Mechanisms underlying neural circuit remodeling in *Toxoplasma gondii* infection

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ABSTRACT

The central nervous system (CNS) is protected by a vascular blood-brain barrier that prevents many types of pathogens from entering the brain. Still, some pathogens have evolved mechanisms to traverse this barrier and establish a long-term infection. The apicomplexan parasite, *Toxoplasma gondii*, is one such pathogen with the ability to infect the CNS in virtually all warm-blooded animals, including humans. Across the globe, an estimated 30% of the human population is infected with *Toxoplasma*, an infection for which mounting evidence suggests increases the risk for developing neurological and neuropsychiatric disorders, like seizures and schizophrenia. In my dissertation, I investigate the telencephalic neural circuit changes induced by long-term *Toxoplasma* infection in the mouse brain and the neuroimmune signaling role of the complement system in mediating microglial remodeling of neural circuits following parasitic infection.

While there has been keen interest in investigating neural circuit changes in the amygdala – a region of the brain involved in fear response and which *Toxoplasma* infection alters in many species of infected hosts – the hippocampus and cortex have been less explored. These are brain regions for which *Toxoplasma* also has tropism, and moreover, are rich with fast-spiking parvalbumin perisomatic synapses, a type of GABAergic synapse whose dysfunction has been implicated in epilepsy and schizophrenia. By employing a range of visualization techniques to assess cell-to-cell connectivity and neuron-glia interactions (including immunohistochemistry, ultrastructural microscopy, and microglia-specific reporter mouse lines), I discovered that long-term *Toxoplasma* infection causes microglia to target and ensheath neuronal somata in these regions and subsequently phagocytose their perisomatic inhibitory synapses. These findings provide a novel model by which *Toxoplasma* infection within the brain can lead to seizure susceptibility and a wider range of behavioral and cognitive changes unrelated to fear response.

In the *Toxoplasma* infected brain, microglia, along with monocytes recruited to the brain from the periphery, coordinate a neuroinflammatory response against pathogenic invasion. This is characterized by a widespread activation of these cells and their increased interaction with

neurons and their synaptic inputs. Yet, whether *T. gondii* infection triggers microglia and monocytes (i.e. phagocytes) to target, ensheath, and remove perisomatic inhibitory synapses on neuronal somata indiscriminately, or whether specificity exists in this type of circuit remodeling, remained unclear. Through a comprehensive assessment of phagocyte interactions with cortical neuron subtypes, I demonstrate that phagocytes selectively target and ensheath excitatory pyramidal cells in long-term infection. Moreover, coupling of *in situ* hybridization with transgenic reporter lines and immunolabeling revealed that in addition to phagocytes, excitatory neurons also express complement component C3 following infection (while inhibitory interneurons do not). Lastly, by employing targeted deletion of complement components, C1q and C3, I show that complement is required for phagocyte ensheathment of excitatory cells and the subsequent removal of perisomatic inhibitory synapses on these cells (albeit not through the classical pathway). Together, these studies highlight a novel role for complement in mediating synapse-type and cell-type specific circuit remodeling in the *Toxoplasma* infected brain.

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GENERAL AUDIENCE ABSTRACT

Parasites are microorganisms that rely on other living organisms (called hosts) for their survival. Although some parasites only live on their hosts, others have developed ways to establish infections and obtain the nutrients that keep them alive from host cells. My Ph.D. research has focused on studying one of these parasites, *Toxoplasma gondii* (commonly referred to as *Toxo*), that has evolved the unique ability to establish brain infections in almost all animals around the world, from rodents to humans. Recent discoveries show that brain infection with this parasite can cause seizures, an imbalance in the way that specialized cells of the brain (called neurons) communicate with each other, causing harmful hyperactivity within the brain. *Toxo* infection can also cause behavioral and cognitive changes in infected animals, making them more susceptible to predation. In humans, infection with *Toxo* increases their risk for developing different types of mental illness, such as schizophrenia.

The focus of my Ph.D. research has been in trying to understand, at the cellular and molecular level, how infection with this parasite can lead to seizures and behavioral changes, by using mice as a model. Mice have a similar brain structure to humans, and over the years, scientists have developed many tools that allow us to visualize and study the connections between neurons (called synapses). I'm interested in understanding how changes in these connections affect how neurons communicate with each other, and ultimately, how we behave and think.

I have been studying a type of connection that, if lost or damaged, can lead to seizures and some types of mental illness. These connections are called 'perisomatic inhibitory synapses', and they form on many distinct types of neurons, but specifically on the cell bodies of these neurons. They act as a traffic light, informing neurons when and for how long to 'slow down' their activity. I discovered that after the parasite enters the brain, it causes another type of cell in the brain, called microglia, to extensively interact with neurons in the cortex and hippocampus (areas of your brain important for thinking, executing behavior, and learning). Microglia are immune cells of the brain that inspect the brain for anything damaged or that doesn't belong (like

parasites) and removes them from the brain. By performing experiments where I delete individual immune molecules from mice, I found that one immune molecule, called 'complement component C3' acts as cue for microglia to find these cells, wrap around them, and permanently remove these important connections. Surprisingly, however, microglia don't remove these connections from all neurons, indiscriminately, they do so only on one specific cell type called 'excitatory pyramidal neurons,' and as the name implies, they're the ones who drive activity in the brain.

My half-a-decade's worth of research helps us understand parasitic infections in the brain in a couple of ways: First, I have discovered one of the mechanisms by which neuronal connections are lost in the *Toxo*-infected brain (which is a mechanism that leads to loss of neuronal connections in the injured and aging brain as well). This is significant because it might provide insight into why some people who are infected with *Toxo* develop seizures or mental illness, while others don't. More importantly, *Toxo*-infection causes changes in the brain that are very specific, in terms of both the type of neuronal connection that is affected and the type of cell that is affected. Why these changes are so specific remain to be uncovered, but it suggests that *Toxo* can either a) trigger a unique immune response in the brain that leads to very precise changes in neuron-to-neuron connections and signaling or b) the parasite, while hiding inside of neurons, may hijack the machinery of certain cell types in a way that helps them survive longer.

DEDICATION

Con mucho cariño, se los dedico a las tres mujeres más fuertes de mi familia

A mi mamá, Betty
A mi abuelita, Ventura
y
A mi abuelita, Luisa

Sus sacrificios, fortaleza, y amor a Dios y a mi, son las razones por las cual yo estoy aquí hoy.
Gracias por darme la oportunidad de tener una educación. Este es nuestro logro.
Las quiero mucho, siempre

To the three strong women of my family.

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developing research skills takes time. I sat there for hours, on my birthday, and gave you some nearly perfect slides. You showed Mike and said something along the lines of "oh, she's good!" and that was the beginning of our wonderful, almost decade-long time working side by side (literally, because I refuse to sit anywhere else that's not directly behind you). You are one of the best, if not the best, teacher I have ever had. I will always be indebted to you for taking the time to explain experiments in great detail and sharing all of your top-secret pro tips with me (even if I earn back questions by helping you with Prism and Illustrator). Which reminds me, I still have about 1,000 questions I can ask you before I leave. Lastly, thank you for sharing your confocal time with me, donating some of your reagents, tissues, and probes, helping me take care of my animals, troubleshooting cloning with me, and not making me clean my messy bench until after I defend (I promise I'll get to that August 2nd). I really couldn't have made it through these last few months without you. I hope you're proud of how far I've come and I look forward to reading your CNS papers (and hopefully collaborating together in the future). And in totally, Jianmin fashion, good luck with your science, sir!

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Chapter 1:

INTRODUCTION

Toxoplasma gondii: the exception to every rule

The apicomplexan parasite, *Toxoplasma gondii (T. gondii)*, was first discovered in 1908 by French scientists, Charles Nicolle and Louis Herbert Manceaux, in the tissues of a laboratory animal, a North African hamster-like rodent named the gundi. First mistaken for a different parasite they were investigating, the *Leishmania*, they quickly realized they had come across a novel organism based on its unfamiliar crescent-shaped morphology. They termed it *Toxo* = arc, *plasma* = life (Nicolle and Manceaux 1908, Nicolle and Manceaux 1908). That same year, in what would later foreshadow the parasite's staggering ability to infect virtually all warm-blooded animals, Brazilian scientist, Alfonso Splendore also independently identified the parasite in a different laboratory animal, the rabbit (Ferguson 2004).

Despite this important discovery, *T. gondii's* medical relevance remained unknown for another 20-30 years, until the first reported sightings of *T. gondii* cysts were found in the retina of an infant, who had misfortunately developed a severe congenital *T. gondii* infection, characterized by hydrocephalus (i.e. fluid build-up in the brain), seizures, and unilateral microphthalmia (i.e. one abnormally small eyeball) (Wolf and Cowen 1937, Wolf et al. 1939). During this time, the pathogenic potential of *T. gondii* became unmistakable as numerous congenitally infected children presented with the classic triad of symptoms: hydrocephalus, retinochoroiditis, and encephalitis (Wolf et al., 1939, Khan and Khan 2018). Yet, the discovery that shocked the world

the most (perhaps until this day and age) came in the early 1950s with the development of a serological dye that could identify *Toxoplasma*-infected humans and animals alike by determining the presence of *Toxoplasma* antibodies in the serum of infected hosts. The Sabin Feldman dye test allowed for large-scale studies that dramatically shifted scientists' and physicians' understanding of *Toxoplasma gondii* from a rare exotic infection to one of the most common human parasitic infections worldwide (Sabin and Fedlman 1948, Desmonts et al. 1965, Dubey 1988, Dubey 1995, Tenter et al. 2000, Dubey 2002, Dubey 2008). In the 1980s, at the height of the AIDS epidemic, *T. gondii* was recognized as an important opportunistic infection and its reliance on the host's immune system was highlighted as patients with immunocompromised systems suffered from rapid systemic dissemination of the parasite, resulting in severe neurological complications and even death (Luft et al. 1983, Porter and Sande 1992, Winstanley 1995). Fast forward to today, and we find ourselves in the era of a 'mind-bending' parasite with the discovery of *Toxoplasma*'s tropism for the brain (Beverley 1976, Dubey and Beattie 1988), preferential invasion of neurons (Melzer et a., 2010, Cabral et al. 2016), and an extensive inventory of human and murine studies linking *Toxoplasma* infection with a whole list of neurological and behavioral changes ranging from seizures (David et al. 2016, Brooks et al., 2015, Wohlfert et al. 2017) to mood and personality disorders (Flegr et al. 1994, Flegr et al. 1996, Flegr et al. 1998, Flegr et al. 2003, Novotna et al. 2005, Lindova et al. 2006) to debilitating psychiatric disorders like schizophrenia (Grande et al. 2017, Buoli et al. 2017, Elsheikha and Zhu 2016, Lucchese 2017). This is precisely where my research lies.

Parasite biology and life cycle: evolving specialized armor

As with other protozoa parasites, *Toxoplasma gondii*, is a microscopic unicellular organism that relies entirely on its host for its nutrition, survival, and replication (Englund and Sher 1988, Goldsmith and Heyneman 1989). *T. gondii* exists in three distinct infective stages: the crescent-shaped and rapidly dividing tachyzoite, the slow-dividing bradyzoite that exists encased in tissue cysts, and the environmental sporozoite that lives protected inside the oocyst (Dubey et al. 2007, Al-Malki 2020).

The definitive host for *T. gondii* is the feline (domestic cat and their relatives) (Frenkel et al. 1970, Jewel et al. 1972). Upon infecting its felid definitive host, *T. gondii* undergoes sexual reproduction in the intestinal epithelium, producing oocysts that are excreted with the feces and

sporulate in the environment (Dubey et al. 1970, Sheffield 1970, Piekarski et al. 1971, Ferguson et al. 1974, Ferguson et al. 1975, Ferguson et al. 1979, Christie et al. 1978, Dubey et al. 2005, Shapiro et al. 2019). Sporulated oocysts are able to survive in the environment (in soil, water, and on vegetation surfaces) for prolonged periods of time, over a year, due to key structural and biophysical attributes of the oocyst, including the presence of a bilayered sporocyst wall that protects the parasite from temperature variations, ultraviolet radiation, and desiccation (Ferguson et al. 1975, Ferguson et al. 1979, Dubey et al. 1988, Fritz et al. 2012, Dumètre et al. 2013). From there, other warm-blooded animals can ingest the oocysts and subsequently become infected (Weinman and Chandler 1954, Benenson et al. 1982). In these other hosts (or in other tissues aside from the feline intestinal epithelium), *T. gondii* multiplies by asexual reproduction (Cleary et al. 2002).

The tachyzoite form of *T. gondii* is known as the proliferative form, dividing by a specialized process called endodyogeny (Gustafson et al. 1954, Frenkel 1973, Sheffield et al. 1968). This is a form of asexual reproduction favored by parasites in which internal budding of a tachyzoite parent produces two daughter cells. As the mother tachyzoite is incorporated into the daughter cells, it is ultimately destroyed in the replication process (Sheffield et al. 1968, Smith 1995, Dubey et al. 1998, Joiner et al. 1994). *T. gondii* tachyzoites have a specialized inner membrane complex that permits its movement through the host and also aids in its invasion of host cells (Kasper et al. 1994, Bonhomme et al. 2012). One key feature of this complex is the release of specialized secretory vesicles, called micronemes, from the parasite's apical tip (Morrisette et al. 1997). Micronemes contain a variety of proteins, including adhesins and perforins, that upon ejection, allow the parasite to attach to the host cell and propel itself forward by a process termed "gliding motility" (Dobrowoski et al. 1997, Carruthers and Sibley 1997, Soldati et al. 2001). This gliding movement is generated by a series of steps whereby adhesin proteins are released and translocated to the posterior end by actomyosin motors, creating powerful forward movement. Nichols et al. 1987, Frixione et al. 1996, Dobrowolski and Subley 1996, Dobrowolski et al. 1997). The second set of key membrane complex features are the adaptable cytoskeletal and the additional secretory organelles released for host cell invasion, called rhoptries. Upon recognizing and attaching to the host cell via receptors for adhesins, *T. gondii* tachyzoites will project rhoptries into the membrane of the host cell, allowing them to create very tight junctions to propel themselves into the host cell. Importantly, rhoptries also contain membranous materials that aid

in forming the parasitophorous vacuole that protects the parasite once inside the host cell (Dubremetz 2007, Broothroyd and Dubremetz 2008). During the acute phase of infection, *T. gondii* tachyzoites are susceptible to elimination by the host immune system, and therefore utilize its quick asexual replication and its ability to infect all nucleated cells as strategies to increase their survival within the host (Vismarra et al. 2020).

As *T. gondii* tachyzoites disseminate throughout the host, they home into various tissues, including muscles and the brain (Dubey et al. 1998), where they infect nucleated cells (Bonhomme et al. 1992, Dubey et al. 1998) and transform into slow-replicating bradyzoites that encyst (Dubey et al., 1998), essentially encasing themselves in a protective barrier. Originally, it was thought that because of the complexity of the differentiation from tachyzoite to bradyzoite, several transcription factors were likely needed to simultaneously elicit minor effects towards the overall transition. Now, there is evidence for a master regulator of *T. gondii* differentiation (Waldman et al. 2020).

Tropism for the brain: establishing unique residency

Crossing the blood brain barrier

The central nervous system (CNS) is protected by a vascular blood-brain barrier that homeostatically regulates the CNS transport of nutrients and other small molecules important for neuronal function, while restricting the entry of peripheral immune cells, macromolecules, and pathogens that may cause neural tissue damage (Zlokovic 2008, Daneman 2012, Daneman and Prat 2015). This stringent double barrier consists of both physiological and immunological defenses. Endothelial cells, held together by tight junctions, form the walls of blood vessels and acts as the main physical barrier (Brightman and Reese 1969, Daneman and Prat 2015). An ensheathing barrier, comprised of mural cells (namely pericytes – a type of smooth muscle cell associated with blood capillaries) partially wraps around endothelial cells (Daneman and Prat 2015), while astrocytes (a type of glia cell found within the CNS) extend projections called end-feet to further wrap around the vessels (Abbott et al. 2006). Although endothelial cells alone can fulfill the functions of the BBB given their specialized properties in the brain (i.e. gap junctions to serve as physical barriers for the spaces between endothelial cells, efflux and nutrient transporters to regulate passage of molecules, and enzymes to inactivate various drugs, toxins, and even

neurotransmitters), their interactions with the adjacent mural cells and astrocytes are required for the proper maintenance and regulation of the barrier (Janzer and Raff 1987, Biegel et al., 1995, Abbot et al. 2006, Armulik et al. 2010, Daneman and Prat 2015).

Most pathogens that do enter the brain, typically only do so when the host is immunodeficient or if the pathogen is highly virulent. In these cases, the integrity of the blood-brain barrier is compromised (i.e. leaky) and thus allows for easier CNS invasion by pathogens (Dando et al. 2014, Xiao et al. 2020, Profaci et al. 2020). However, in immunocompetent hosts, some pathogens, like *Toxoplasma gondii*, are still able to breach these barriers without causing lethality. Three mechanisms have been proposed for *T. gondii* entry to the CNS: 1) paracellular crossing 2) transcellular crossing and 3) the "Trojan horse" mechanism of entry.

Paracellular crossing: Although *T. gondii* tachyzoites do not possess cilia or flagella to facilitate their mobility, they are in fact able to propel themselves by use of their actin-myosin motors, described above, to generate a gliding movement (Nichols et al. 1987, Frixione et al. 1996, Dobrowolski and Subley 1996, Dobrowolski et al. 1997). This gliding motility is what is thought to aid *T. gondii* tachyzoites to move across the epithelium of the small intestine (the first barrier they encounter upon entering the host) (Barragan and Sibley 2002). Importantly, the gut epithelial barriers share many features with the blood-brain barrier, including tight junctions and regulation barrier properties (Weight et al. 2015, Daneman and Prat 2015, Jones et al 2017). In respect to the blood-brain barrier, current evidence shows that *T. gondii* tachyzoites are capable of adhering to and migrating on vascular endothelium in live-cell microfluidic chambers that mimic physiological conditions of the blood-brain barrier (Harker et al., 2014).

Transcellular crossing: Another proposed method by which *T. gondii* tachyzoites traverse the blood-brain barrier is by exploiting their capabilities of cell invasion. In fact, *T. gondii* tachyzoites are able to infect endothelial cells in multiple organs, including the brain (Konradt et al. 2016). Studies employing reporters that allowed for the visualization of both *T. gondii* tachyzoites and endothelial cells in real-time (via multiphoton in *vivo* imaging), showed that tachyzoites circulating in the bloodstream are able to adhere to and invade CNS endothelial cells, where they replicate until eventually egressing from these cells and depositing these parasites into the CNS (Konradt et al. 2016). This mechanism is proposed to primarily occur in smaller diameter vessels where the physiological shear force conditions are more ideal (Konradt et al.

2016). Moreover, some studies suggest that this mechanism of entry to the brain may precede infected immune-cell infiltration into the CNS (i.e. Trojan horse) (Conley and Jenkins, 1981, Mendez and Koshy 2017, Ross et al., 2020)

The 'Trojan horse' mechanism of entry: *Toxoplasma gondii* is unique in that it has evolutionarily developed several mechanisms to exploit the host immune response to infection to increase its chances of survival (Bhandage et al., 2019). One such mechanism is invasion of immune cells. Upon infection, the host will mount a strong immune response in an attempt to quickly clear the pathogen (Sasai and Yamamoto 2019). *T. gondii tachyzoites* will subsequently invade these immune cells, including dendritic cells and inflammatory monocytes, causing their hypermigration across the blood-brain barrier (Lambert et al. 2006, Ueno et al. 2014, Courret et al. 2006, Lambert et al. 2010, Bhandage et al. 2019, Ross et al. 2021)

Invasion of neurons

Once in the CNS parenchyma, *T. gondii* tachyzoites will persist in the brain by invading CNS cells and establishing intracellular cysts (ranging in size from 5-50 um in diameter) that evade clearance by the host immune system (Sahm et al. 1997, Sullivan et al. 2012). Given the implications of a CNS infection, the localization of cysts throughout the brain and the CNS cell types *T. gondii* invades has been a great area of interest in research. Human data on CNS regions that are susceptible to *T. gondii* cysts primarily come from postmortem tissue from AIDS patients, where *T. gondii* lesions have been found mostly in the cerebral cortex and basal ganglia, with fewer lesions in the cerebellum, brainstem, and spinal cord (Luft and Remington, 1992, Brightbill et al, Maeda et al. 2006, Schroeder et al. 2006, Suzuki et al. 2010). In mouse models of *T. gondii* long-term infection, cysts are observed to be distributed throughout the brain and selective tropism of the *T. gondii* toward a particular brain region or functional system is not observed (Berenreiterová et al 2011). However, in some studies, some brain regions have consistently appeared more infected (by the presence of *T. gondii* cysts) than others. These include the olfactory bulb, entorhinal cortex, somatosensory cortex, motor and orbital cortex, frontal association cortex, visual cortex, hippocampus, and amygdala, although it remains unclear if this is in part due to increased regional area and higher probability for cyst formation, or if these brain regions are more susceptible to *T. gondii* tachyzoite breach of the blood-brain barrier (Berenreiterová et al 2011, McConkey et al. 2013, Hermes et al. 2008, Vyas et al. 2007). Importantly, regions of the brain

with high density of myelinated axons show less incidence of tissue cysts (Berenreiterová et al., 2011). Furthermore, studies have shown that although *T. gondii* cysts are found in all nucleated CNS cells, they primarily form within neurons (Ferguson and Hutchinson, 1987, Sims et al. 1989, Mezler et al. 2010, Cabral et al. 2016). Originally, it was believed this was due to the inability of neurons to eliminate the pathogen from within (while glia cells like astrocytes and microglia could), therefore leaving neurons to be the main host cell for persistent infection. However, the development of a *T. gondii*-Cre *in vivo* system in which parasite entry of cells triggers a permanent host-cell expression of green fluorescent protein (GFP) (even when a *T. gondii* cell infection doesn't lead to cyst formation), demonstrated that *T. gondii* predominantly and almost exclusively interacts with neurons, although the majority of these interactions do not lead to intracellular cysts (Koshy et al., 2010). It is unclear whether these other interactions between *T. gondii* and neurons (in which T. gondii tachyzoites inject parasitic proteins into the cell) are a different type of host-cell invasion that serves to facilitate *T. gondii* persistence within the host, or whether these are a result from unsuccessful attempts at establishing a long-term latent infection in the form of intracellular *T. gondii* cysts.

Neurological consequences and behavioral changes associated with *Toxoplasma gondii*

Toxoplasmic encephalitis: setting the brain on fire

Within the United States, Toxoplasmic encephalitis (TE) affects roughly 25-50% of HIV-infected patients who are seropositive for *Toxoplasma* (Luft and Remington 1992, Zengerle et al., 1991). TE is also readily observed in patients who are severely immunocompromised for other reasons, such as cancer patients and those requiring organ transplants. In immunocompromised individuals, TE can result in headaches, focal neurological deficits, seizures, hemiparesis (slight one-sided weakness), and aphasia (inability to communicate). In severe cases, TE can result in death. It is postulated that a weakened adaptive immune response underlies the reactivation of latent tissue cysts, whereby a decrease in immune function causes the rupture of tissue cysts, releasing bradyzoites into the extracellular environment where they can then differentiate into the tachyzoite stage and once again replicate and spread, causing severe damage in the CNS. However, how a weakened adaptive immune response triggers the differentiation of *T. gondii* from the so called "inactive" bradyzoite cysts to the tachyzoite stage is not yet well understood. Although presentations of Toxoplasmic encephalitis are most seen in immunocompromised

individuals, some cases have also been observed in immunocompetent individuals. In these cases, patients only presented with headaches and/or some unilateral weakness, yet MRIs revealed brain lesions and biopsies of the brain parenchyma revealed acute inflammation, diffuse encephalitis pattern, and the presence of *T. gondii* cysts (Ferreira Lima, et al. 2021, Ramachandran et al. 2014, Vastava et al. 2002, Carme et al. 2002, Cureus et al. 2017). Although patients showed an increase in IgG and IgM antibodies against *T. gondii*, it is unclear whether this is indicative of active tachyzoite proliferation in the brain (either from an initial acute infection or from reactivation of tissue cysts) (as only elevated levels of *Toxoplasm*a IgG and IgA, but not IgM, antibody levels are seen significantly higher in immunocompetent mice with cerebral proliferation of tachyzoites during the chronic stage of the infection (Singh et al. 2010, Liu et al. 2015), or due only to the presence of *T. gondii* cysts within the brain. In either case, the fact that *T. gondii* can cause life-threatening inflammation in the brains of immunocompromised patients and can also induce neuroinflammation in immunocompetent individuals (and perhaps more so than we suspect), is significant as neuroinflammation is a common etiopathogenic factor in many neurological and neurodegenerating disorders (Degen et al. 2018, Skaper et al. 2018, Gilhus and Deuschl 2019, Zindler and Zipp 2010).

Behavioral changes in chronic infection

Infection with *Toxoplasma gondii* has been linked to a number of behavioral changes in all warm-blooded animals studied, including rodents, non-human primates, and strikingly, humans. In the wild, rodents infected with *T. gondii* have been reported to demonstrate reduced aversion to predator (feline) odors, and in fact, appear to actually be attracted to such odors (Berdoy et al., 2000). In mouse studies, chronic infection with *T. gondii* blocks the natural aversion to cat pheromones, while leaving other non-predator fear-related behaviors, such as learned fear and anxiety-like behaviors, completely intact (Vyas et al. 2007). A similar lack of fear to predator odor (or rather attraction) has been observed in chimpanzees infected with *T. gondii* (Poirotte et al. 2016). In other rodent studies, *T. gondii*'s effects appear to be more syndromic, affecting host defensive behaviors more widely and indiscriminately. In these cases, the increase in exploratory behaviors of infected rodents more closely resembles greater risk-taking and impulsivity in humans (Boillat et al. 2020, Eells et al. 2015, Gulinello et al. 2010, Ihara et al. 2016, Mahmoud et al. 2017, Mahmoud et al. 2016). In humans, over 50 clinical studies in the last 20 years have found chronic *T. gondii* infection to be a risk factor for impaired psychomotor performance (Havlikev et

al. 2001, Flegr et al. 2001, Yereli et al. 2006), personality disorders (Flegr et al. 1994, Flegr et al. 1996, Flegr et al. 1998, Flegr et al. 2003, Novotna et al. 2005, Lindova et al. 2006) and even more severe psychiatric disorders and behaviors such as depression, bipolar disorder, schizophrenia, and suicidality (Arling et al., 2009, Okusaga et al., 2011, Torrey et al. 2012, Groet et al. 2011, Pearce et al. 2012, Dickerson et al. 2014, Hamdani et al. 2013, Postolache and Cook 2013, Ling et al. 2011, Pearce et al. 2013).

What underlies these behavioral changes? One hypothesis, termed 'behavioral manipulation', proposes that parasites can alter host behavior, specifically, to ensure its survival and increase its chances of transmitting the infection to other animals (Thomas and Adamo et al. 2005, Klein 2005). In regard to behavioral changes that closely relate to or mimic reduced fear responses, there has been much interest in assessing neural circuits comprising the medial hypothalamic zone, septohippocampal pathway, and amygdalar pathway (all pathways which are involved in innate fear, conditioned fear, learned fear, and unconditioned anxiety) (Blanchard et al. 2005, Canteras et al. 1997, Dielenberg et al. 2001, Markham et al. 2004, McGregor et al. 2004, Adamec et al. 2005, Dayas et al. 1999, Pawlak et al. 2003, Vazdarjanova et al. 2001). It is proposed that *T. gondii's* tropism to the amygdala could potentially influence innate fear via specific modulation of these pathways. Yet, although some studies do show a slight increase in cyst density in medial and basolateral amygdala (Vyas et al. 2007) compared to other regions, *T. gondii* does have tropism for many other regions of the brain, including the hypothalamus and cortex (Berenreiterová et al. 2011, Carrillo et al. 2020). Modulation of these circuits, whether due to specific manipulation of the parasite, or by infection-driven inflammation in these regions, could lead to a wider range of behavioral changes and mental illness.

GABAergic circuits and their role in neurological and neuropsychiatric disorders

In the mammalian brain, most complex behaviors are orchestrated between multiple brain regions that are connected by precise neuronal networks. The ability of neurons to communicate effectively within these networks is, in large part, dependent on the homeostatic regulation of neurotransmission. The inhibitory neurotransmitter, gamma-aminobutyric acid (GABA), is a key player in regulating excitatory neurotransmission of pyramidal neurons in the adult brain, by

hyperpolarization of the membrane potential and by shunting inhibition on excitatory inputs (Mueller et al. 1984, Freund and Buzsaki 1996, Buzsaki and Draguhn, 2004, Ben-Ari 2014). It is important to note, however, that GABA's role as an inhibitory neurotransmitter is acquired during early postnatal development. Before then, GABAergic neurotransmission is excitatory, with the capacity to increase intracellular calcium concentrations and trigger action potentials (Luhmann and Prince et al. 1991, Reichling et al. 1994, Chen et al. 1996, Ganguly et al. 2001). Traditionally, dysfunction or loss of GABAergic interneurons have been proposed to lead to seizure activity as a result of decreased inhibition on excitatory pyramidal neurons, although, it is possible GABAergic interneurons may provide context-dependent excitatory roles and contribute to seizure development in that manner (Ye and Kaszuba 2017).

Several distinct types of GABAergic interneurons can be found distributed throughout the brain. In the cerebral cortex, over 20 different subtypes of GABAergic interneurons exist, each with unique morphologies, electrical and chemical properties, firing patterns, and connectivity across the cortical layers (Lorente de Nó 1992, Cauli et al. 1997, Somogyi and Klausberger, 2005, Gonchar et al. 2007, Ascoli et al. 2008). Three main subtypes account for all inhibitory cortical interneurons (or nearly all known to date) and have been identified based on the calcium-binding proteins they express: parvalbumin-expressing interneurons (PV), somatostatin-expressing interneurons (SST), and 5HT3aR-expressing interneurons (Butt et al., 2005, Miyoshi et al., 2007). Over 70% of cortical inhibitory interneurons are accounted for by PV^+ and SST^+ interneurons, two distinct non-overlapping populations. $5HT3aR^+$ interneurons account for the rest of the inhibitory interneurons found in the cortex. Each of these interneuron subtypes, however, can be further categorized into subpopulations, illustrating not only their heterogeneity, but also the diverse roles they play in cortical regulation and function (Lee et al. 2010, Kelsom and Lu 2014).

Parvalbumin-expressing interneurons represent a population of GABAergic cells that can dramatically influence neurotransmission throughout the brain, and particularly within the cortex, and thus, have unique implications in the development of neurological and neuropsychiatric disorders. Despite only representing a small population of all neurons in the cortex, they alone account for roughly 40% of the GABAergic interneurons here. PV interneurons are fast-spiking, firing at a continuous high frequency (> 50 Hz) that is much higher than the firing rates of excitatory pyramidal neurons (Nahar et al. 2021). Moreover, they are uniquely known for having low input resistance and high-amplitude rapid after-hyperpolarization. These

unique electrophysiological and biochemical properties allow them to control the timing of spikes and modulate glutamatergic neurotransmission in nearby excitatory pyramidal neurons with great precision (Kim et al. 2015, Jouhanneau et al. 2018). Like other GABAergic interneurons, PV interneurons can be further categorized into subtypes based on their morphology and their synaptic targets (Ferguson and Gao 2018, Nahar et al. 2021). PV chandelier cells innervate the axons, including the axon initial segment, of excitatory neurons, while PV basket cells innervate the soma and proximal dendrites of both excitatory pyramidal neurons and other PV inhibitory interneurons. Thus, PV basket cells provide strong inhibitory postsynaptic effects that decrease overall excitatory neurotransmission, directly and indirectly (Ferguson and Gao 2018, Nahar et al. 2021. Armstrong and Soltesz).

