS in the culture media for a 24hr period, after which culture medium was collected from each well, and later assayed for the presence of IL-6, TNF-α and IL-1β using ELISAs. Values with median ± IQR, N = 8 - 16, *$p \leq 0.05$, **$p \leq 0.01$, ***$p \leq 0.001$, Kruskal-Wallis test with Dunn's multiple comparison post-hoc test C) Release of pro-inflammatory cytokines by hippocampal organotypic brain slice cultures in the presence of increasing doses of zymosan A. On day 6 *in vitro*, hippocampal organotypic cultures were exposed to various concentrations of zymosan A in the culture media for a 24-hr period, after which culture media were collected, and later assayed for the presence of IL-6 and TN-Fα using ELISAs. Values with median ± IQR, N = 1 except for controls where N = 16. D) Release of pro-inflammatory cytokines by hippocampal organotypic brain slice cultures in the presence of increasing doses of zymosan A. On day 6 *in vitro*, hippocampal organotypic cultures were exposed to various concentrations of polyinosinic-polycytidylic acid (poly(I:C)) in the culture media for a 24hr period, after which culture media were collected, and later assayed for the presence of IL-6 and TN-Fα using ELISAs. Values with median ± IQR, N = 1 except for controls where N = 16.

5.3 *TAENIA CRASSICEPS* LARVAL EXTRACTS CONTAIN BOTH ENDOTOXINS AND HOST CYTOKINES.

To investigate the ways in which *Taenia* larvae may modulate host immunity I utilised an *in vitro* hippocampal organotypic brain slice culture model. Before doing so, however, I decided to determine whether the larval extracts contain host pro-inflammatory cytokines and/or contaminating lipopolysaccharides (also known as endotoxins) due to environmental contact. Either of these substances could induce the release of pro-inflammatory cytokines by hippocampal brain slices, which would confound the effect of larval-derived substances on the release of such cytokines. ELISAs for IL-6, TNF-α and IL-1β were done on whole cyst homogenate and on "excretory/secretory extracts for cytokine content determination" derived from several separate harvests of *T. crassiceps* larvae. These revealed that both the whole cyst homogenate and the excretory/secretory extracts contained all three cytokines, although the concentration of these cytokines varied greatly between harvest batches (**Suppl table 5.2A, Fig. 5.2A**). Despite these large variances, Mann-Whitney U tests revealed that both IL-6 and TNF-α were significantly higher (relative to total protein content) in excretory/secretory extracts than in homogenate ($p \leq 0.05$, **Fig. 5.2A**). Endotoxin assays showed that *T. crassiceps* extracts also contain endotoxins, although this was low in the concentrated excretory/secretory extracts (2.0 EU/ml, N = 1), somewhat higher in cyst membrane and cyst vesicular fluid (35 EU/ml and 129 EU/ml, respectively, N = 1) and extremely high in the homogenate (1660.0 EU/ml) (**Fig. 5.2B**). Endotoxin units can be converted to pg/ml (of LPS) using the rough estimate of 1 EU = 100 pg. Thus, the homogenate would have an estimated concentration of endotoxin roughly equivalent to 166 ng/ml (Stromberg *et al.*, 2017).

Having discovered that *T. crassiceps* larval excretory/secretory extracts contain host cytokines, I was curious as to whether this occurs due to the transfer of the larvae to a different medium *in vitro*, or whether the larvae release these cytokines over a longer period. To do this I measured the cytokine concentration in "excretory/secretory extracts for cytokine content determination" (see **Table 2.2**) from two different harvests using ELISAs. The results suggest that, for IL-6 and TNF-α, most of the host cytokines do appear to be released within the first three days *in vitro*, with reduced release thereafter, although the release of IL-1β followed a less predictable pattern (**Suppl table 5.2C, Fig. 5.2C**).

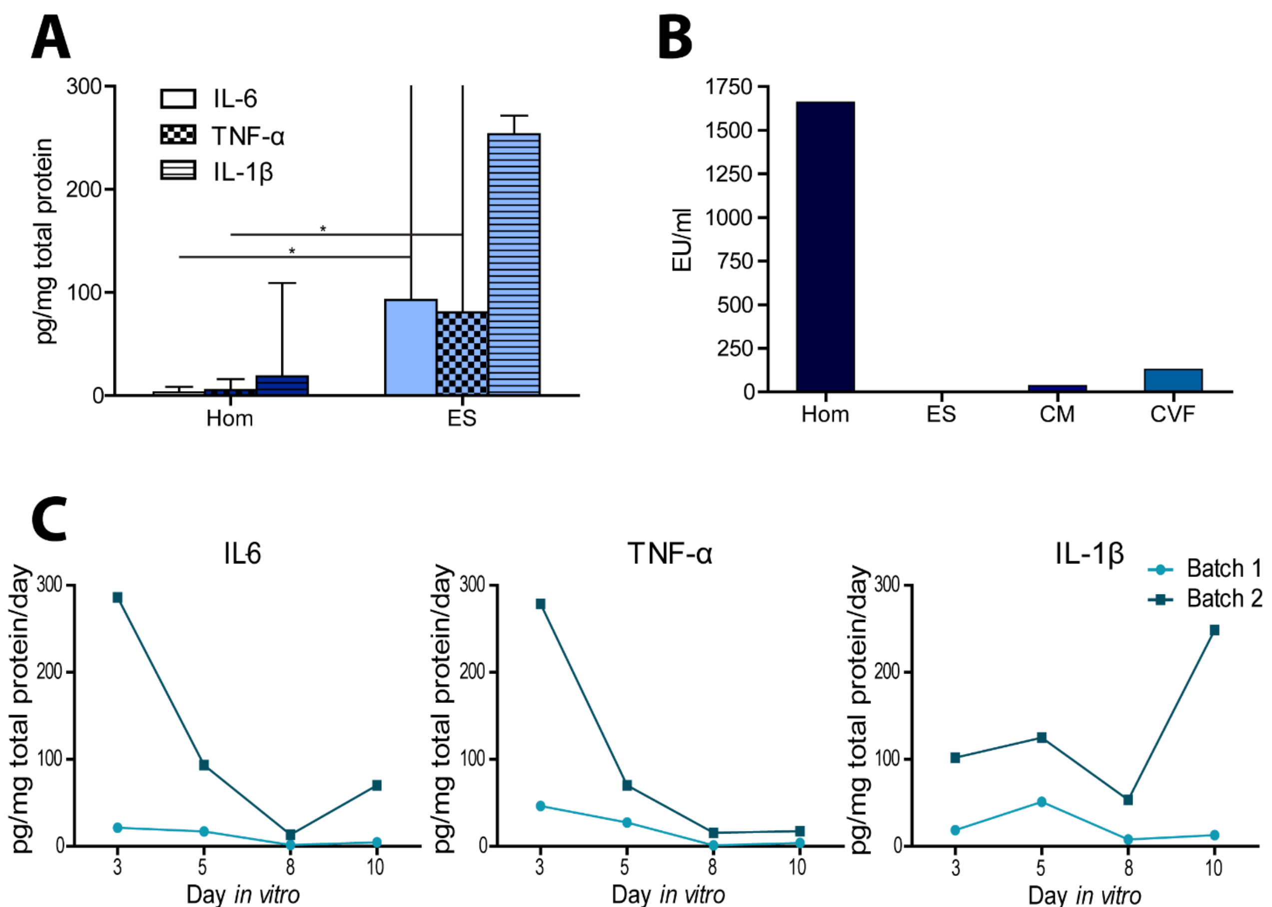


Figure 5.2: Presence of pro-inflammatory cytokines and endotoxins in *T. crassiceps* extracts. A) Different batches *T. crassiceps* whole cyst homogenate (Hom) and excretory/secretory extracts (ES) were assessed for the presence IL-6, TNF-α and IL-1β using enzyme linked immunosorbent assays (ELISAs). Cytokine concentrations are reported relative to total protein content of the *T. crassiceps* extracts (pg of cytokine/mg total protein). Values with median ± Range, * $p \leq 0.05$, Mann-Whitney U tests, N = 8 for Hom and N = 3 for ES. B) Endotoxin content of *T. crassiceps* Hom, ES, cyst membrane (CM) and cyst vesicular fluid (CVF) was quantified utilising a Pierce™ LAL Chromogenic Endotoxin Quantitation Kit. N = 1. C) The *in vitro* release of proinflammatory cytokines by two separate batches of *T. crassiceps* larvae in serum free culture medium over a 10-day period. The culture medium was replaced on D3, D5, D8 and D10 and each collection was later assessed for IL-6, TNF-α and IL-1β using ELISAs. Cytokine concentrations are reported relative to total protein concentration in the media, and these were divided by the number of days between each collection, such that cytokine production is expressed as pg of cytokine produced/mg total protein/day.

5.4 *TAENIA CRASSICEPS* EXTRACTS LARGELY FAIL TO INDUCE THE RELEASE OF PRO-INFLAMMATORY CYTOKINES BY HIPPOCAMPAL ORGANOTYPIC BRAIN SLICE CULTURES.

Having established the baseline cytokine release of hippocampal organotypic brain slice cultures, as well as the cytokine and endotoxin content of the *T. crassiceps* extracts, I proceeded to explore whether *T. crassiceps* extracts may induce the release of pro-inflammatory cytokines by hippocampal organotypic brain slice cultures. First, I investigated cytokine release by hippocampal brain slices when exposed to 150 µg/ml homogenate or concentrated excretory/secretory extracts for 24 hrs on day 6 *in vitro* (**Fig. 5.3A**). ELISAs on the medium collected after 24 hrs revealed that neither homogenate nor excretory/secretory extracts induced the release of large amounts of IL-6, TNF-α or IL-1β (**Suppl table 5.3A, Fig. 5.3A**). A Kruskal Wallis test with Dunn's multiple comparison post-hoc test did demonstrate, however, that IL-6 release is slightly, but significantly, increased when hippocampal slices are exposed to *T. crassiceps* homogenate.

Having found that *T. crassiceps* homogenate and excretory/secretory extracts largely fail to induce a notable increase in the release of IL-6, TNF-α or IL-1β, I decided to perform a pilot study investigating whether *T. crassiceps* extracts - either from larvae that had been killed with an anthelminthic; larvae that had been killed through starvation; late-stage larval granulomas; or larval homogenate solubilised with DMSO - could induce cytokine release where the homogenate and excretory/secretory extracts had failed (see **Table 2.1** for the rationale behind the use of each of these extracts). Again, larval extracts were added to the slice culture medium on day 6 *in vitro* for a 24-hour period, after which the medium was collected and analysed for IL-6 and TNF-α using ELISAs. The ELISA results demonstrated that none of the abovementioned *T. crassiceps* extracts induced the release of large amounts of cytokines, except for the DMSO solubilised extracts (**Suppl table 5.3B, Fig. 5.3B**). Although the DMSO homogenate extract did result in increased cytokine production, this did not reach statistical significance, nor was statistical significance found between any of the other treatment groups ($p > 0.05$, Kruskal-Wallis with Dunn's multiple comparison post hoc).