PV interneurons, specifically, have been extensively implicated in a number of neuropsychiatric diseases of both developmental (i.e. autism spectrum disorders) and mature onsets (schizophrenia and Alzheimer's) (Lewis et al. 2012, Gonzalez et al. Marin 2012, Ogiwara 2007, Filice et al. 2016). Many of the specialized properties that that allow these cells to have such a vast influence on widespread excitatory neurotransmission also make them extremely vulnerable to stressors. For example, they are part of very intricate, highly interconnected networks (Galarreta and Hestin, 2002), where individual PV cells provide synaptic inputs onto almost all nearby excitatory pyramidal cells, and in turn, local pyramidal neurons provide synaptic inputs on PV interneurons. In fact, pyramidal cells provide the most excitatory drive onto these interneurons than any other inhibitory interneuron in the cortex, placing them under a significant amount of stress (Behrens et al. 2007, Gulyas et al. 1999, Ruden et al. 2021). Other characteristics that increase their susceptibility to stressors include their dependence on NMDA receptor function (of which dysregulation is also prevalent in schizophrenia) and their high energy requirements to support their high metabolic activity and protection against stress from increased excitatory input (Kann et al. 2014, Whittaker et al. 2011, Behrens et al., 2007, Ruden et al. 2021)

In the context of parasitic infection, there is evidence to suggest that *Toxoplasma gondii* has evolutionarily developed mechanisms to alter GABAergic circuitry. For example, in studies of acute *T. gondii* infection, tachyzoite invasion of host immune cells causes these infected-immune cells to dramatically increase their production of GABA, specifically by increasing the production of GABA metabolites (Gad65 and Gad67) and decreasing the production of catabolites (GABA-

T) (Bhandage and Barragan 2021, Bhandage et al. 2020). In the infected mouse brain, changes in the distribution of GAD67 (a key enzyme that catalyzes the synthesis of GABA) throughout regions of the telencephalon that are dense with perisomatic synapses originating from fast-spiking PV basket interneurons, leads to spontaneous seizures and increased seizure susceptibility (Brooks et al. 2015). The discovery of these changes in GABAergic circuitry following T. gondii infection, and their potential implication in the development of neurological and neuropsychiatric disorders is the foundation to my dissertation.

Questions, approaches, and key findings

My dissertation research is divided into two main chapters. In Chapter 1 (Carrillo et al., 2020), I employ ultrastructural microscopy (serial block face scanning electron microscopy), transgenic reporter mouse lines, and microbiology techniques to assess GABAergic synaptic connectivity in long-term brain infection. I demonstrate that the altered localization of the GABAergic enzyme, GAD67, in the chronically infected mouse brain (Brooks et al. 2015), is a result of perisomatic inhibitory synapse loss within the hippocampus and neocortex, and that a key neuron-glia interaction, termed 'ensheathment' underlies the phagocytosis of these synapses by microglia and infiltrating monocytes. In Chapter 2 (Carrillo et al. 2022 – which has been recently submitted for peer-review), I uncover the mechanism by which microglia (and monocytes) ensheath neurons and permanently remove perisomatic inhibitory synapses. In this study, I take advantage of mouse models with targeted deletion of immune molecules to show that complement is required for the loss of perisomatic inhibitory synapses in *T. gondii* infection. Additionally, I demonstrate cell-type specificity in phagocyte ensheathment and perisomatic inhibitory synapse loss of excitatory cortical neurons following infection.

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Chapter 2:

Toxoplasma infection induces microglia-neuron contact and the loss of perisomatic inhibitory synapses

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ABSTRACT

Infection and inflammation within the brain induces changes in neuronal connectivity and function. The intracellular protozoan parasite, *Toxoplasma gondii*, is one pathogen that infects the brain and can cause encephalitis and seizures. Persistent infection by this parasite is also associated with behavioral alterations and an increased risk for developing psychiatric illness, including schizophrenia. Current evidence from studies in humans and mouse models suggest that both seizures and schizophrenia result from a loss or dysfunction of inhibitory synapses. In line with this, we recently reported that persistent *Toxoplasma gondii* infection alters the distribution of glutamic acid decarboxylase 67 (GAD67), an enzyme that catalyzes GABA synthesis in inhibitory synapses. These changes could reflect a redistribution of presynaptic machinery in inhibitory neurons or a loss of inhibitory nerve terminals. To directly assess the latter possibility, we employed serial block face scanning electron microscopy (SBFSEM) and quantified inhibitory perisomatic synapses in neocortex and hippocampus following parasitic infection. Not only did persistent infection lead to a significant loss of perisomatic synapses, it induced the ensheathment of neuronal somata by myeloid-derived cells. Immunohistochemical, genetic, and ultrastructural analyses revealed that these myeloid-derived cells included activated microglia. Finally, ultrastructural analysis identified myeloid-derived cells enveloping perisomatic nerve terminals, suggesting they may actively displace or phagocytose synaptic elements. Thus, these results suggest that activated microglia contribute to perisomatic inhibitory synapse loss following parasitic infection and offer a novel mechanism as to how persistent *Toxoplasma gondii* infection may contribute to both seizures and psychiatric illness.

INTRODUCTION

Toxoplasma gondii is a widespread obligate, intracellular protozoan parasite that infects roughly one-third of the world's population (Pappas et al. 2009; Montoya and Liesenfeld 2004). The parasite reproduces sexually in the felid intestine or asexually in most warm-blooded animals. Following digestion of the resulting oocysts (generated by sexual reproduction) or tissue cysts (generated in intermediate host tissues), intestinal enzymes degrade the walls of these structures freeing sporozoites or bradyzoites, respectively, which then infect intestinal epithelial

cells and differentiate into virulent tachyzoites (Montoya and Liesenfeld 2004). Inflammatory cells recruited to the site of initial infection become infected by these tachyzoites and assist in their dissemination throughout the body of the intermediate host, including to the brain, retina and skeletal muscle (Lambert et al. 2006; Courret et al. 2006, Coombes et al. 2013). Within these tissues, tachyzoites convert to slowly dividing bradyzoites that develop into tissue cysts that may persist for the lifetime of the intermediate host.

In the brain, after the parasite triggers an initial inflammatory response, bradyzoite-containing cysts preferentially form within neurons (Sims et al. 1989; McConkey et al. 2013; Cabral et al. 2016). A growing body of literature suggests that persistent *Toxoplasma gondii* infection induces behavioral changes in intermediate hosts, including humans (Poirotte et al. 2016; Berdoy et al. 2000; Vyas et al. 2007; Beste et al. 2014; Stock et al. 2014; Boillat et al. 2020). Furthermore, over four dozen studies have identified *Toxoplasma gondii* infection as a risk factor for developing schizophrenia (Wang et al. 2019, Xiao et al. 2018, Burgdorf et al. 2019; Dickerson et al. 2017, 2014, 2018; Kano et al. 2018), a complex disorder characterized by altered cognitive performance, the acquisition and expression of behaviors absent from healthy individuals (such as hallucinations), and the loss of behaviors normally present in healthy individuals (leading to social withdrawal, apathy, and neglect). A number of cellular and molecular mechanisms have been proposed to underlie behavioral changes in schizophrenia, many of which point to alterations in the assembly, maintenance and function of synapses (Birnbaum and Weinberger 2017). In addition to its association with schizophrenia, persistent *Toxoplasma* infection in animal models leads to seizures (Brooks et al. 2015; David et al. 2016), altered neurotransmitter levels (Alsaady et al. 2019; Skallova et al. 2006; Martin et al. 2015; Gatkowska et al. 2013), altered neural connectivity (Brooks et al. 2015; Ihara et al. 2016; Parlog et al. 2015; Lang et al. 2018) and altered neuronal function (Haroon et al. 2012), all phenotypes that are associated with impaired synaptic organization or function.

Synapses can be broadly categorized into those whose activity increases the probability of activity in a postsynaptic neuron (i.e. excitatory synapses) and those whose activity reduces the probability of activity in a postsynaptic neuron (i.e. inhibitory synapses). Although inhibitory synapses comprise only a fraction (~20%) of all synapses in neocortex or hippocampus, they are essential for controlling the flow of information transfer in the brain and their dysfunction has

been associated with neurological and neuropsychiatric disorders (Marin 2012). In fact, we previously identified alterations in the distribution of glutamic acid decarboxylase 67 (GAD67), the enzyme required to generate the inhibitory neurotransmitter GABA, in *Toxoplasma*-infected mice (Brooks et al. 2015). These changes could reflect a redistribution of presynaptic machinery in inhibitory neurons or a loss of inhibitory nerve terminals. This motivated us to directly test whether *Toxoplasma gondii* infection leads to the loss of inhibitory synapses. We focused our attention on perisomatic inhibitory synapses formed by Parvalbumin-expressing, fast-spiking interneurons in the cerebral cortex and hippocampus as the loss or impairment of these specific synapses has been associated with seizures, schizophrenia, and schizophrenia-related behaviors (Schwaller et al. 2004; Belforte et al. 2010; Gonzalez-Burgos et al. 2010, 2011; Gonzalez-Burgos and Lewis 2012; Lewis et al. 2011; Wohr et al. 2015; Hamm et al. 2017; Mukherjee et al. 2019). Using serial block face scanning electron microscopy (SBFSEM) and immunolabeling, we discovered *Toxoplasma* infection leads to a significant loss of these perisomatic inhibitory synapses. Moreover, we observed ensheathment of neuronal somata (and perisomatic nerve terminals) by activated microglia, suggesting that these cells may contribute to the loss of inhibitory synapses following long-term infection. Thus, these data suggest that *Toxoplasma* may increase the risk of developing seizures and schizophrenia by contributing to microglial activation and the loss of perisomatic inhibitory synapses.

MATERIALS AND METHODS

Animals. Male and female C57BL/6J mice were purchased from Jackson Laboratories (Bar Harbor, ME) and *Cx3cr1-GFP* mice (Jackson Laboratories, Stock# 005582) were provided by Dr. M. Theus (Virginia Tech). All analyses conformed to National Institutes of Health guidelines and protocols approved by the University at Buffalo and Virginia Polytechnic Institute and State University Institutional Animal Care and Use Committees.

Parasite infections. Male and female mice (8-12 weeks of age) were infected with Type II strains (ME49 and ME49-RFP) of *Toxoplasma gondii* as previously described (Brooks et al. 2015). The total number of *Toxoplasma gondii* cysts within 20µl of brain homogenate was counted by brightfield or fluorescence microscopy, respectively. Mice were interperitoneally infected with 5 cysts of either in PBS. Unless specifically noted, all data and analyses in this manuscript refer to mice

infected with Type 2 strains (ME49 and ME49-RFP) of *Toxoplasma gondii.* For the one experiment with a Type III (CEP) strain of *Toxoplasma gondii,* CEP parasites were cultured in Human foreskin fibroblasts -1 (HFF-1) cells grown at 37C in 5% CO2 in Dulbecco's modified Eagle's Medium (DMEM) supplemented with 10% fetal calf serum, 1% L-Glutamine, and 1% Penicillin/Streptomycin. CEP parasites were harvested by syringe lysis of infected HFF-1 cells through a 27 gauge need and passed through a 20 μm filter. Following resuspension, CEP parasites were counted and resuspended in PBS for intraperitoneal (I.P.) administration into mice at either 100 and 10,000 tachyzoites, to produce mice with low or high cyst burdens, respectively. Brains from chronically infected mice (30 to 90 days post-infection) were isolated and homogenized in phosphate-buffered saline pH 7.4 (PBS). After 30 days, brains from *Toxoplasma gondii*-infected or mock-infected (which received IP injection of PBS) were collected for immunohistochemistry (IHC) or serial block face scanning electron microscopy (SBFSEM) analysis.

Immunohistochemistry. IHC was performed as described previously (Su et al. 2010, 2016; Brooks et al. 2015). Anesthetized mice were transcardially perfused with PBS pH 7.4 followed by 4% paraformaldehyde in PBS (PFA; pH 7.4). Post-fixed brains (12-16 hrs at 4°C in 4% PFA) were harvested, cryopreserved in 30% sucrose for 3 days, and embedded in Tissue Freezing Medium (Electron Microscopy Sciences, Hatfield, PA). Brains were coronally cryostectioned at 16μm and were air dried for 15 min at room temperature before incubation with blocking buffer (5% normal goat serum-2.5% bovine serum albumin-0.1% Triton X-100-PBS) for 60 min. Primary antibodies were diluted in blocking buffer as follows: GAD67 1:500 (Millipore MAB5406); IBA1 1:800 (Wako, Richmond, VA; 019-19741); CD68 1:1000 (Abcam, Cambridge, UK; AB53444); NeuroTrace 1:300 in PBS (ThermoFisher, Waltham, MA; N-21480) and incubated on coronal brain sections overnight at 4°C. Slides were washed in PBS and incubated with fluorescently conjugated secondary antibodies obtained from Molecular Probes/Invitrogen (diluted 1:1,000 in blocking buffer). After washing in PBS, slides were mounted with Vectashield (Vector Laboratories, Burlingame, CA) and images were acquired on a Zeiss LSM 700 confocal microscope. Identical imaging parameters were used for the acquisition of images for different experimental conditions. A minimum of three animals and three image fields per brain region (per experimental condition) were compared in all experiments.

Image analysis. For binary quantification of fluorescent images, single channels of each image obtained after immunohistochemistry (as described above) were converted to binary images and the percent field occupied by immunoreactivity was obtained using ImageJ software (see Brooks et al. 2015; Su et al. 2016; Monavarfeshani et al. 2018). For quantifying the ratio of immunoreactivity, the average fluorescent intensity was measured in images of stratum pyramidalis and in the surrounding hippocampal layers (SR and SO). DAPI labeling was used to differentiate these layers. Average fluorescent intensity was calculated using ImageJ software. Ratios of the fluorescent intensities in these regions was calculated and a student's *t* test was used to test for statistical significance. Quantification of the number of cells ensheathed by $Iba1^+$ myeloid-derived cells was performed using maximum-projection images of merged channels. Ensheathed cells were identified by observing myeloid-derived cells ($Iba1^+$ or GFP^+ cells) that were both in direct contact with non-phagocytes ($Iba1^-/DAPI^+$ or $GFP^-/DAPI^+$ cells) and extended processes around a fraction of those cell somas. Examples of are shown in Supplemental Figure 4. Total ensheathed cells were counted in each field of view for each condition. Quantification of the density of $GAD67^+$ perisomatic nerve terminals or the surface area contacted by GFP^+ cells along the surface of neuronal somata (or both measures in the same cells) was performed on high resolution, single optical sections of confocal images of layer V of cortex from mock- and ME49-infected *Cx3cr1-GFP* mice. Five images were acquired per animal (and 3-5 animals per condition) and every cell (~20) in the field of view was analyzed. Data was measured and analyzed in ImageJ (Su et al. 2016). For all analyses, three to six mice were used per condition with a minimum of three images per brain region.

***In situ* hybridization (ISH).** ISH was performed on 16 µm coronal sections as described previously in (Su et al. 2010; Monavarfeshani et al. 2018). Briefly, tissue slides were incubated with diluted and heat-denatured *Gad1* and *Syt1* riboprobes (1-2µm of riboprobe in 60 µl of hybridization solution heated for 12 min in 70°C) overnight in 60°C incubation. Bound riboprobes were detected by staining with Tyramide Signal Amplification (TSA) system. Immunohistochemistry was immediately performed following TSA system signal detection as described above. Images were obtained on a Zeiss LSM700 confocal microscope. A minimum of three animals per condition were compared in ISH experiments.

Serial block face scanning electron microscopy. SBFSEM was performed as described previously (Hammer et al. 2014, 2015; Monavarfeshani et al. 2018). Briefly, male mice were perfused with 0.1M sodium cacodylate buffer containing 4% PFA and 2.5% glutaraldehyde and immediately 300 µm vibratomed coronal sections were collected. CA1 regions of hippocampus and layer V of neocortex were microdissected and sent to Renovo Neural Inc. (Cleveland, OH) for staining and processing as described previously (Mukherjee et al. 2016; Hammer et al. 2014, 2015; Monavarfeshani et al. 2018). Images were acquired on a Thermofisher/FEI Apreo VolumeScope at Renovo Neural Inc. Datasets represented regions that were 50µm x 50-150µm and consisted of ~500 serial sections (with each section being 50-60 nm thick). SBFSEM images were imaged at a 5nm/pixel resolution. Serial image stacks were manually traced and analyzed using TrakEM2 in Fiji (Cardona et al., 2012). Perisomatic nerve terminals were identified based on direct apposition to neuronal somata, the presence of synaptic vesicles, and the clustering of synaptic vesicles on the presynaptic membrane (i.e. active zones). Perisomatic synapses were quantified around at least nine cells from mock- or ME49-infected datasets (n=3 animals per condition). Student *t* tests were used to assess statistical significance. Microglia and macrophages-derived from infiltrating monocytes were identified based on the presence of electron dense cytoplasm, electron-dense heterochromatin near the nuclear membrane, lysosomes / phagosomes, mitochondria and long stretches of endoplasmic reticulum (Yamasaki et al. 2014; Savage et al. 2018). Microglia have previously been differentiated from infiltrating monocytes based on nuclear shape, osmophilic granules and microvilli, and process number / complexity (Yamasaki et al. 2014).

RESULTS

Toxoplasma infection leads to the loss of perisomatic inhibitory synapses

To assess how parasitic infection affects GABAergic inhibitory synapses in the mammalian brain, adult mice were mock-infected or infected with the type II ME49 *Toxoplasma* strain. Thirty days after infection, brains were harvested and GABAergic synapses in hippocampus and neocortex were analyzed. These regions were selected since they contain a high concentration of perisomatic inhibitory synapses formed by fast-spiking, Parvalbumin (Parv)-expressing interneurons. Additionally, while *Toxoplasma gondii* cysts have been found in most (if not all) regions of the rodent brain, the parasite has an increased tropism for infecting cells in the

telencephalon, which includes both neocortex and hippocampus (Berenreiterova et al. 2011). While cyst density has been described as high to very high in these brain regions (Berenreiterova et al. 2011; Boillat et al. 2020), cysts remain vastly outnumbered by the neural cells in these regions (Supplemental Figure 1). For example, we failed to observe more than three cysts in any given hippocampal section, and the majority of sections either lacked an identifiable cyst or had only one.

In mock-infected controls, punctate GAD67-immunoreactivity labels inhibitory synapses throughout the brain, however, in the Cornu Ammonis (CA) regions of hippocampus, the majority of these inhibitory synapses are concentrated in stratum pyramidalis (SP) (Figure 1A,B). In neocortex, GAD67-labeled inhibitory synapses are distributed in all cortical layers, although an increased density is observed in layer V (and in layer II/III), where a high proportion of these synapses surround neuronal cell bodies (Figure 1E,F). Similar to what we previously observed in the CA3 subregion of ME49-infected hippocampus (Brooks et al. 2015), data presented here revealed GAD67 immunoreactivity is less punctate and more diffusely localized in other hippocampal layers and in neocortex following ME49 infection (Figure 1A-K). Not only did we observe this redistribution of GAD67 immunoreactivity, we observed a significant loss of GAD67$^+$ terminals surrounding neuronal somas in ME49-infected tissues (Figure 1L-Q). We interpret this data to suggest a loss of GABAergic perisomatic synapses following persistent *Toxoplasma* infection. While significant changes in the density of perisomatic synapses was observed following infection with Type II strains of *Toxoplasma gondii*, similar synaptic changes were not observed following infection with a Type III strain of this parasite (CEP)(Figure 1P,Q)(Brooks et al. 2015).

In both stratum pyramidalis of the hippocampus and layer V of cerebral cortex, Parv$^+$ interneurons generate GAD67$^+$ perisomatic synapses onto both excitatory pyramidal neurons and other Parv$^+$ inhibitory interneurons (Pi et al. 2013; Pfeffer et al. 2013). To assess whether the loss of GAD67$^+$ perisomatic synapses was cell-specific or occurred on both excitatory and inhibitory neurons, we immunolabeled GAD67$^+$ terminals and performed *in situ* hybridization for either *Synaptotagmin1* (*Syt1*) mRNA or *Glutamic Acid Decarboxylase 1* (*Gad1)* mRNA (which encodes GAD67) to discriminate between excitatory and inhibitory postsynaptic neurons, respectively. *Syt1* mRNA is transcribed by a large subset of excitatory neurons (and some interneurons) in

stratum pyramidalis of CA1 and layer V of cortex (Marqueze et al. 1995; Su et al. 2010). In contrast, *Gad1* mRNA is present in most, if not all, inhibitory neurons (and in no excitatory neurons) in these regions. We observed abundant GAD67$^+$ nerve terminals surrounding both *Syt1*$^+$ or *Gad1*$^+$ neurons in controls, but few GAD67$^+$ terminals were observed surrounding either populations of neurons in *Toxoplasma*-infected mice (Supplemental Figure 2). These data suggest a loss of GABAergic perisomatic synapses onto both pyramidal neurons and interneurons in hippocampus and neocortex of *Toxoplasma*-infected brains.

While we used GAD67-immunoreactivity as a marker of inhibitory nerve terminals, it was not clear whether the absence of GAD67$^+$ terminals reflected the loss of perisomatic nerve terminals or merely the loss of GAD67-immunoreactivity within these nerve terminals. To circumvent this issue, we employed serial block face scanning electron microscopy (SBFSEM), which allows for large volumes of serial, ultrastructural data to be collected and analyzed in 3D (Denk and Horsfmath 2014). SBFSEM datasets were generated for both CA1 and neocortex of mock-infected and ME49-infected mice. At low magnification, we identified regions of stratum pyramidalis (SP) in CA1 or layer V in neocortex to analyze in detail (Figure 2A,B, and data not shown). Once regions of interest were selected, serial sections were obtained at high resolution (5nm/pixel) (Figure 2C).

SBFSEM images resemble data from traditional transmission electron microscopy allowing us to use traditional ultrastructural morphological criteria to identify neurons, glial cells, blood vessels, synapses and parasite cysts (Figure 2C) (Savage et al. 2018; Hammer et al. 2014; David et al. 2016). Perisomatic synapses in hippocampus and cerebral cortex were identified by the presence of mitochondria, synaptic vesicles, and active zones that opposed adjacent neuronal somata (Figure 3A-H). To quantify perisomatic synapse number, we identified nine neurons in mock- and ME49-infected stratum pyramidalis whose entire somata were present in the 3D SBFSEM datasets and manually traced all of the perisomatic synapses onto these somata (Figure 3A-D,I). Our analyses revealed that infection significantly reduced the numbers of hippocampal perisomatic synapses (Figure 3I). Persistent parasitic infection has been shown to cause neuronal changes, including neuronal loss due to excitotoxicity (David et al. 2016; Torres et al. 2018), therefore we also addressed the possibility that the decrease in perisomatic synapses was due to changes in neuronal size. We analyzed the number of serial sections per neuron in mock- and

ME49-infected hippocampus and observed a modest decrease in neuronal size following infection (Figure 3J). However, even when we accounted for this difference, a significant reduction in the ratio of perisomatic synapses per section was observed in stratum pyramidalis (Figure 3K).

Similarly, it appeared as though fewer perisomatic synapses were present on neocortical neurons following *Toxoplasma* infection (Figure 3E-H). However, while the high density of neurons in stratum pyramidalis ensured we were able to identify neurons whose entire somas fell within the ~500 section SBFSEM datasets, the same was not true in neocortex. The sparser distribution of neurons in layer V of neocortex and the large size of the neurons meant few, if any, neurons could be analyzed in their entirety by SBFSEM. For this reason, we quantified the ratio of perisomatic synapses to the number of sections for neurons whose somas were mostly captured in the SBFSEM datasets (which we assessed based upon their entire nucleus being present in the dataset; for example, see Figure 4). As was the case in hippocampus, this revealed a significant reduction in the density of perisomatic synapses in neocortex following persistent *Toxoplasma* infection (Figure 3L). Together with GAD67-immunoreactivity data, these results reveal a significant loss of GABAergic perisomatic synapse following *Toxoplasma* infection.

Neuronal ensheathment by microglia following Toxoplasma infection

In assessing perisomatic synapses in the SBFSEM datasets from *Toxoplasma*-infected tissue, we found that the somata of neurons were contacted and ensheathed by non-neuronal cells (something we failed to observe in mock-infected datasets). Ultrastructural analysis of these ensheathing cells revealed that they contain relatively dark cytoplasm, electron dense heterochromatin near the nuclear membrane, lysosomal dense granules and prominent endoplasmic reticulum and Golgi apparati (Figures 2C,4, Supplemental Figure 3, and Supplemental Movie) – all morphological features of myeloid-derived cells (i.e. resident microglia or macrophages-derived from infiltrating monocytes) (Tremblay et al. 2010; Chen et al. 2014; Savage et al. 2018; Yamasaki et al. 2014). Based on the presence of long and intricate processes of some of these cells, as well as their nuclear morphology, many of these myeloid-derived cells appeared to be microglia (Figure 4 and Supplemental Figure 3).

Ensheathment of neuronal somas by these microglia / myeloid-derived cells following *Toxoplasma* infection was extensive and, in several cases, multiple cells ensheathed a single neuron. Figure 4 shows such an example from ME49-infected neocortex in which a single neuron is ensheathed by at least three distinct myeloid-derived cells. In such cases, little somal surface of the neuron remained free of myeloid-derived cell-contact, and thus, little space of the neuronal cell surface remained available for contact by perisomatic nerve terminals. Remarkably, an individual myeloid-derived cell's reach was not limited to a single neuronal soma but each often ensheathed portions of multiple somas (Figure 4).

While SBFSEM datasets provide a wealth of ultrastructural data, they are limited in scope and these observations could therefore represent rare events that we were fortunate to capture in these datasets. To assess larger regions of hippocampus and neocortex for myeloid-derived cell-neuron interactions, we immunolabeled mock- and ME49-infected tissue with antibodies against Ionizing Calcium Binding Adapter 1 (Iba1) which is expressed by resident microglia in the healthy brain and by both reactive microglia and macrophages derived from infiltrating monocytes, following injury, infection, or inflammation (Yamasaki et al. 2014). In mock-infected mice, $Iba1^+$ cells exhibited stellate morphologies, a hallmark of surveillant microglial phenotypes. Moreover, few $Iba1^+$ resident microglia were observed in SP of CA1 in control mice (Figure 5A). In contrast, in ME49-infected mice, $Iba1^+$ cells resembled the morphology of both activated microglia (i.e. increased cell size and thicker, shorter processes; Ransohoff and Perry 2019) and macrophages derived from in filtrating monocytes (i.e. stellate in shape with few processes; Yamasaki et al. 2014) and were abundant in SP of CA1 and all other regions of hippocampus and neocortex (Figure 5B,D,F,H,J,L). Moreover, this analysis showed widespread $Iba1^+$ cells in close apposition with other brain cells (labeled either by their nuclei [DAPI] or with NeuroTrace) in *Toxoplasma*-infected neocortex and hippocampus (Figure 5A-D, G-J). We quantified the number of $Iba1^+$ cells that were surrounding or contacting $Iba1^-/DAPI^+$ cells (see arrows in Figure 5B,H). This quantification confirmed widespread, significant contact and ensheathment of neurons by $Iba1^+$ cells in the hippocampus and neocortex of ME49-infected brains (Figure 5M,N and Supplemental Figure 4).

Since Iba1-immunoreactivity alone cannot distinguish reactive resident microglia from macrophages derived from infiltrating monocytes, we assessed phagocytic ensheathment of

neurons in *Cx3cr1-GFP* mice infected with *Toxoplasma gondii*. Microglia, but not infiltrating monocytes, have been reported to express high levels of GFP in inflamed brains of these transgenic reporter mice (Yamasaki et al. 2014; Greter et al. 2015). In mock-infected *Cx3cr1-GFP*, GFP^+ cells exhibited characteristic surveillant microglia morphology in all brain regions and few GFP^+ cells were present in SR of CA1 (Figure 5,6). In contrast, GFP^+ cells appeared strikingly different in infected mice. First, in SP of CA1 and layer V of neocortex, GFP+ cells appeared to have larger cell bodies and thicker, shorter processes following infection (Figure 5,6 and Supplemental Figure 6). Second, as described in previously reported models of inflammation, both high and low GFP-expressing cells were present in *Toxoplasma*-infected *Cx3cr1-GFP* mice (Supplemental Figure 5). Cells ensheathing neurons in *Toxoplasma*-infected *Cx3cr1-GFP* mice expressed high levels of GFP and were process bearing (Supplemental Figure 5). Together these data, coupled with the ultrastructural morphology of these cells in SBFSEM datasets (Figure 4, Supplemental Figure 3, and Supplemental Movie), suggest that a significant fraction of the neuron-ensheathing myeloid-derived cells in *Toxoplasma*-infected brains are activated microglia. Third, GFP^+ microglia appeared strikingly different in distinct hippocampal layers of infected *Cx3cr1-GFP* mice, with rod-like microglia being present in stratum radiatum (SR) and stratum lacunose-moleculare (SLM) (Supplemental Figure 6). Similar rod-like, $Iba1^+$ cells were observed in SR of *Toxoplasma*-infected mice (see asterisk in Figure 5B) (also see Wyatt-Johnson et al. 2017). Fourth, and perhaps most importantly, GFP^+ microglia not only ensheathed GFP^- cells in ME49-infected hippocampus and cortex (Figure 5L,O-T), but increasing coverage of neuronal somas by GFP^+ microglia coincided with decreased perisomatic inhibitory synapses number on these cells (Figure 5S,T).

Finally, we sought to determine whether activated microglia ensheath both excitatory and inhibitory neurons following persistent *Toxoplasma* infection. As described above, we used *in situ* hybridization for *Syt1* and *Gad1* mRNA to label these populations of neurons. *In situ* hybridization in *Cx3cr1-GFP* mock- and ME49-infected tissue revealed that GFP^+ cells ensheath both excitatory and inhibitory neurons in hippocampus and neocortex (Figure 6).

Widespread microglial removal of perisomatic synapses following Toxoplasma infection

Microglia have been implicated in synaptic remodeling both in development and various models of injury and disease (Kettenmann et al. 2013). Since immunolabeling and ultrastructural data revealed inhibitory perisomatic synapse loss and microglial activation following persistent *Toxoplasma gondii* infection, it seemed plausible that these microglia may be involved in the loss of inhibitory perisomatic nerve terminals. To begin to test this, several approaches were employed. First, we used SBFSEM datasets to demonstrate that microglia contact, surround, and potentially displace perisomatic synapses (Figure 7A-C). Similar observations in SBFSEM datasets in other models of inflammation have been suggested to support synaptic displacement by microglia (Chen et al. 2014). Moreover, SBFSEM images illustrated frequent examples of phagosomes in microglial processes adjacent to neuronal somata in ME49-infected mice (Figure 7B, 8A,B). Furthermore, we observed GAD67-immunoreactive puncta within GFP^+ microglia in ME49-infected *Cx3Cr1-GFP* mice (something we failed to observe in mock- or uninfected controls)(Figure 8). Finally, we employed immunohistochemistry against Cluster of Differentiation 68 (CD68), a marker of lysosomal activity often employed to assess the phagocytic nature of myeloid-derived cells following infection, inflammation or injury (Simpson et al. 2007; Waller et al. 2019). Enhanced CD68-immunoreactivity was observed in hippocampus and cortex of *Toxoplasma gondii*-infected brains, compared with mock-infected controls (Figure 8C-J). Colocalization of CD68 with $Iba1^+$ myeloid-derived cells and GFP^+ microglia (in *Cx3cr1-GFP* mice) confirmed that microglia in ME49-infected tissue were not only activated, but exhibited enhanced lysosomal/phagocytic activity compared to those in controls (Figure 8C-J). Taken together, these results strongly suggest activated microglia participate in the loss of inhibitory synapses following persistent *Toxoplasma gondii* infection.

DISCUSSION

Synaptic changes following parasitic infection

Here, we report that the number of inhibitory perisomatic synapses is significantly reduced following *Toxoplasma gondii* infection. In hippocampus and neocortex, perisomatic synapses are generated by Parv-expressing, fast-spiking interneurons (Kawaguchi 1993; Kawaguchi and Kubota 1998,1997; Pi et al. 2013; Pfeffer et al. 2013). Although $Parv^+$ interneurons represent only a fraction of the total neurons in hippocampus or neocortex (<10%)(Freund and Buzsaki 1996; Tremblay et al. 2016), the perisomatic synapses they generate on excitatory

pyramidal neurons and other Parv$^+$ interneurons (Pi et al. 2013) play essential roles in controlling the spread of neural activity (Hensch 2005), in regulating oscillatory network activity (Buzsaki and Wang 2012; Tukker et al. 2007; Bartos et al. 2002), and in regulating neural plasticity during development, learning and plasticity (Fagiolini et al. 2004; Donato et al. 2013; Kuhlman et al 2013). Disruption of Parv$^+$ synapses (or Parv$^+$ interneurons) in neocortex or hippocampus has been associated with epileptiform activity (Schwaller et al. 2004; Paz and Huguenard 2015; Su et al. 2016) and optogenetic stimulation of these interneurons at the onset of seizure activity is sufficient to halt electrographic seizures and reduce behavioral seizures (Krook-Magnuson et al. 2013). Thus, the loss of GABAergic perisomatic synapses reported here following persistent *Toxoplasma* infection is likely to underlie spontaneous seizures and enhanced susceptibility to drug-induced seizures observed in infected mice (Brooks et al. 2015; David et al. 2016). It is important to point out, however, that alternative mechanisms underlying epileptiform activity following chronic *Toxoplasma gondii* infection have been proposed, including the downregulation of astrocyte-derived excitatory amino acid transporter 2 (EAAT2, also called Glt1), which is essential for clearing extracellular glutamate from the synaptic cleft following neurotransmission (David et al. 2016; Wohlfert et al. 2017). Together, the loss of both perisomatic inhibitory synapses and elevated levels of extracellular glutamate (due to the loss of astrocyte-derived EAAT2; David et al. 2016) offer a two-hit model for how seizures develop in patients suffering from Toxoplasmic encephalitis.

In addition to seizures, a large number of studies, including a recent case-control study with over 80,000 individuals, have revealed a strong association between *Toxoplasma gondii* infection and neuropsychiatric disorders, including schizophrenia (Dickenson et al. 2007, 2014; Flegr and Horacek 2018; Burgdorf et al. 2019). Given that *Toxoplasma* parasites are known to increase dopamine metabolism in their hosts and even generate dopamine themselves (Prandovszky et al. 2011; Martin et al. 2015; Ihara et al. 2016; Gaskell et al. 2009) and neuropsychiatric illnesses have long been attributed to dopamine dysfunction (Brisch et al. 2014), it has been hypothesized that the association between persistent *Toxoplasma* infection and schizophrenia is due to altered dopamine signaling (Skallova et al. 2006; Elsheikha et al. 2016; Stock et al. 2017; but see also Wang et al. 2015, 2017). However, it is noteworthy that the impairment of Parv$^+$ interneurons and GABAergic perisomatic synapses has also been strongly associated with the development of neuropsychiatric disorders, such as schizophrenia (Belforte

et al. 2010; Marin 2012; Gonzalez-Burgos et al. 2010, 2011; Sgado et al. 2011; Gonzalez-Burgos and Lewis 2012; Lewis et al. 2012). Similarly, defects in Parv$^+$ interneurons and synapses have been reported to promote the onset of epilepsy (Jiang et al. 2016), which has been reported to be a risk factor in *Toxoplasma*-infected individuals (Ngoungou et al. 2015; Palmer 2007). Thus, results presented here offer an alternative mechanism by which persistent *Toxoplasma* infection may contribute to the development of neuropsychiatric and neurological illnesses.

Finally, while we focus on the loss of perisomatic synapses in this study, it is likely that far more inhibitory synapses are affected or lost following parasitic infection than just perisomatic synapses formed by Parv$^+$ interneurons. Thus, *Toxoplasma gondii* infection likely has a far wider influence on inhibitory synapses and neurotransmission than reported here. This hypothesis stems from the observation that persistent ME49 infection results in altered GAD67 immunoreactivity (i.e. the loss of its punctate appearance) in regions that lack these Parv$^+$ perisomatic inhibitory synapses. This raises the question of what widespread redistribution of GAD67 following persistent *Toxoplasma* infection might reflect. Data presented here suggests that the loss of punctate GAD67-immunoreactivity reflect the loss of inhibitory synapses. But what about the increased and diffuse nature of GAD67-immunoreactivity outside of these synaptic zones (Brooks et al. 2015)? One possibility is that GAD67 may be upregulated by other cells following parasitic infection. In fact, one recent study suggested that *Toxoplasma* infection induces an upregulation of GAD67 by microglia (Bhandage et al. 2019). Unfortunately, our *in situ* hybridization studies do not support expression of *Gad1* mRNA by hippocampal or neocortical microglia following persistent ME49 infection (Figure 6B,D,F,H). Instead, we suspect that the diffuse localization of GAD67 immunoreactivity following ME49 infection indicates reduced levels of GAD67 from remaining inhibitory nerve terminals and its increase in other compartments of the neurons (i.e. neurites and somata). During development, components of the presynaptic machinery, including GAD67, appear diffusely localized throughout the neuron, and only become concentrated at the presynaptic terminal following the process of synaptic differentiation and maturation (Fox and Umemori 2006; Fox et al. 2007). Since inhibitory interneurons exhibit plasticity following activity, disease or injury – including the ability to integrate into new circuits in the adult brain (Baraban et al. 2009; Donato et al. 2013; Tang et al. 2014; Mukherjee et al. 2019) – the redistribution of GAD67 following persistent *Toxoplasma* infection may reflect the re-initiation of a developmental program to promote inhibitory synapse

formation or plasticity. As such, the infected brain may be undergoing cycles of losing and regenerating inhibitory synapses as a result of inflammation or the parasite itself.

Loss of perisomatic synapses coincides with microglial ensheathment of neuronal somata in parasite-infected brains

While our initial intent was to assess perisomatic synapse density following parasite-infection using a state-of-the-art ultrastructural imaging modality (SBFSEM), we unexpectedly discovered that non-neuronal, myeloid-derived cells ensheathed the somata of neurons in both hippocampus and neocortex of infected mice. The electron dense cytoplasm of these process-bearing cells, the presence of lysosomes and phagosomes, the relatively sparse volume of cytoplasm, and the distribution of electron dense heterochromatin along the nuclear membrane all suggest these are microglia (Savage et al. 2018). This suggestion was supported by results demonstrating Iba1^{+} and *Cx3cr1-GFP*$^{+}$ cells are closely apposed to excitatory and inhibitory neurons following persistent parasitic infection. The association of activated, phagocytic microglia with neuronal somata is not unique to animals with latent *Toxoplasma gondii* infection, as these cellular interactions have been observed in a number of neurodegenerative and neuroimmune disorders (Haga et al. 1989; Peterson et al. 2001; Neumann et al. 2006). While results presented here implicate microglia in neuronal ensheathment (and potentially synaptic loss), they do not rule out roles for macrophages derived from infiltrating monocytes which are notoriously difficult to unambiguously differentiate from resident microglia following injury, infection, or inflammation (Koeniger and Kuerlen 2017; but see Yamasaki et al. 2014). In fact, although CD68-immunoreactivity is often used as a marker of microglial cells, our results identified numerous GFP^{-}/CD68^{+} cells in ME49-infected *Cx3cr1-GFP* mice which may represent phagocytic infiltrating monocytes in neocortex and hippocampus of parasite-infected brains.

Why might microglia ensheath neuronal cell bodies following parasitic infection? Over half a century ago, microglia were observed ensheathing lower motoneuron somas following axotomy and this coincided with the loss of perisomatic synapses onto these cells (Blinzinger and Kreutzberg 1968). It was hypothesized that these microglia played an active role in the displacement and removal of these synapses. The ability of microglia to either phagocytose or displace synaptic elements has since been observed not only after axotomy or injury, but also

following infection or inflammation (Di Liberto et al. 2018; Kreutzfeldt et al. 2013; Trapp et al. 2007; Chen et al. 2014), and it involves not only excitatory perisomatic synapses (like those on lower motoneurons; Blinzinger and Kreutzberg 1968) but also inhibitory perisomatic synapses (like those in neocortex and hippocampus; Di Liberto et al. 2018; Chen et al. 2014). While activation of microglia following insult and inflammation may lead to synaptic loss, it does not in all cases (Siskova et al. 2009; Perry and O'Conner 2010), nor is activation a requirement for microglial to phagocytose or displace synaptic elements. During development, non-activated microglia remove supernumerary excitatory synapses (or reshape excitatory nerve terminals through trogocytosis) in the thalamus, neocortex and hippocampus (Tremblay et al. 2010; Schafer et al. 2012; Weinhard et al. 2018).

Toxoplasma infection is known to induce microglial activation (Dellacasa-Lindberg et al. 2011; Li et al. 2019), although it is unclear whether this is the result of direct parasite-derived signals, direct neuron-derived signals (Di Liberto et al. 2018), altered oscillatory network activity (Iaccarino et al. 2016), or other indirect pathways (such as inflammation). Here, we show that these reactive microglia not only ensheath excitatory and inhibitory neurons, but enwrap, and potentially phagocytose, perisomatic inhibitory synapses. Although we previously failed to observe changes in excitatory nerve terminals following parasitic infection (Brooks et al. 2015), other groups have reported altered connectivity, reduced levels of excitatory synaptic machinery, and reduced dendritic spine density (David et al. 2016; Mitra et al. 2013; Lang et al. 2018; Parlog et al. 2014; Wang et al. 2018), all suggesting these reactive microglial may phagocytose more than just inhibitory synapses following persistent parasite infection. The unique morphologies we observed for reactive microglia in different sublamina of the hippocampus, where excitatory and inhibitory synapses are largely restricted to non-overlapping regions, certainly support roles for these microglia beyond phagocytosing perisomatic inhibitory synapses following *Toxoplasma gondii* infection.

The ability of microglia to phagocytose or displace synaptic elements has been proposed to be both pathological (as in Alzheimer's Disease, Amyotrophic Lateral Sclerosis, and Lupus; Hong et al. 2016; Shi et al. 2017; Paolicelli et al. 2017; Krasemann et al. 2017; Bialas et al. 2017) and neuroprotective (Chen et al. 2014). At present it remains unclear whether the loss of perisomatic synapses following *Toxoplasma gondii* infection is pathological (and the direct cause of

epileptiform activity) or beneficial (and may be a homeostatic response to other alterations following infection or inflammation). Insight into this question may come from assessing molecular signatures of the populations of microglia present in the parasite-infected brain. In other conditions, such analysis has shed light on whether microglia are pro- or anti-inflammatory, scavenging debris, or promoting tissue homeostasis (Butovsky and Weiner 2018). In cases where microglia have been implicated in the pathological loss of synapses, it appears to be mediated through the complement pathway (Hong et al. 2016; Sekar et al. 2016; Vasek et al. 2016; Hammond et al. BioRxiv), a similar pathway used to prune the excess of excitatory synapses during development (Schafer et al. 2012; Stevens et al. 2007). In contrast, the proposed neuroprotective role for microglial synaptic stripping appear to be complement-independent (Chen et al. 2014; Di Liberto et al. 2018). While future studies will be needed to resolve the beneficial or pathological consequences of synapse loss reported here, recent studies showing activation of the complement pathway in microglia following *Toxoplasma gondii* infection (Li et al. 2019; Xiao et al. 2016) suggest that the actions of microglia in this case may be pathological.

FIGURES

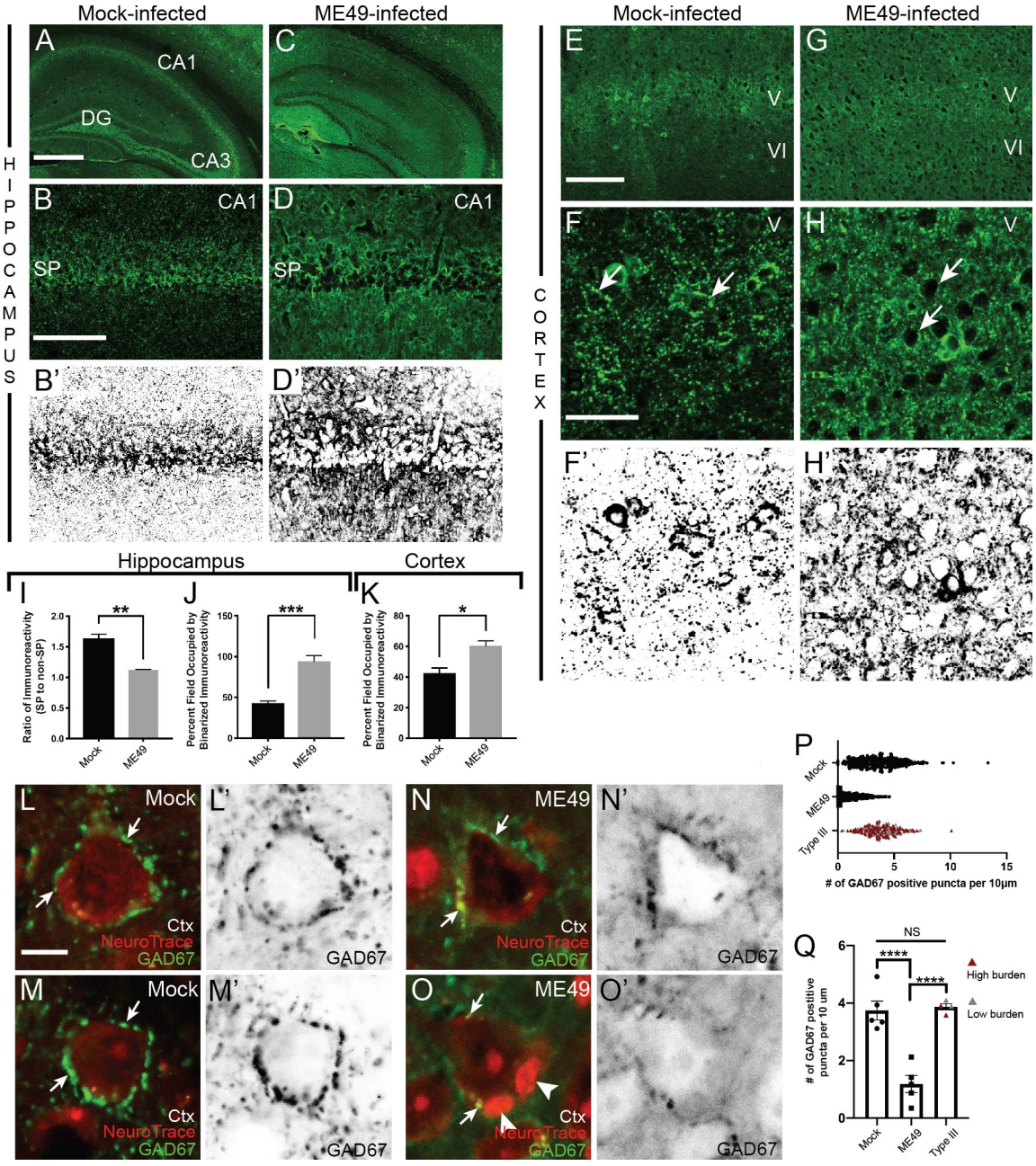


Figure 2.1. Disruption of inhibitory synapses in hippocampus and neocortex of *Toxoplasma*-infected mice.

A-D. GAD67-immunostaining of inhibitory synapses in mock- (**A**) and ME49- (**C**) infected hippocampi. Hippocampal regions CA1, CA3, and dentate gyrus (DG) are shown in **A**. ME49 infection induced a redistribution of GAD67-immunoreactivity, as can be seen in high

magnification images of the CA1 region of hippocampus (**B,D**). Binarized, inverted images of GAD67-immunoreactivity in CA1 shows a concentration of inhibitory synapses in stratum pyramidalis (SP) of mock-infected tissue (**B'**). In contrast, this signal is redistributed out of SP in ME49-infected mice (**D'**). **E-H**. GAD67-immunostaining of inhibitory synapses in layer V of mock- (**E**) and ME49- (**G**) infected neocortex. High magnification images of layer V in mock-infected tissue (**F**) shows GAD67$^+$ perisomatic synapses clustering around cell bodies (arrows). Fewer GAD67$^+$ perisomatic synapses are present in ME49-infected layer V (**H**). **F',H'** show binarized, inverted images of GAD67-immunoreactivity in layer V of mock- and ME49-infected mice. **I**. Quantification of GAD67-immunoreactivity in SP (versus non-SP regions) of the CA1 region of hippocampus of mock and ME49-infected mice. Bars represent means +/- SEM. ** indicates $p<0.01$ by Students T-test (n=6 animals per condition). **J,K.** Quantification of the area of binarized, inverted GAD67-immunoreactivity in CA1 (**J**) and layer V of neocortex (**K**) of mock and ME49-infected mice. Bars represent means +/- SEM. * indicates $p<0.05$ and *** indicates $p<0.001$ by Students T-test (n=3 animals per condition). **L-O.** High magnification images of GAD67$^+$ inhibitory terminals on NeuroTrace-labeled somas in layer V of mock- **(L,M)** and ME49- **(N,O)** neocortex. **I'-L'** show binarized, inverted images of GAD67-immunoreactivity in mock- **(L',M')** and ME49- **(N',O')** neocortex. Arrows indicate GAD67$^+$ perisomatic synapses. Arrowheads in **L** highlight two small, NeuroTrace-labeled cells contacting the soma of a pyramidal neuron. **P**. Quantification of the density of GAD67$^+$ perisomatic nerve terminals along the surface of neuronal somata in mock, ME49 or Type III (CEP) *Toxoplasma*. Each data point represents one cell. In the case of the Type III (CEP) *Toxoplasma* red data points depict cells from animals with high cyst burden mice and gray data point depict cells from low cyst burden mice. **Q.** Average density of GAD67$^+$ perisomatic nerve terminals along the surface of neuronal somata in mock, ME49 or Type III (CEP) *Toxoplasma* from data plotted in **P**. Bars represent means +/- SEM. **** indicates $p<0.0001$ by Students T-test (n=4-5 animals per condition).

Scale bar in **A** = 500 μm for **A,C**; in **B** = 100 μm for **B,B',D,D'**; in **E** = 200 μm for **E,G**; in **F** = 50 μm for **F,F',H,H'**; in **L** = 10 μm for **L-O**; **L'-O'**.

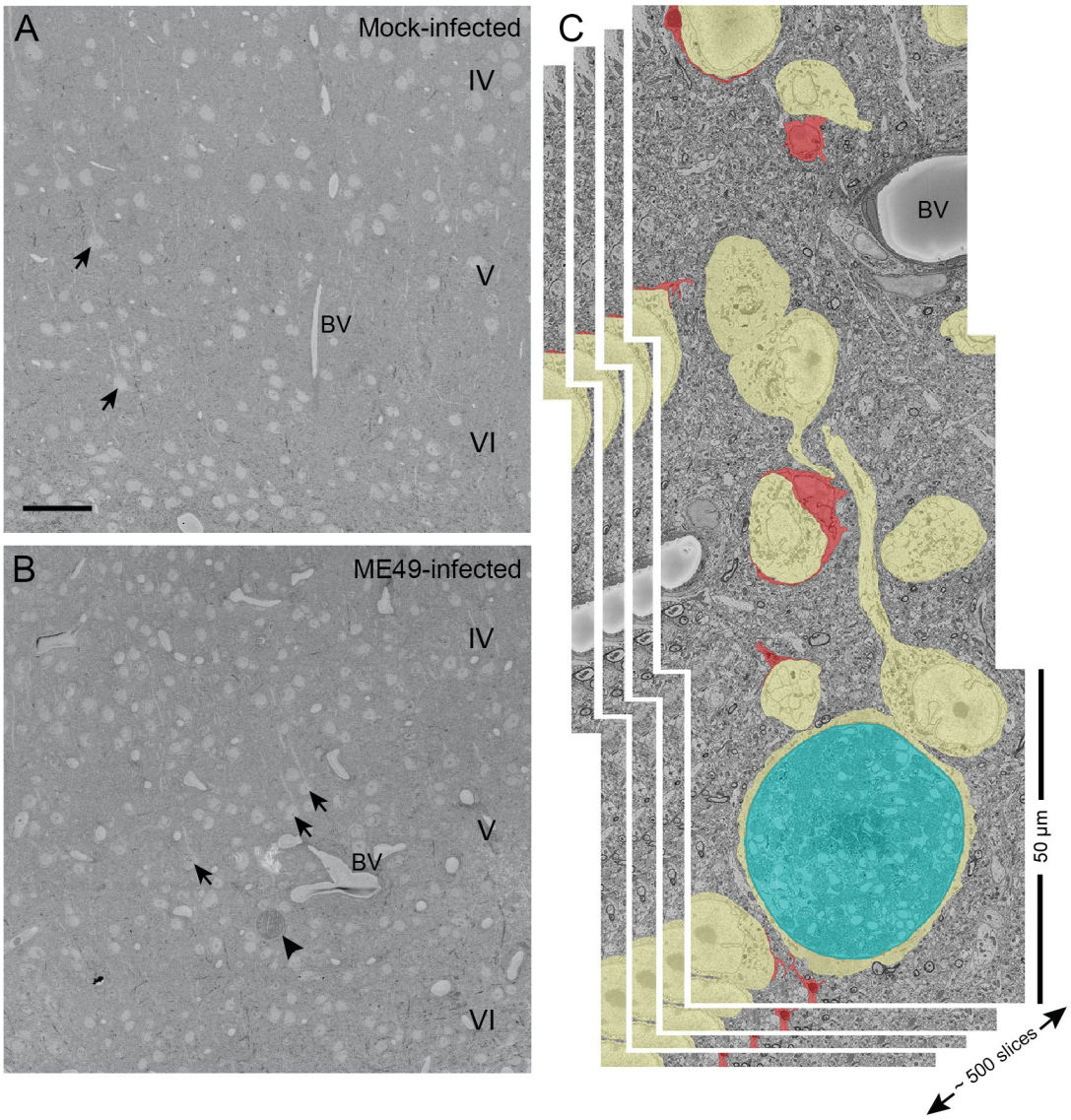


Figure 2.2. Serial block face scanning electron microscopic (SBFSEM) analysis of mock- and ME49-infected brain tissue.

A, B. Low magnification SBFSEM images of cerebral cortex of mock- (**A**) and ME49- (**B**) infected mice. Cortical layers IV, V, and VI are labeled. Arrows highlight layer V pyramidal neurons. Arrowhead in **B** highlights a *Toxoplasma gondii* cyst. BV – blood vessels. **C.** High magnification montage of 3 adjacent SBFSEM datasets. Each dataset consists of ~500 serial 50 µm x 50 µm micrographs, each 60 nm thick. In these micrographs, neurons are pseudocolored yellow, myeloid-derived cells / microglia are red, and a *Toxoplasma gondii* cyst is pseudocolored blue.

Scale bar in **A** = 50 µm for **A,B**.

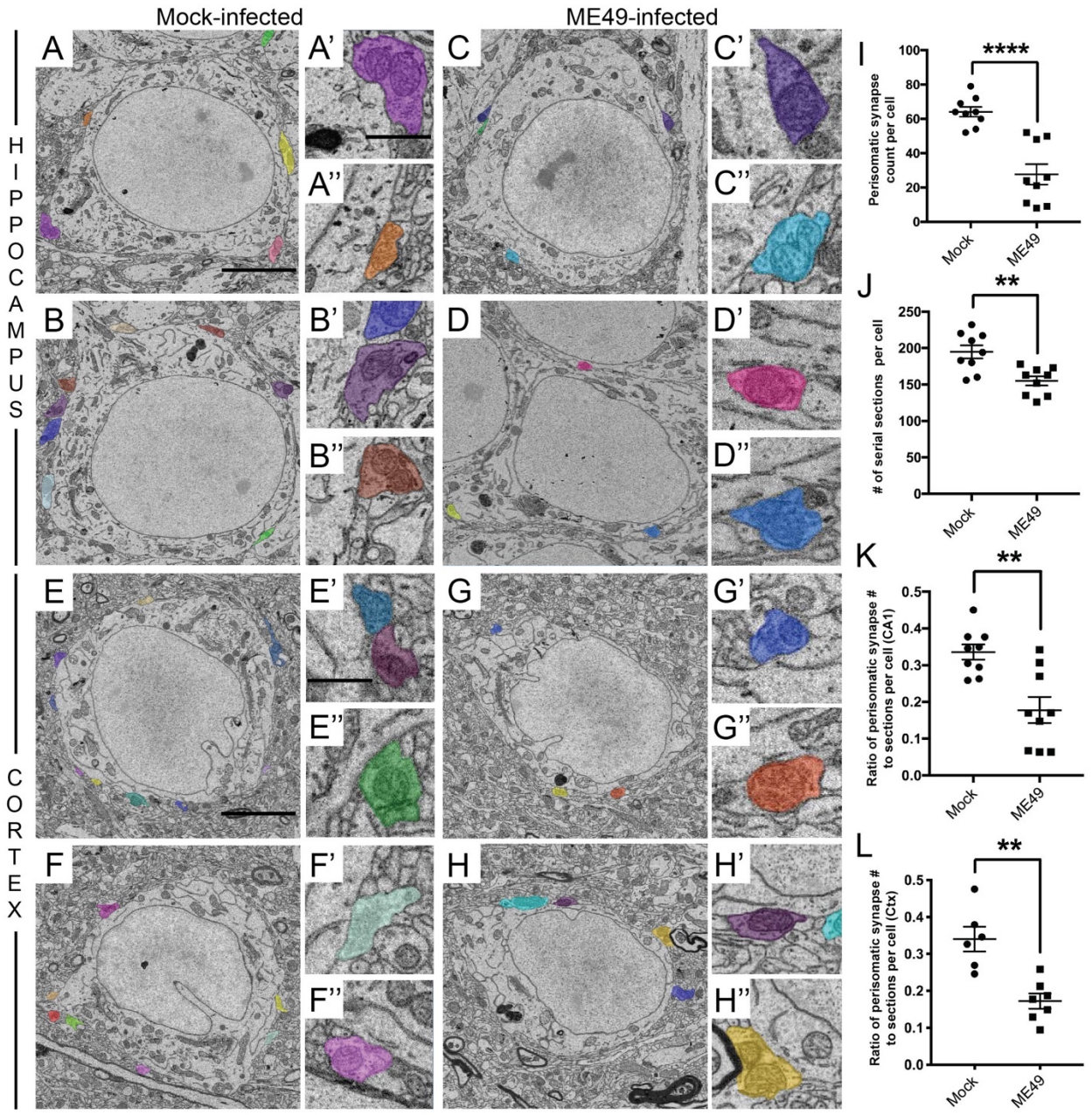


Figure 2.3. ***Toxoplasma*** **infection leads to a loss of perisomatic synapses in hippocampus and layer V of neocortex**.

A-D. SBFSEM micrographs of neurons in stratum pyramidalis of the CA1 region of hippocampus of mock- (**A,B**) and ME49- (**C,D**) infected mice. Perisomatic nerve terminals are pseudocolored and shown in the insets in **A′-D′** and **A′′-D′′**. Scale bar in **A** = 7 µm for **A-D** and in **A′** = 850 nm for **A′-D′** and **A′′-D′′**. **E-H.** SBFSEM micrographs of neurons in layer V of the neocortex of mock- (**E,F**) and ME49- (**G,H**) infected mice. Perisomatic nerve terminals are pseudocolored and shown

in the insets in **E′-H′** and **E′′-H′′**. Scale bar in **E** = 5 μm for **E-H** and in **E′** = 150 nm for **E′-H′** and **E′′-H′′**. **I.** Quantification of perisomatic nerve terminals on entire neuronal somata in stratum pyramidalis of the hippocampus of mock- and ME49- infected mice. Each data point represents the total perisomatic synapses on one cell. Lines represent means +/- SEM. **** indicates $p<0.0001$ by Student's t-test (n= 9 total cells; 3 cells from 3 animals per condition). **J.** Quantification of cell thickness in stratum pyramidalis of mock- and ME49- infected hippocampi. Each data point represents the number of serial sections per cell. Lines represent means +/- SEM. ** indicates $p<0.01$ by Student's t-test (n= 9 total cells; 3 cells from 3 animals per condition). **K.** Despite reduced neuronal thickness in ME49-infected tissue, the ratio of perisomatic nerve terminal number to soma thickness is significantly reduced in parasite infected hippocampi. Each data point represents the ratio of perisomatic synapses to somata thickness per cell. Lines represent means +/- SEM. ** indicates $p<0.01$ by Student's t-test (n= 9 total cells; 3 cells from 3 animals per condition). **L**. The ratio of perisomatic nerve terminal number to soma thickness is significantly reduced in layer V of ME49-infected neocortex. Each data point represents the ratio of perisomatic synapses to somata thickness per cell. Lines represent means +/- SEM. ** indicates $p<0.01$ by Student's t-test (n=7 total cells from 3 animals per condition).