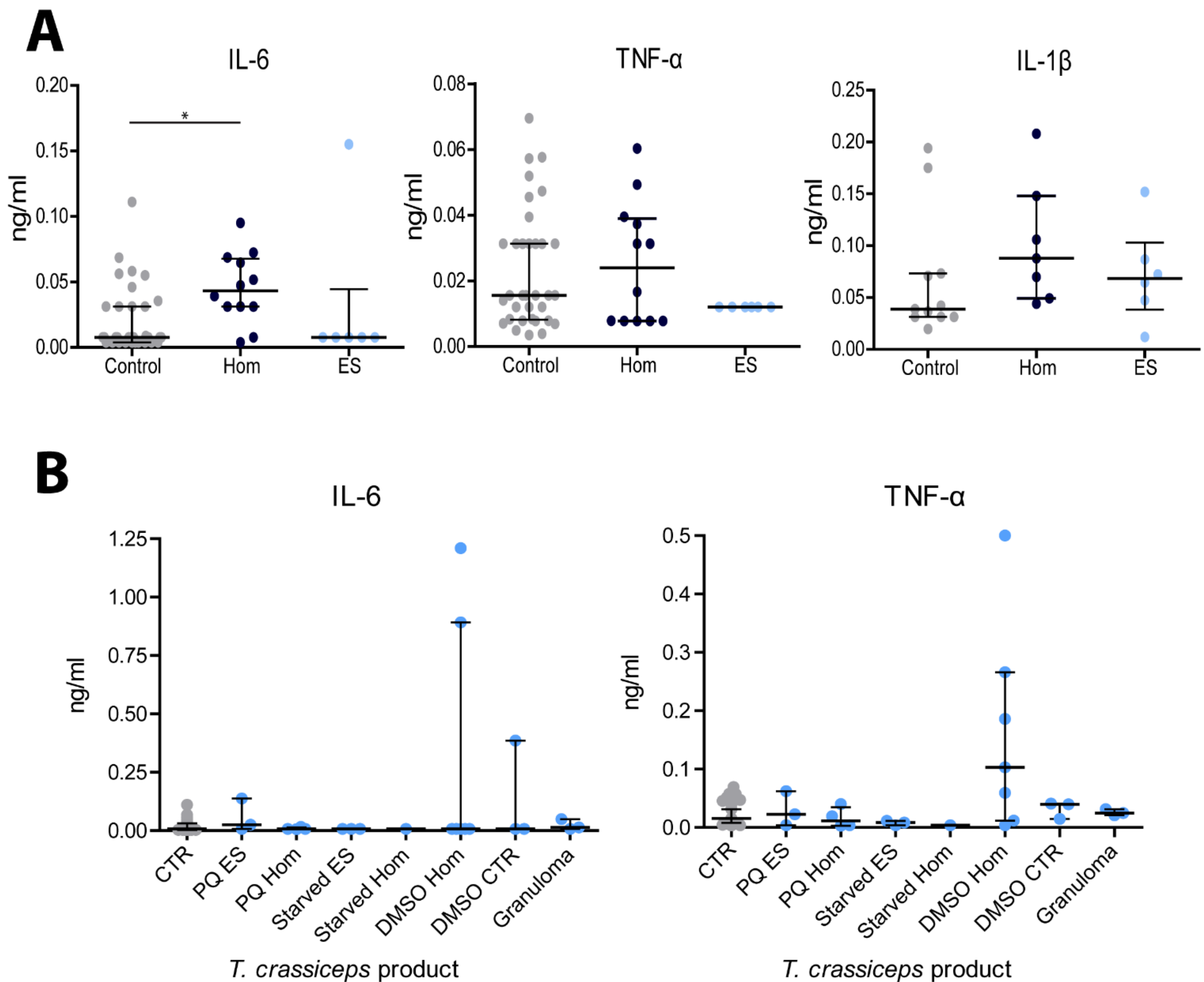


Figure 5.3: *T. crassiceps* extracts largely fail to induce the release of pro-inflammatory cytokines by hippocampal organotypic brain slice cultures. A) The release of pro-inflammatory cytokines by hippocampal organotypic brain slice cultures when exposed to *T. crassiceps* whole cyst homogenate (Hom) or excretory/secretory extracts (ES). Hippocampal organotypic cultures were prepared from 7-9-day old mice and cultured on 6-well-plate Millicell cell culture inserts (6 slices per insert with 1.2 ml of culture medium). On day 6 *in vitro* slices were exposed to150 µg/ml of either *T. crassiceps* Hom (N = 12) or ES (N = 6) for 24 hours. Thereafter media were collected and later assessed for the presence of IL-6, TNF-α and IL-1β using enzyme linked immunosorbent assays (ELISAs). Values with median ± IQR, N = 35 for control, *$p \leq 0.05$, Kruskal-Wallis test with Dunn's multiple comparison post-hoc test. B) The release of pro-inflammatory cytokines by hippocampal organotypic brain slice cultures when exposed to various additional preparations of *T. crassiceps* larval extracts. On day 6 *in vitro* wells containing hippocampal brain slices were exposed to either: the excretory/secretory extracts of *T. crassiceps* larvae that had been treated with the anthelminthic drug praziquantel (PQ ES) (N = 3); the whole cyst homogenate of *T. crassiceps* larvae that had been treated with the anthelminthic drug praziquantel (PQ Hom) (N = 4); the excretory/secretory extracts of *T. crassiceps* larvae that were left in culture until they lost viability (starved E/S) (N = 3); the whole cyst homogenate of *T. crassiceps* larvae that were left in culture until they lost viability (starved Hom) (N = 1); the whole cyst homogenate of *T. crassiceps* larvae to which the solvent DMSO had been added (DMSO Hom) (N = 7); a vehicle control for DMSO (DMSO CTR) (N = 3) or the homogenate of calcified cyst granulomas (Granuloma) (N = 3). After 24 hrs of exposure the culture medium from each well was collected. Later, the concentration of IL-6 and TNF-α in the culture media were determined using ELISAs. Values with median ± IQR. N = 35 for control, Kruskal-Wallis test with Dunn's multiple comparison post-hoc test revealed no significant differences.

5.5 *T. CRASSICEPS* EXTRACTS STRONGLY ATTENUATE LIPOPOLYSACCHARIDE-INDUCED RELEASE OF PRO-INFLAMMATORY CYTOKINES BY HIPPOCAMPAL ORGANOTYPIC BRAIN SLICE CULTURES.

Having established that *T. crassiceps* extracts do not cause the release of large amounts of cytokines, I next set out to explore whether these extracts may, instead, be able to attenuate LPS-induced pro-inflammatory cytokine release by hippocampal organotypic brain slice cultures. After 6 days *in vitro*, brain slices were exposed to 10 ng/ml LPS together with 150 µg/ml of either homogenate, excretory/secretory extracts, cyst membrane or cyst vesicular fluid. After 24 hrs the culture medium was collected from each well and later used to detect IL-6 and TNF-αlevels, using ELISAs. The ELISAs revealed that *T. crassiceps* extracts do, in fact, all seem to have the ability to dramatically attenuate LPS-induced release of pro-inflammatory cytokines (**Suppl table 5.4A, Fig. 5.4A**). Kruskal Wallis test with Dunn's multiple comparison post-hoc test confirmed that 10 ng/ml LPS on its own induced a significant increase in the release of IL-6 and TNF-α, as compared to control slices ($p \leq 0.001$, **Suppl table 5.4A, Fig. 5.4A)** and showed that the addition of homogenate or cyst vesicular fluid statistically significantly reduced the release of LPS-induced pro-inflammatory cytokines ($p \leq 0.001$, **Suppl table 5.4A, Fig. 5.4A).**

With the knowledge that *T. crassiceps* extracts can attenuate LPS-induced release of pro-inflammatory cytokines, I wanted to test the robustness of this ability. To do so, I exposed hippocampal organotypic slices to increasing doses of LPS on day 6 *in vitro*, either alone or together with 150 µg/ml homogenate. After 24hrs the medium from each well was collected and later used for ELISAs for IL-6 and TNF-α. The ELISA results showed that at 10^1 ng/ml LPS the *T. crassiceps* homogenate can almost completely suppress the release of IL-6 and TNF-α (IL-6 = 96.5 %; TNF- α = 95.8 %), but that this ability is impaired with increasing doses of LPS (**Suppl table 5.4B, Fig. 5.4B**). Despite this, the homogenate still causes a significant reduction in the release of IL-6 at both 10^2 ng/ml and 10^3 ng/ml LPS ($p \leq 0.05$, Mann-Whitney tests, **Suppl table 5.4B, Fig. 5.4B**) and in TNF-α at 10^2 ng/ml LPS ($p \leq 0.05$, Mann-Whitney test, **Suppl table 5.4B, Fig. 5.4B**).

I was also curious as to whether the *T. crassiceps* homogenate may induce the release of the anti-inflammatory cytokine, IL-10, either on its own or when it was applied together with different doses of LPS. ELISAs for IL-10 on control samples (N = 5), samples where slices had been exposed to *T. crassiceps* homogenate on its own (N = 6), samples exposed to 10^1, 10^2, 10^3 or 10^4 ng/ml LPS (N = 3 for all doses) or samples exposed to 10^1, 10^2, 10^3 or 10^4 ng/ml LPS together with 150 µg/ml homogenate (N = 3 for all groups) revealed that all the samples had a concentration of IL-10 that was below the assay detection limit (31 pg/ml).