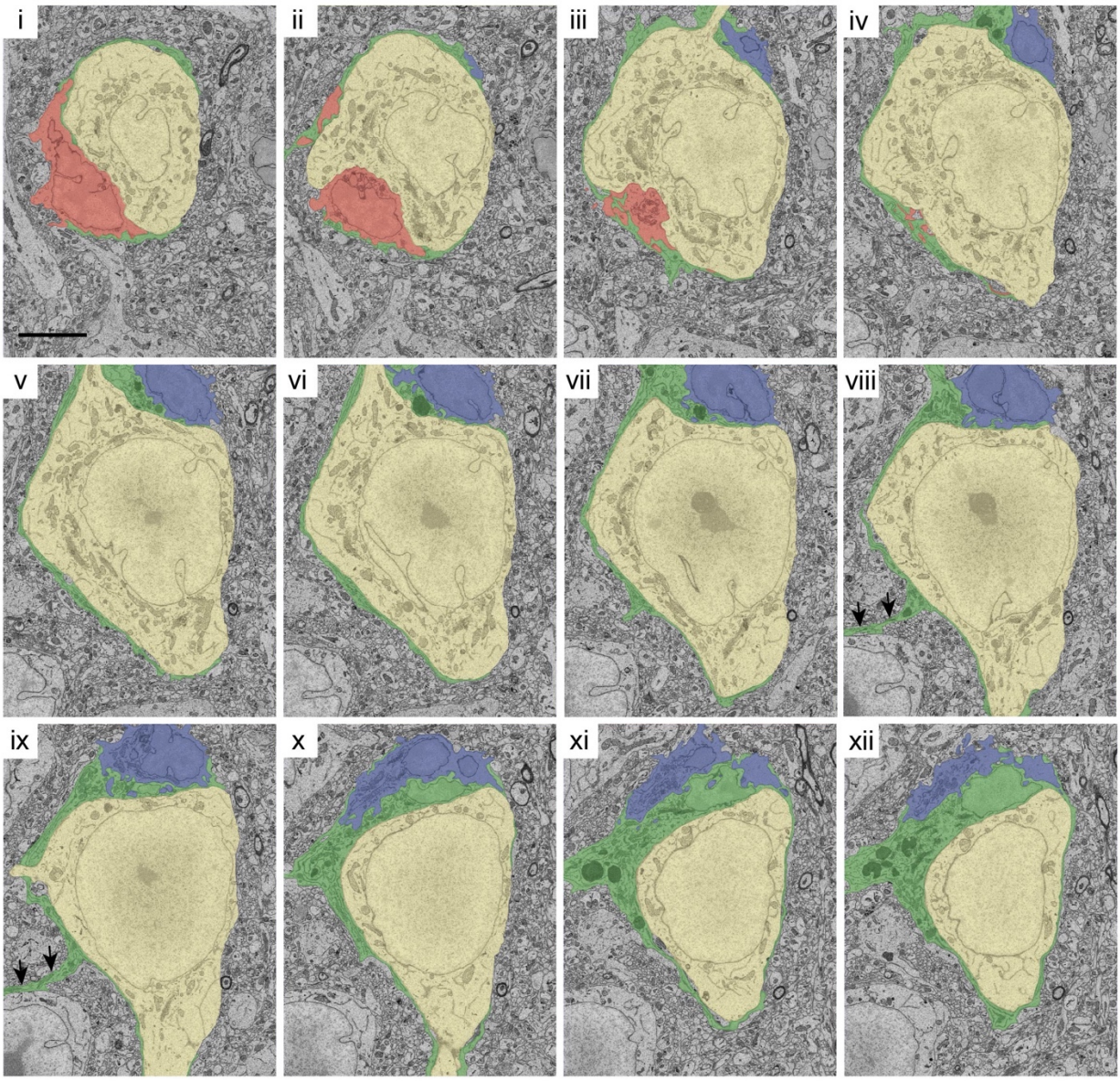
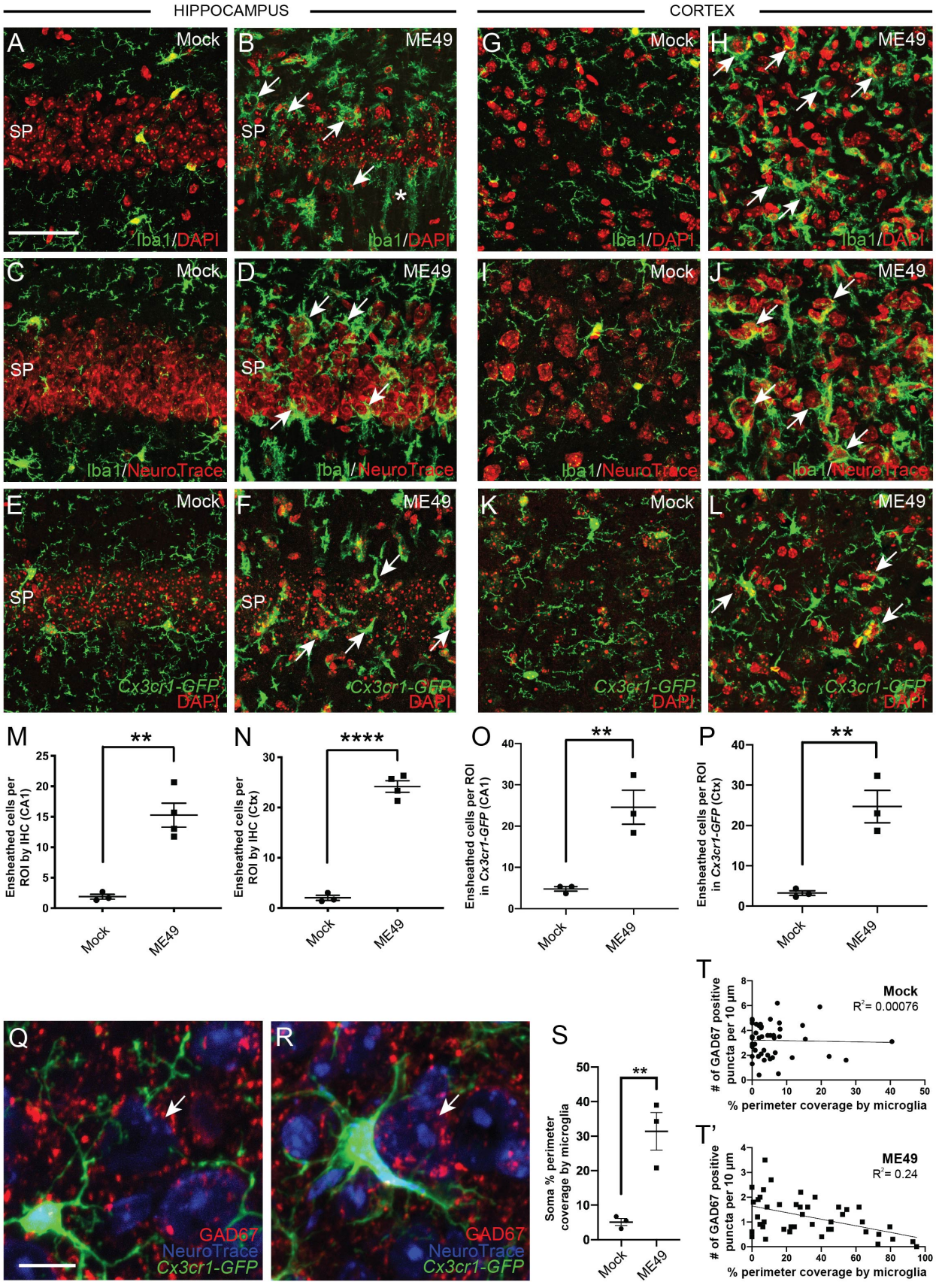


Figure 2.4. Myeloid-derived cells ensheath neuronal somata in *Toxoplasma*-infected neocortex.

A. Twelve SBFSEM micrographs through the same pyramidal neuron (pseudocolored yellow) in layer V of ME49-infected neocortex. The three myeloid-derived cells ensheathing this neuron are pseudocolored blue, green, and red.

Scale bar = 2.5 μm for all panels.


HIPPOCAMPUS
CORTEX
A
Mock
SP
Iba1/DAPI
B
ME49
SP
*
Iba1/DAPI
G
Mock
Iba1/DAPI
H
ME49
Iba1/DAPI
C
Mock
SP
Iba1/NeuroTrace
D
ME49
SP
Iba1/NeuroTrace
I
Mock
Iba1/NeuroTrace
J
ME49
Iba1/NeuroTrace
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SP
Cx3cr1-GFP
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Cx3cr1-GFP
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**
Ensheathed cells per ROI by IHC (CA1)
25
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Mock
ME49
N
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Ensheathed cells per ROI by IHC (Ctx)
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Ensheathed cells per ROI in Cx3cr1-GFP (CA1)
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ME49
P
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Ensheathed cells per ROI in Cx3cr1-GFP (Ctx)
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30
20
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0
Mock
ME49
Q
GAD67
NeuroTrace
Cx3cr1-GFP
R
GAD67
NeuroTrace
Cx3cr1-GFP
S
**
Soma % perimeter coverage by microglia
50
40
30
20
10
0
Mock
ME49
T
Mock
R2= 0.00076
# of GAD67 positive puncta per 10 µm
8
6
4
2
0
0
10
20
30
40
50
% perimeter coverage by microglia
T'
ME49
R2= 0.24
# of GAD67 positive puncta per 10 µm
4
3
2
1
0
0
20
40
60
80
100
% perimeter coverage by microglia

Figure 2.5. Widespread ensheathment of cells by microglia in the hippocampus and neocortex of *Toxoplasma*-infected mice.

A,B. Confocal images of immunolabeling of myeloid-derived cells with antibodies against Iba1 and labeling of nuclei with DAPI in mock- (**A**) and ME49- (**B**) infected CA1 of hippocampus. SP denotes stratum pyramidalis. Arrows highlight cells ensheathed by Iba1$^+$ cells. **C,D.** Iba1-immunolabeling and cell labeling with NeuroTrace in mock- (**C**) and ME49-(**D**) infected CA1 of hippocampus. Arrows highlight cells ensheathed by Iba1$^+$ cells in ME49-infected tissue. **E,F.** Genetic labeling of microglia in mock- (**E**) and ME49- (**F**) infected CA1 of *Cx3cr1-GFP* mice. Arrows highlight cells ensheathed by GFP$^+$ myeloid-derived cells / microglia in ME49-infected tissue. **G,H.** Immunolabeling of myeloid-derived cells with antibodies against Iba1 and labeling of nuclei with DAPI in mock- (**G**) and ME49- (**H**) infected layer V of neocortex. Arrows highlight cells ensheathed by Iba1$^+$ cells. **I,J.** Iba1-immunolabeling and cell labeling with NeuroTrace in mock- (**I**) and ME49- (**J**) infected layer V of neocortex. Arrows highlight cells ensheathed by Iba1$^+$ cells in ME49-infected tissue. **K,L.** Genetic labeling of myeloid-derived cells / microglia in mock- (**K**) and ME49- (**L**) infected neocortex of *Cx3cr1-GFP* mice. Arrows highlight cells ensheathed by GFP$^+$ cells in ME49-infected tissue. **M,N.** Quantification of the number of DAPI$^+$ cell somas ensheathed by Iba1$^+$ cells in stratum pyramidalis of CA1 (**M**) or layer V of neocortex (**N**). Lines represent means +/- SEM. ** indicates $p<0.01$ and **** indicates $p<0.0001$ by Student's t-test (n=4 animals per condition). **O,P**. Quantification of the number of DAPI$^+$ cell somas ensheathed by GFP$^+$ cells in stratum pyramidalis of CA1 (**O**) or layer V of neocortex (**P**). Lines represent means +/- SEM. ** indicates $p<0.01$ by Student's t-test (n=3 animals per condition). **Q,R**. GFP+ cells ensheath neuronal somas (labeled with NeuroTrace) in ME49-infected tissue. **S**. Quantification of the percent soma surface area of NeuroTrace$^+$ cells (arrows) ensheathed by GFP$^+$ cells in layer V of neocortex of mock or ME49-infected *Cx3cr1-GFP* mice. (**Q,R**). Lines represent means +/- SEM. ** indicates $p<0.01$ by Student's t-test (n=3 animals per condition). **T,T'**. Individual NeuroTrace$^+$/GFP$^-$ cells from mock (**T**) or ME49-infected (**T'**) *Cx3cr1-GFP* mice (from data in **S**) plotted based on percent somal surface area covered by GFP$^+$ cells versus number of GAD67$^+$ perisomatic synapses.

Scale bar in A = 50 μm for A-L, in Q = 10 μm for Q,R..

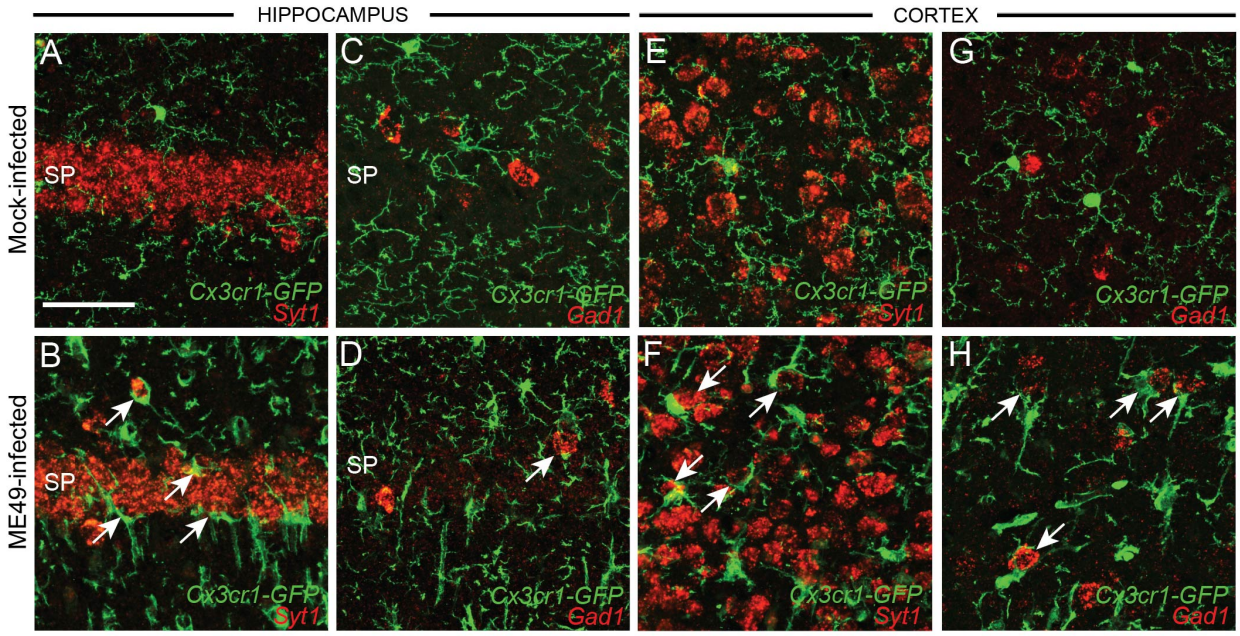

Figure 2.6. Microglia ensheath both excitatory and inhibitory neurons in parasite-infected hippocampus and neocortex.

A-D. *In situ* hybridization for *Syt1* mRNA and *Gad1* mRNA to label excitatory and inhibitory neurons, respectively, in CA1 of mock- or ME49-infected *Cx3cr1-GFP* mice. Arrows highlight *Syt1*$^+$ and *Gad1*$^+$ cells ensheathed by GFP$^+$ microglia. **E-H**. *In situ* hybridization for *Syt1* mRNA and *Gad1* mRNA to label excitatory and inhibitory neurons, respectively, in cortical layer V of mock- or ME49-infected *Cx3cr1-GFP* mice. Arrows highlight *Syt1*$^+$ and *Gad1*$^+$ cells ensheathed by GFP$^+$ microglia.

Scale bar in **A** = 50 μm for all panels.

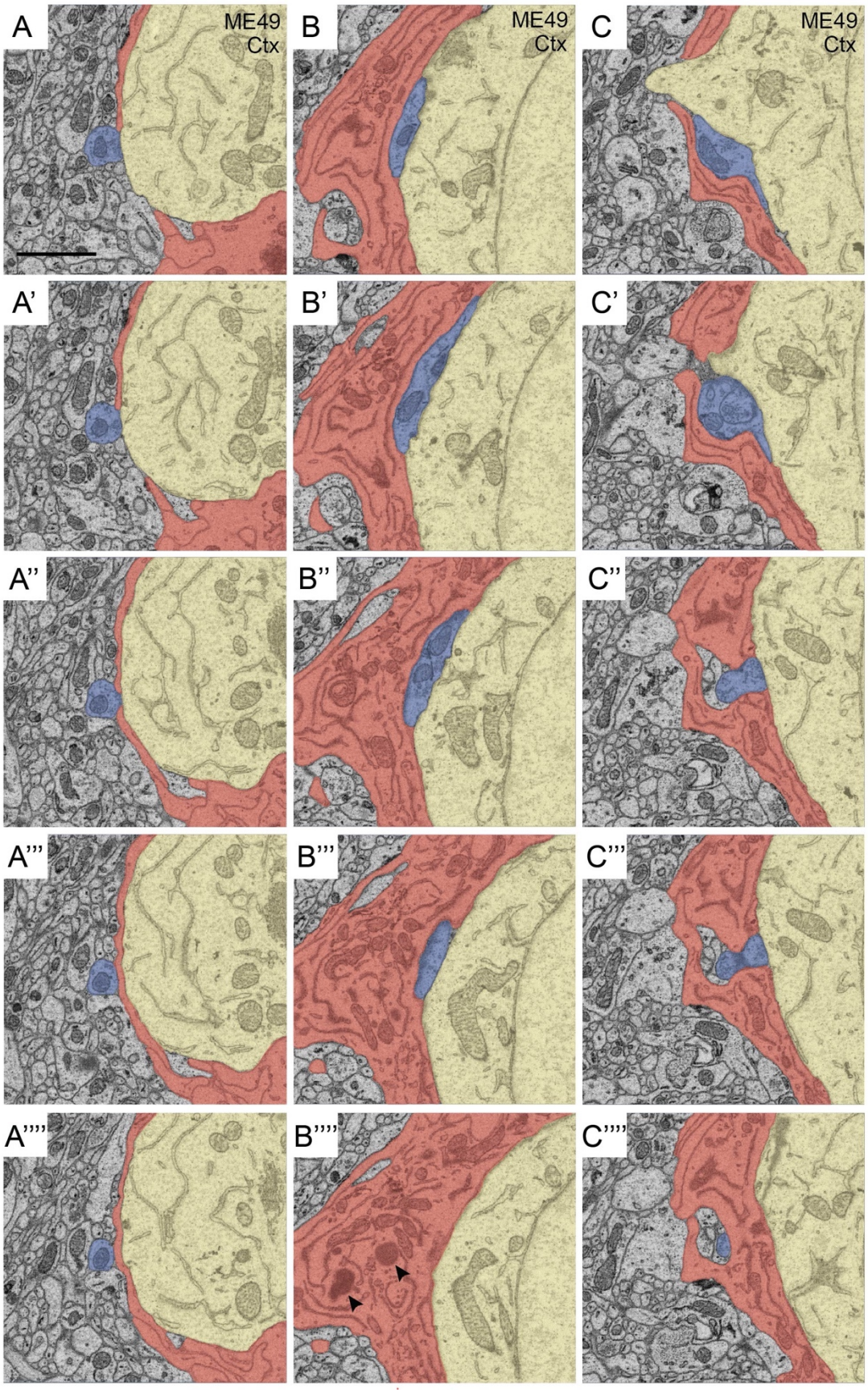
A
ME49
Ctx
B
ME49
Ctx
C
ME49
Ctx
A'
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Figure 2.7. Perisomatic synapses are ensheathed by microglia in *Toxoplasma*-infected brain tissue.

A-C. Examples of 3 perisomatic nerve terminals (in blue) ensheathed by microglia (in red) on a neocortical neuron (yellow). 5 micrographs shown for each example from this SBFSEM dataset. Black arrowheads indicate electron dense lysosomes.

Scale bar in **A** = 1 μm for all panels.

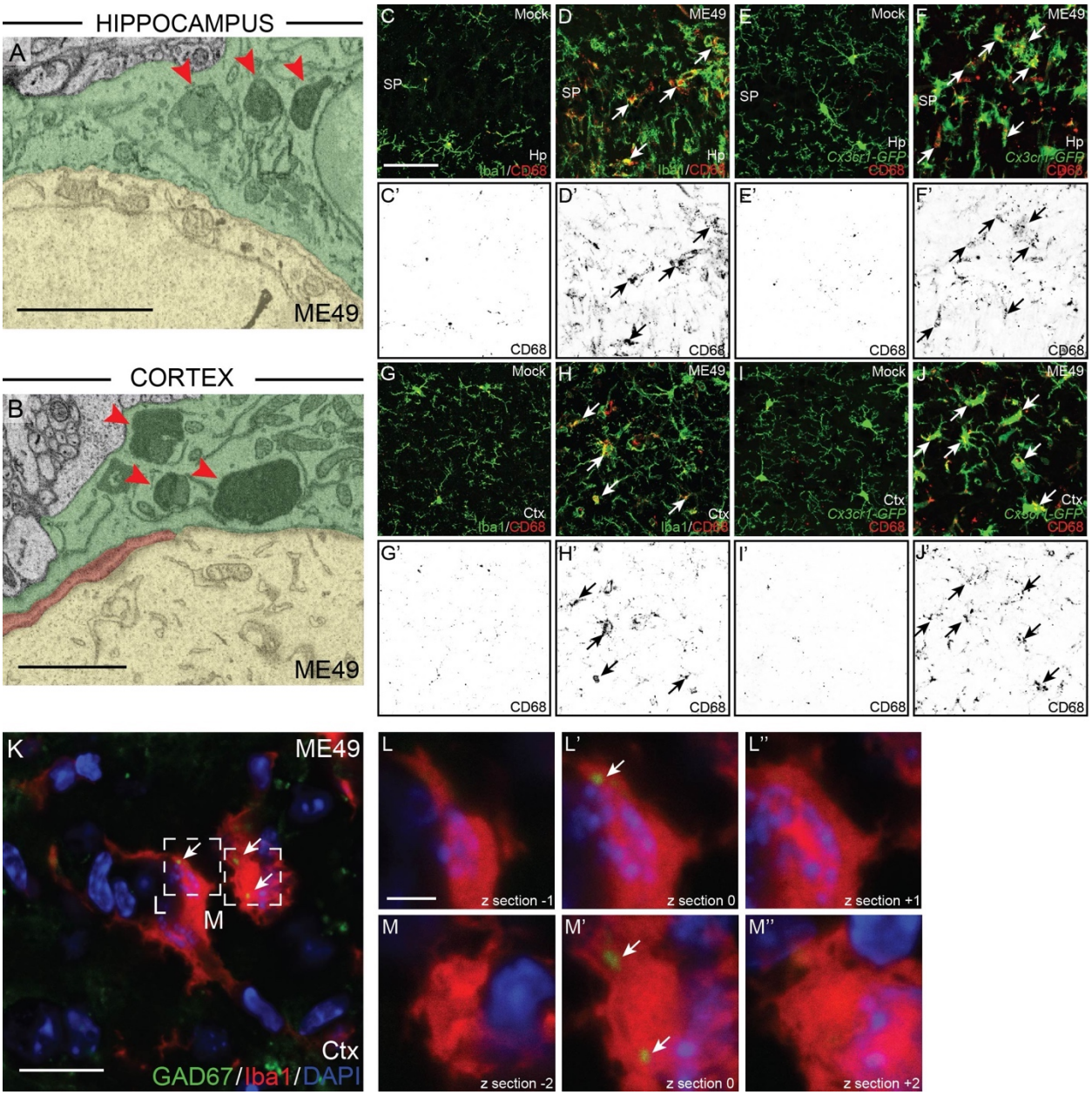

Figure 2.8. Phagocytic microglia ensheath neurons in *Toxoplasma*-infected brain tissue.

A,B. SBFSEM micrographs show lysosomes/phagosomes within the microglial ensheathing neuronal cell somas in ME49-infected hippocampus (**A**) and cortex (**B**). Electron dense lysosomes highlighted by red arrowheads. Neuronal somas pseudocolored in yellow. Microglia pseudocolored in green and red. **C-J**. Immunolabeling for CD68 in stratum pyramidalis (SP) of CA1 (Hp) and layer V of neocortex (Ctx) in mock- or ME49-infected *Cx3cr1-GFP* mice. Maximum projection confocal images shown. Arrows highlight microglia with elevated levels of CD68, a

marker of lysosomal activity and phagocytosis, in ME49-infected cortex and hippocampus. **K.** Confocal image showing Iba1$^+$ cells that ensheath neuronal somata contain GAD67$^+$ puncta in ME49-infected neocortex. **L-L", M-M"**. High magnification consecutive single optical sections (from **K**) highlighting GAD67$^+$ puncta (arrows) within the Iba1$^+$ cells.

Scale bar in **A** = 1 μm; in **B** = 1.25 μm; in **C** = 50 μm for **C-J**; in **K** = 10 μm; in **L** = 2 μm for **L-L", M-M"**.

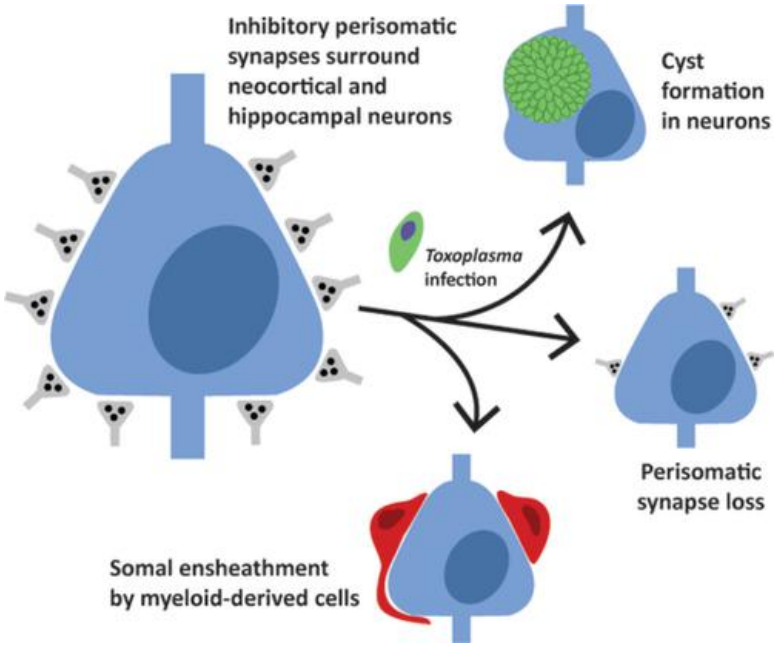

Figure 2.9. *Toxoplasma* infection induces microglia-neuron contact and the loss of perisomatic inhibitory synapses

Schematic summarizing key events during long-term *Toxoplasma* infection: neuronal cyst formation, microglial and monocytic ensheathment of neurons, and subsequent perisomatic inhibitory synapse.

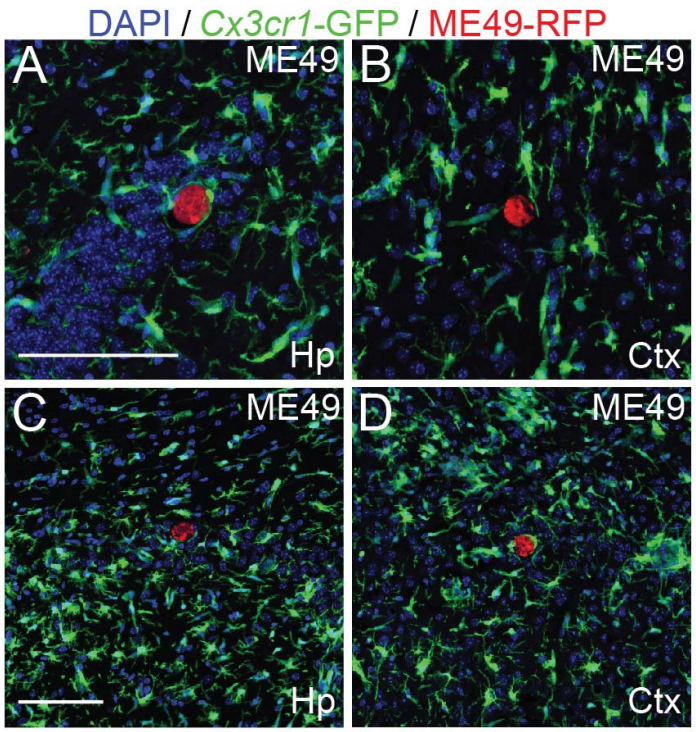

Figure 2.10. [Supplemental Figure] Distribution of *Toxoplasma gondii* in mouse hippocampus and neocortex.

Me49-RFP cysts in infected hippocampus (Hp; a, c) and cortex (Ctx; b, d) of *Cx3cr1-GFP* mice. Few ME49-RFP cysts are present in any single coronal section of hippocampus or cortex. DAPI labels all nuclei. GFP labels myeloid-derived cells.

Scale bar in a = 60 µm for a, b, in c = 60 µm for c, d

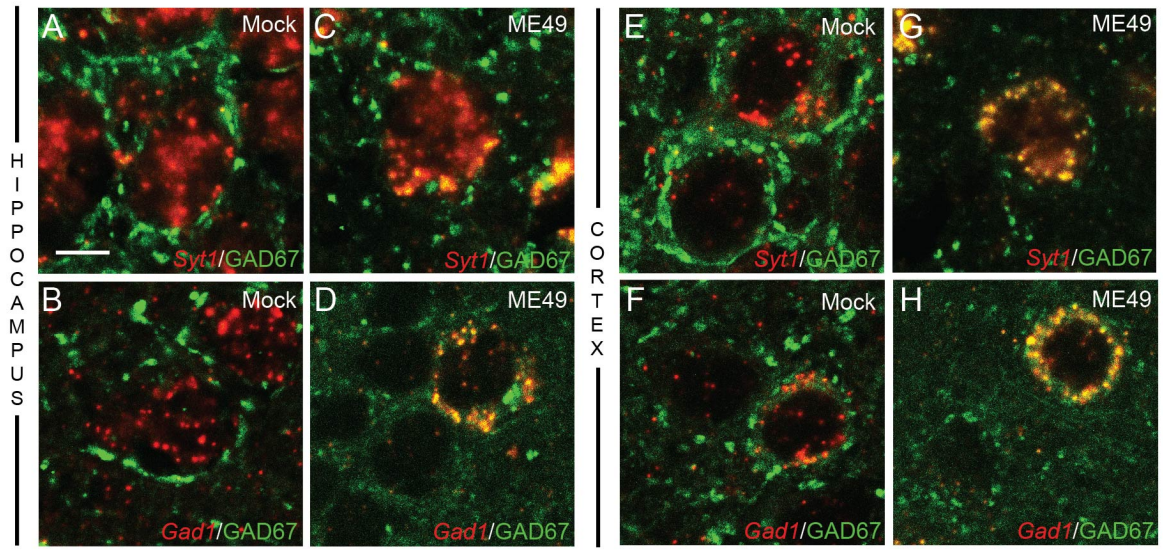

Figure 2.11 [Supplemental Figure] Loss of perisomatic synapses from excitatory and inhibitory postsynaptic neurons of *Toxoplasma*-infected mice.

(a–h) GAD67 immunolabeling and in situ hybridization for *Syt1* mRNA and *Gad1* mRNA (to label excitatory and inhibitory neurons, respectively). These data reveal reduced numbers of perisomatic GAD67^{+} synapses on both *Syt1*- and *Gad1*-expressing neurons in CA1 (c,d) and Layer V of neocortex (g,h) of ME49-infected mice.

Scale bar in a = 5 μm for all panels

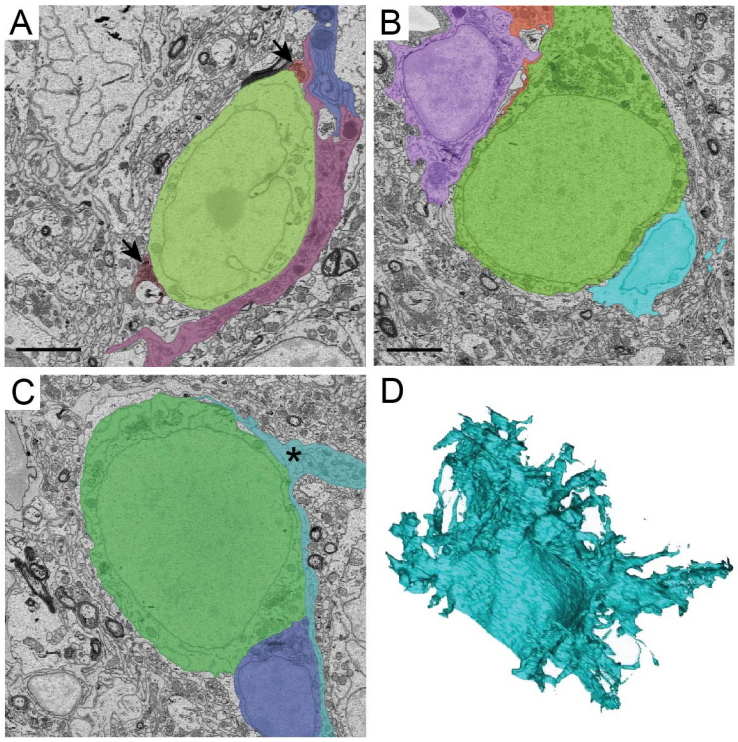

Figure 2.12 [Supplemental Figure] Microglial ensheathment of neuronal somata in *Toxoplasma*-infected hippocampus.

(a–c) Examples of neurons (pseudo-colored green) contacted by phagocytic cells in SBFSEM of ME49-infected hippocampus. In a, two axosomatic synapses are labeled brown and coral (arrows), and two phagocytic cells are labeled blue and burgundy. In b, three phagocytic cells, pseudo-colored purple, blue and red, contact the surface of the neuron. In c, two phagocytic cells, pseudo-colored in different shades of blue, contact the surface of the neuron. Panel d depicts a 3D reconstruction of the microglia pseudo-colored light blue and labeled with an asterisk in c.