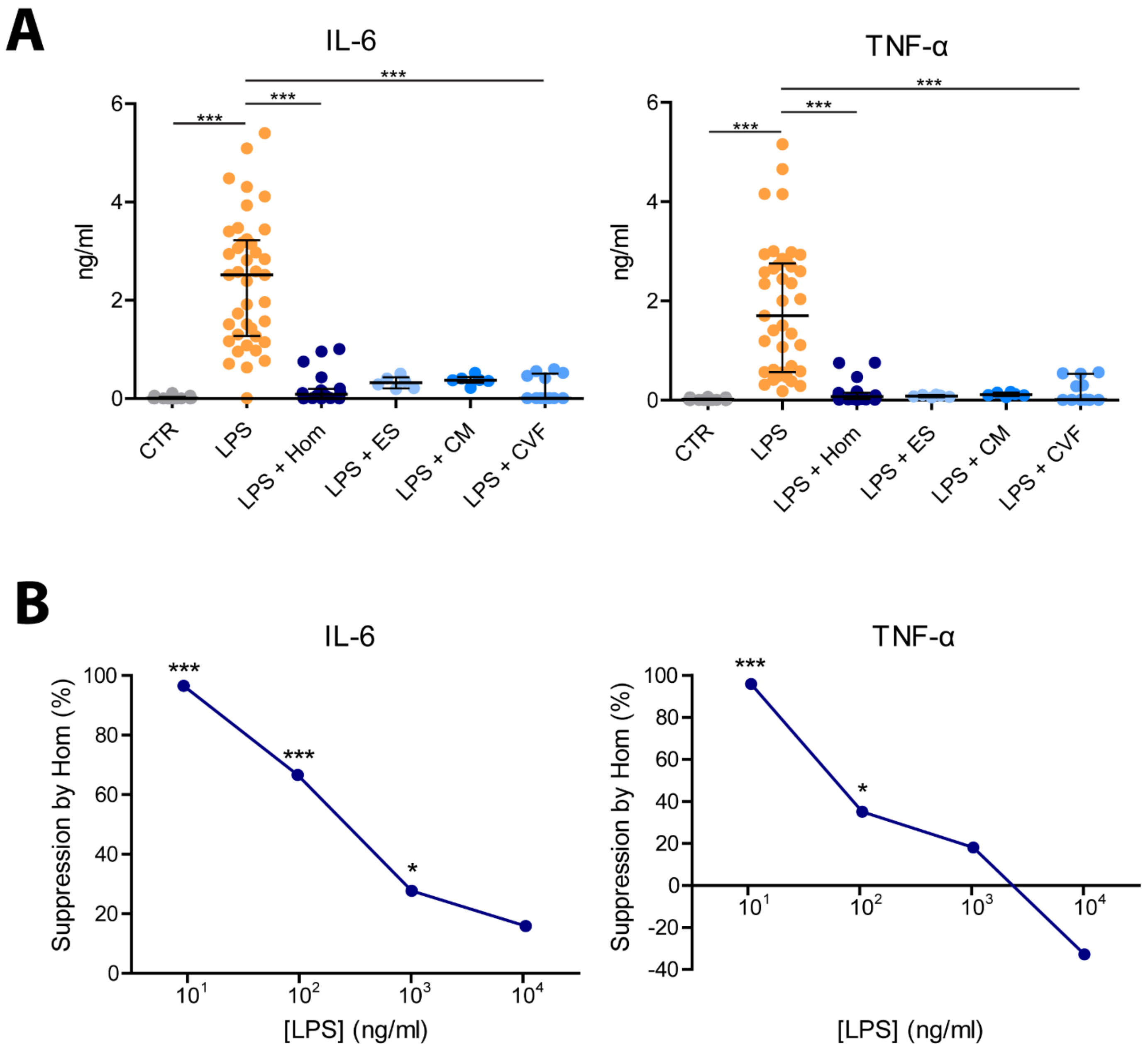


Figure 5.4: *T. crassiceps* extracts strongly attenuate lipopolysaccharide-induced release of pro-inflammatory cytokines by hippocampal organotypic brain slice cultures. A) Release of pro-inflammatory cytokines by hippocampal brain slices when exposed to lipopolysaccharide (LPS) alone, or together with *T. crassiceps* extracts. Hippocampal organotypic cultures were prepared from 7-9-day old mice and cultured on 6-well-plate Millicell cell culture inserts (6 slices per insert with 1.2 ml of culture medium). On day 6 *in vitro* slices were exposed to either 10 ng/ml LPS alone (N = 40), or LPS together with 150 µg/ml of either *T. crassiceps* whole cyst homogenate (Hom) (N =20), excretory/secretory extracts (ES) (N =6), cyst membrane (CM) (N =6) or cyst vesicular fluid (CVF) (N =12) for 24 hours. Thereafter the medium from each well was collected and later assessed for the presence of IL-6 and TNF-α using enzyme linked immunosorbent assays (ELISAs). Values with median ± IQR, N = 35 for control, ***$p \leq 0.001$, Kruskal-Wallis test with Dunn's multiple comparison post-hoc test. B) The percentage suppression of LPS-induced cytokine release by *T. crassiceps* Hom. On day 6 *in vitro* hippocampal brain slices were exposed to various concentrations of LPS either on their own (N = 40 for 10^1 ng/ml LPS, N = 8 for 10^2 ng/ml LPS, N = 8 for 10^3 ng/ml LPS, N = 15 for 10^4 ng/ml LPS), or together with 150 µg/ml Hom (N = 20 for 10^1 ng/ml LPS + Hom, N = 8 for 10^2 ng/ml LPS + Hom, N = 8 for 10^3 ng/ml LPS + Hom, N = 7 for 10^4 ng/ml LPS + Hom). After 24 hours the medium from each well was collected and later assessed for the presence of IL-6 and TNF-α using ELISAs. The amount of residual cytokine release in the presence of LPS together with Hom was statistically compared to that produced by LPS alone for each concentration of LPS using Mann-Whitney tests. Values = 100% - % mean residual cytokine release. *$p \leq 0.05$, **$p \leq 0.01$, ***$p \leq 0.001$.

5.6 *TAENIA CRASSICEPS* EXTRACTS ATTENUATE THE RELEASE OF PRO-INFLAMMATORY CYTOKINES INDUCED BY ZYMOSAN A.

Having revealed that *T. crassiceps* homogenate is able to significantly attenuate lipopolysaccharide-induced release of pro-inflammatory cytokines by hippocampal organotypic brain slice cultures, I further wished to investigate whether the homogenate could also attenuate the release of pro-inflammatory cytokines induced by zymosan A and poly(I:C). To do so, hippocampal brain slices were exposed to either 5 µg/ml zymosan A or poly(I:C) on their own, or together with 150 5 µg/ml homogenate, on day 6 *in vitro*, for a 24-hour period. After the exposure period, the medium from each well was collected and later analysed for the presence of IL-6 and TNF-α, using ELISAs. The ELISAs revealed that both 5 µg/ml zymosan A and 5 µg/ml poly(I:C) resulted in significant increases in IL-6 and TNF-α (Kruskal-Wallis tests with Dunn's multiple comparison post-hoc tests, $p \leq 0.001$, **Suppl table 5.5A & B, Fig. 5.5A&B**). Further, the addition of homogenate with zymosan A resulted in a significant decrease in the median concentration of both IL-6 and TNF-α in the culture medium (Kruskal-Wallis tests with Dunn's multiple comparison post-hoc tests, $p \leq 0.001$, **Suppl table 5.5A, Fig. 5.5A**). A similar trend was seen when homogenate was added with poly(I:C), with the median concentration of both IL-6 and TNF-α being lower than when just poly(I:C) was added (**Suppl table 5.5B, Fig. 5.5B**). Statistical analysis showed, however, that this decrease was not significant for either cytokine ($p > 0.05$), and that the concentrations of cytokine produced were still significantly higher than that of control samples for both IL-6 ($p \leq 0.001$) and TNF-α ($p \leq 0.01$) (Kruskal-Wallis tests with Dunn's multiple comparison post-hoc tests, **Suppl table 5.5B, Fig. 5.5B**).

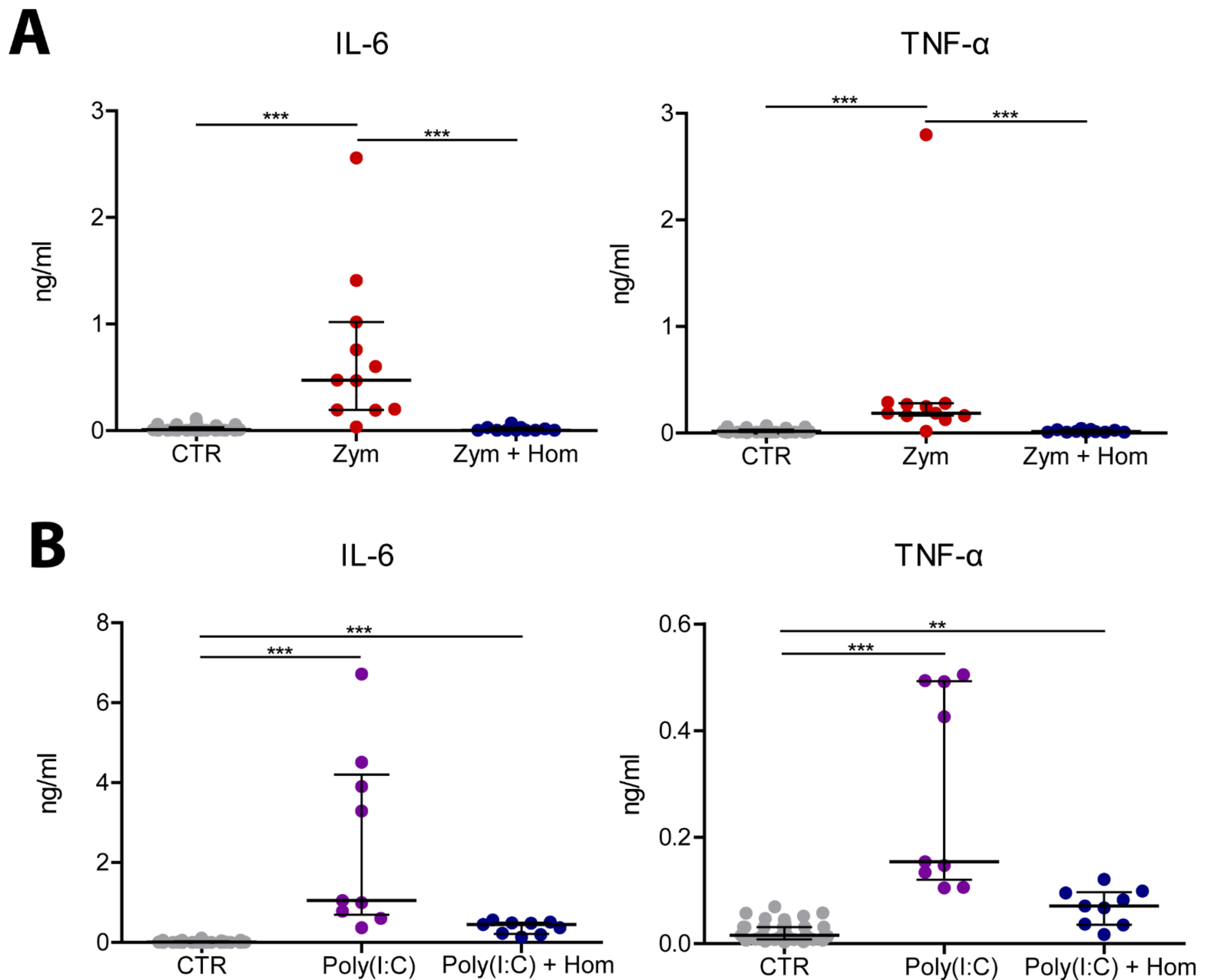


Figure 5.5: *T. crassiceps* extracts can also attenuate the release of pro-inflammatory cytokines induced by other immunogenic substances. A) Release of pro-inflammatory cytokines by hippocampal brain slices when exposed to zymosan A (Zym) alone, or together with *T. crassiceps* whole cyst homogenate (Hom). Hippocampal organotypic cultures were prepared from 7 - 9-day old mice and cultured on 6-well-plate Millicell cell culture inserts (6 slices per insert with 1.2 ml of culture medium). On day 6 *in vitro* slices were exposed to either 5 µg/ml Zym alone (N = 11), or together with 150 µg/ml Hom (N =11) for 24 hours. Thereafter the medium from each well was collected and later assessed for the presence of IL-6 and TNF-α using enzyme linked immunosorbent assays (ELISAs). Values with median ± IQR, N = 35 for control, ***$p \leq 0.001$, Kruskal-Wallis test with Dunn's multiple comparison post-hoc test. B) Release of pro-inflammatory cytokines by hippocampal brain slices when exposed to polyinosinic-polycytidylic acid (poly(I:C)) alone, or together with *T. crassiceps* Hom. On day 6 *in vitro* slices were exposed to either 5 µg/ml poly(I:C) alone (N = 9), or together with 150 µg/ml Hom (N =9) for 24 hours. Thereafter the medium from each well was collected and later assessed for the presence of IL-6 and TNF-α using ELISAs. Values with median ± IQR, N = 35 for control, **$p \leq 0.001$, ***$p \leq 0.001$, Kruskal-Wallis test with Dunn's multiple comparison post-hoc test.

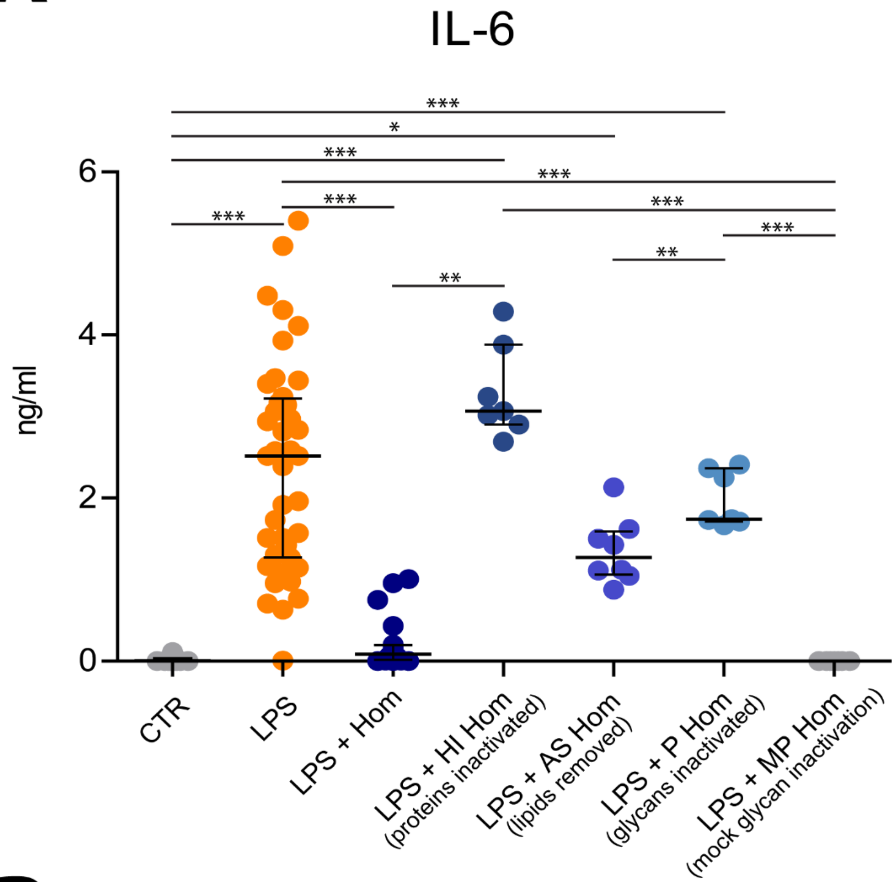
5.7 Manipulating lipids, carbohydrates or proteins in *Taenia crassiceps* extracts can impair their ability to attenuate LPS-induced release of pro-inflammatory molecules in hippocampal organotypic brain slice cultures.

Having established that *T. crassiceps* homogenate can significantly attenuate both LPS- and zymosan A-induced release of pro-inflammatory cytokines by hippocampal organotypic cultures, I went on to explore what component of the homogenate may be responsible for the apparent anti-inflammatory action. This involved manipulating the homogenate in three different ways before exposing hippocampal organotypic slices to the homogenate: the first was the heat inactivation of protein components at 100°C for 30 min (HI homogenate); the second was the removal of lipids using an ammonium sulfate protein precipitation protocol (AS homogenate); and the third a periodate-mediated inactivation of glycans (P homogenate). I also performed a "mock periodate" treatment (MP homogenate) as a control for the periodate treatment, where homogenate was exposed to all the steps of the periodate treatment, apart from the step where the periodate itself is added (see **Table 2.1** for a summary of the preparation and use of these manipulated extracts). Again, hippocampal brain slices were exposed to 10 ng/ml LPS together with the various manipulated *T. crassiceps* extracts (150 µg/ml) on day 6 *in vitro*, for a 24-hour period, after which the medium from each well was collected.

ELISA for IL-6 and TNF-α on the media from slices treated with LPS + HI revealed that heat-inactivation of the homogenate appears to impair the ability of the homogenate to attenuate LPS-induced release of IL-6 and TNF-α. Slices treated with LPS + HI homogenate displayed significantly higher median release of IL-6 and TNF-α than control slices, slices treated with LPS + unaltered homogenate and slices treated with LPS + MP homogenate ($p \leq 0.05$, Kruskal-Wallis test with Dunn's multiple comparison post-hoc test, **Suppl table 5.6, Fig. 5.6**). Slices treated with LPS + AS homogenate displayed a similar trend, with the median amount of both IL-6 and TNF-α released being statistically significantly higher than that produced by control slices and slices treated with LPS + MP homogenate ($p \leq 0.05$ for IL-6 and TNF-α, Kruskal-Wallis test with Dunn's multiple comparison post-hoc test, **Suppl table 5.6, Fig. 5.6**), although not statistically significantly higher than that of slices treated with LPS + unaltered homogenate ($p \geq 0.05$ for IL-6 and TNF-α, Kruskal-Wallis test with Dunn's multiple comparison post-hoc test, **Suppl table 5.6, Fig. 5.6**)

Slices treated with LPS + P homogenate again displayed an impaired ability to attenuate LPS-induced production of cytokines (**Suppl table 5.6, Fig. 5.6**). These slices produced

significantly more IL-6 and TNF-α than control slices and slices treated with LPS + MP homogenate ($p \leq 0.01$ for IL-6 and TNF-α, Kruskal-Wallis test with Dunn's multiple comparison post-hoc test, **Suppl table 5.6, Fig. 5.6**). For TNF-α, the amount of cytokine produced by slices exposed to LPS + P homogenate was also significantly higher than that produced by slices exposed to LPS + unaltered homogenate ($p \leq 0.01$, Kruskal-Wallis test with Dunn's multiple comparison post-hoc test, **Suppl table 5.6, Fig. 5.6**). The MP homogenate did not appear to be at all impaired in its ability to attenuate LPS-induced release of IL-6 or TNF-α, with median release of these cytokines by slices treated with LPS + MP homogenate not differing significantly from that of control slices or slices treated with LPS + unaltered homogenate ($p \geq 0.05$ for IL-6 and TNF-α, Kruskal-Wallis test with Dunn's multiple comparison post-hoc test, **Suppl table 5.6, Fig. 5.6**).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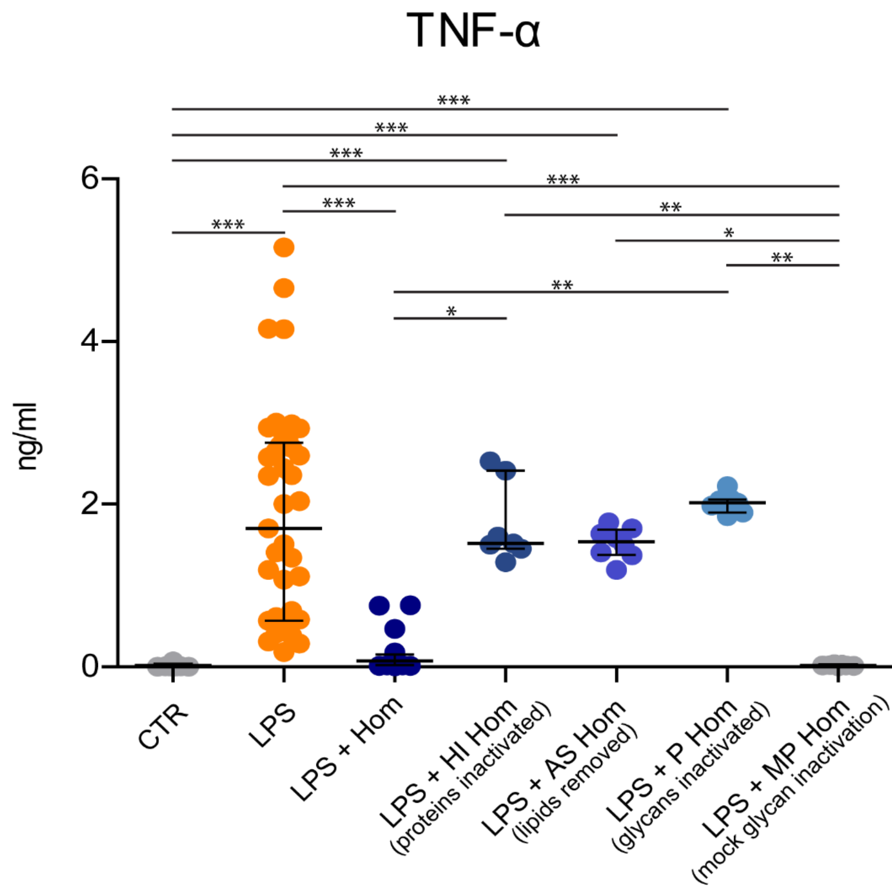
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LPS + AS Hom
(lipids removed)
LPS + P Hom
(glycans inactivated)
LPS + MP Hom
(mock glycan inactivation)

Figure 5.6: The attenuation of lipopolysaccharide-induced pro-inflammatory cytokine release by *Taenia crassiceps* whole cyst homogenate is impaired when lipid, carbohydrate, or protein components are disrupted. Hippocampal organotypic cultures were prepared from 7 - 9-day old mice and cultured on 6-well-plate Millicell cell culture inserts (6 slices per insert with 1.2 ml of culture medium). On day 6 *in vitro* slices were exposed to either 10 ng/ml lipopolysaccharide (LPS) alone (N = 40), or together with 150 µg/ml of: *Taenia crassiceps* whole cyst homogenate (Hom); Hom in which the proteins had been heat inactivated (HI Hom) (N =7); Hom from which lipids had been removed with an ammonium sulfate precipitation protocol (AS Hom) (N = 8); Hom in which glycans were inactivated using a periodate treatment (P Hom) (N = 7); or a control for the P Hom that was subjected to the same protocol but with the periodate omitted (mock periodate Hom – MP Hom) (N = 7). After 24 hours the medium from each well was collected and later assessed for the presence of IL-6 (A) and TNF-α (B) using enzyme linked immunosorbent assays (ELISAs). Values with median ± IQR, N = 35 for control, *$p \leq 0.05$, **$p \leq 0.01$, ***$p \leq 0.001$, Kruskal-Wallis test with Dunn's multiple comparison post-hoc test.

5.8 DISCUSSION

In the previous two chapters I demonstrated ways in which *Taenia* larval extracts may act directly on neurons, or act on the neurotransmitter acetylcholine to disrupt normal neuronal signaling. In this chapter I aimed to establish whether *Taenia* larval extracts alter the production of cytokines by hippocampal brain slices, as changes in cytokine levels could also affect neuronal signaling.

There is increasing evidence suggesting that neuroinflammation, including the release of pro-inflammatory cytokines by innate brain immune cells, may be major players in epileptogenesis (Rana and Musto, 2018). It has also been reported that there is a robust activation of innate and adaptive host immune responses when cysts begin to degenerate in NCC (Restrepo *et al.*, 2001, 1998; Singh *et al.*, 2013). The mechanisms by which *Taenia* cysts induce host immune responses, and how these responses may result in seizures in NCC is still largely unknown. Additionally, it has been shown that *Taenia* larvae shift the immune system to an anti-inflammatory state, in order to favour their survival while the cysts are viable (Peon *et al.*, 2016). Again, many of the mechanisms that enable this immune modulation are, as yet, undiscovered. I therefore set out to establish a model of NCC using hippocampal organotypic brain slice cultures which would allow us to explore the release of cytokines by innate immune cells in response to various larval extracts.

To establish the effect that *Taenia* larval extracts have on proinflammatory cytokine release by hippocampal brain slices, I first had to establish the baseline release of cytokines by hippocampal brain slices over the first six days in culture. I found that the release of IL-6 and TNF-α on day 1 *in vitro* was significantly higher than on subsequent days (**Fig. 5.1A**). This is likely due to a transient inflammatory response caused by the trauma of slicing (Huuskonen *et al.*, 2005). Baseline production of IL-1β was consistently below the detection limit of the ELISA (7.8 pg/ml). I further wanted to confirm that I could induce a robust inflammatory response in this model, both to ensure that I was able to detect an inflammatory effect, should any of the *Taenia* larval extracts induce one, and to utilise in the exploration of the anti-inflammatory potential of the *Taenia* larval extracts.

When I exposed brain slices to different doses of LPS, I found that IL-6 and TNF-α release showed largely linear dose-response curves, whilst the levels of IL-1β released by the brain slices remained very low (**Fig. 5.1B**). Based on the dose-response curves of IL-6 and TNF-α I opted to use a concentration of 10 ng/ml LPS for subsequent experiments where I utilised LPS, as I wanted to use the lowest dose that resulted in a significant release of pro-inflammatory cytokines. I also went on to perform similar dose-response experiments using the inflammatory agents zymosan A and poly(I:C). These both displayed similar dose-response curves in IL-6 and TNF-α release. It is important to note that the zymosan A and poly(I:C) curves were produced using only a single sample per dose, so the reliability of these is limited, but they informed my choice of dose for subsequent investigations into the anti-inflammatory ability of *T. crassiceps* whole cyst homogenate (**Fig. 5.5**).

Huuskonen *et al.* [2005] performed a similar characterisation of baseline cytokine levels, as well as LPS responses in the hippocampal organotypic model, utilising rat brain slices. They report a similar transient increase in the release of IL-6 and TNF-α in the first day/s of culture, and a similar, robust increase in the release of IL-6 when slices are exposed to different doses of LPS. In studies by Fu *et al.* [2010] and Yousif *et al.* [2018], they report an increase in the release of IL-6 and TNF-α by mouse hippocampal brain slices in response to the application of 10 ng/ml LPS of a similar magnitude to that which I report. These corroborating findings confirms that the hippocampal brain slice culture model releases pro-inflammatory cytokines in response both to the trauma of slicing, and more notably, to the inflammatory stimuli.

Interestingly, another study using rat hippocampal organotypic cultures detected much higher concentrations of IL-1β (around 400 pg/ml/culture) and IL-10 (around 1600 pg/ml/culture) released by control and LPS-treated slices than I report here, although they similarly report no significant increase in the release of these cytokines when slices were exposed to LPS (Papageorgiou *et al.*, 2016). Another study utilising mouse slices, however, reports IL-1β levels of similar magnitude to what I report in Figure **5.1A** (Bernardino *et al.*, 2008), which suggests that there may exist some differences in the release of certain cytokines by innate immune cells of the two species.