Scale bar in a = 4 µm for a,c and in b = 4 µm

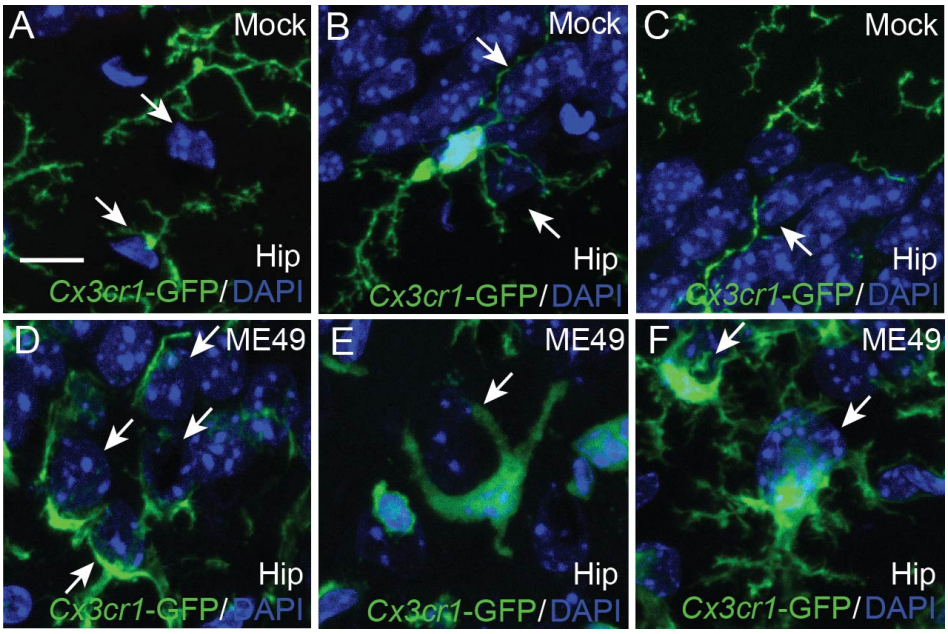

Figure 2.13 [Supplemental Figure] Neuronal contact and ensheathment by microglia in confocal images of mock- and ME49-infected hippocampus.

Maximum projection confocal images of GFP$^+$ myeloid-derived cells in mock- (a–c) and ME49 (d–f)-infected *Cx3cr1-GFP* mice. DAPI labels all nuclei. Arrows in a–c depict GFP$^+$ resident microglia whose processes appear to contact to GFP−/DAPI+ cells in mock-infected hippocampus. Arrows in d–f depict GFP$^+$ myeloid-derived cells (activated microglia or macrophages derived from infiltrating monocytes) whose cell bodies ensheath the cell bodies of GFP−/DAPI$^+$ cells in ME49-infected hippocampus.

Scale bar in a = 10 μm for all panels

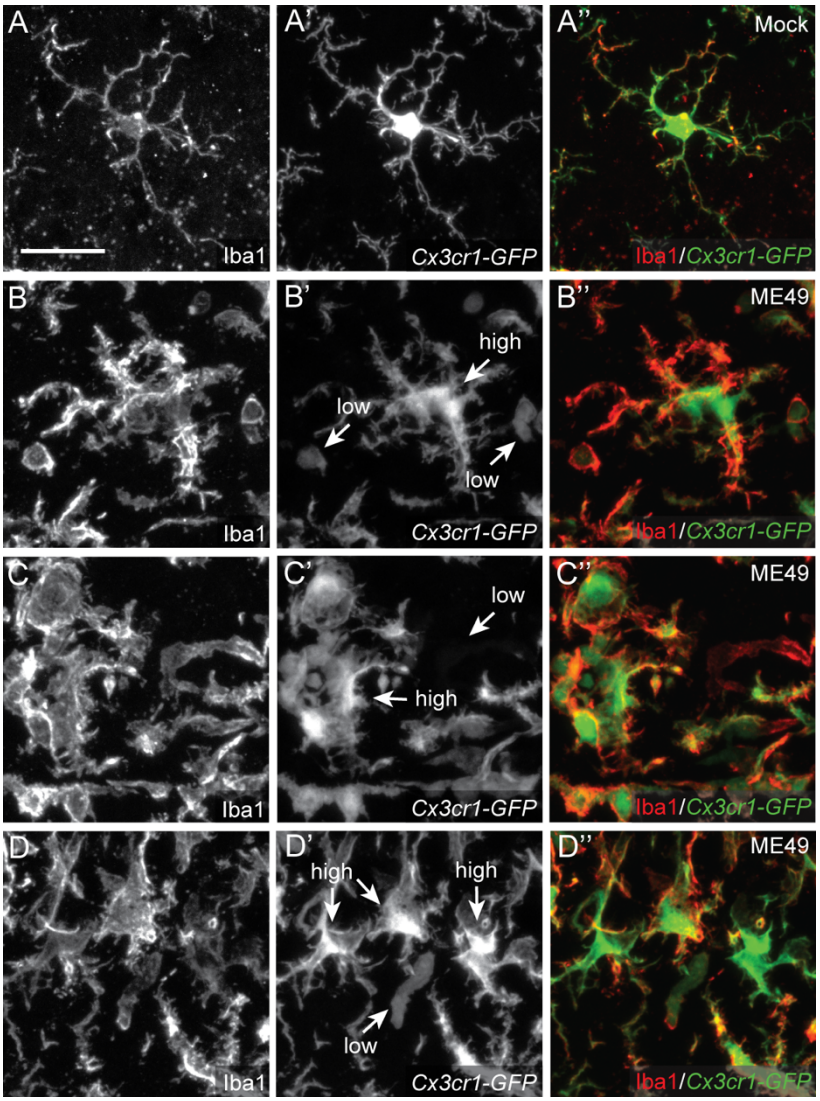

Figure 2.14 [Supplemental Figure] High and low GFP expression in myeloid-derived cells in *Toxoplasma*-infected *Cx3cr1-GFP* mice.

(a) Maximum projection confocal image of Iba1-immunostaining of resident microglia in mock-infected *Cx3cr1-GFP* mice. (b–d) Maximum projection confocal images of Iba1-immunostaining of myeloid-derived cells in ME49-infected *Cx3cr1-GFP* mice. Microglia have been reported to express high levels of GFP in inflamed brains of these transgenic reporter mice, whereas macrophages derived from infiltrating monocytes have been reported to express low levels of GFP (Yamasaki et al., **2014**). Here, we highlight Iba1^{+}, process-bearing cells with high levels of

GFP and Iba1^{+}, ameboid-shaped cells with low levels of GFP expression following infection with *Toxoplasma gondii*.

Scale bar in a = 20 µm for all images

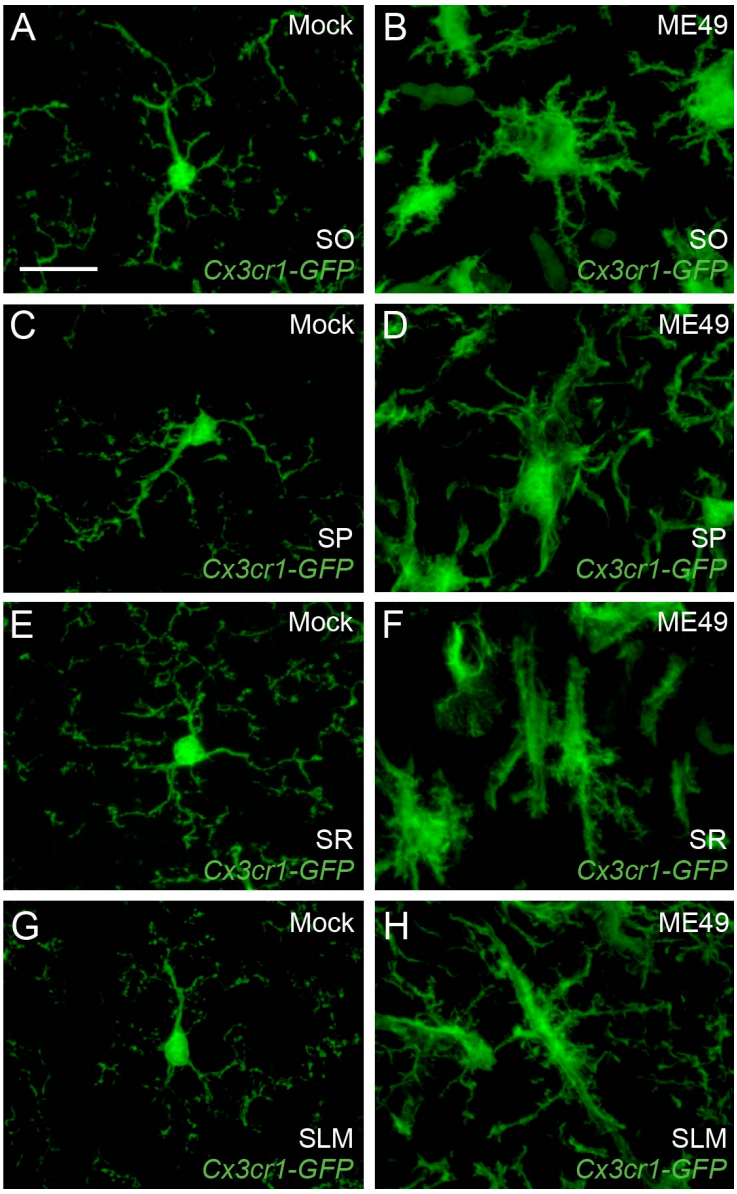

Figure 2.15 [Supplemental Figure] Microglial morphology in distinct layers of the CA1 region of the hippocampus in *Toxoplasma*-infected *Cx3cr1-GFP* mice.

Panels a,c,e,g depict examples of GFP$^+$ microglia in the stratum oriens (SO), stratum pyramidalis (SP), stratum radiatum (SR) and stratum lacunosum-moleculare (SLM) of mock-infected *Cx3cr1-GFP* mice. Panels b,d,f,h depict examples of GFP$^+$ microglia in SO, SP, SR, and SLM of ME49-infected *Cx3cr1-GFP* mice.

Scale bar = 15 µm for all panels

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Chapter 3:

Complement-dependent loss of inhibitory synapses on pyramidal neurons following *Toxoplasma gondii* infection

Previously published in BioRxiv:

Carrillo GL, Su J, Cawley ML, Wei D, Simran K, Blader IJ, Fox MA. (2022) Phagocytic cells selectively target pyramidal neurons and contribute to complement-dependent perisomatic synapse loss in *Toxoplasma gondii* infection.

ABSTRACT

The apicomplexan parasite *Toxoplasma gondii* has developed mechanisms to establish a central nervous system infection in virtually all warm blooded animals. Acute *T. gondii* infection can cause neuroinflammation, encephalitis, and seizures. Meanwhile, studies in humans, non-human primates, and rodents have linked chronic *T. gondii* infection with altered behavior and increased risk for neuropsychiatric disorders, including schizophrenia. We previously demonstrated that *T. gondii* infection triggers the loss of perisomatic inhibitory synapses, an important source of inhibition on excitatory pyramidal cells, and a type of synapse that is disrupted in neurological and neuropsychiatric disorders. Similar to other instances of inflammation and neurodegeneration, we showed that phagocytic cells (including microglia and infiltrating monocytes) contribute to the loss of these inhibitory synapses. However, in the case of *T. gondii*-induced synapse loss, phagocytic cells target and ensheath the cell bodies of telencephalic neurons. Here, we show that these phagocytic cells specifically ensheath excitatory pyramidal neurons, leading to the preferential loss of perisomatic synapses on these neurons. In contrast, inhibitory cortical interneuron subtypes are not extensively ensheathed by phagocytic cells following infection. Moreover, we show that infection induces expression of complement C3 protein by these excitatory neurons and that C3 is required for the loss of perisomatic inhibitory synapses, albeit not through activation of the classical complement pathway. Together, these findings provide evidence that *T. gondii* induces changes in excitatory pyramidal neurons that trigger selective removal of inhibitory perisomatic synapses and provide a novel role for complement in remodeling of inhibitory circuits in the infected brain.

INTRODUCTION

The central nervous system (CNS) is protected by a vascular blood-brain barrier (BBB) that prevents pathogens from entering the brain. However, in some cases, select pathogens have evolved mechanisms to traverse this barrier and invade the CNS. The apicomplexan parasite, *Toxoplasma gondii*, is one such pathogen that enters the brain and can establish a long-lasting CNS infection in almost all warm-blooded animals, including humans (Dubey, 2009, Montoya and Liesenfield, 2004). In fact, it is estimated that over 30% of the global human population are chronically infected with *Toxoplasma* (Flegr et al., 2014, Pappas, Roussos, and Falagas, 2009). Although there are several ways in which humans come into contact with *T. gondii*, we most

commonly become infected by ingestion of raw or undercooked meat contaminated by *T. gondii* tissue cysts or by consumption of vegetables and water that have *T. gondii* oocysts shed from the feline definitive host (Hajimohammadi et al., 2022, Jones and Dubey, 2005, Dabritz et al., 2007). Upon oral ingestion, *T. gondii* cysts and oocysts rupture and transform into tachyzoites, the form of the parasite that rapidly grows and disseminates throughout the host (Montoya and Liesenfield, 2004).

This acute phase of the infection is characterized by a severe inflammatory response aimed at controlling the infection, and the tachyzoite form of the parasite responds by differentiating into bradyzoites and forming intracellular cysts. These cysts can persist for the host's lifetime and they can reactivate. If reactivation occurs in an immunocompetent individual the tachyzoites that emerge will activate a cellular immune response that rapidly and efficiently controls the acute infection. In the absence of such an immune response, or if the immune response is too robust, disease will ensue. In such cases, the brain is the most commonly affected tissue, with patients developing life-threatening seizures and other neurological sequelae (Wong and Remington, 1993, Suzuki and Remington, 1993, Montoya and Liesenfeld, 2004).

Although tachyzoites can invade any nucleated cell, in the brain, they preferentially infect neurons where they transform into cyst-encased bradyzoites that are protected from immune-clearance. (Dubey, 2009, Melzer et al., 2010, Cabral et al., 2016, McConkey et al., 2013, Sims et al., 1989). A substantial body of evidence suggests that chronic *T. gondii* infection alters behaviors in infected hosts (Berdoy et al., 2000, Beste et al., 2014, Vyas et al., 2007). Moreover, although it is not yet understood why, dozens of studies, including a large-scale retrospective study of over 80,000 individuals, indicate a strong association between *T. gondii* infection and an increased risk for developing neuropsychiatric disorders, including schizophrenia (Burgdorf et al., 2019, Dickerson et al., 2014, 2017, 2018, Kano et al., 2020, Wang et al., 2019, Xiao et al., 2018).

Animal models of *T. gondii* infection highlight the impact that this parasite has on brain function by demonstrating that infection leads to seizure development (Brooks et al., 2015, David et al., 2016). In these models, *T. gondii* infection also leads to changes in neurotransmitter synthesis and release (Alsaady et al., 2019, Gatkowska et al., 2016, Martin et al., 2015, Skallova et al., 2006) and altered neural circuits (Brooks et al., 2015, Ihara et al., 2016, Lang et al., Parlog et al., 2015).

This is especially true for the inhibitory neurotransmitter, gamma-aminobutyric acid (GABA), and GABAergic circuits in the telencephalon (including the hippocampus and cerebral cortex). For example, studies show *T. gondii* is able to modulate the synthesis of GABA by upregulating expression of GABA synthesis enzymes, GAD65 and GAD67, and downregulating GABA-T, the enzyme responsible for breaking down GABA (Fuks et al., 2012, Bhandage et al., 2020). Additionally, we recently demonstrated that *T. gondii* infection leads to the loss of perisomatic GABAergic synapses from hippocampal and cortical neurons (Carrillo et al., 2020).

The loss of these inhibitory perisomatic synapses following *T. gondii* infection involves the activation of phagocytic cells, which in the infected brain, include both activated microglia and macrophages derived from infiltrating monocytes. We recently showed that these phagocytic cells ensheath neuronal somata and subsequently phagocytose perisomatic inhibitory synapses in the *T. gondii*-infected neocortex (Carrillo et al., 2020). Yet, whether perisomatic inhibitory synapse loss is an indiscriminate process or specific to cell types, and the mechanism underlying synapse loss has remained unclear. Here we sought to address these two important questions. We show that these phagocytic cells preferentially ensheath the somata of excitatory pyramidal neurons in neocortex and that these excitatory neurons differentially express complement C3 in *T. gondii*-infected brains. Moreover, conditional deletion of C3, but not C1qa, rescued the loss of perisomatic inhibitory synapses following infection and substantially reduced neuronal ensheathment, but had no significant effect on the initial phagocyte targeting of these neurons. Thus, our findings demonstrate a novel role for complement in inducing cell-type specific ensheathment and phagocytosis of GABAergic perisomatic synapses in *T. gondii* CNS infection.

MATERIALS AND METHODS

Key resources table

Reagent type (species) or resource	Designation	Source or reference	Identifiers
Parasite	Type II ME49	Blader Lab	N/A
Mouse	C57BL/6J	The Jackson Laboratory	JAX#000664; RRID:IMSR_JAX:000664
Mouse	*PV-Cre-YFP*	The Jackson Laboratory	N/A
Mouse	C3 $^{-/-}$	The Jackson Laboratory	JAX # 029661 RRID:IMSR_JAX:029661
Mouse	C1qa $^{-/-}$	The Jackson Laboratory	JAX #031675 RRID:IMSR_JAX:031675
antibody	mouse anti-GAD67 (1:1000 dilution)	EMD Millipore	MAB1501 RRID:AB_2223041
antibody	rabbit anti-IBA1 (1:700 dilution)	Wako	019–19741 RRID:AB_839504
antibody	rat anti-CD68 (1:1000 dilution)	Abcam	AB53444; RRID:AB_869007
antibody	rat anti-CTIP2 (1:500 dilution)	Abcam	AB18465; RRID:AB_2064130
antibody	mouse anti-CALB (1:500 dilution)	Swant	McAB300
antibody	mouse anti-SST (1:200)	Millipore Sigma	MAB354; RRID:AB_2255365
antibody	rabbit anti-GFP GFP (1:200 dilution)	Thermo Fisher Scientific	Cat#A-11122; RRID:AB_221569
antibody	NeuroTrace	Thermo Fisher Scientific	Cat# N21483; RRID:AB_2572212
antibody	anti-Diogoxigenin-POD (1:2000 dilution)	Millipore Sigma	Cat#11207733910; RRID:AB_514500
antibody	anti-Fluorescein-POD (1:3000 dilution)	Millipore Sigma	Cat#11426346910; RRID:AB_840257
commercial assay or kit	pGEM-T Easy Vector Systems	Promega	A1360
commercial assay or kit	Ambion MAXIscript T7 In Vitro Transcription Kit	Thermo Fisher Scientific	AM1312
commercial assay or kit	Tyramide Signal Amplification (TSA) systems	PerkinElmer	NEL75300 1KT

chemical compound, drug	Fluorescein RNA Labeling Mix	Roche	11685619910
chemical compound, drug	DIG RNA Labeling Mix	Roche	11277073910
chemical compound, drug	Proteinase K	Thermo Fisher Scientific	EO0491
chemical compound, drug	Prehybridization Solution	Sigma	P1415
chemical compound, drug	Yeast RNA	Roche	10109223001
chemical compound, drug	Blocking reagent (ISH)	Roche	11096176001
chemical compound, drug	Paraformaldehyde, EM grade	EMS	12300
chemical compound, drug	Tissue Freezing Medium	EMS	16220
recombinant DNA reagent	Image clone for *C3*	Horizon Discovery	MMM1013-202768722
Recombinant DNA reagent	Image clone for *Npnt*	Horizon Discovery	MMM1013-202708550
recombinant DNA reagent	Image clone for *Syt1*	Horizon Discovery	MM1013-9199901
sequence-based reagent	Cloning primers for *C1qa:* F: ATGGGGCTCCAGGAAATC R: AGTCCTCAGTGCCCTCCC	Integrated DNA Technologies	N/A
sequence-based reagent	Cloning primers for *Gad1:* F: TGTGCCCAAACTGGTCCT; R:TGGCCGATGATTCTGGTT	Integrated DNA Technologies	N/A
sequence-based reagent	Cloning primers for *Vglut1:* F: CAGAGCCGGAGGAGATGA R: TTCCCTCAGAAACGCTGG	Integrated DNA Technologies	N/A
sequence-based reagent	Genotyping primers for C3: Comm: ATCTTGAGTGCACCAAGCC Wt:GGTTGCAGCAGTCTATGAAGG Mut:GCCAGAGGCCACTTGTGTAG	Integrated DNA Technologies	N/A
sequence-based reagent	Genotyping primers for C1qa: F:TGCATCCTGCCATCTCCT R:GAAAGTGCTTAAAGAAACCACTG	Integrated DNA Technologies	N/A
sequence-based reagent	Genotyping primers for Cre: F: CGTACTGACGGTGGGAGAAT R:T GCATGATCTCCGGTATTGA	Integrated DNA Technologies	N/A

sequence-based reagent	Genotyping primers for: YFP F: AAGTTCATCTGCACCACCG R: TCCTTGAAGAAGATGGTGCG	Integrated DNA Technologies	N/A
software, algorithm	ImageJ	NIH	Version 1.52p; RRID: SCR_003070
software, algorithm	Prism	GraphPad	Version 8.0; RRID: SCR_002798
software, algorithm	Adobe Photoshop	Adobe Inc.	Version: 21.1.2

Animals

C57/BL6 mice C3$^{-/-}$ mice (JAX # 029661, genetic background: C57BL/6J), C1qa$^{-/-}$ mice (JAX #031675, genetic background: C57BL/6J) were obtained from Jackson Laboratory. *PV-Cre-YFP* mice were generated from crossing *Parv-Cre*$^{KI/KI}$ mice (JAX #008069, genetic background: C57BL/6J) with *Thy1-stop-yfp15* mice (JAX# 005630, genetic background: C57BL/6J) for several generations. All mice were genotyped before infection and experimentation (C3 genotyping primers: Comm: ATCTTGAGTGCACCAAGCC, Wt: GGTTGCAGCAGTCTATGAAGG, Mut: GCCAGAGGCCACTTGTGTAG); C1qa genotyping primers (F: TGCATCCTGCCATCTCCT, R: GAAAGTGCTTAAAGAAACCACTG); Cre genotyping primers (F: CGTACTGACGGTGGGAGAAT, R: T GCATGATCTCCGGTATTGA); YFP genotyping primers: F: AAGTTCATCTGCACCACCG, R: TCCTTGAAGAAGATGGTGCG) all purchased from Integrated DNA Technologies). Both sexes were used for all experiments. Prior to infection, mice were housed in the same ABSL-1 temperature-controlled room with a 12 hr dark/light cycle and *ad libitum* access to food and water. Upon infection, mice were moved to an ABSL-2 room with similar temperature-control, 12 hr dark/light cycle, and they continued to have *ad libitum* access to food and water, supplemented with wet food. Experiments were performed in compliance with National Institute of Health (NIH) guidelines and protocols were approved by the Institutional Animal Care and Use Committee at Virginia Tech.

Parasite infections

As previously described, age-matched male and female mice (~8 weeks of age) were infected with the Type II ME49-RFP strain of *T. gondii* (Brooks et al., 2015, Carrillo et al., 2020). The total number of *T. gondii* cysts within 20µl of infected brain homogenate was determined by fluorescence microscopy. Mice were interperitoneally infected with 2 ME49-RFP cysts in PBS. For

time course experiments, brains were collected after 7 days, 12 days, 21 days, and 30 days of infection. For long-term infection, brains were collected 30 days following infection.

Tissue Preparation

Tissue was prepared as previously described in Su et al., 2010. Briefly, an intraperitoneal injection of tribromoethanol at a concentration of 12.5 ug/ml was used to anesthetize mice. Mice were perfused with 10 mL of DEPC-treated 1x PBS followed by DEPC-treated 4% PFA pH 7.4. Brains were kept overnight at 4°C in 4% DEPC-PFA and then transferred to 30% DEPC-sucrose for at least 4 days. Fixed brains were embedded in Tissue Freezing Medium (Electron Microscopy Sciences), frozen overnight, and coronally sectioned on a Leica CM1850 cryostat at 16µm thickness. Prepared tissue slides were kept in -20°C for later IHC and ISH experiments.

Immunohistochemistry (IHC)

Tissue slides were air-dried at room temperature for 15 minutes. Slides were incubated in IHC blocking buffer (2.5% bovine serum albumin, 5% normal goat serum, 0.1% Triton-X in PBS) for 1 hour. Primary antibodies were diluted in blocking buffer as follows: GAD67 1:1000 (Millipore MAB5406); IBA1 1:700 (Wako, Richmond, VA; 019-19741); CD68 1:1000 (Abcam Cambridge, UK; AB53444); CTIP2 1:500 (Abcam, AB18465); CALB 1:500 (Swant, McAB300); SST 1:200 (Millipore, MAB354); GFP (Thermo Fisher Scientific, Waltham, MAA-11122). Tissue slides were incubated in diluted primary antibody at 4°C for a minimum of 15 hrs. After removal of primary antibody, slides were washed in 1x PBS for an hour, followed by incubation with fluorophore-conjugated secondary antibodies diluted IHC blocking buffer at 1:1000 for 1 hour room temperature. Lastly, after several washes in 1x PBS, tissue sections were either stained with 4',6-diamidino-2-phenylindole (DAPI) or with NeuroTrace 640/660. NeuroTrace staining was performed as follows: First, slides were incubated in 0.1% Triton-X in PBS for 10 minutes, washed in 1x PBS twice for 5 minutes each, incubated in NeuroTrace (diluted 1:250 in PBS (Thermo Fisher, N21483)) for 35 minutes in room temperature, washed in 0.1% Triton-X in PBS for 10 minutes, and two final washes in 1x PBS.

Riboprobe production

pCMV-SPORT6 plasmids carrying *C3* (cat # 5134713), *Syt1* (cat # 5363062), and *Npnt* (cat # 5149204) were obtained from Horizon Discovery (previously GE Dharmacon). *Gad1* cDNA,

C1qa cDNA, and *Vglut1* cDNA were generated and amplified as previously described in Monavarfeshani et al., 2018. Primers are as follows: *Gad1* primers (F: TGTGCCCAAACTGGTCCT; R:TGGCCGATGATTCTGGTT); *C1qa* primers (F: ATGGGGCTCCAGGAAATC, R: AGTCCTCAGTGCCCTCCC); Vglut1 primers (Cloning primers for *Vglut1: (*F: CAGAGCCGGAGGAGATGA, R: TTCCCTCAGAAACGCTGG) all obtained from Integrated DNA Technologies, Coraville, IL. All riboprobes were generated as described in Monavarfeshani et al., 2018.

In situ hybridization (ISH)

ISH was performed using the in-house generated riboprobes on fixed tissue, prepared with DEPC-treated reagents as described above. Tissue sections on slides were fixed in 4% DEPC-PFA for 10 minutes at room temperature, washed with 1x DEPC-PBS, and incubated with proteinase K solution (1ug/mL in 50 mM Tris pH 7.5, 5 mM EDTA) for 10 minutes at room temperature. Slides were then washed with 1x DEPC-PBS, incubated in 4% DEPC-PFA for 5 minutes at room temperature, and incubated in acetylation solution (0.25% acetic anhydride, 20 mM hydrochloric acid and 1.33% triethanolamine) for 10 minutes at room temperature. To permeabilize the tissue, slides were incubated in 1% triton in DEPC-PBS for 30 minutes room temperature. To block endogenous peroxidase, slides were incubated in 0.3% hydrogen peroxide in DEPC-PBS for 30 minutes at room temperature, followed by DEPC-PBS washes. Tissue sections were equilibrated in hybridization solution (40 mL of prehybridization solution, 1.6 mL of 5 mg/mL, and 25 mg Roche yeast RNA #10109223001) for 1 hour at room temperature. Riboprobes were denatured for 10 minutes at 80°C, applied to tissue sections, cover slipped, and incubated overnight at 65°C (probes were denatured for 10 minutes at 80°C). On day 2, coverslips were removed by slide incubation in 2x saline-sodium citrate solution for 5 min in 65°C, and slides underwent several 45 minute-long washes at 65°C, with a final wash at room temperature, followed by rinsing with 0.2x saline-sodium citrate solution in tris-buffered saline (TBS) at room temperature. Slides were incubated in ISH blocking buffer (10% lamb serum 0.2% Roche blocking reagent in TBS) for 1 hour at room temperature and then incubated overnight at 4°C in HRP-conjugated anti-DIG or anti-FL antibodies (DIG RNA Labeling Mix #11277073910; Fluorescein RNA Labeling Mix #11685619910, Roche). On day three, riboprobes were identified using a Tyramide Signal Amplification (TSA) system (#NEL75300 1KT, PerkinElmer). For double riboprobe ISH, the first riboprobe antibody was quenched by incubation in 2% hydrogen peroxide in TBS for 1 hour and 15 minutes at room temperature. After washing with TBS, slides

were incubated in ISH blocking buffer for 1 hour at room temperature and incubated overnight at 4°C in the second HRP-conjugated anti-DIG or anti-FL antibody. On day four, the second riboprobe was identified with the TSA system and then washed with TBS. For IHC following ISH, slides were incubated in IHC blocking buffer and IHC was performed as described above. IHC antibody dilutions following ISH were as follows: rabbit anti-GFP dilution: 1:200, rabbit anti-IBA1 dilution: 1:600).

Imaging

Images were acquired with a Zeiss LSM700 confocal microscope. Representative images shown in figures are of a maximum intensity projection, unless otherwise noted. Analyses were performed with single optical section images.

Density of cells (*Vglut1*$^+$, *Gad1*$^+$, *PV-Cre-YFP*$^+$, IBA1$^+$)

For assessment of cell numbers in cortical layer V, images were obtained using 40x magnification (20x 2 zoom). Counts were performed manually. The "Count Tool" function in Adobe Photoshop (Adobe Inc., version: 21.1.2) was used to keep track of cell counts and colocalized counts. Layer IV – VI cell counts were obtained from 20x images. Cell counts are normalized by the ROI area described as μm^2.

Cell type ensheathment analysis

Excitatory and inhibitory cell types were labeled either by immunohistochemistry (CTIP2, SST, CALB) or by use of a transgenic reporter (*PV-Cre-YFP*; details in 'Animals' section). Tissue was co-stained with NeuroTrace for visualization of the entire neuronal soma (except for *PV-Cre-YFP)*. Images were taken at 40x magnification to capture only cortical layer V at 1µm intervals. Cells of interest were identified by colocalization of cell type marker with NeuroTrace, and the % somata coverage by IBA1$^+$ cell was calculated by length of IBA1$^+$ cell or process contact neuronal soma (µm) / neuronal soma perimeter (µm) for each optical section where the cell's NeuroTrace signal was visible. Length and perimeter measurements were taken with ImageJ. % soma coverage calculations on individual optical sections were averaged to obtain % soma coverage for each cell. Data is shown as quantification of biological replicates (mean of all cells from each animal) and plotted as individual cell data points to show distribution across all animals.

Inhibitory perisomatic synapse analysis

Cells were labeled transgenically (*PV-Cre-YFP*) or by NeuroTrace. Inhibitory perisomatic synapses were identified with GAD67 immunolabeling. Images were taken at 40x magnification to capture only cortical layer V at 0.5µm intervals. The number of GAD67$^+$ puncta contacting the perimeter of NeuroTrace-labeled neuronal soma were counted manually and normalized to the neuronal soma perimeter (measured by ImageJ) at each optical section. Calculations from optical sections were averaged to obtain a single calculation of the number of GAD67$^+$ puncta / 10µm perimeter of neuronal soma. Data is shown as quantification of biological replicates (mean of all cells from each animal) and plotted as individual cell data points to show distribution across all animals.

Colocalization analysis

All ISH colocalization (*Syt1* and *C3, Syt1* and *C1qa*, *Npnt* and *C3*, *Gad1* and *C3*, *Npnt* and *Vglut1*, *Npnt* and *Gad1*) was determined by single optical image sections. The "Count Tool" function in Adobe Photoshop was used to keep track of cell counts and colocalized counts.