In Figure **5.1A&B** it is evident that hippocampal organotypic slice cultures display highly variable responses to inflammatory stimuli. There are several factors that may contribute to this. The first is that each sample represents media collected from a culture well containing six mouse hippocampal brain slices and considering that one typically only obtains between 18 and 24 slices from the hippocampi of one mouse pup, all experimental groups consist of samples from several different animals, which likely introduces a certain amount variability. Further to this, there is some variability in slice size, which means that not all the culture wells will contain identical amounts of tissue. Additionally, both horse serum and B27 are used in

the culture medium, the composition of which can vary between batches, which can also account for some of this variability. Finally, it is likely that not all samples contain identical numbers of immune cells, nor respond identically to culture and inflammatory conditions. This variability is particularly evident in the response to inflammatory stimuli (see **Fig. 5.1B, Fig. 5.4A & Fig. 5.5A&B**). An additional factor in these cases may be that the suspensions of the inflammatory agents may not be entirely homogenous and may vary slightly in concentration between different batches.

Next, to exclude effects induced by host cytokines taken up by the larvae, or by contaminating endotoxins, I explored whether the *T. crassiceps* larval extracts has detectable amounts of these. I discovered that both the whole cyst homogenate and the excretory/secretory extracts > 3kDa contained detectable amounts of IL-6, TNF-α and IL-1β (**Suppl table 5.2A, Fig. 5.2A**). The amount of IL-6 and TNF-α in the excretory/secretory extracts showed large variability, mainly because one batch of excretory/secretory extracts had drastically higher concentrations of these cytokines (**Suppl table 5.2A**). The sample size for the assays (N=3) was quite small, and could, in future investigations, be improved by adjusting the collection method of the excretory/secretory extracts to one where larvae from different mice are kept separate and cultured in smaller batches in small amounts of media, each of which could be separately assayed. Interestingly, a similarly large variance is seen between the two batches utilised to determine the daily release of cytokines by the larvae over a ten-day period (**Suppl table 5.2C, Fig. 5.2C**). Again, these experiments would benefit from an adjusted excretory/secretory extracts collection technique allowing for greater sample size. Endotoxin assays showed that *T. crassiceps* extracts also contain endotoxins, although this was very low in the concentrated excretory/secretory extracts (2.0 EU/ml, N = 1), somewhat higher in cyst membrane and cyst vesicular fluid (35 EU/ml and 129 EU/ml, respectively, N = 1) and extremely high in the homogenate (1660.0 EU/ml) (**Fig. 5.2B**). These results meant that if any inflammatory immune response were to be found using the whole cyst homogenate, they would need to be interpreted with caution, and protocols to remove endotoxins from the *T. crassiceps* extracts may need to be considered.

Having determined baseline cytokine release by hippocampal brain slices, as well as host cytokine and endotoxin content of *T. crassiceps* larval extracts, I then explored the inflammatory potential of *T. crassiceps* larval extracts in the hippocampal organotypic slice culture model. The incubation of hippocampal slices with 150 µg/ml of *T. crassiceps* whole cyst homogenate or excretory/secretory extracts for 24 hrs did not result in any large increases in the release of IL-6, TNF-α or IL-1β (**Fig 5.3A**). Statistical analysis did reveal that the small increase in IL-6 release induced by *T. crassiceps* whole cyst homogenate was significant, but this increase is very small, when compared to that induced by even the lowest concentration

of LPS and could potentially even be attributed to the endotoxin content of this extract. Disappointingly, even when I treated slices with *T. crassiceps* extracts from larvae that had been killed (either with an anthelminthic or through starvation), from larval late-stage granulomas, or from *T. crassiceps* extracts solubilised in DMSO, no significant increase in the release of IL-6 or TNF-α was produced. A trend towards an increased release of TNF-α by slices treated with DMSO-solubilised whole cyst homogenate was observed, which perhaps warrants repeat experiments, and the exploration of agents which can assist in the solubilisation of hydrophobic components of the larval extracts.

Future studies could also investigate whether a longer incubation of brain slices with larval extracts, or a greater concentration of larval extracts in the culture media, can elicit the release of proinflammatory cytokines. Alternatively, a model in which multiple whole praziquantel-killed or starved larvae are added to the culture medium, such that no components are lost through the homogenisation process, may also be worth exploring.

I next investigated the anti-inflammatory potential of the *T. crassiceps* larval extracts by exposing hippocampal slices to 10 ng/ml LPS together with 150 µg/ml of either whole cyst homogenate, excretory/secretory extracts, cyst membrane extract, or cyst vesicular fluid. All the larval extracts caused a large drop in the median release of both IL-6 and TNF-α by hippocampal slices, although this reduction was only significant for the whole cyst homogenate and the cyst vesicular fluid, likely because the sample sizes for these two extracts were slightly larger (**Suppl Table 5.4A, Fig. 5.4A**). These results suggest that all the *T. crassiceps* larval extracts have anti-inflammatory potential, which suggests that either the active inflammatory agent occurs in all components of the larvae, or that there are multiple anti-inflammatory agents involved in the effect. This result may also help to explain why I was unable to detect large increases in cytokine release by any *T. crassiceps* extracts (despite the high endotoxin content in some), as, even if these extracts contain inflammatory agents, the effect of these would potentially be masked by anti-inflammatory components of the extracts.

The ability of *T. crassiceps* product to suppress the production of inflammatory cytokines has been previously reported in models utilising murine macrophages (Landa *et al.*, 2019; Martínez-Saucedo *et al.*, 2019), murine spleen cells (Gómez-García *et al.*, 2006), murine dendritic cells (Terrazas *et al.*, 2011, 2010) and even human dendritic cells (Terrazas *et al.*, 2013). To my knowledge, however, this is the first time this effect has been show in a more complex, brain- specific, *ex-vivo* mouse model.

To test whether the suppression of cytokine release by *T. crassiceps* larval extracts would prevail under exacerbated inflammatory conditions, I explored the ability of 150 µg/ml of whole cyst homogenate to suppress cytokine release by increasing doses of LPS (10^1, 10^2, 10^3 and

10^4 ng/ml). Impressively, this concentration of whole cyst homogenate significantly supressed cytokine release even at high LPS concentrations (10^2 ng/ml for IL-6 TNF-α and at 10^3 ng/ml for IL-6, **Suppl Table 5.4B, Fig. 5.4B**). Interestingly, at a concentration of 10^4 ng/ml the whole cyst homogenate seemed almost to exacerbate TNF-α release, although this did not prove to be statistically significant. These results suggest that the anti-inflammatory components of *T. crassiceps* larvae do not have an "all or nothing" effect, but rather a more graded effect.

The inflammatory effects of lipopolysaccharides appear to be mediated by TLR4 (Vargas-Caraveo *et al.*, 2020). I decided to further investigate whether *T. crassiceps* whole cyst homogenate can attenuate inflammation induced by antigens that act on other toll-like receptors. When I exposed slices to 5 µg/ml zymosan A, a TLR2 ligand, it induced a significant increase in the release of IL-6 and TNF-α (**Suppl Table 5.5A, Fig. 5.5A**). The addition of 150 µg/ml homogenate was able to reduce this release of IL-6 and TNF-α to levels comparable to that of the control slices (**Suppl Table 5.5A, Fig. 5.5A**), which demonstrates that the anti-inflammatory effect of *T. crassiceps* whole cyst homogenate is not exclusively TLR4 dependent. Interestingly, when I performed the same experiments using the TLR3 ligand, poly(I:C), the *T. crassiceps* homogenate seemed unable to suppress the induction of IL-6 and TNF-α release by poly(I:C) as strongly as it did that by LPS and zymosan A, with the release of both IL-6 and TNF-α by slices treated with poly(I:C) + homogenate still being significantly higher than that of control slices (**Suppl Table 5.5B, Fig. 5.5B**). This suggests that the *T. crassiceps* homogenate is not as efficient at ameliorating TLR3-mediated inflammation as it is at ameliorating TLR4 and TLR2 mediated inflammation. Further, the reduction observed in the median release of poly(I:C)-induced IL-6 and TNF-α when *T. crassiceps* homogenate was added proved not to be significant, but I believe that this is due to the high variability in the poly(I:C)-induced inflammatory response, which likely resulted due to the aging of the poly(I:C) I utilised. I think that expanding the sample size in this experiment and using freshly prepared poly(I:C), would likely reveal a significant attenuation of the poly(I:C)-induced release of IL-6 and TNF-α by *T. crassiceps* homogenate.

A study by Terrazas *et al.* (2010) similarly demonstrated that *T. crassiceps* excretory/secretory extracts were able to attenuate inflammatory cytokine release by murine dendritic cells in response to several TLR agonists and another by Sun *et al.* (2014) showed this to also be true when the whole cyst homogenate of *M. corti* was utilised to inhibit the release of pro-inflammatory cytokines by murine microglia. These results are interesting, as a number of studies have reported that polymorphisms in the TLR4 gene are related to susceptibility to acquiring NCC, as well as susceptibility to developing symptomatic NCC (Lachuriya *et al.*, 2016; Singh *et al.*, 2015; Verma *et al.*, 2011, 2010). My results suggest that the anti-inflammatory immune-mediation by *Taenia* larvae does not seem to be exclusively TLR4

dependant and a study in a mouse model of NCC also reported that the gene and protein expression of all toll-like receptors, not just TLR4, increased several fold in the brains of animals intracranially infected with *M. corti* (Mishra *et al.*, 2006). Together, these findings suggest that further investigation into the involvement of other toll-like receptors in NCC, in addition to TLR4, may be warranted.

I next wanted to examine the involvement of glycan, protein, and lipid components in mediating the suppressive effect of *T. crassiceps* homogenate on the release of pro-inflammatory cytokines by mouse hippocampal brain slices. Intriguingly, the manipulation of any one of these three components impaired the ability of the *T. crassiceps* homogenate to suppress the release of both IL-6 and TNF-α (**Suppl Table 5.6B, Fig. 5.6B**). The involvement of glycans in the anti-inflammatory properties of *T. crassiceps* and *M. corti* have been previously demonstrated (Gómez-García *et al.*, 2006; Sun *et al.*, 2014; Terrazas *et al.*, 2010; E. Vendelova *et al.*, 2016b). Interestingly, whilst the Terrazas *et al.* (Terrazas *et al.*, 2010) study found a very similar impairment in the ability of *T. crassiceps* extracts to prevent LPS-induced release of pro-inflammatory cytokines when glycan moieties were modified, they found no impairment in this ability when the *T. crassiceps* extracts were subjected to heat inactivation. My findings may differ from theirs perhaps because I utilised different culture models (hippocampal slices versus bone-marrow derived dendritic cells), or perhaps because I investigated different extracts of *T. crassiceps* (whole cyst homogenate versus excretory/secretory extracts).

Contrastingly, Vendelova *et al.* (2016) found that both glycan modification, and heat inactivation of proteins, impaired the ability of *M. corti* excretory/secretory extracts to suppress LPS-induced release of pro-inflammatory cytokines by murine dendritic cells, but that the removal of lipids through ammonium sufate precipitation did not. They suggest, following these findings, that the active anti-inflammatory component in the *M. corti* extract was likely a glycoprotein. I found that the manipulation of glycans, proteins, or lipids in the *T. crassiceps* homogenate impaired its anti-inflammatory properties. If a single molecule were to be responsible for this effect, it would have to be a non-polar molecule (as the ammonium sulfate precipitation targets polar molecules) that nonetheless has glycan, protein, and lipid components. It seems more likely that there is more than one anti-inflammatory agent at play. Additional experiments involving enzymatic digestions (such as with proteinase K) or the fractionation of, or extraction of particular components from, the homogenate using chromatographic methods could help to elucidate what the active agent/s are (see, for example, methods used by Emilia Vendelova *et al.*, 2016). It would further be useful to explore by what anti-inflammatory pathways the *T. crassiceps* homogenate exerts its effects.

In this chapter I explore the usefulness of hippocampal organotypic brain slices treated with *Taenia* larval extracts as a model of NCC in which to study innate immune responses to *Taenia* cysts. I demonstrate that after day one *in vitro* the slices show very low baseline release of IL-6 and TNF-α, but that the innate immune cells respond to inflammatory stimuli by drastically increasing the release of these cytokines. Despite treating the larvae in several ways, none of the larval extracts I prepared were able to induce an appreciable release of IL-6 and/or TNF-α. I did, however, find that the whole cyst homogenate robustly inhibits the release of IL-6 and TNF-α induced by LPS or zymosan A; and that the manipulation of proteins, glycans, or lipids, in the whole cyst homogenate compromised its anti-inflammatory ability. In this chapter I demonstrate that the hippocampal organotypic brain slice culture model is a powerful model in which to examine and characterise innate immune responses in NCC. I additionally describe, and perform some characterisation of, the compelling anti-inflammatory potential of *T. crassiceps* whole cyst homogenate, which could have exciting applications as an anti-inflammatory agent to treat central nervous system inflammation.

Chapter 6

OVERALL DISCUSSION AND CONCLUSIONS

The mechanisms underlying seizures in neurocysticercosis (NCC) remain largely unknown, even though this presents a significant global health burden. As such, in this thesis I set out to "fill" several gaps in the current collective knowledge regarding seizures in NCC, namely: how parasite-derived products directly affect neuronal excitability (Chapter 3); whether larvae can indirectly affect neuronal excitability through the production of AChE enzymes (Chapter 4); and, finally, whether larvae can alter cytokine release by resident brain immune cells which, in turn, may alter neuronal excitability (Chapter 5). There is a scarcity of model systems for NCC which allow for the evaluation of electrophysiological effects. As such, to achieve the above aims, I utilised several iterations of a novel model system for NCC in which rodent hippocampal organotypic brain slice cultures were combined with several different preparations/extracts of *T. crassiceps* larvae. The investigations performed using these models revealed much about their utility and limitations.

6.1 WHAT THE INVESTIGATIONS REPORTED IN THIS THESIS REVEAL ABOUT ORGANOTYPIC BRAIN SLICE CULTURES AS A SYSTEM TO MODEL NEUROCYSTICERCOSIS

6.1.1 Organotypic brain slice culture systems have many merits that are useful in modelling neurocysticercosis.

The patch-clamp experiments which I performed in Chapters 3 & 4, together with the local field-potential recordings reported in Chapter 3, underscore a major strength of this model, which previous studies that have utilised the same experimental system have also demonstrated – the largely unrestricted electrical access to brain tissue (Holopainen, 2005; Liu *et al.*, 2017; Park *et al.*, 2015; Raimondo *et al.*, 2016; Ziobro *et al.*, 2011). The calcium imaging performed in Chapter 3 also serves as a demonstration of the ease of genetic manipulation in this culture model (Opitz-Araya and Barria, 2010), which grants the model much potential for future studies into the cellular and molecular mechanisms involved in NCC-related neuropathology. Another strength of these model systems, as demonstrated by the addition of tetrodotoxin in many of the patch-clamp experiments described in Chapter 3, is that they allow for manipulations in brain tissue which may not be possible *in vivo*, as they may

result in unwanted confounding side-effects or fatality in live animals (Hwang and Noguchi, 2007).

The versatility and fairly rapid throughput of this system (as compared to *in vivo* models), and the fact that these enable fairly large sample sizes, without being majorly resource or time consuming, were other aspects of these model systems that proved useful. This is notable in the patch-clamp experiments performed in Chapter 3, for example, where the excitatory mechanism of *T. crassiceps* homogenate was sequentially narrowed down using a series of slices as opposed to a series of animals. This was further demonstrated in Chapter 5, where the pre-screening of zymosan A and poly-IC doses in a small number of samples informed the appropriate dose for later experiments (**Fig. 5.1 C&D**). The pro-inflammatory properties of a whole range of *T. crassiceps* extracts were easily assessed within the relatively short time frame of a few weeks (**Fig. 5.3**). The cytokine ELISAs further demonstrate the advantage of these models for easily assessing changes in the production of particular molecules of interest by the brain slices simply by analysing the culture medium. This would be possible with numerous other biochemical assays and, although I did not demonstrate this here, the tissue itself can be utilised for biochemical assays, or for various staining and microscopic imaging protocols (Opitz-Araya and Barria, 2010).

In Chapter 3 I further demonstrated that the organotypic brain slice culture model allows for co-culture with live *Taenia* larvae, and whilst this did not result in any observable changes in brain network excitability in my model, it is a very promising model which could in future help to elucidate the cellular and molecular effects that viable larvae have on the brain and could even be used to further explore the immune-suppressive described in Chapter 5, above.

6.1.2 There are several adaptations that could be made to organotypic brain slice culture systems that would greatly increase their utility in studying neurocysticercosis.

The experiments performed in this thesis, whilst demonstrating several strengths of the model systems, also highlighted several limitations and possible areas for improvements. A major drawback of the models utilised in this thesis is that they employ an alternative parasite to model the human disease. The data describing both glutamate content and release (Chapter 3), and AChE activity and its localisation (Chapter 4) in *T. crassiceps* and *T. solium* larvae show that, however closely related the parasites may be, there are still significant differences in their biology. This raises the concern that experimental discoveries made using a model parasite may not have great clinical relevance. Organotypic brain slice culture models for NCC would therefore be greatly strengthened by the use of *T. solium* larvae/larval extracts in place

of *T. crassiceps*, which could potentially be procured from infected pigs in the Eastern Cape province of South Africa (Sithole *et al.*, 2019).

The fact that none of the larval extracts tested in this thesis induced the release of inflammatory cytokines by hippocampal organotypic brain slices was somewhat unexpected, as *Taenia* cysts have clinically been reported to induce a strong pro-inflammatory host immune response upon degeneration (Carpio, 2002; Garcia and Del Brutto, 2017). This could be explained by the possibility that *Taenia* antigens need to be recognised by peripheral immune cells, such as T cells or lymphocytes to prompt an inflammatory host response. The brain parenchyma proper is isolated from the peripheral immune system, but when the blood-brain barrier is compromised due to autoimmune disease, infection, or injury, adaptive immune cells from the periphery are able to infiltrate the parenchyma (Engelhardt *et al.*, 2017; Prinz and Priller, 2017). It has been well established that the blood-brain barrier becomes compromised surrounding *Taenia* brain cysts due to factors like rapid angiogenesis and the disruption of junctional complex proteins (Alvarez and Teale, 2007; Carmen *et al.*, 2018; Sikasunge *et al.*, 2009). It has also been demonstrated that peripheral immune cells are abundant in the tissue surrounding inflamed *T. solium* cysts in human patients (Alvarez *et al.*, 2002a; Restrepo *et al.*, 2001, 1998). It could therefore be hypothesised that, in the case of parenchymal NCC, it is only once breakdown of the blood-brain barrier occurs, and adaptive immune cells infiltrate the brain and encounter larval elements, that the characteristic inflammatory host immune response is mounted.

This hypothesis could potentially be explored using hippocampal organotypic slice culture models where peripheral immune cells are introduced into the hippocampal brain slices. A study by Ling *et al.* [2008] have demonstrated that it is possible to effectively integrate antigen-specific T cells into hippocampal brain slice cultures, as the T cells become activated and migrate to the sites of their specific antigens when applied to hippocampal brain slice cultures. If this could be achieved with various other peripheral immune cells, it could be a very compelling model in which to dissect the contribution of various immune cell types to the cumulative inflammatory response in NCC, as well as in other inflammatory neuropathologies.

Another unexpected finding in this thesis is that T. crassiceps larvae did not alter seizure susceptibility when co-cultured with organotypic hippocampal brain slices. Organotypic hippocampal brain slices represent a small part of the brain in isolation, and it is possible that other parts of the brain are involved in the generation of seizures in neurocysticercosis. The use of extended network slices (such as thalamo-cortical, limbic, and cortico-cortical slices) (Dulla *et al.*, 2018) to study neurocysticercosis in vitro may therefore be recommended.

Another adaptation to the current model that would greatly improve its utility in studying NCC, would be to utilise human brain tissue as opposed to mouse brain tissue. In recent years, the use of "access" human brain tissue (acquired from patients who undergo clinically necessary brain surgery) to produce organotypic brain slice cultures has been proven to be very promising for electrophysiological and imaging investigations, as well as for genetic and optogenetic manipulations (Andersson *et al.*, 2016; Eugène *et al.*, 2014; Le Duigou *et al.*, 2018; Schwarz *et al.*, 2017; Ting *et al.*, 2018). During the course of my PhD, I have assisted in the establishment of human organotypic brain slice cultures in the Raimondo lab, and I succeeded in maintaining such cultures for multiple days, detecting cytokines in the culture medium of slices (unpublished data). Utilising human tissue for organotypic brain slice cultures will greatly increase the chances that discoveries made in this model may be applicable in a clinical context.

6.2 POTENTIAL CLINICAL IMPLICATIONS OF THE FINDINGS OF THIS THESIS

6.2.1 Parasite-derived glutamate could contribute to seizurogenesis in neurocysticercosis and may be released to benefit parasite survival.

In Chapter 3, using patch-clamp electrophysiology, calcium-imaging, and glutamate assays, my collaborators and I showed that acute exposure to *T. crassiceps* larval extracts had an excitatory effect on pyramidal neurons, and that this was the result of activation of glutamate receptors by glutamate in the parasitic extracts. Further, utilising a novel brain slice-larvae co-culture model and local field potential recordings, I illustrated that chronic exposure to live larvae did not result in altered network excitability in hippocampal brain slices.

Glutamatergic signaling has been established to be central in the initiation and spread of seizures, regardless of the primary cause (Chapman, 2000). Indeed, in major pathologies causing adult acquired epilepsy, such as; stroke, traumatic brain injury, and brain tumours (Forsgren *et al.*, 2005; Hauser *et al.*, 1991; Preux and Druet-Cabanac, 2005), glutamatergic signaling and glutamate excitotoxicity are thought to underlie seizure occurrence (Buckingham *et al.*, 2011; Lai *et al.*, 2014; Yi and Hazell, 2006).

The release of large amounts of endogenous parasitic glutamate upon cyst degeneration could result in seizures and excitotoxicity.

Glutamate excitotoxicity is the phenomenon whereby depolarized, damaged, or dying neurons release large amounts of glutamate, which bind to N-methyl-D-aspartate (NMDA) and α-Amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptors on surrounding neurons. This results in prolonged neuronal depolarization, Ca^{2+} influx and subsequent activation of necrotic or apoptotic pathways (Ankarcrona *et al.*, 1995). The dying cells, in turn,

release large amounts of glutamate thereby propagating the excitotoxic process. Some neurons may survive the prolonged exposure to glutamate, but such cells have been shown to have undergone changes that cause them to become hyperexcitable, making the network more prone to seizures (DeLorenzo *et al.*, 1998; Lee *et al.*, 2011; Sun *et al.*, 2001; Terunuma *et al.*, 2010). Given the finding that *Taenia* larvae contain significant quantities of glutamate, it seems quite plausible that the degeneration of the cyst and subsequent release of the parasite's ample endogenous glutamate could cause seizures (due to prolonged neuronal depolarization), and further initiate an excitotoxic cascade, in much the same way as abrupt cell damage does in cases of stroke or traumatic brain injury (Lai *et al.*, 2014; Yi and Hazell, 2006). If the release of endogenous parasitic glutamate does, in fact, contribute to seizures in NCC, it could explain why seizures often only present once cysts start to degenerate.