IBA1 masked analysis (*C1qa* and CD68)

IBA1$^+$ cells were masked using the 'threshold' and 'analyze particles' functions in ImageJ. The masks were superimposed to either the CD68 channel, or the *C1qa* channel and the mean signal intensity was calculated within the masks, and outside the masks (by inverting the ROI).

Expression analysis (% of IBA1$^+$ cells express *C1qa*, % of *Npnt*$^+$ cells express *Vglut1* or *Gad1*)

Quantifications for the percentage of IBA1$^+$ cells that express *C1q* were determined by maximum intensity projection images (and confirmed by single optical sections). The number of IBA1$^+$ cells with colocalized *C1q* signal was divided by the total number of IBA1$^+$ cells per image area (µm^2). For % of *Npnt*$^+$ cells that express *Vglut1* or *Gad1,* the number of *Vglut1*$^+$ cells (or the number of *Gad1*$^+$ cells) that colocalized with *Npnt*$^+$ cells were divided by the total number of *Npnt*$^+$ cells per image area (µm^2). All cells were counted manually and the "Count Tool" function in Adobe Photoshop was used to keep track of cell counts and colocalized counts.

Quantifications and statistics

All analyses were performed with 3-4 biological replicates per genotype and condition. Both sexes were used in the quantification. No data or animals were excluded. No sex-specific differences were observed with the number of animals used. Statistical analyses (Student's *t*-test or one-way ANOVA with Sidak's correction for multiple comparisons as indicated in figure legends) were performed using GraphPad Prism (version 8.0; RRID: SCR_002798). $P < 0.5$ values were considered to be significantly different (*P* values included in figure legends). Data points represent biological replicates or individual cells analyzed as described in figure legends and are plotted as ± SEM or mean, respectively.

RESULTS

Differential ensheathment of cortical neurons by IBA1$^+$ phagocytes in parasite-infected neocortex

We previously discovered that long-term infection with type II ME49 *Toxoplasma gondii* (> 30 days post infection; Fig 1A) results in the development of seizures and the loss of perisomatic inhibitory synapses in several regions of the mouse telencephalon, including in stratum pyramidalis of the hippocampus and layer V of the cerebral cortex (Carrillo et al., 2020, Brooks et al., 2015). The loss of perisomatic synapses co-occurred with the ensheathment of neuronal cell bodies by IBA1$^+$ phagocytic cells (which likely includes both resident microglia and macrophages-derived from peripheral monocytes that infiltrate the brain during infection (Carrillo et al., 2020). The ensheathment of neuronal somata by IBA1$^+$ cells in the telencephalon of infected mice is dramatic and noteworthy for at least 3 reasons: 1) neuronal ensheathment and perisomatic synapse loss was widespread throughout the telencephalon at 4 weeks post-infection; 2) some neurons appeared to be almost entirely ensheathed by IBA1$^+$ cells; and 3) not all neurons appeared to be ensheathed (Figure 1B). This led to a number of questions: *When do phagocytic cells ensheath neurons following infection? What types of neurons are ensheathed? And what mechanisms drive neuronal ensheathment and perisomatic synapse loss?* Here, we have addressed these important questions.

First, we addressed the timing of neuronal ensheathment by phagocytic cells by assessing the distribution of IBA1$^+$ cells in the neocortex each week after infection. We show that

phagocytes begin to target neurons between 12 and 21 days post infection (dpi), with significant ensheathment of neuronal somata observed at 21 dpi (Figure 1C).

Next, we sought to address whether there was any specificity in terms of which cell types were targeted and ensheathed by IBA1$^+$ phagocytes following infection. It is noteworthy that in layer V of neocortex of infected mice, the neurons ensheathed by IBA1$^+$ cells appeared pyramidal in morphology (Figure 1B), suggesting excitatory neurons may be preferentially targeted by these phagocytes. To determine if this was indeed the case, brains from infected mice were immunostained for IBA1 and a number of cell-type specific markers allowing us to differentiate between types of excitatory and inhibitory neurons. In layer V of neocortex (where we focus all of these studies), we show that COUP TF1-interacting protein 2$^+$ (CTIP2) expressing excitatory pyramidal neurons are extensively ensheathed by phagocytes following infection (Figure 2A-C; Arlotta et al., 2005, Lodato et al., 2011). We measured the extent of the somal surface of these neurons ensheathed by IBA1$^+$ cells and found, on average, the surface of pyramidal neurons was over 40% covered by IBA1$^+$ cells (Figure 2B, C). Next, we used immunostaining and genetic reporter lines to assess ensheathment of GABAergic interneurons. A subset of somatostatin$^+$ (SST), calbindin$^+$ (CALB), and parvalbumin$^+$ (PV) GABAergic interneurons were contacted by phagocytes in *Toxoplasma*-infected cortex, although this is also the case in mock-infected brains. While all 3 types of interneurons exhibited higher percentage of ensheathment in infected brains than in mock-infected brains (Figure 2D-L), the level of ensheathment remained significantly lower (~15% ensheathed per cell) compared to pyramidal neurons (Figure 2). Thus, it appears that excitatory neurons are preferentially targeted and ensheathed by IBA1$^+$ phagocytes following long-term infection with *Toxoplasma gondii*.

It is possible that certain cell types, such as GABAergic interneurons, are preferentially lost following infection, ensheathment, and synapse loss. This could lead to lower numbers of highly ensheathed GABAergic interneurons in our counts (and to alterations in GAD67 distribution (Brooks et al., 2015)). To test this possibility, we used riboprobes against *Gad1* and *Vglut1* to label inhibitory and excitatory neurons, respectively, by *in situ* hybridization (ISH). In line with our previous assessment of neuronal loss in the infected neocortex (Carrillo et al., 2020), we did not observe significant changes in the number of excitatory or inhibitory cells in layer V at 30 days post infection (Figure S1).

Perisomatic inhibitory synapses are not lost from the somata of PV$^+$ interneurons following infection

We interpret the above results to suggest that pyramidal neurons most likely express or release a factor that attracts phagocytes to ensheath their somata. However, the somata of these neurons are studded with perisomatic synapses, so an alternative possibility is that phagocytes are instead attracted to these synapses following infection. In layer V, the majority of perisomatic synapses on excitatory pyramidal cells are formed by fast-spiking, PV-expressing interneurons. Importantly, however, PV$^+$ cells also provide substantial perisomatic inhibition onto other PV$^+$ cells (Figure 3A-B). Since PV$^+$ interneurons are not dramatically ensheathed by IBA1$^+$ cells following infection (Figure 2J-L), this suggests that perisomatic nerve terminals are not attracting IBA1$^+$ cells to ensheath neuronal somata and phagocytose these nerve terminals following infection. To directly test the latter possibility, we took advantage of our genetic reporter line labeling PV cells to probe whether perisomatic synapse loss also occurred on these inhibitory PV$^+$ interneurons. We observed no significant decrease in the number of GABAergic perisomatic synapses on PV$^+$ interneurons (labeled by immunostaining for glutamic acid decarboxylase 67; GAD67) between mock- and ME49-infected brains (Figure 3C-E). Additionally, we quantified the number of PV$^+$ interneurons in layer V and in the neighboring cortical layers (IV-VI) and found no change in PV$^+$ cell numbers following infection (Figure 3F-G). Together, these results show that PV$^+$ cells are not extensively ensheathed by IBA1$^+$ phagocytes following infection, nor do they lose perisomatic inhibitory inputs form their somata. This suggests that post-synaptic signals arising from excitatory pyramidal cells are likely serving as cues for microglia to ensheath excitatory somata and remove inhibitory inputs.

Differential expression of complement components following *T. gondii* infection

We next sought to identify what some of the cues might be that are necessary for IBA1$^+$ phagocyte ensheathment of excitatory pyramidal neurons and the targeted loss of perisomatic synapse on these neurons. Activation of the classical complement pathway has been shown to control synaptic pruning during developmental refinement as well as in the neurodegenerating brain (Stevens et al., 2007, Cong et al., 2021, Alawieh et al., 2021, Werneburg et al., 2020, Hammond et al., 2020). Moreover, several studies report upregulation of complement components in the *Toxoplasma*-infected brain (Shinjyo et al., 2020, Huant et al., 2018, Xiao et al., 2016). We similarly observed significant increase of classical components *C1qa* and *C3* in infected

cortex by RT-qPCR (*C1qa*: mock = 1 ± 1.3 fold-change; ME49 = 6.5 ± 4.2 ; *C3*: mock = 1 ± .95 ; ME49 = 267 ± 13.5). Based on this, we hypothesized that these complement pathway components might be involved. In the inflamed or injured brain, microglia provide a major source of *C1qa* mRNA, however it remains unknown which cells express *C1qa* and *C3* in the *Toxoplasma*-infected brain. To determine the source of complement components in the *T. gondii*-infected brain, we generated riboprobes against *C1qa* and *C3* and performed ISH. In the infected brain, *C1qa* mRNA expression patterns resembled phagocyte morphology. Immunolabeling for IBA1 following *in situ* hybridization confirmed the presence of *C1qa* mRNA within IBA1$^+$ cells in both mock- and ME49-infected brains. In fact, the majority of IBA1$^+$ cells in both sets of brains expressed *C1qa* although expression appeared higher in the infected brain (Figure 4A-C). Additionally, a population of cells expressing *C1qa* that are not labeled with IBA1 are present (Figure S3A). ISH against *C3* mRNA similarly revealed expression by IBA1$^+$ phagocytes in the infected brain. However, in contrast to *C1qa* mRNA, *C3* mRNA expression was not detectable in the mock-infected brain.

We next sought to examine neuronal subtype expression for *C1qa* and *C3* in mock- and ME49-infected brains. To accomplish this, we performed *in situ* hybridization for *C1qa* and *C3* and genes expressed by all neurons (Synaptotagmin 1; *Syt1*), GABAergic neurons (Glutamic acid decarboxylase 1; *Gad1*) or layer V pyramidal neurons (Nephrenectin; *Npnt*) (Figure S2; Su et al., 2021). While we observed instances of *C1qa* expression by non-IBA1$^+$ cells, we failed to observe any *C1qa* expression by *Syt1*$^+$ neurons (Figure S3). However, we did find several instances of *C3* mRNA expression by both *Syt1*$^+$ and *Npnt*$^+$ neurons in the ME49-infected cortex (Figure 5A, B). Importantly, we did not observe *C3* expression by *Gad1*$^+$ inhibitory cells (Fig 5C).

C3 is required for inhibitory perisomatic synapse loss in a non-classical pathway-dependent manner

The select expression of C3 by some pyramidal neurons, but not interneurons, suggests that the complement pathway may contribute to neuronal ensheathment by IBA1$^+$ phagocytes and perisomatic synapse loss. To test this, we assessed infection-induced IBA1$^+$ ensheathment of neurons and perisomatic synapse loss in mice lacking C3. Following most types of infection or injury, resident microglia quickly expand in the brain, and monocytes from the periphery are recruited into the brain to aid with pathogen and debris clearance (D'Mello et al., 2009). We observe this same process following *T. gondii* infection in WT mice (Fig 6A, B). Interestingly, in

mice that lack C3 globally, we also see an increase in the number of phagocytes following infection, however, the number is slightly but significantly less than in WT mice (Figure 6 A,B). We failed to observe significant differences in the percentage of IBA1^{+} phagocytes that contact neurons in WT and C3$^{-/-}$ infected brains. However, we observed a significant decrease in neuronal ensheathment by IBA1^{+} cells (Figure 6C-F). In fact, loss of C3 led to ensheathment levels that quantitatively and qualitatively resembled what levels we observed on inhibitory interneurons in WT infected mice (compares Figure 2 and 6).

Next, to determine if the reduced ensheathment of neurons in the absence of C3 resulted in less phagocytosis and loss of synapses following infection, we assessed the distribution of CD68 (a marker of phagosomes) and of inhibitory perisomatic synapses (Carrillo et al., 2020). In C3 $^{-/-}$ infected mutants, we saw a decrease in the distribution of CD68 immunoreactivity within layer V (Figure 7A-B). Importantly, we found that not all phagocytes showed a loss or reduction in CD68 expression. Areas of the cortex where phagocytes accumulated (perhaps denoting regions of monocyte infiltration or macrophage response to tachyzoites within the brain parenchyma), for example, still expressed high levels of CD68, suggesting that the increase in phagocytic activity in ensheathing phagocytes (but not all phagocytes) was dependent on C3 activation (Figure 7C). We next assessed the number of GABAergic inhibitory synapses and saw no significant loss of perisomatic synapses in the neocortex of infected C3 $^{-/-}$ mutants (Figure 7D-F). Since the number of perisomatic synapses in C3 $^{-/-}$ mock-infected cortex was unchanged from WT mock-infected cortex, we conclude that these findings demonstrate C3 is required for the phagocytosis of inhibitory perisomatic synapses in *T. gondii* infection.

After establishing that *T. gondii* infection-induced synapse loss is C3-dependent, we sought to determine if this was mediated through activation of the classical pathway. A number of studies have demonstrated that synapse loss in the diseased or inflamed brain involve the classical complement pathway (Hong et al., 2016, Wang et al., 2020, Datta et al., 2020, Carvalho et al., 2019). To test this, we used targeted mouse mutants that lack C1qa, as enzymatic cleavage of this protein activates the classical pathway. We found that removal of C1qa did not prevent *T. gondii*-induced synapse loss (Figure S4A-C). Thus, the classical complement pathway is not involved in the loss of synapses in the *Toxoplasma*-infected brain, similar to what is seen in neurodegeneration (Werneburg et al., 2020).

DISCUSSION

CNS infection with *Toxoplasma gondii* can lead to behavioral alterations, seizures, and increased risk for the development of several neuropsychiatric disorders, some, or all, of which may arise from dysfunction in GABAergic neurotransmission and circuitry. Data from our previous studies demonstrate that infection by type II ME49 *T. gondii* leads to the loss of GABAergic perisomatic synapses and the acquisition of spontaneous seizures (Brooks et al., 2015, Carrillo et al., 2020). Dysfunction or perturbation of these inhibitory perisomatic synapses have been linked to both seizures and neuropsychiatric disorders, such as schizophrenia (Belforte et al., 2010, Gonzalez-Burgos et al., 2011, Gonzalez-Burgos et al., 2010, Gonzalez-Burgos and Lewis, 2012, Hamm et al., 2017, Lewis et a., 2011, Mukherjee et al., 2019, Schwaller et al., 2004, Wohr et al., 2015). In the case of *T. gondii* infection, activated microglia and/or peripheral monocytes that infiltrate the brain following infection, ensheath neuronal somata and phagocytose a significant number of the GABAergic perisomatic synapses on these cells (Carrillo et al., 2020). Here, we discovered that the loss of GABAergic perisomatic synapses in the *T. gondii*-infected cerebral cortex occurs in a cell-type specific manner in that phagocytic cells preferentially ensheath excitatory pyramidal cells, leading to the loss of inhibitory perisomatic synapses on this cell type, but not on others. We further demonstrate that this process is complement-dependent but is not mediated by the classical pathway. Together, these findings highlight a new role for complement in orchestrating phagocyte cell-type specific remodeling in the *T. gondii*-infected brain.

Cell-type specific neuron-glia interactions in parasite-infected brain

Microglia are key regulators of neural circuitry with established roles in immunosurveillance and circuit remodeling in the central nervous system. In the developing brain, microglia eliminate excess neural precursor cells and synapses to maintain homeostasis and refine circuitry (Cunningham et al., 2013, Paolicelli et al., 2011, Li et al., 2012, Schafer et al., 2012). Upon injury or disease to the brain, microglia can temporarily or permanently remodel circuitry by employing both pathogenic and protective mechanisms (Chen et al., 2014, Chen et al., 2012, Kerschensteiner et al., 2003, Hellwig et al., 2013). At the initial stages of the infection, *T. gondii* infection follows a similar pattern of widespread microglial activation and increase in phagocytic cells (here, likely attributed to both microglial proliferation and monocyte recruitment from the periphery), as is commonly seen in other, but not all, types of neuroinflammation or

neurodegeneration (Borges et al., 2003, Feng et al., 2019, Hagan et al., 2020). Moreover, *T. gondii* infection induces microglia to extensively ensheath the somata of neurons, a process first described in the peripheral central nervous system following injury to the facial nerve (Blinzinger and Kreutzberg, 1968, Shibasaki et al., 2007, Chen et al., 2014, Wan et al., 2020). Interestingly, ensheathment of neurons in models of induced epilepsy has been described as a transient process, whereby microglia temporarily displace perisomatic synapses as a response to aberrant GABAergic signaling from presynaptic nerve terminals, thus serving as a protective mechanism to the neuron (Wan et al., 2020). Furthermore, displacement of GABAergic perisomatic synapses results in an increase in synchronized neuronal activity which triggers neuronal expression of anti-apoptotic and neurotrophic molecules as neuroprotection (Chen et al., 2014). In *T. gondii* infection, however, we find that GABAergic perisomatic synapse loss is not a result of temporary displacement, but rather is a process where presynaptic nerve terminals are removed by phagocytosis. If widespread perisomatic synapse loss continues throughout the course of the infection, it may contribute to neuronal death seen in later stages of chronic *T. gondii* infection (Cabral et al., 2016, Mendez et al., 2021).

In the current study, we discovered *T. gondii* infection triggers microglia to specifically ensheath excitatory pyramidal neurons in neocortex and that GABAergic perisomatic synapse loss occurs on these excitatory cells. These findings are in line with other models of epilepsy that show preferential ensheathment of excitatory cortical neurons and subsequent displacement of GABAergic perisomatic synapses from these cells (Wan et al., 2020). Microglia, however, are not the only ones that preferentially interact with excitatory neurons. Recent studies assessing neurons that are injected with *T. gondii* proteins (but do not contain the parasite itself) shows the parasite, like microglia, also has preference for targeting excitatory neurons (Koshy et al., 2012, Mendez et al., 2021). Thus, excitatory cells could be secreting signaling molecules such as neurotrophins and neuropeptides that attract both microglia and parasites to these cells. Alternatively, phagocytes may be attracted to these cells as a response to parasite-modulation of host cell machinery or neuronal stress within *T. gondii*-protein-injected neurons. A third possibility is that instead of being attracted to excitatory cells, microglia are attracted to the inhibitory perisomatic synapses. Microglia express a variety of receptors for neurotransmitters, including GABA receptors, that allow them to sense neuronal activity (Pocock and Kettenmann, 2007, Krabbe et al., 2012, Seifert et al., 2011, Kuhn et al., 2004). In *T. gondii* infection, microglia

upregulate the expression of GABA-A receptors, which aids in their migration, therefore, it is possible that perisomatic synapses could be releasing an increase in GABA that attracts phagocytes to neuronal somata (Bhandage et al., 2019, Bhandage et al., 2020). However, this is unlikely to be the case since perisomatic inhibitory synapses remain on PV inhibitory interneurons following *T. gondii* infection.

Differential expression of complement as a cue for microglia ensheathment of excitatory neurons leading to perisomatic synapse phagocytosis

An important discovery in these studies is that along with microglia and monocyte expression of complement components, *T. gondii* infection also induces the differential expression of C3 by excitatory pyramidal cells. Neuronal expression of complement is not an entirely novel concept. In animal models of Alzheimer's, where synapse loss is an early manifestation of the neurodegenerative process via the complement pathway, neuronal expression of complement C1q has been reported and linked to synapse elimination (Selkoe 2002, Bialas and Stevens, 2013, Hong et al., 2016). Similarly, in chronic cases of multiple sclerosis, microglial clusters (although less extensive than we see in *T. gondii* infection), are associated with neuronal production of C3. In these studies, expression of C3 and microglial clusters only occurred after prolonged disease, and was not observed during acute disease (Michailidoi et al., 2016). In our studies, it is important to note that several instances of neuronal C3 production (by excitatory pyramidal cells only) were observed at 30 days after infection, a timepoint of long-term infection where significant perisomatic inhibitory synapse loss already occurs. This raises important questions as to the timing and duration of complement expression by neurons in the *T. gondii* infected brain. *In vivo* assessment of neuronal C3 expression throughout the course of the infection, and targeted downregulation of C3 in neurons, will be important in establishing neuronal C3 as a driver in *T. gondii*-induced synapse loss. Since phagocytes were also observed to upregulate expression of C3 in *T. gondii* infection, the role of phagocyte C3 in inducing synapse loss should also be carefully examined. This raises the possibility that excitatory pyramidal neuron expression of C3 may also be accompanied by inhibitory neuron upregulation of complement regulators as a protective mechanism against microglia secreted C3 (Zhu et al., 2020, Gonzalez, et al., 2021). Of note, *T. gondii* tachyzoites are able to resist complement-mediated killing by recruiting host-derived complement regulatory proteins (Factor H regulator of the alternative pathway and C4b-binding protein of the classical and lectin pathways) to the parasite's surface and inactivate surface-bound

C3 (Fuhrman and Joiner, 1989, Sikorski et al., 2020). It is possible that intracellular *T. gondii* cysts may employ a similar strategy to avoid complement-mediated phagocytosis or lysis of their neuronal host. Overall, our findings suggest that neurons might be playing a more significant role in initiating circuit remodeling in degeneration or prolonged inflammation and should be examined in other models of neurodegeneration and disease.

Our data suggest that C3 is required for neuronal ensheathment and phagocytosis of perisomatic inhibitory synapses, but is not required for the initial targeting of excitatory pyramidal neurons. This was surprising to us based on the role of C3 in chemoattraction (Chen et al., 2021). This begs the question, what are the chemoattractants that initiate selective microglial targeting of excitatory cells in the *T. gondii*-infected brain? Under pathological conditions, activated microglia will migrate towards the site of injury by detecting different classes of membrane-bound and secreted chemoattractants (Fan et al., 2017, Hu et al., 2014, Mazaheri et al., 2017). One example of these is the inhibitory neurotransmitter, GABA. In the developing cortex, secreted GABA will attract $GABA_B$ receptive microglia, and subsequently induce a transcriptional synapse remodeling program within these immune cells to trigger synapse phagocytosis (Favuzzi et al., 2021). In *T. gondii* infection, GABA-induced chemotaxis of microglia may account for the selective phagocytosis of inhibitory perisomatic synapses, but seems unlikely to explain selectivity for excitatory pyramidal neurons. The identification of such cell-type specific chemoattractants in *T. gondii* infection will require further investigation.

FIGURES

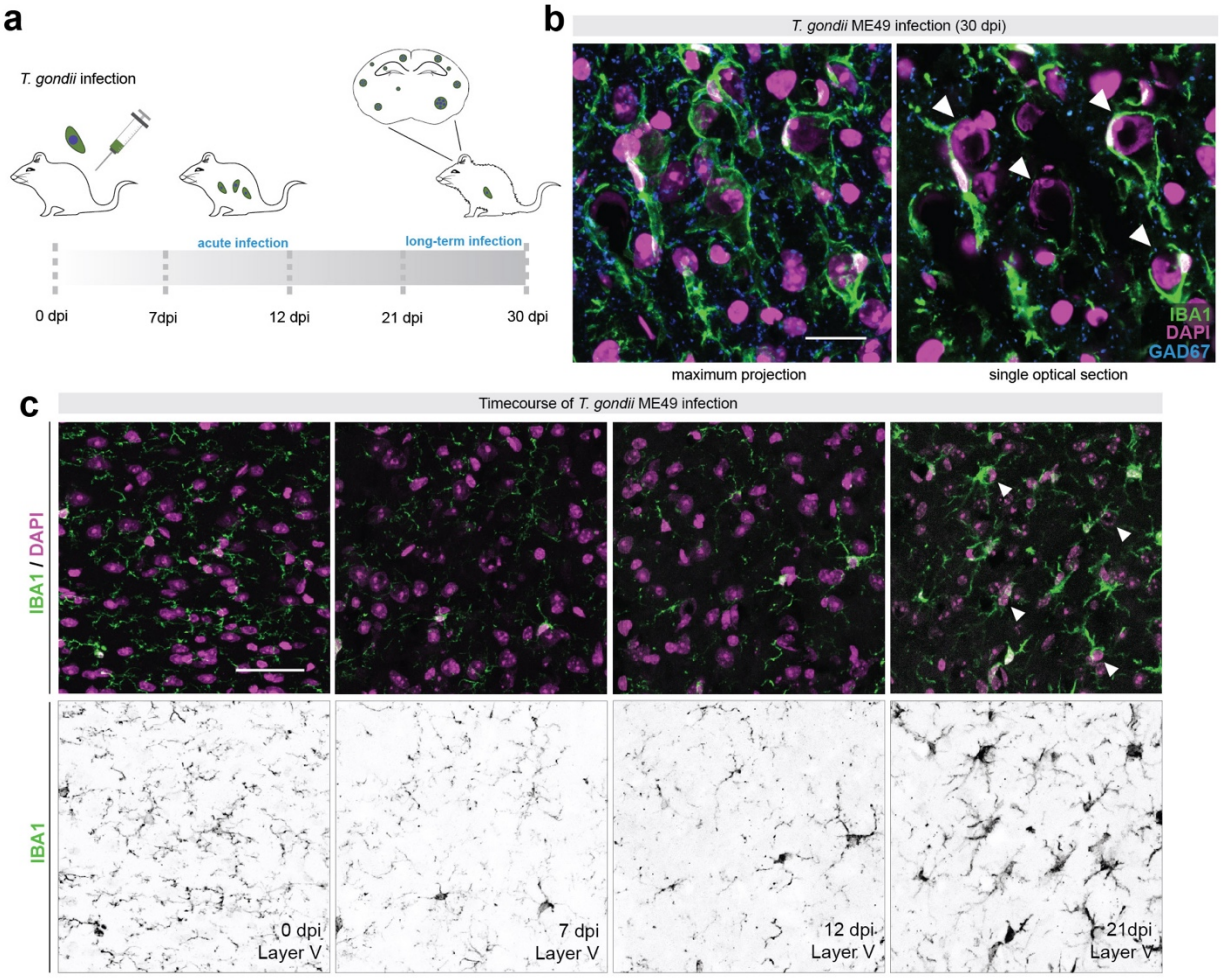

Figure 3.1. Inhibitory synapse loss and microglial targeting of neuronal somata begins weeks after infection

(a) Schematic representation of *T. gondii* ME49 infection following intraperitoneal injection in adult C57BL/6J mice.

(b) Immunostaining of IBA1⁺ phagocytes and GAD67⁺ inhibitory synapses in layer V of neocortex of ME49-infected (30 dpi) mice. Single optical section is shown to highlight cells ensheathed by IBA1⁺ cells (arrows).

(c) IHC for IBA1 and DAPI in layer V of cortex at 0 dpi, 7 dpi, 12 dpi, and 21 dpi infection. Arrows depict ensheathed cells at 21 dpi.

Scale bars in B: 20 µm and in C: 40 µm

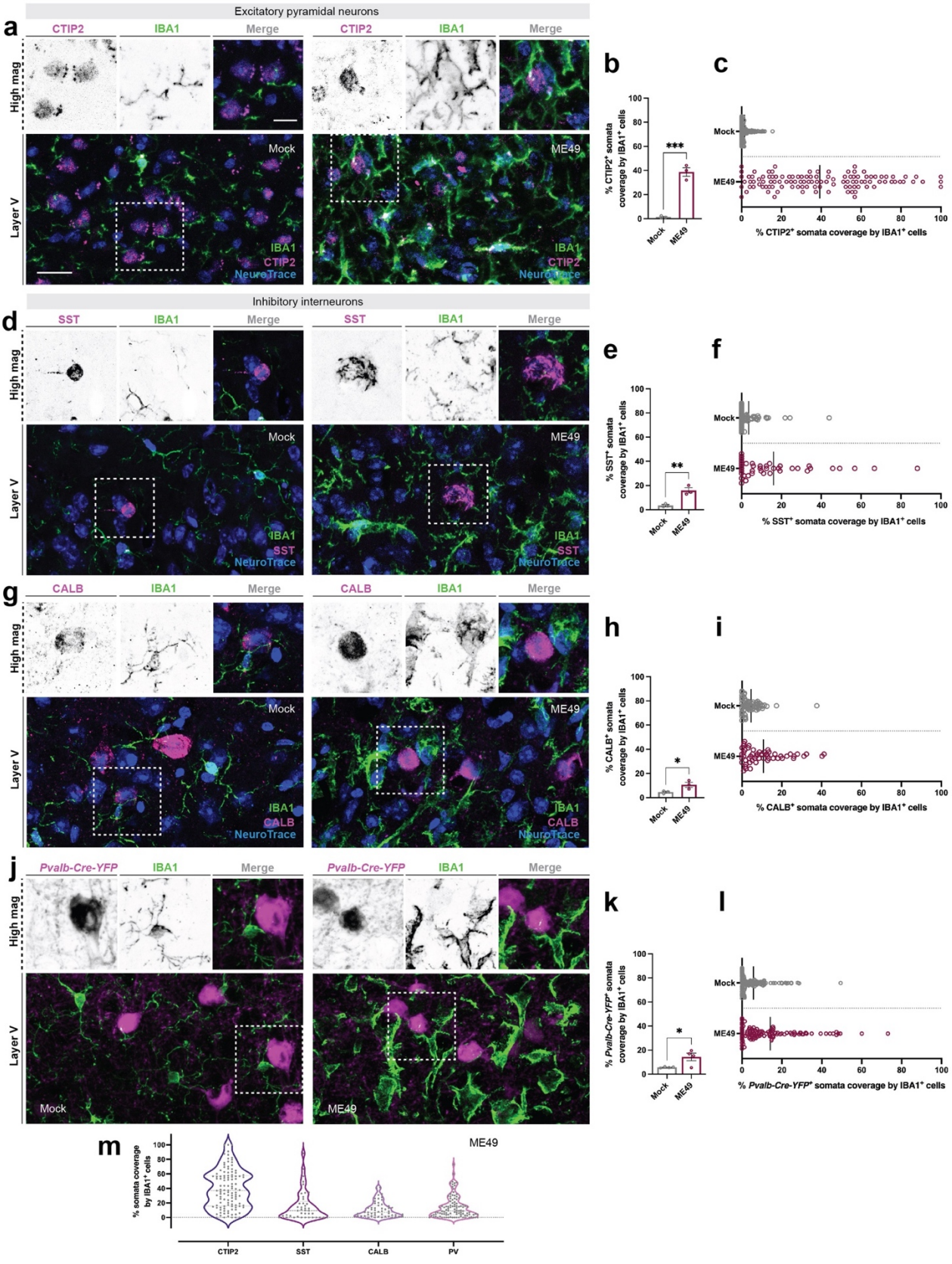
Excitatory pyramidal neurons
a
CTIP2
IBA1
Merge
High mag
Layer V
Mock
ME49
IBA1
CTIP2
NeuroTrace
b
% CTIP2+ somata coverage by IBA1+ cells
***
Mock
ME49
c
% CTIP2+ somata coverage by IBA1+ cells
Inhibitory interneurons
d
SST
e
% SST+ somata coverage by IBA1+ cells
**
f
% SST+ somata coverage by IBA1+ cells
g
CALB
h
% CALB+ somata coverage by IBA1+ cells
*
i
% CALB+ somata coverage by IBA1+ cells
j
Pvalb-Cre-YFP
k
% Pvalb-Cre-YFP+ somata coverage by IBA1+ cells
l
% Pvalb-Cre-YFP+ somata coverage by IBA1+ cells
m
% somata coverage by IBA1+ cells
CTIP2
SST
CALB
PV

Figure 3.2. Excitatory pyramidal neurons are preferentially ensheathed by microglia

(a) IHC for layer V neurons (CTIP2), NeuroTrace and IBA1 in layer V of neocortex at 30 dpi (ME49) compared to mock-infection (Mock).

(b) Quantification of $CTIP2^+$ $NeuroTrace^+$ soma coverage by $IBA1^+$ cells in mock- and ME49-infected neocortex. Each data point represents the average of one biological replicate and bars depict mean ± SEM. Asterisks (***) indicate $P < 0.001$ by Student's *t*-test (n=3 mice per condition).