The excitotoxic effects of the release of endogenous parasitic glutamate upon cyst degeneration could be amplified in NCC by the presence of reactive astrocytes. "Reactive astrocytosis" describes a state in which astrocytes have undergone a phenotypical change in response to central nervous system pathology (Buckingham and Robel, 2013). Reactive astrocytes have long been associated with epilepsy, and studies in numerous epilepsy models have found that glutamate uptake/buffering by reactive astrocytes is disrupted (Crunelli *et al.*, 2015). Notably, reactive astrocytosis has also been described to be a common host response to larval cysts in NCC (Fleury *et al.*, 2016). This could potentially result increased susceptibility of pericystic brain regions to damage mediated by the release of endogenous parasitic glutamate, as the ability of pericystic astrocytes to buffer glutamate in the extracellular space may be impaired.

Continual release of glutamate by viable Taenia cysts may eventually overwhelm the brain's ability to buffer extracellular glutamate and may further "prime" the brain for seizures.

In addition to the finding that viable *Taenia* larvae contain large amounts of glutamate, I also report that they steadily release glutamate over several days in culture. If larvae lodged in the brain consistently release glutamate into the extracellular space, this could have significant implications. Under normal conditions, extracellular glutamate concentrations are tightly regulated to prevent excitotoxicity, primarily by the uptake and breakdown of glutamate by astrocytes (Coulter and Eid, 2012). It is possible, however, that glutamate released by *Taenia* could potentially overwhelm the ability of astrocytes to take up glutamate, leading to a gradual increase in extracellular glutamate levels. Indeed, one study, in which glioma cells (which continually release glutamate) and astrocytes were co-cultured, reports that as the glioma/astrocyte ratio was increased the levels of glutamate in the culture medium also increased – demonstrating that astrocytes have a limited ability to take up excess glutamate

(Yao *et al.*, 2014). Increased extracellular glutamate is known to precede seizures (During and Spencer, 1993; Vespa *et al.*, 1998), which could potentially explain why, on occasion, seizures occur in patients with only viable cysts (Fleury *et al.*, 2004; Garcia and Del Brutto, 2017).

Additionally, the prolonged presence of elevated extracellular glutamate could "prime" the brain for seizures, by, for instance, acting on astrocytes. Glutamate is known to cause Ca^{2+} influx in astrocytes, and it has been shown that astrocytes with high basal levels of intracellular Ca^{2+} can initiate seizures when exposed to any stimulus that causes an additional Ca^{2+} influx (Crunelli *et al.*, 2015). It is therefore plausible that glutamate produced by viable *Taenia* cysts in the brain could make the surrounding cellular networks more susceptible to hyperexcitability and seizures, both by increasing extracellular glutamate concentrations and by increasing basal astrocytic Ca^{2+} levels, such that an additional excitatory stimulus (such as an inflammatory reaction to, or glutamate from, a degenerating cyst) may be sufficient to induce seizures, where it may not have been under non-pathological conditions.

Taenia larvae may release glutamate to promote their survival.

The discovery that *Taenia* larvae can consistently produce glutamate also poses the question of what function this serves for the parasite itself. Interestingly, in gliomas, it has been found that the release of glutamate is necessary both to create space into which the tumour can expand (by killing surrounding neurons in an excitotoxic manner), and to activate signaling pathways that result in cell growth, motility and invasion (de Groot and Sontheimer, 2011). Perhaps *Taenia* larvae release glutamate in a similar attempt to expand and survive. Glutamate blockers are currently being explored as a novel therapy for gliomas (Liubinas *et al.*, 2014). The prospect of glutamate blockers as an anti-helminthic treatment is alluring, as the evidence does suggest that it could also be of potential therapeutic value in the prevention of NCC-related seizures.

6.2.2 Parasite-derived acetylcholinesterases could contribute to seizurogenesis in neurocysticercosis.

In Chapter 4, using Ellman's assays and AChE activity stains, I demonstrated that both *T. crassiceps* and *T. solium* larvae produce AChEs with notable activity and using additional patch-clamp experiments I present evidence that suggests that these have sufficient activity to modulate neuronal AChE signaling in hippocampal organotypic brain slices.

Taenia larval-derived acetylcholinesterases could contribute to seizurogenesis in neurocysticercosis by disrupting peri-cystic neuronal acetylcholine signaling.

The fact that *Taenia* larval-derived AChEs have sufficient activity to break down acetylcholine at a concentration which induces changes in neuronal signaling has potential implications in

the context of NCC. These experiments demonstrate that although the activities of *Taenia* larval-derived AChEs may not seem substantial when compared to that of other parasitic worms, such as *Nippostrongylus brasiliensis,* they may well be substantial enough to exert an effect in the sensitive brain environment (*Nippostrongylus brasiliensis* has a reported AChE activity of 5.3 $\mu mol.min^{-1}.mg^{-1}$ in it excretory/secretory extracts (Grigg *et al.*, 1997), versus the much lower 14.0 nmol min^{-1} mg^{-1} activity of the membrane and scolex extract of *T. solium* that I report in Chapter 4). In the clinical context, *T. solium* larvae interface directly with brain tissue. It is plausible, then, that upon degeneration of the cyst, membrane AChEs could impact pericystic cholinergic neurotransmission in a direct, spatially concentrated manner.

In this thesis I have shown that AChEs reduced acetylcholine-induced pyramidal cell depolarization, an inhibitory action. Interestingly, however, Zimmerman *et al.* (2008) reported that in epileptic rats there was an increase in extracellular AChEs in the brain and that this was associated with an increased sensitivity to acetylcholine which in turn resulted in acetylcholine signaling inducing seizure activity in the epileptic animals. It seems conceivable, then, that an increase in AChEs in the extracellular brain environment as a result of cyst degeneration could similarly induce greater acetylcholine sensitivity and could contribute to the generation of seizure activity in NCC, particularly if combined with other seizure-promoting processes. Although I have demonstrated in this study that larval-derived AChEs have sufficient activity to break down acetylcholine at a concentration which induces changes in neuronal signaling in an *ex vivo* brain slice model, further electrophysiological investigations, preferably using purified *T. solium* AChE applied in an *in vivo* model, are required to determine whether the activity of larval-derived AChEs can, in fact, functionally alter neuronal signaling.

Taenia larval-derived acetylcholinesterases could contribute to seizurogenesis in neurocysticercosis by impairing acetylcholine-mediated anti-inflammatory immune signalling.

In the brain, microglia and astrocytes regulate inflammatory signaling via, amongst other mechanisms, acetylcholine receptor-dependent signaling (Carnevale *et al.*, 2007). The activation of acetylcholine receptors on microglia and astrocytes has been shown to strikingly impair acute phase inflammatory responses (Binning *et al.*, 2020; Patel *et al.*, 2017). One might hypothesise, then, that exposure of the brain to *T. solium* membrane AChEs during cyst degeneration could exacerbate perilesional inflammation if the larval AChEs break down acetylcholine molecules involved in anti-inflammatory signaling. Based on this supposition, future investigations involving the addition of AChE inhibitors to NCC model systems that present with severe perilesional inflammation, could be of potential value.

6.2.3 Data describing and characterising the immunomodulatory abilities of Taenia larvae can inform treatment strategies for neurocysticercosis, as well as for other inflammatory central nervous system disorders.

In Chapter 5, I demonstrate that extracts from viable *T. crassiceps* larvae ameliorate the release of pro-inflammatory cytokines by hippocampal brain slices upon exposure to inflammatory stimuli. This phenomenon has previously been reported in a number of experimental models (Gómez-García *et al.*, 2006; Landa *et al.*, 2019; Martínez-Saucedo *et al.*, 2019; Terrazas *et al.*, 2013, 2011, 2010), but to my knowledge I am the first to demonstrate this ability in the hippocampal organotypic brain slice model. These results serve as a demonstration that viable *Taenia* larvae induce significant immunomodulatory changes in their host (Peon *et al.*, 2016). In this experimental set-up, the *Taenia* larval extracts induced a state of immune hyporesponsiveness, a phenomenon seen in many other helminthic infections (Maizels and McSorley, 2016). It has been well established that symptomatic NCC is generally accompanied by a strong, even excessive, inflammatory host immune response in the brain (Nash *et al.*, 2015a). It seems plausible, then, that upon the degeneration of *Taenia* larvae, an exaggerated, inflammatory host immune response occurs, in part, because of the cessation of immune modulatory strategies employed whilst the larvae are viable. This is a possibility which does not yet seem to have been considered in existing literature on NCC, but one that I believe warrants further investigation.

In Chapter 5 I further demonstrated that extracts from viable *T. crassiceps* larvae can reduce the release of pro-inflammatory cytokines induced by agonists of several different TLRs, and that this ability appears to be mediated by protein, glycan, and lipid elements. These are valuable contributions to the field of NCC immunology, as they contribute to a greater understanding of how *Taenia* larvae modulate the host immune system. The fact that the manipulation of protein, glycan or lipid elements impaired the ability of *Taenia* derived extracts to suppress cytokine release suggests, for example, that *Taenia* larvae may have a level of redundancy in their strategies to modulate components of the immune response which could lead to their elimination. A comprehensive understanding of NCC immunology can ultimately inform the development of superior treatment and management strategies for clinical NCC.

In recent years, there has been a growing urgency to elucidate the mechanisms of immune modulation by helminthic parasites for another reason – the immune hyporesponsiveness induced by helminths has been proposed to hold great therapeutic potential in the treatment of a variety of allergic, autoimmune and inflammatory diseases (Maizels and McSorley, 2016). In Chapter 5 of this thesis, I demonstrate that the extracts of viable *T. crassiceps* larvae can prevent the release of TNFα by the innate immunocompetent cells of the CNS. TNFα has been implicated in the neuropathology and excitotoxic processes in various neurological disorders,

including; traumatic brain injury, ischemia, Alzheimer's disease, Parkinson's disease, multiple sclerosis, and amyotrophic lateral sclerosis (Olmos and Lladó, 2014). There is, then, the very exciting prospect that, if the data presented here were expanded upon and the individual agents/fractions responsible for the amelioration of TNFα release isolated, they could potentially offer a novel therapy for a host of neuropathologies, including symptomatic NCC. Drugs inspired by the strategies employed by helminths to regulate the host immune response are of particular interest, as they are likely to be safe and have minimal immunogenicity (Ryan *et al.*, 2020).

6.3 Conclusion

In this doctoral thesis I explore how *Taenia* larvae may facilitate seizures by acting directly on neurons/neuronal networks (Chapter 3), by acting on a neurotransmitter (Chapter 4), and by acting on the innate immunocompetent cells of the brain (Chapter 5). In collaboration with colleagues, I report the novel finding that both *T. crassiceps* and *T. solium* larvae produce and release significant amounts of the excitatory neurotransmitter glutamate, which is sufficient to cause large depolarisations of neurons *ex vivo*, and I discuss several mechanisms by which this could viably initiate or potentiate seizures in NCC. I also detail the production, release, location, and activity of AChE enzymes in these two species of larvae and similarly outline ways in which the activity of *T. solium* AChEs could contribute to seizures in NCC. I further demonstrate, for the first time in an *in vitro* brain model, that extracts of viable *T. crassiceps* larvae have a robust ability to prevent the release of pro-inflammatory cytokines by brain innate immune cells after exposure to agonists of several different toll-like receptors. I show that this ability is mediated by protein, glycan, and lipid elements. I also raise the possibility that this ability could potentially be harnessed for the development of novel therapeutic agents for a variety of neuroinflammatory pathologies.

Two major insights become apparent when considering the investigations explored in this thesis as a whole: The first is that seizures secondary to NCC are complex and multi-faceted, with many potential contributing mechanisms. The second is that seizurogenesis in NCC seems to be inextricably linked to host immune responses. These observations underscore the importance of continued work towards the understanding of seizurogenesis and host immune responses in NCC. Together, the insights provided by this thesis present novel and valuable contributions to our collective understanding of the pathogenesis of NCC. They also serve to inform future investigations in the field and highlight several exciting and promising avenues for future inquiry.

Supplemental Table 5.1A: Baseline cytokine release of hippocampal organotypic brain slice cultures.