(c) Distribution plot of individual $CTIP2^+$ $NeuroTrace^+$ somata analyzed and pooled from biological replicates in (B). Bar represents mean percent somata coverage by all $IBA1^+$ cells.

(d) IHC for SST, NeuroTrace, and IBA1 in layer V of neocortex (ME49) compared to mock-infection (Mock).

(e) Quantification of SST^+ $NeuroTrace^+$ soma coverage by $IBA1^+$ cells in mock- and ME49-infected neocortex. Each data point represents the average of one biological replicate and bars depict mean ± SEM. Asterisks (**) indicate $P < 0.01$ by Student's *t*-test (n=3 mice per condition).

(f) Distribution plot of individual SST^+ $NeuroTrace^+$ somata analyzed and pooled from biological replicates in (E). Bar represents mean percent somata coverage by all $IBA1^+$ cells.

(g) IHC for CALB, NeuroTrace, and IBA1 in layer V of neocortex (ME49) compared to mock-infection (Mock).

(h) Quantification of $CALB^+$ $NeuroTrace^+$ soma coverage by $IBA1^+$ cells in mock- and ME49-infected neocortex. Each data point represents the average of one biological replicate and bars depict mean ± SEM. Asterisks (*) indicate $P < 0.05$ by Student's *t*-test (n=3 mice per condition).

(i) Distribution plot of individual $CALB^+$ $NeuroTrace^+$ somata analyzed and pooled from biological replicates in (H). Bar represents mean percent somata coverage by all $IBA1^+$ cells.

(j) IHC for IBA1 in *Pvalb-Cre-YFP* shows minimal ensheathment of PV^+ inhibitory interneurons in layer V of neocortex at 30 dpi (ME49) compared to mock-infection (Mock).

(k) Quantification of *Pvalb-Cre-YFP*$^+$ soma coverage by $IBA1^+$ cells in mock- and ME49-infected neocortex. Each data point represents the average of one biological replicate and bars depict mean ± SEM. Asterisks (*) indicate $P < 0.05$ by Student's *t*-test (n=4 mice per condition).

(l) Distribution plot of individual *Pvalb-Cre-YFP*$^+$ somata analyzed and pooled from biological replicates in (K). Bar represents mean percent somata coverage by all $IBA1^+$ cells.

(m) Violin-plot showing the distribution of ensheathment by $IBA1^+$ phagocytes (as a % of somal coverage) for 4 types of neocortical neurons (Data from c, f, i, l).

Scale bars in A, D, G, J: 20 µm and in A, D, G, J high magnification images: 10 µm

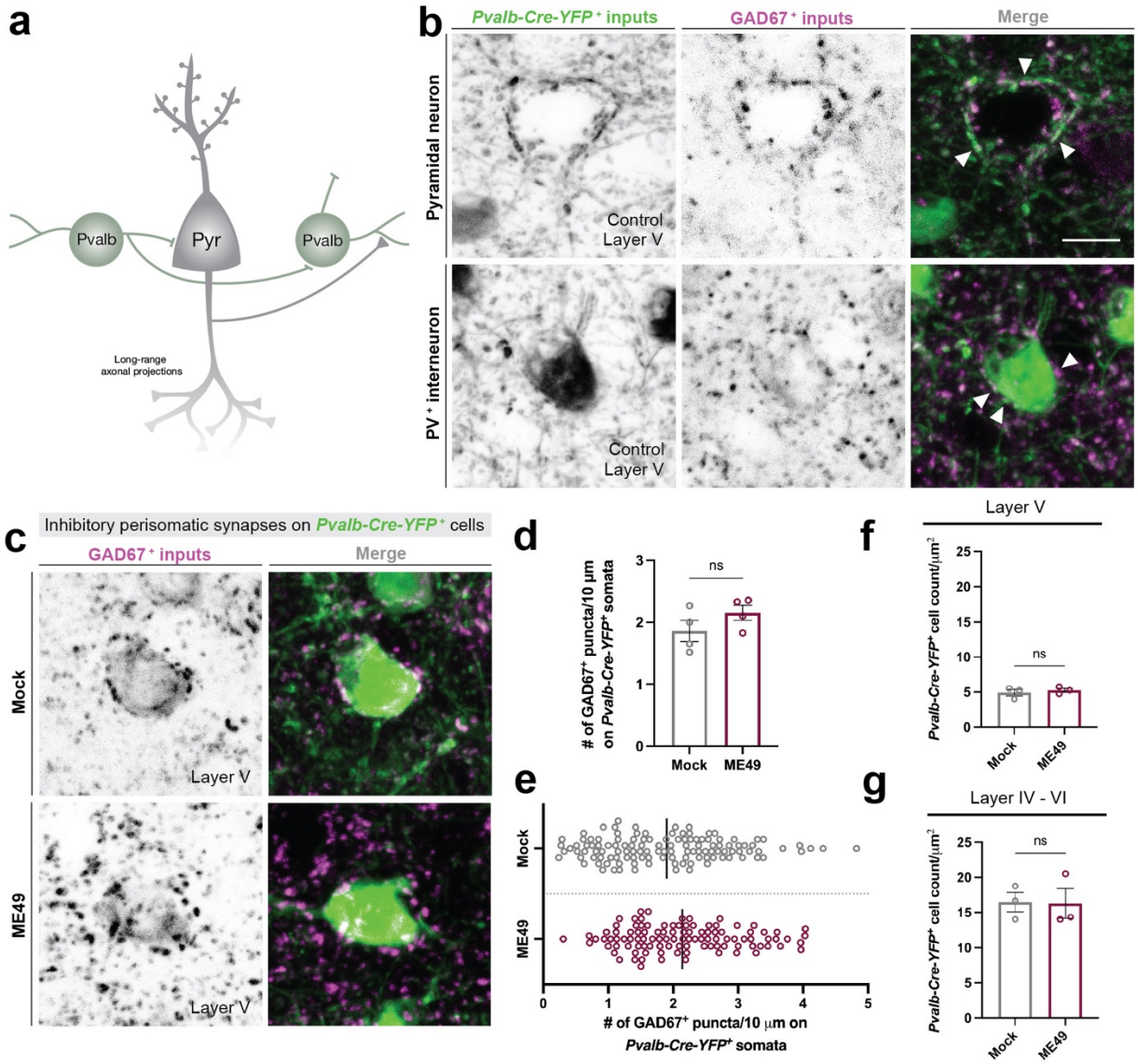

Figure 3.3. Inhibitory perisomatic synapses are not lost on PV⁺ interneurons

(a) Schematic illustrating PV inhibitory interneuron and excitatory pyramidal connectivity in cortical layer V.

(b) IHC for GAD67 in *Pvalb-Cre-YFP* mice shows *YFP*+ inputs on both pyramidal neurons (based on morphology) and on PV interneurons. White arrowheads indicate inhibitory perisomatic synapses.

(c) IHC for GAD67 in mock- and ME49-infected (30 dpi) *Pvalb-Cre-YFP* mice.

(d) Quantification of GAD67$^+$ synapses on *YFP*$^+$ somata in mock- and ME49-infected Pvalb-Cre-YFP mice. Each data point represents one biological replicate and bars depict mean ± SEM. No significant difference (ns) indicates $P > 0.05$ by Student's *t*-test (n=4 mice per condition).

(e) Distribution plot of *YFP*$^+$ somata analyzed and pooled from biological replicates in (D). Bar represents mean number of GAD67$^+$ synapses per *Pvalb-Cre-YFP*$^+$ somata in mock- or ME49-infected neocortex.

(f) Quantification of *Pvalb-Cre-YFP*$^+$ cell count in layer V of mock- and ME49-infected cortex. Each data point represents one biological replicate and bars depict mean ± SEM. No significant difference (ns) indicates $P > 0.05$ by Student's *t*-test (n=3 mice per condition).

(g) Quantification of *Pvalb-Cre-YFP*$^+$ cell count in layer IV - VI of mock- and ME49-infected cortex. Each data point represents one biological replicate and bars depict mean ± SEM. No significant difference (ns) indicates $P > 0.05$ by Student's *t*-test (n=3 mice per condition).

Scale bars in B and C: 10 µm

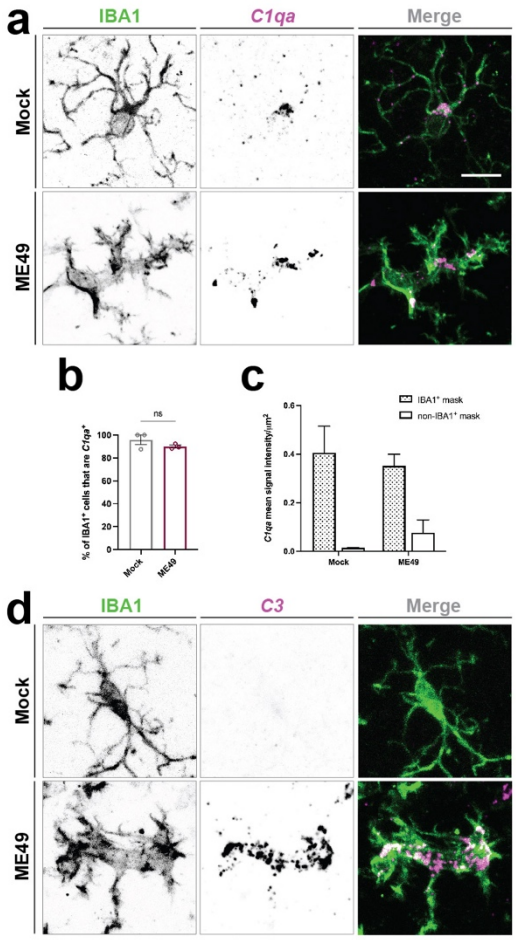

Figure 3.4. Complement mRNA is upregulated by immune cells in the infected brain

(a) ISH for *C1qa* and IHC for IBA1 in layer V of neocortex of mock- and ME49-infected mice.

(b) Quantification of IBA1$^+$ cells expressing *C1qa* mRNA in mock- and ME49-infected cortex. Each data point represents one biological replicate and bars depict mean ± SEM. No significant difference (ns) indicates $P > 0.05$ by Student's *t*-test (n=3 mice per condition).

(c) Quantification of *C1qa* mRNA signal intensity within IBA1$^+$ cells and outside of IBA1$^+$ cells. Each data point represents one biological replicate and bars depict ± SEM.

(d) ISH for *C3* and IHC for IBA1 in layer V of neocortex of mock- and ME49-infected mice.

Scale bars in A and D: 10 µm

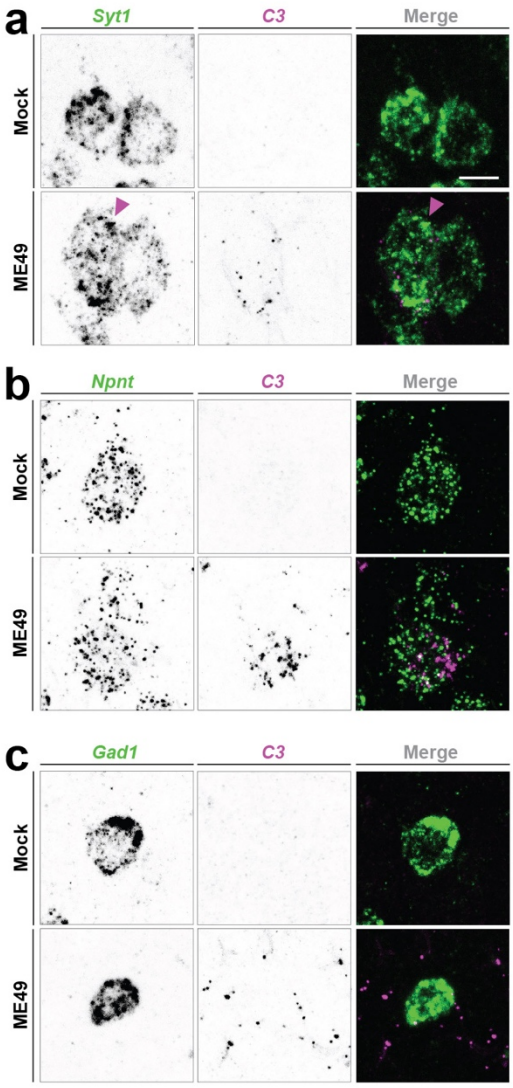

Figure 3.5. C3 is expressed by some excitatory pyramidal neurons following *T. gondii* infection

(a) ISH for *Syt1* and *C3* in mock- and ME49-infected mice. Neuronal expression of *C3* mRNA was detected in some neurons in ME49-infection.

(b) ISH for *Npnt* and *C3* in mock- and ME49-infected mice. *C3* mRNA was detected in some *Npnt*-expressing pyramidal neurons in layer V of neocortex.

(c) ISH for *Gad1* and *C3* show no expression of *C3* mRNA by inhibitory neurons in ME49-infection.

Scale bars in A, B and C: 10 µm

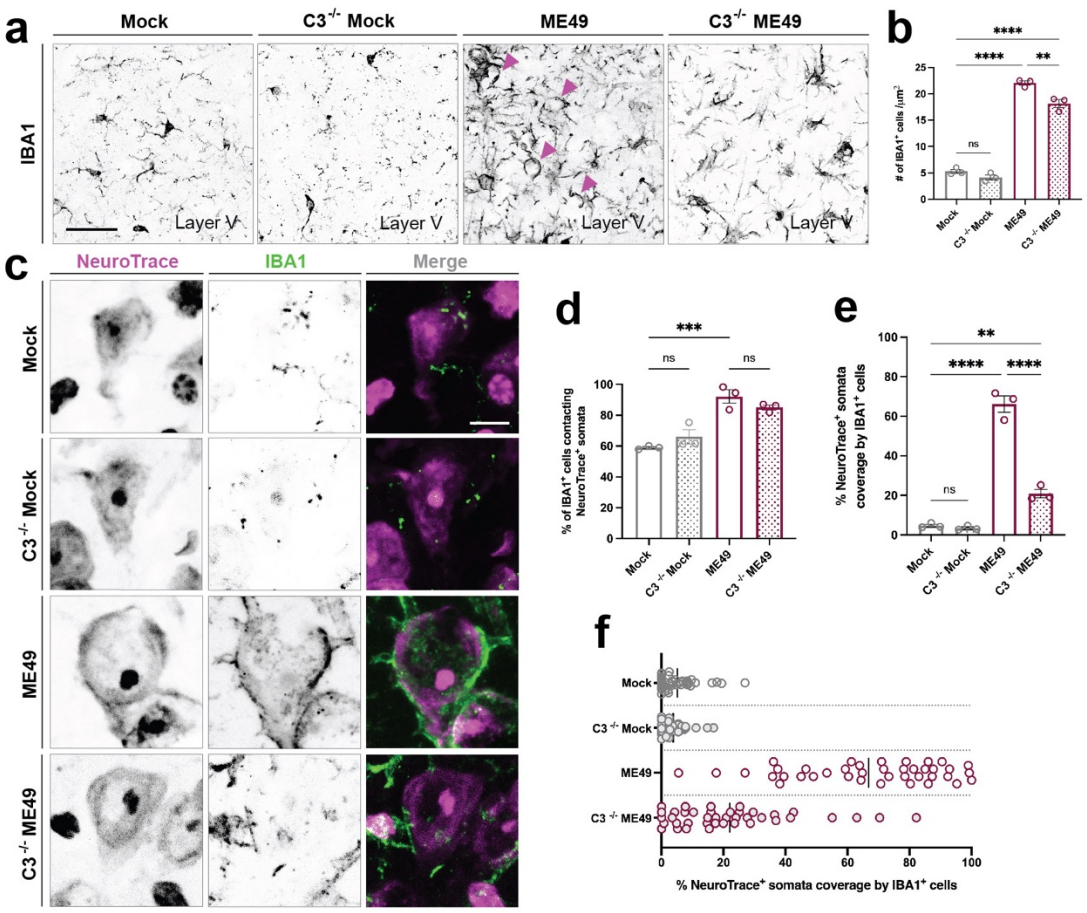

Figure 3.6. Reduced neuronal ensheathment by IBA1⁺ phagocytes in the absence of complement C3

(a) IHC for IBA1 in layer V of neocortex of C3$^{-/-}$ and littermate control mice mock-infected and infected with ME49 parasites.

(b) Quantification of the number of IBA1⁺ cells in layer V of neocortex in C3$^{+/+}$ mock-, C3$^{-/-}$ mock, C3$^{+/+}$ ME49-, and C3$^{-/-}$ ME49-infected brains. Asterisks (**) indicate $P < 0.01$, (****) indicate $P < 0.0001$ by one-way ANOVA with Sidak's multiple comparison correction (n=3 mice per condition).

(c) IHC for IBA1 and NeuroTrace in layer V of neocortex of C3$^{+/+}$ mock-, C3$^{-/-}$ mock, C3$^{+/+}$ ME49- and C3$^{-/-}$ ME49-infected mice.

(d) Quantification of the percentage of IBA1^{+} cells contacting NeuroTrace^{+} somata in cortical layer V in in C3$^{+/+}$ mock-, C3$^{-/-}$ mock, C3$^{+/+}$ ME49-. and C3$^{-/-}$ ME49-infected brains. Each data point represents one biological replicate and bars depict mean ± SEM. Asterisks (***) indicate $P < 0.001$ and (ns) indicates $P > 0.05$ by one-way ANOVA with Sidak's multiple comparison correction (n=3 mice per condition).

(e) Quantification of the percentage of NeuroTrace^{+} soma coverage by IBA1^{+} cells in cortical layer V in C3$^{+/+}$ mock-, C3$^{-/-}$ mock, C3$^{+/+}$ ME49-. and C3$^{-/-}$ ME49-infected brains. Each data point represents one biological replicate and bars depict mean ± SEM. Asterisks (**) indicate $P < 0.01$, (****) indicate $P < 0.0001$ by one-way ANOVA with Sidak's multiple comparison correction (n=3 mice per condition).

(f) Distribution plot of individual NeuroTrace^{+} somata analyzed and pooled from biological replicates in (D). Bar represents mean percent somata coverage by IBA1^{+} cells.

Scale bars in A: 40 µm and in C: 10 µm

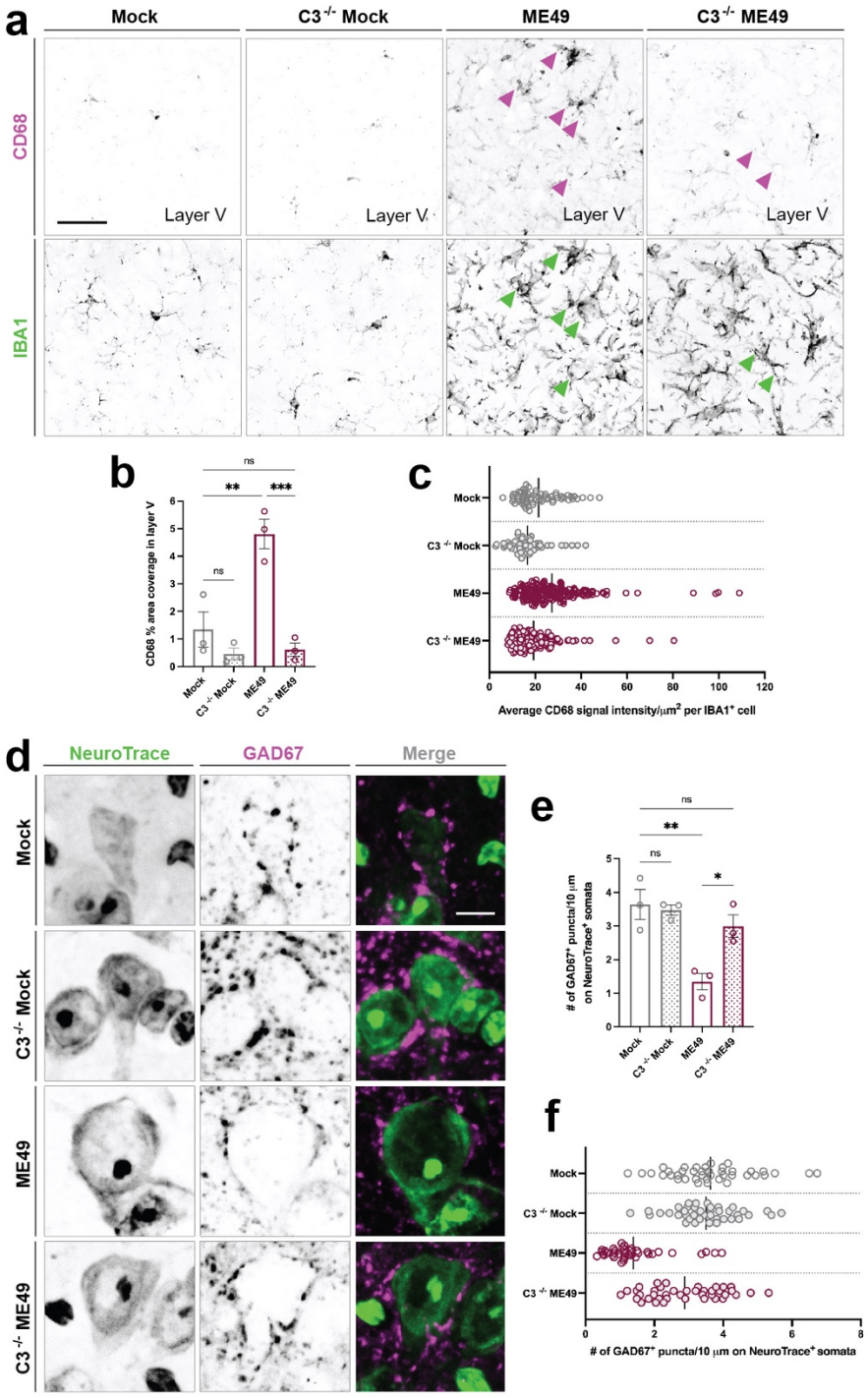

Figure 3.7. C3 is required for phagocytosis of inhibitory perisomatic synapses

(a) IHC for IBA1 and CD68 show decreased expression by IBA1^{+} cells in layer V of neocortex in C3$^{-/-}$ mice following *T. gondii* infection compared to C3$^{+/+}$ ME49-infected mice. Magenta arrowheads point to CD68 expression with IBA1^{+} cells. Green arrowheads point to IBA1^{+} cells

(b) Quantification of CD68 immunoreactivity percentage area coverage in cortical layer V in C3$^{+/+}$ mock-, C3$^{-/-}$ mock, C3$^{+/+}$ ME49-. and C3$^{-/-}$ ME49-infected brains. Each data point represents one biological replicate and bars depict mean ± SEM. Asterisks (**) indicate $P < 0.01$, (***) indicate $P < 0.001$, and (ns) $P > 0.05$ by one-way ANOVA with Sidak's multiple comparison correction (n=3 mice per condition).

(c) Distribution plot of individual IBA1^{+} cells analyzed and pooled from images in (B). Bar represents mean number of CD68 signal intensity within IBA1^{+} cells.

(d) IHC for GAD67 and NeuroTrace in layer V of neocortex of C3$^{+/+}$ mock-, C3$^{-/-}$ mock, C3$^{+/+}$ ME49-. and C3$^{-/-}$ ME49-infected mice.

(e) Quantification of GAD67^{+} synapses on NeuroTrace^{+} somata in cortical layer V in C3$^{+/+}$ mock-, C3$^{-/-}$ mock, C3$^{+/+}$ ME49-. and C3$^{-/-}$ ME49-infected brains. Each data point represents one biological replicate and bars depict mean ± SEM. Asterisks (*) indicate $P < 0.05$, (**) indicate $P < 0.01$, and (ns) $P > 0.05$ by one-way ANOVA with Sidak's multiple comparison correction (n=3 mice per condition).

(f) Distribution plot of individual NeuroTrace^{+} cells analyzed and pooled from images in (E). Bar represents mean number of GAD67^{+} synapses on NeuroTrace^{+} somata.

Scale bars in A: 40 µm and in D: 10 µm

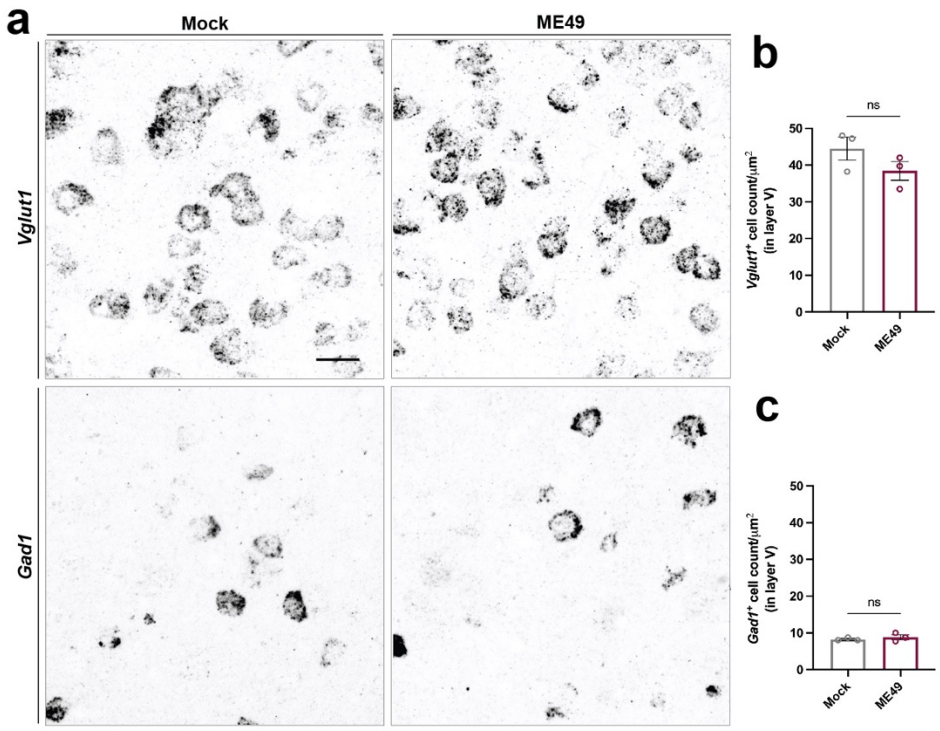

Figure 3.8 [Supplemental Figure] No excitatory or inhibitory neuron loss occurs at time of perisomatic synapse loss

(a) ISH for *Vglut1* and *Gad1* in layer V of mock- and ME49-infected neocortex reveals no significant decrease in the number of excitatory or inhibitory cells following infection.

(b) Quantification of *Vglut1*$^+$ cell count in layer V of mock- and ME49-infected cortex. Each data point represents one biological replicate and bars depict mean ± SEM. No significant difference (ns) indicates $P > 0.05$ by Student's *t*-test (n=3 mice per condition).

(c) Quantification of *Gad1*$^+$ cell count in layer V of mock- and ME49-infected cortex. Each data point represents one biological replicate and bars depict mean ± SEM. No significant difference (ns) indicates $P > 0.05$ by Student's *t*-test (n=3 mice per condition).

Scale bar in A: 20 µm

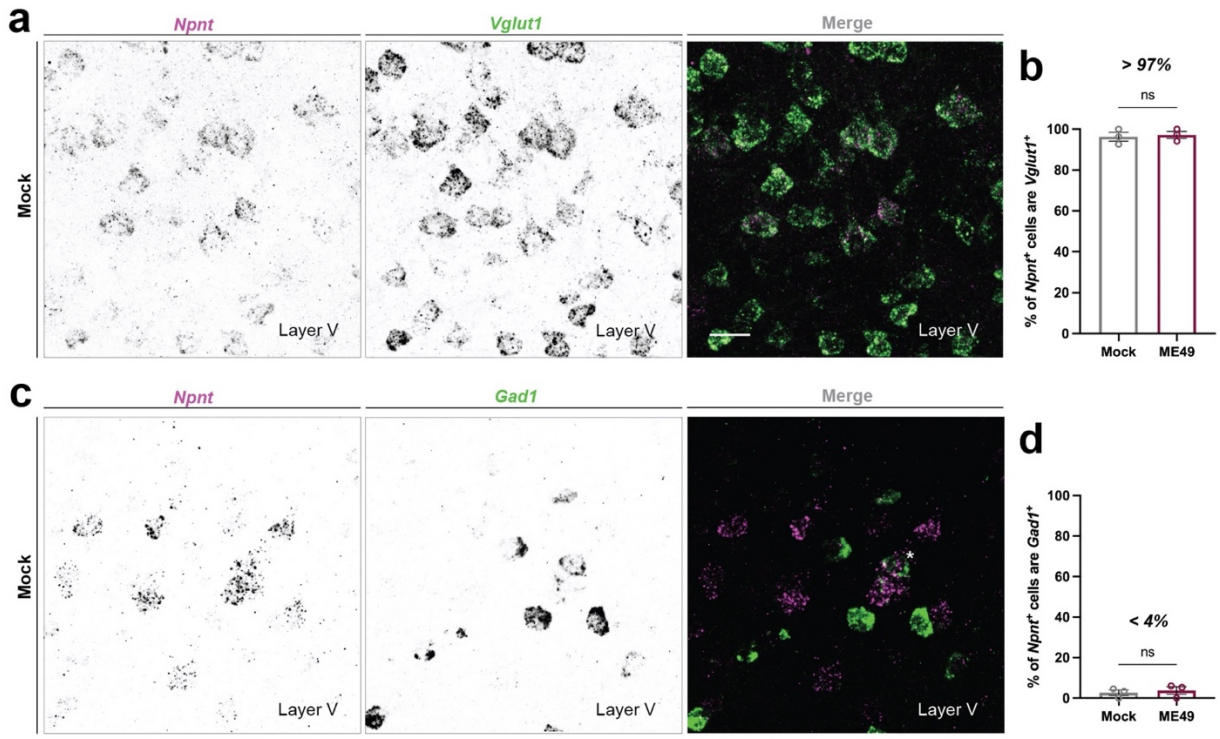

Figure 3.9 [Supplemental Figure] NPNT is a faithful marker of excitatory cells in cortical layer V

(a) ISH for *Vglut1* and *Npnt* in wildtype mouse neocortex shows most excitatory cells in layer V express *Npnt* mRNA.

(b) Quantification of the percentage of *Npnt*+ cells in layer V that express *Vglut1* mRNA in mock- and ME49-infected cortical layer V. Each data point represents one biological replicate and bars depict ± SEM. No significant difference (ns) indicates $P > 0.05$ by Student's *t*-test (n=3 mice per condition).

(c) ISH for *Gad1* and *Npnt* shows very few inhibitory cells in layer V express *Npnt* mRNA. Each data point represents one biological replicate and bars depict ± SEM. No significant difference (ns) indicates $P > 0.05$ by Student's *t*-test (n=3 mice per condition).

(d) Quantification of the percentage of *Npnt*+ cells in layer V that express *Gad1* mRNA in mock- and ME49-infected cortical layer V.

Scale bars in A and C: 20 µm

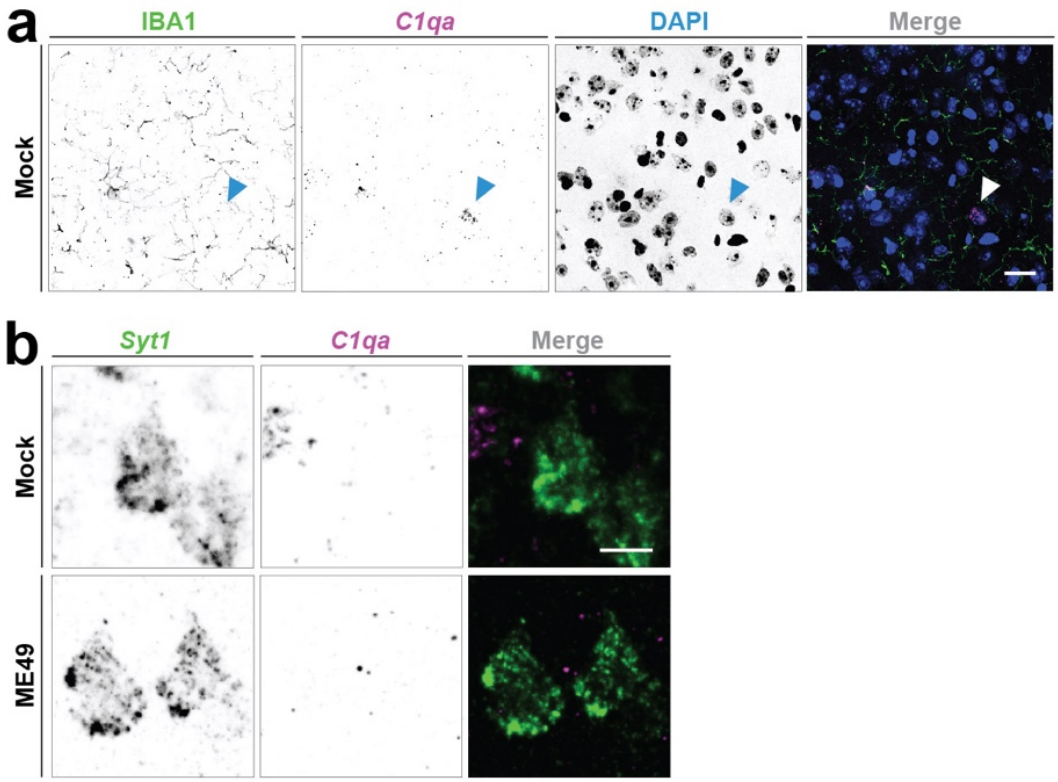

Figure 3.10 [Supplemental Figure] *C1qa* is not expressed by neurons

(a) ISH for *C1qa* and IHC for IBA1 and DAPI revealed *C1qa* mRNA expression by non-IBA1$^+$ cell in mock-infected layer V.

(b) ISH for *Syt1* and *C1qa* showed no neuronal expression of *C1qa* mRNA in both mock- and ME49-infected layer V.

Scale bars in A: 20 µm and in B: 10 µm

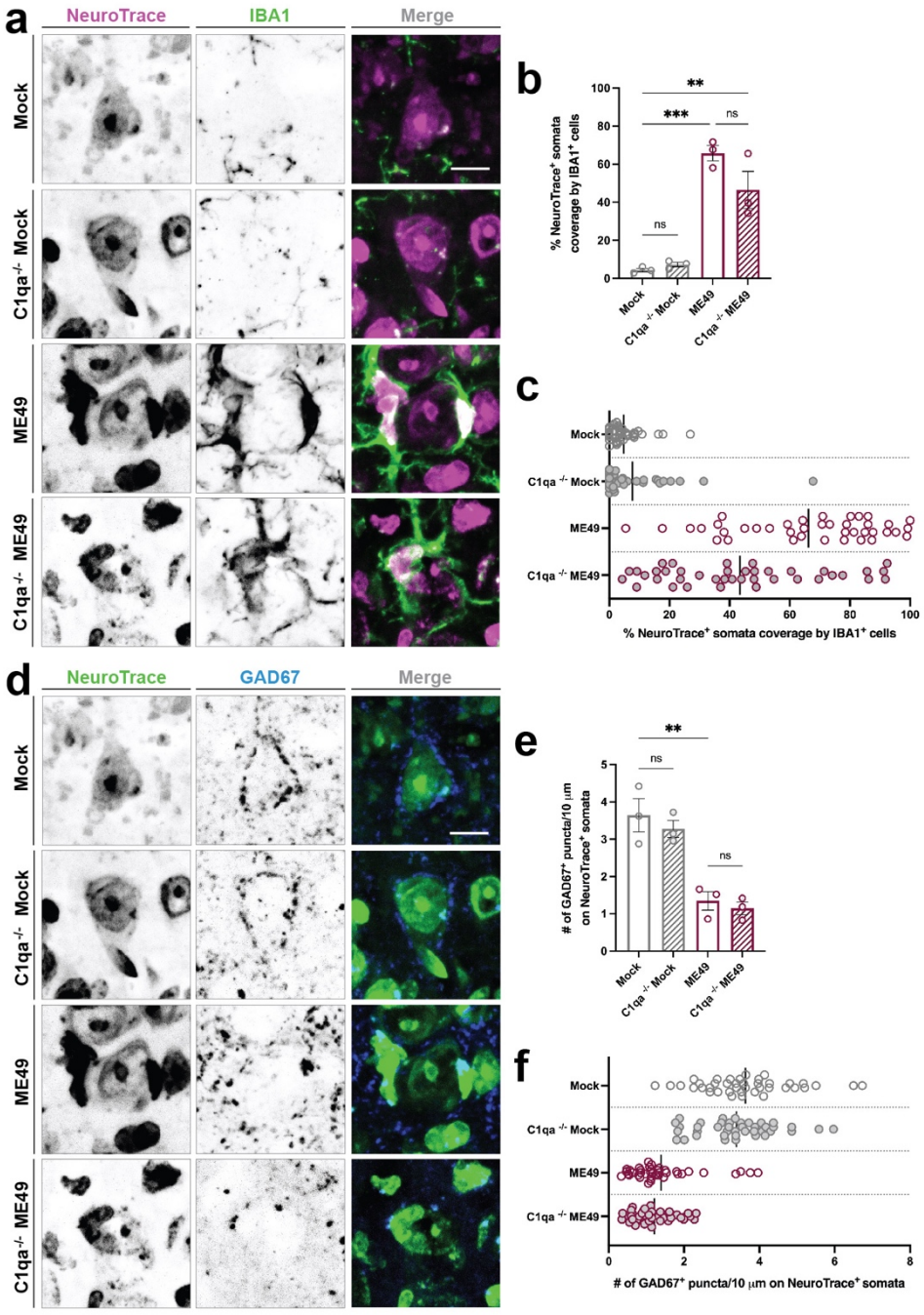

Figure 3.11 [Supplemental Figure] Inhibitory perisomatic synapse loss following *T. gondii* infection is not mediated by the classical pathway of complement activation

(a) IHC for IBA1 shows no reduction in microglial ensheathment of NeuroTrace+ neurons in C1qa-/- ME49-infected cortex.

(b) Quantification of percentage of NeuroTrace^{+} soma coverage by IBA1^{+} cells in cortical layer V in C1qa$^{+/+}$ mock-, C1qa$^{-/-}$ mock, C1qa$^{+/+}$ ME49-. and C1qa$^{-/-}$ ME49-infected brains. Each data point represents one biological replicate and bars depict mean ± SEM. Asterisks (**) indicate $P < 0.01$, (***) indicate $P < 0.001$, and (ns) $P > 0.05$ by one-way ANOVA with Sidak's multiple comparison correction (n=3 mice per condition).

(c) Distribution plot of individual NeuroTrace^{+} somata analyzed and pooled from biological replicates in (B). Bar represents mean percent somata coverage by IBA1^{+} cells.

(d) IHC for GAD67 and NeuroTrace shows no reduction in inhibitory perisomatic synapse loss in C1qa$^{-/-}$ ME49 infection compared to C1qa$^{+/+}$ ME49-infection.

(e) Quantification of GAD67^{+} synapses on NeuroTrace^{+} somata in cortical layer V in C1qa$^{+/+}$ mock-, C1qa$^{-/-}$ mock, C1qa$^{+/+}$ ME49-. and C1qa$^{-/-}$ ME49-infected brains. Each data point represents one biological replicate and bars depict mean ± SEM. Asterisks (**) indicate $P < 0.01$ and (ns) $P > 0.05$ by one-way ANOVA with Sidak's multiple comparison correction (n=3 mice per condition).

(f) Distribution plot of individual NeuroTrace^{+} cells analyzed and pooled from images in (E). Bar represents mean number of GAD67^{+} synapses on NeuroTrace^{+} somata.

Scale bars in A and D: 10 µm

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Chapter 4:

DISCUSSION

Over the years, infection with the parasite, *Toxoplasma gondii*, has garnered much attention due to its ability to alter behaviors in infected animals and its established risk factor for developing neuropsychiatric disorders in humans. Despite its significance in the central nervous system, however, much remains to be elucidated regarding how long-term *Toxoplasma* infection alters distinct neural circuits, and how such changes may give rise to altered behaviors, neurological disorders, and neuropsychiatric disorders associated with *Toxoplasma* infection. My dissertation research sought to uncover some of these changes to neural circuitry, and the neuroimmune mechanisms underlying them, in the *Toxoplasma*-infected brain.

Key Contributions to the Field

1. *Toxoplasma gondii* infection induces the loss of perisomatic inhibitory synapses

An important initial discovery I made was demonstrating the loss of perisomatic inhibitory synapses from neurons in multiple telencephalic regions (hippocampus and neocortex) following *Toxoplasma* infection with the type II ME49 strain (Chapter 2). This followed previous work in the Fox lab that showed long-term *Toxoplasma* infection in the brain (by this low virulent strain, but not another low virulent strain) led to onset of seizures and altered distribution of GAD67, a key enzyme that catalyzes the principle inhibitory neurotransmitter, GABA (Brooks et

al., 2015). As GAD67 is localized in the nerve terminals, these changes could signify a loss of perisomatic synapses, or alternatively, a redistribution of GAD67 throughout the neuropil, signaling a re-employment of programs seen during critical periods of development (Pinal and Tobin, 1998, Begym and Sng, 2017). By use of ultrastructural analysis, I resolved that perisomatic GABAergic synapses were, in fact, lost following brain infection, and importantly, that microglia served a critical role in permanently eliminating these types of inhibitory inputs in multiple regions of the telencephalon by ensheathing neuronal somata and subsequently phagocytosing a significant number of perisomatic inhibitory synapses. This contrasts with other types of insults to the brain that induce seizures, where ensheathment of neuronal somata only temporarily displaces perisomatic inhibitory synapses in a process termed, 'synaptic stripping' (Blinzinger and Kreutzberg, 1968, Shibasaki et al., 2007, Chen et al., 2014, Wan et al., 2020). In these cases, neuroinflammation and activation of microglia was experimentally induced by either low dose administration of lipopolysaccharide for 5 consecutive days or prolonged hypothermia (~15 minutes) to induce febrile seizures (Chen et al., 2014, Wan et al., 2020). In our models of long-term *T. gondii* infection, we see significant neuronal ensheathment 21 days after intraperitoneal injection with a low count of *T. gondii* cysts, with cysts already present in the brains of newly infected mice as early as 14 days post infection (Chapter 3). No significant microglial activation and ensheathment was observed at 12 days post infection, and therefore, it is unclear how soon after tachyzoite infiltration and intracellular cyst formation ensheathment and perisomatic inhibitory synapse loss occurs. An additional shorter-interval time course assessment of cyst presence, ensheathment, and perisomatic synapse loss will be needed to determine if intracellular cyst formation precedes neuronal ensheathment, and if so, how long after it occurs. It remains unclear as to whether the perisomatic inhibitory synapse loss (and neuronal ensheathment) observed in *T. gondii* infection is due to tachyzoite presence in the brain, or neuronal infection of *T. gondii* cysts, therefore determining the timing of cyst formation in relation to neuronal ensheathment might provide some insight into this outstanding question. In regard to other models of induced epilepsy, it is also important to note that microglia were observed to slowly retract (and no longer ensheath neurons), and revert back to surveillant states after seizing administration of LPS or induction of hypothermia (Chen et al., 2014, Wan et al., 2020). In *T. gondii*-infected brains, microglia appear to remain in an activated state for weeks following infection, with neuronal ensheathment still visible at 6 months post infection, raising the possibility that perisomatic inhibitory synapses are phagocytosed (and not simply temporarily

displaced) due to the prolonged neuroinflammation and microglial activation in the *T. gondii* infected brain. It is also necessary to highlight that despite showing these synapses are in fact phagocytosed at a relatively early timepoint of chronic infection (30 days post infection), it is unclear whether there is regrowth of nerve terminals and re-established or newly established perisomatic synapses on these neurons at later stages. Although some studies do suggest there is some neuronal loss in later stages of the infection (Cekanaviciute et al., 2014, Mendez et al., 2021)), I have not observed such loss even at 6 months post infection, raising the possibility that microglial ensheathment may be spatiotemporally dynamic. Perhaps one single neuron is not permanently ensheathed throughout the course of the infection, but rather ensheathed for prolonged periods of time (enough to induce phagocytosis of perisomatic synapses) and then they retract to target and ensheath a different excitatory cell, permitting the cell to re-establish sufficient neural connectivity to ensure their survival.

2. Specificity in neuron-glia interactions in the *Toxoplasma* infected brain

A second important finding was the discovery that in the infected brain, microglia do not indiscriminately ensheath all neuron subtypes, but rather selectively ensheath excitatory pyramidal cells. Previous assessment of neuronal ensheathment showed increased microglial contact with both inhibitory and excitatory neurons (Chapter 1), originally leading me to believe that infection-induced activation of microglia was causing uncontrolled and imprecise remodeling of circuits. To determine if this was the case, I assessed perisomatic inhibitory synapses and neuronal ensheathment in *Palv-Cre-YFP* mice (as PV interneurons also receive the same GABAergic perisomatic input as excitatory cells in layer V of neocortex), and to my surprise, I saw that *T. gondii* infection did not cause loss of perisomatic inhibitory synapses on PV interneurons. Moreover, although microglia clearly contacted PV interneurons, the level of contact starkly contrasted the extensive ensheathment of neighboring non-PV^+ neurons. This led me to become quite interested in how microglia might interact distinctly with excitatory cells and the different inhibitory interneurons subtypes found in layer V of neocortex, following infection. Comprehensive assessment of neuronal ensheathment between various cortical neuron subtypes showed that microglia preferentially target and ensheath excitatory cells (Chapter 3). This is in line with some recent studies of induced-epilepsy that show microglia preferentially removing (albeit, temporarily as mentioned above), perisomatic inhibitory synapses from glutamatergic

excitatory cells following microglial activation (Wan et al., 2020). The removal of perisomatic inhibitory inputs onto excitatory neurons has been shown to increase synchronized neuronal activity, which subsequently triggers neuronal expression of anti-apoptotic and neurotrophic molecules, suggesting this process may be a neuroprotective response to neuroinflammation (Chen et al., 2014). Whether this process is protective or pathogenic in *T. gondii* infection, however, is still unclear. Preliminary unpublished studies assessing seizure susceptibility in infected mice lacking complement component C3 (which I show the absence of prevents infection-induced loss of perisomatic inhibitory synapses in Chapter 3), showed that mock-infected mice lacking C3 already had increased seizure susceptibility. This is not surprising as complement proteins are important for developmental pruning of excess excitatory synapses during critical periods of refinement (Stevens et al., 2007, Schafer et al., 2012), and thus, eliminating C3 too early can lead to enhanced synaptic connectivity and hyperexcitability in the brain (Chu et al., 2010). A more targeted approach to deleting C3, such as tamoxifen-induced deletion of C3 immediately following *T. gondii* infection, will be needed to determine if the absence of C3 prevents onset of seizures and increased seizure susceptibility in infection. Importantly, assessment of neuronal integrity (i.e. density, pro- and anti-apoptotic molecules, electrophysiological measures) and behaviors normally seen altered in mice infected with *T. gondii* (fear response, exploratory behaviors, and anxiety behaviors) should be assessed following long-term infection in tamoxifen conditional deletion of C3 and littermate controls to better demonstrate whether this process is pathogenic or protective.

3. Complement C3 is necessary for neuronal ensheathment and loss of perisomatic inhibitory synapses

The third significant contribution of my dissertation work is the identification of complement C3 as one of the neuroimmune mechanisms involved in neural circuit remodeling in the parasite infected brain (Chapter 3). The complement system is an evolutionarily conserved first line of defense that is quickly activated within the host, both in the peripheral and central nervous systems, in response to invading pathogens (Sikorski et al., 2021). Within the brain, two complement components, C1qa (the initiating protein of the classical pathway) and C3 (the driver effector protein for all pathways), have been shown to have roles in opsonization and recruitment of phagocytes to lyse or phagocyte pathogens, cells, and synapses (Stevens et al., 2007, Schafer et

al., 2012, Wang et al., 2020, Sirkorski et al., 2020, Sikorski et al., 2021). In the *Toxoplasma gondii*-infected brain, we see significant upregulation of these complement components within the neocortex, similarly to what other studies have observed (Chapter 3, Shinjyo et al., 2021). Although upregulation of complement had been observed by others following *T. gondii* infection, the source of complement proteins in the infected brain was not known. In these sets of studies, I showed that within the neocortex, *C1qa* is predominantly expressed by phagocytes in both the mock-infected (by microglia) and *T. gondii* infected (by microglia and monocytes) brains. In the adult brain, neuronal expression of *C1qa* has been reported, although it represents only a minimal source of *C1qa* as compared to glial expression (Fonseca et al., 2017). In mock-infected brains, I observed *C1qa* expression by non-IBA1$^+$ DAPI$^+$ cells, however, I failed to identify any *C1qa* expression by *Syt1*$^+$ neurons in both mock- and ME49-infected brain. It's possible that since *C1qa* and *C3* mRNAs are so highly upregulated in ME49-infected mice that detection by *in situ* hybridization (and likely subsequent confocal imaging parameters) might not be capturing lower levels of mRNA expression. Thus, employment of single-cell RNA sequencing or RNAScope are likely better strategies to assess such drastic transcriptomic changes we see in *T. gondii* infection. Importantly, although phagocytes also express C3 in the infected-brain, I demonstrate that T. gondii induces differential expression of C3 by excitatory pyramidal cells (but not GABAergic interneurons). This is significant as C3 expression by excitatory pyramidal cells, specifically, has been observed in neurodegeneration, where synapse loss also occurs in a complement-dependent manner, such as in Alzheimer's and Multiple Sclerosis (Shen et al., 1997, Michailidoi et al., 2015, Werneburg et al., 2020). I next sought to determine if complement components were involved in the loss of perisomatic inhibitory synapses following *T. gondii* infection. I assessed perisomatic inhibitory synapses and neuronal ensheathment in mock-infected and ME49-infected mice lacking C1q or C3, by immunohistochemistry as I had done previously (Carrillo et al., 2020). To my surprise, I found that C3 but not C1q was required for the *T. gondii*-infection induced loss of these perisomatic synapses, demonstrating that although complement is involved, it is not through the classical pathway. It's likely that the alternative pathway is involved in perisomatic synapse loss as recent studies have demonstrated this to be the case in MS models of neurodegeneration (Werneburg et al., 2020). Importantly, these analyses were performed on cells that morphologically resembled excitatory pyramidal neurons in the cortex (and not by CTIP2$^+$ immunolabeling for excitatory cells) as it was important for me to perform ensheathment and synapse analyses on the same cells (and I was limited by the number of fluorophores I could use).

Subsequent perisomatic inhibitory synapse analyses on excitatory-labeled neurons will be needed.

Toxoplasma gondii: a model for neuroinflammation?

Are the behavioral and cognitive changes seen in *Toxoplasma gondii* infection due to chronic widespread inflammation within the brain? Or did *Toxoplasma gondii* evolve to manipulate certain regions of the brain? These are questions that have perplexed the field for decades, with evidence to support both sides. I will elaborate on these possibilities in the context of my dissertation findings.

Infection within the brain induces such widespread microglial activation (here, characterized by morphological changes such as reduced ramification), that at the start of my dissertation, I believed the entire brain (and perhaps many regions of the CNS) were equally affected, and equally 'damaged.' Yet, a closer look at region-specific microglial morphologies, and importantly, their interactions with neurons seemed to imply otherwise. In the infected brain, microglia interact with neurons in distinct ways. I've highlighted an interaction termed 'ensheathment' whereby microglia extensively wrap around neuronal somata (including the axon hillock). Importantly, the dosage of *T. gondii* infection does not appear to impact if ensheathment occurs or the level of ensheathment that takes place, on an individual cell level but also throughout the brain (that is IP injection of 1 cyst versus 50 cysts did not make a difference).

There are regions of the brain where ensheathment is most prevalent, such as the stratum pyramidalis of hippocampus and layer V of neocortex (and not surprisingly, these were also areas where alterations of GABAergic perisomatic synapses were most apparent, and areas that are dense with inhibitory perisomatic synapses on excitatory pyramidal cells). Yet, some microglia display unique activated morphologies in other regions of the hippocampus, such as in the stratum ratiatum and stratum lacunsom-moleculare. How microglia might interact with neurons (or distinct neuronal processes such as distal axons) and whether they alter synapses in these regions remains to be carefully investigated. Preliminary assessment of neuron-glia interactions in these regions, and other layers of the cerebral cortex, however, reveal much less ensheathment

and cell-to-cell contact. Moreover, my studies show that microglia preferentially target and ensheath excitatory pyramidal neurons, already showing a level of specificity.

Neuronal ensheathment is also not specific to the telencephalon. Although perhaps not as prevalent, ensheathment of neuronal somata is observed in the lateral geniculate nucleus, extensively in the spinal cord on motor neurons, and in some rare cases on retinal ganglion cells in the retina. Interestingly though, in these regions, distinct types of synapses seem to be affected, if at all. In the spinal cord, preliminary unpublished studies show dramatic changes in cholinergic inputs onto motor neurons, but alterations in GABAergic and glutamatergic inputs are not clear and some rather appear unaffected. Moreover, *T. gondii* infection leads to significant loss of motor neurons, whereas we do not see loss of neurons in the cortex at this timepoint of chronic infection. In the retina, despite the significance of ocular toxoplasmosis in human patients, and some instances of ensheathment (or at minimum increased neuron-glia contact), retinal circuits appear to be completely untouched, with no observable changes in cell types or synapses. In the lateral geniculate nucleus, preliminary assessments of neuronal ensheathment show that, similar to the cortex, PV^+ inhibitory interneurons are not extensively ensheathed, again indicating specificity in *T. gondii*-induced microglial targeting of neurons. It is unclear whether perisomatic synapse loss occurs here, although prior studies of GAD67 localization suggest it might. Could differences in *T. gondii*'s tropism for the retina, brain, and spinal cord account for differences in microglia activation and circuit changes? It's possible that *T. gondii*'s tropism for the retina might be the highest during congenital infection, when the host immune response is still maturing (Holladay and Smialowicz 2000). However, this does not seem to be the case for the spinal cord, where *T. gondii* cysts are prevalent (Möhle et al., 2012), highlighting how tropism for distinct regions can lead to distinct neural circuit changes, despite widespread neuroinflammation.

As with everything in biology, this is likely not a case of one or the other. It seems more likely that strain-specific modulation of neuroinflammatory pathways (Glausen et al., 2021), combined with region-specific neuroinflammatory responses (Jung et al., 2022) (whether from uninfected host cells or as a result of *T. gondii* hijacking of host cell machinery remains to be determined) play a role in such specificity in neural circuit changes.

Employment of strategies for pathogen survival: a shift to mutualism?

Effectively all animals, including humans, are home to symbiotic microorganisms (i.e. bacteria, viruses and parasites). Three categories of symbiotic relationships exist: mutualism, where both host and microorganism benefit; commensalism, where the microorganisms' benefit has no impact on the host; and parasitism, where the microorganisms' benefit is harmful to the host. Yet, these categories are not binary, nor fixed. Instead, they represent positions on a spectrum, whereby a symbiotic organisms' relationship with their host can evolutionarily shift (Drew et al., 2021). And growing evidence suggests these shifts can occur rather quickly due to the increased rate by which microorganisms are introduced to new contexts and their fluid ability to adapt to these changing environments (Shu et al., 2018, Kümmerli et al., 2009). *What causes microorganisms to shift on the mutualism-to-parasitism spectrum?* It is thought that the relative costs and benefits to each of the organisms, both symbiont and host, play a crucial role, and that these may be further influenced by environmental interactions, such as transmission routes and ecological contexts (Ewald 1987, Bronstein, 1994, Thrall et al., 2007, Chamberlain et al., 2014).

Toxoplasma gondii, like all other symbiotic microorganisms, was at one point a free-living organism, meaning that it was not associated with any host (Sachs et al., 2011). At some point, it formed a host association (or rather many), and rather than maintaining a facultative association with the hosts, it evolved in such a way that its survival is now dependent on its hosts. This was likely evolutionarily advantageous given the wide range of hosts it can infect and exploit resources from (virtually all warm-blooded animals). Where does *Toxoplasma gondii* stand on this spectrum of symbiotic relationships? *Toxoplasma gondii* is a parasite, but it is possible it could be shifting to a more mutualistic relationship with its hosts.

It is in the parasite's best interest, especially as it is completely dependent on its hosts' intracellular resources, for the host it invades to stay alive, healthy, and mobile. One way in which it appears to be accomplishing this is by providing host immune protection against other pathogens. For example, animals chronically infected with *Toxoplasma gondii* demonstrate enhanced protective immune response against other infections, such as the influenza virus (O'Brien et al., 2011).

Could a central nervous system infection with *Toxoplasma gondii* be another example of a shift towards a mutualistic relationship? At first thought, the answer would likely be no. Behavioral changes seen in some animals infected with *Toxoplasma gondii* (such as decreased fear response, and perhaps attraction to predators) is too costly for the infected host (Berdoy et al., 2000, Vyas et al., 2007, Ihara et al., 2016). Yet, not all hosts (like us humans) may suffer the same consequences. Some studies suggest *Toxoplasma gondii* might confer benefits to human hosts. For example, one study found a negative association with *Toxoplasma gondii* infection and the development of Multiple Sclerosis (MS). Moreover, MS patients who were found to be chronically infected with *Toxoplasma gondii* showed lower disability scores and fewer relapses (Koskdereliouglu et al., 2017). On the contrary, studies supporting a protective role against neurodegenerative diseases are outnumbered by others suggesting *Toxoplasma gondii* increases the risk for dementia and Alzheimer's Disease (among many other neurodegenerative and neurological disorders) (Torres et al., 2018, Nayeri et al., 2021, Kusbeci et al., 2011, Yang et al., 2021, Rubio et al., 2012).

At the cellular and synaptic level, *Toxoplasma gondii* infection of the brain appears to be mostly pathogenic. As I show in Chapter 2, mice chronically infected with *Toxoplasma gondii* lose perisomatic inhibitory synapses in several regions of the telencephalon, including the hippocampus and neocortex. Of importance, these synapses originate from a subset of GABAergic interneurons that are essential for homeostatic regulation of excitatory neurotransmission and are highly vulnerable to stressors, the fast-spiking parvalbumin interneuron (PV) (Ruden et al., 2021). PV interneurons (and their synaptic inputs) have been implicated in many neuropsychiatric disease states, from Alzheimer's, to autism spectrum disorders, to schizophrenia (Konradi et al., 2011, Lewis et al., 2012, Marin 2012, Gonzalez-Burgos et al., 2015, Mahar et al., 2016, Takahashi et al., 2012, Wohr et al., 2015). In a similar manner, the mechanism by which these synapses are lost in the *Toxoplasma gondii*-infected brain (complement C3) seems to signal pathogenicity, as many studies demonstrating C3-mediated synapse loss show neurodegeneration (Shi et al., 2017, Hong et al., 2016, Wu et al., 2019, Fonseca et al., 2004, Michailidoi et al., 2015, Werneburg et al., 2020). However, it is important to note that *Toxoplasma gondii* has also evolved mechanisms to intracellularly regulate the host immune response in order to increase its survival, and as an obligate intracellular parasite, has direct consequences for the host. At the stage of the tachyzoite, in modulates GABAergic signaling in infected-immune cells

to increase its dissemination throughout its host and reach host tissues, including the brain, more quickly and thereby evade immune clearance by the host (Fuks et al., 2012, Bhandage et al., 2019, Bhandage et al., 2020). During this stage, it also employs multiple strategies to both inactivate and regulate the innate complement immune response and avoid serum killing early on. These include recruiting host complement regulator proteins to the parasite's surface, expressing complement regulator protein orthologs, and expressing parasite-encoded proteins, all with the ultimate goal of inactivating host complement proteins that are attempting to eliminate the parasite from the host (Shao et al., 2019, Sikorski et al., 2021).

There is much to be elucidated about *Toxoplasma gondii's* intracellular regulation of neurons, and even more so, regulation of the immune system from within neurons. It is unclear whether *Toxoplasma gondii* forms cysts preferentially in one neuronal subtype over another, but some studies do provide evidence for increased interaction with excitatory pyramidal cells (and that these cells are infected with *Toxoplasma gondii* proteins; called *Toxoplasma*-injected neurons (TINs) (Mendez et al., 2021). This is congruent with findings I described in Chapter 3, where I show microglia preferentially target and ensheath excitatory pyramidal cells. Moreover, I demonstrate that *Toxoplasma gondii*-infection can induce the neuronal expression of C3 by excitatory pyramidal cells (but not GABAergic cells). *I wonder, could Toxoplasma infection (either by intracellular cyst formation or TINs) be modulating the host neuroimmune response from within neurons? And if so, what benefit could it have for the host?* In other animal models of induced epilepsy, microglia similarly ensheath and remove inhibitory perisomatic synapses from excitatory neuronal somata as what is purported to be a protective response. Such changes in GABAergic circuitry result in the neuron's expression of anti-apoptotic and neurotrophic molecules, suggesting neuroprotection against neuroinflammation (Chen et al., 2014), although it is possible that this is a neuron's final attempt at survival. Either way, neuronal survival is key to the survival of *Toxoplasma gondii*, especially considering it has preference for invading neurons in the CNS (Cabral et al, 2016).

Some characteristics of *Toxoplasma gondii* suggest that perhaps it does not have a need to shift to a more mutualistic relationship. The mode by which microorganisms are transmitted from one host to another (i.e. vertical or horizontal) seems to predict whether an organism might evolve to be more parasitic or more mutualistic (Ewald et al., 1987). In the case of microorganisms who

are transmitted horizontally, and that can form associations with many unrelated hosts (such as *Toxoplasma gondii* and its ability to infect various species), the cost of high virulence, and thus, more harm to the host seems to dampen (Anderson et al., 1982, Ewald et al., 1983, Bull 1994, Drew et al., 2021). Although vertical transmission of *Toxoplasma gondii* does happen, congenital infections account for only a small part of infection rates as this primarily occurs when females are infected during pregnancy or if immunosuppression during pregnancy reactivates a chronic infection with *Toxoplasma gondii* (and very rarely happens in chronically infected immunocompetent pregnant females) resulting in a relatively narrow timeframe in which vertical transmission can take place (McAuley, 2014, Kodjikian et al., 2004). Horizontal transmission of *Toxoplasma gondii* (via carnivorism and environmental contamination) is much more prevalent, and, coupled with its extensive host repertoire, might not be as dependent on the survival of one species of host. So, perhaps its teeter-tottering between parasitism and mutualism. Or, perhaps this is why various strains of *Toxoplasma gondii* have evolved in the last century since its discovery.

Not all *Toxoplasma gondii* strains are created equal. *Toxoplasma gondii* strains vary in virulence but also in geographical prevalence, with type I strains (the most virulent) predominating in South America and type II strains (of lower virulence) found in the United States and Europe (Howe and Sibley, 1995, Sibley et al., 2009, Khan et al., 2011). Of noteworthy importance, several studies suggest infection with type I *Toxoplasma gondii* may account for the development of more severe psychiatric disorders (Xiao et al., 2009, Groer et al., 2011) as opposed to infection with type II *Toxoplasma gondii* which may be more prevent in immunocompromised patients where reactivation of the infection commonly results in epilepsy (Ajzenberg et al., 2009, Khan et al., 2005, Ferreira et al., 2008, Xiao et al., 2015). Strain specific differences in parasitic virulence factors also account for varying susceptibility to seizures among different *Toxoplasma gondii* infections in mice (Brooks et al., 2015, Glausen et al., 2021). Thus, perhaps its relationship with each host species is evolving distinctly along the symbiotic spectrum, and likely in a strain-specific manner. The good news is that phylogenetic studies point to a trajectory favoring mutualism, as most symbiotic organisms that are now classified as 'mutualistic' were once parasitic in nature (Sachs et al., 2011, Sachs et al., 2014, Clayton et al., 2012, Melnyk et al., 2019).

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