		Day in vitro					
		1	2	3	4	5	6
		Cytokine release (pg/ml)					
IL-6	Median	51.0	3.9	3.9	3.9	3.9	3.9
	IQR	26.5 – 72.3	3.7 – 4.4	3.9 – 3.9	3.9 – 3.9	3.9 – 3.9	3.9 – 3.9
TNF-α	Median	9.6	2.4	2.2	2.0	2.0	3.8
	IQR	7.8 – 11.8	2.0 – 7.7	1.9 – 4.2	2.0 – 3.4	2.0 – 2.9	2.4 – 6.3

Supplemental Table 5.1B: Cytokine release by hippocampal organotypic brain slice cultures in response to different doses of lipopolysaccharides.

		Lipopolysaccharide concentration (ng/ml)				
		0	10^1	10^2	10^3	10^4
		Cytokine release (pg/ml)				
IL-6	Median	0.031	1.115	1.135	1.811	4.540
	IQR	0.004 – 0.053	0.751 – 1.511	0.942 – 1.255	1.635 – 2.430	3.470 – 7.020
	# of wells	16	14	8	8	15
TNF-α	Median	0.031	0.562	0.150	0.763	1.190
	IQR	0.007 – 0.051	0.413 – 0.687	0.327 – 0.878	0.713 – 0.933	0.792 – 1.490
	# of wells	16	14	8	8	15
IL-1β	Median	0.039	0.089	-	-	0.038
	IQR	0.031 – 0.073	0.067 – 0.135	-	-	0.031 – 0.113
	# of wells	11	7	-	-	8

Supplemental Table 5.1C: Cytokine release by hippocampal organotypic brain slice cultures in response to different doses of zymosan A.

		Zymosan A concentration (ng/ml)				
		0	10^1	10^2	10^3	10^4
		Cytokine release (pg/ml)				
IL-6	Median	0.031	0.085	0.067	1.060	2.140
	IQR	0.004 – 0.053	-	-	-	-
	# of wells	16	1	1	1	1
TNF-α	Median	0.031	0.198	0.151	0.580	1.590
	IQR	0.007 – 0.051	-	-	-	-
	# of wells	16	1	1	1	1

Supplemental Table 5.1D: Cytokine release by hippocampal organotypic brain slice cultures in response to different doses of polyinosinic–polycytidylic acid (poly(I:C))

		Poly(I:C) concentration (ng/ml)				
		0	10^2	10^3	10^4	10^5
		Cytokine release (pg/ml)				
IL-6	Median	0.031	0.264	1.020	4.000	4.000
	IQR	0.004 – 0.053	-	-	-	-
	# of wells	16	1	1	1	1
TNF-α	Median	0.031	0.169	0.228	1.090	2.800
	IQR	0.007 – 0.051	-	-	-	-
	# of wells	16	1	1	1	1

Supplemental Table 5.2A: Cytokine content of *T. crassiceps* larval extracts

		Cytokine content (pg/mg total protein)	
		Whole cyst homogenate	Excretory/secretory extracts
IL-6	Median	3.1	92.6
	IQR	1.0 – 8.5	5.8 – 1357.0
	# of batches	8	3
TNF-α	Median	5.3	80.8
	IQR	2.2 – 15.8	29.9 – 942.9
	# of batches	8	3
IL-1β	Median	18.7	253.4
	IQR	9.4 – 109.1	23.5 – 271.4
	# of batches	4	3

Supplemental Table 5.2C: Cytokine release by *T. crassiceps* larvae in vitro.

		Day in vitro			
		3	5	8	10
		Cytokine release (pg/mg total protein/day)			
IL-6	Batch 1	21.3	17.2	1.6	4.5
	Batch 2	286.1	93.4	13.3	70.2
TNF-α	Batch 1	46.3	27.3	1.2	3.9
	Batch 2	278.5	70.2	15.7	17.5
IL-1β	Batch 1	18.5	51.1	7.8	12.9
	Batch 2	101.9	124.9	53.5	248.7

Supplemental Table 5.3A: Cytokine release by hippocampal organotypic brain slice cultures when exposed to whole cyst homogenate or excretory/secretory extracts.

		Cytokine production ng/ml)		
		Control	**Whole cyst homogenate**	**Excretory/secretory extracts**
IL-6	Median	0.008	0.043	0.008
	IQR	0.004 – 0.031	0.031 – 0.068	0.008 – 0.045
	# of wells	35	12	6
TNF-α	Median	0.016	0.024	0.012
	IQR	0.008 – 0.031	0.008 – 0.039	0.012 – 0.012
	# of wells	34	12	6
IL-1β	Median	0.039	0.088	0.068
	IQR	0.031 – 0.073	0.049 – 0.015	0.038 – 0.103
	# of wells	11	7	6

Supplemental Table 5.3B: Cytokine release by hippocampal organotypic brain slice cultures when exposed to different preparations of *T. crassiceps* extracts.

		Cytokine production ng/ml)							
		Control	**PQ ES**	**PQ Hom**	**Starved ES**	**Starved Hom**	**DMSO Hom**	**DMSO CTR**	**Granuloma**
Final concentration in medium (µg/ml)		_	100	50	100	65	500	-	100
IL-6	Median	0.008	0.025	0.008	0.008	0.008	0.008	0.008	0.014
	IQR	0.004 – 0.031	0.008 – 0.138	0.007 – 0.014	0.008 – 0.008	-	0.008 -0.892	0.008 – 0.386	0.008 – 0.049
	# of wells	35	3	4	3	1	7	3	3
TNF-α	Median	0.016	0.023	0.012	0.008	0.004	0.103	0.039	0.025
	IQR	0.008 – 0.031	0.004 – 0.062	0.003 – 0.035	0.004 – 0.011	-	0.012 – 0.266	0.015 – 0.041	0.021 – 0.031
	# of wells	34	3	4	3	1	7	3	3

Abbreviations: PQ ES: excretory/secretory extracts of larvae treated with 0.2 µg/ml praziquantel for 1 week; PQ Hom: the whole cyst homogenate of larvae treated with 0.2 µg/ml praziquantel for 1 week; Starved ES: excretory/secretory extracts of larvae “starved” in vitro by not replacing the culture medium for 3 weeks; Starved Hom: whole cyst homogenate of larvae “starved” in vitro by not replacing the culture medium for 3 weeks; DMSO Hom: a solution prepared by solubilising the pellets remaining after the preparation of whole cyst homogenate in 2 % DMSO; DMSO CTR: a control for the DMSO Hom, where the same volume of 2% DMSO was added to culture to control for the effect of DMSO itself on brain slices; Granuloma: this solution was prepared by isolating dense yellow granulomas from T. crass larvae harvests and then homogenising these.

Supplemental Table 5.4A: Cytokine release by hippocampal organotypic brain slice cultures when exposed to 10 ng/ml lipopolysaccharides together with 150 µg/ml of different *T. crassiceps* extracts.

		Cytokine production (ng/ml)					
		Control	**LPS**	**LPS + Hom**	**LPS + ES**	**LPS + CM**	**LPS + CVF**
IL-6	Median	0.008	2.519	0.088	0.320	0.375	0.008
	IQR	0.004 – 0.031	1.274 – 3.223	0.020 – 0.195	0.206 – 0.431	0.320 – 0.435	0.008 – 0.507
	# of wells	35	40	20	6	6	12
TNF-α	Median	0.015	1.700	0.071	0.083	0.112	0.013
	IQR	0.008 – 0.031	0.566 – 2.760	0.024 – 0.153	0.065 – 0.107	0.087 – 0.157	0.008 – 0.531
	# of wells	34	41	22	6	6	11

Abbreviations: LPS: lipopolysaccharides; Hom: whole cyst homogenate; ES: excretory/secretory extracts; CM: cyst membrane; CVF: cyst vesicular fluid.

Supplemental Table 5.4B: Cytokine release by hippocampal organotypic brain slice cultures when exposed to different doses of lipopolysaccharides together with 150 µg/ml of *T. crassiceps* homogenate.

		Cytokine production ng/ml)							
		LPS 10^1 ng/ml	**LPS 10^1 ng/ml + Hom**	**LPS 10^2 ng/ml**	**LPS 10^2 ng/ml + Hom**	**LPS 10^3 ng/ml**	**LPS 10^3 ng/ml + Hom**	**LPS 10^4 ng/ml**	**LPS 10^4 ng/ml + Hom**
IL-6	Median	2.519	0.089	1.135	0.379	1.811	1.310	4.540	3.820
	IQR	1.274 – 3.223	0.020 – 0.195	0.942 – 1.255	0.318 – 0.468	1.635 – 2.430	0.973 – 1.723	3.470 – 7.020	2.190 – 4.410
	# of wells	40	20	8	8	8	8	15	7
	% suppression		96.5		66.6		27.7		15.9
TNF-α	Median	1.700	0.071	0.450	0.292	0.763	0.626	1.190	1.580
	IQR	0.566 – 2.760	0.024 – 0.153	0.327 – 0.878	0.225 – 0.398	0.713 – 0.933	0.529 – 1.002	0.792 – 1.490	1.240 – 1.900
	# of wells	41	22	8	8	9	8	15	7
	% suppression		95.8		35.1		18.0		-32.8

Abbreviations: LPS: lipopolysaccharides; Hom: whole cyst homogenate.

Supplemental Table 5.5A: Cytokine release by hippocampal organotypic brain slice cultures when exposed to 5 µg/ml zymosan A alone, or together with 150 µg/ml *T. crassiceps* whole cyst homogenate.

		Cytokine production (ng/ml)		
		Control	**Zymosan A**	**Zymosan A + Hom**
IL-6	Median	0.008	0.475	0.004
	IQR	0.004 – 0.031	0.195 – 1.020	0.004 – 0.028
	# of wells	35	11	11
TNF-α	Median	0.016	0.188	0.015
	IQR	0.008 – 0.031	0.163 – 0.278	0.008 – 0.032
	# of wells	34	11	11

Abbreviations: Hom: whole cyst homogenate.

Supplemental Table 5.5B: Cytokine release by hippocampal organotypic brain slice cultures when exposed to 5 µg/ml polyinosinic–polycytidylic acid (poly(I:C)) alone, or together with 150 µg/ml *T. crassiceps* whole cyst homogenate.

		Cytokine production (ng/ml)		
		Control	**Poly(I:C)**	**Poly(I:C) + Hom**
IL-6	Median	0.008	1.050	0.450
	IQR	0.004 – 0.031	0.694 – 4.205	0.215 – 0.500
	# of wells	35	9	9
TNF-α	Median	0.016	0.154	0.071
	IQR	0.008 – 0.031	0.150 – 0.493	0.036 – 0.097
	# of wells	34	9	9

Abbreviations: Hom: whole cyst homogenate.

Supplemental Table 5.6A: Cytokine release by hippocampal organotypic brain slice cultures when exposed to 10 ng/ml lipopolysaccharides alone, or together with 150 µg/ml *T. crassiceps* whole cyst homogenate that had been treated to either modify glycan moieties, remove lipids, or inactivate proteins.

		Cytokine production (ng/ml)						
		Control	**LPS**	**LPS + Hom**	**LPS + MP Hom**	**LPS + P Hom**	**LPS + AS Hom**	**LPS + HI Hom**
IL-6	Median	0.008	2.519	0.089	0.004	1.740	1.275	3.070
	IQR	0.004 – 0.031	1.274 – 3.223	0.020 – 0.0196	0.004 – 0.004	1.710 – 2.370	1.065 – 1.590	2.900 – 3.880
	# of wells	35	40	20	7	8	7	7
TNF-α	Median	0.016	1.700	0.071	0.017	2.020	1.535	1.520
	IQR	0.008 – 0.31	0.566 – 2.760	0.024 – 0.153	0.011 – 0.028	1.900 – 2.060	1.380 – 1.685	1.450 – 2.410
	# of wells	34	41	22	7	8	7	7

Abbreviations: LPS: lipopolysaccharides; Hom: whole cyst homogenate, MP Hom: mock periodate treated Hom as a control for P Hom;
P Hom: periodate treated Hom for modification of glycan moieties; AS Hom: ammonium sulfate precipitated Hom for removal of lipid elements;
HI Hom: heat inactivated Hom for denaturing of proteins.

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