THE ROLE OF COMPLEMENT C3 IN THE HIPPOCAMPAL PATHOLOGY OF STATUS EPILEPTICUS
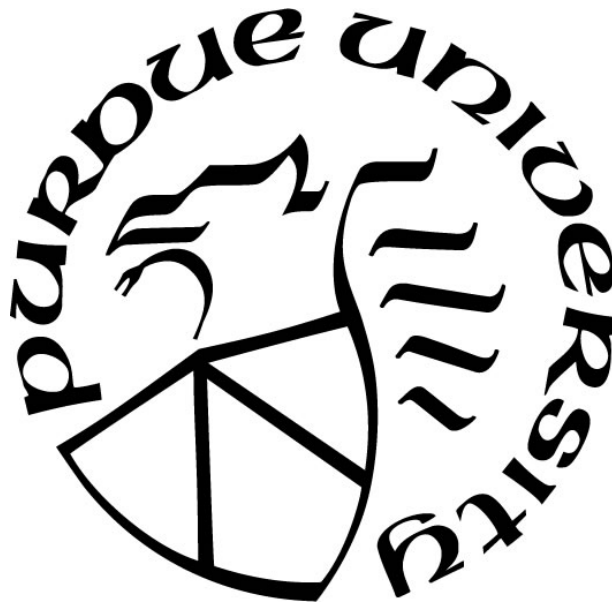
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ABSTRACT

Author: Schartz, Nicole Denise. PhD
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Title: The Role of Complement C3 in the Hippocampal Pathology of Status Epilepticus
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Epilepsy is comorbid with cognitive and psychiatric dysfunctions. This pathophysiology, associated with hippocampal synaptodendritic structural and functional changes, is exacerbated by prolonged seizures (status epilepticus; SE). We found a correlation between hippocampal dendritic loss and microgliosis after SE, along with hyperactivation of the classical complement pathway (C1q-C3). These paralleled increased seizure frequency and memory deficits in a rat model of SE and acquired epilepsy. C1q leads to C3 cleavage into biologically active fragments C3a and C3b. Evidence suggests that C1q and C3b contribute to synaptic stripping by microglia in the developing brain and neurodegenerative disorders. Thus, we hypothesized that SE-induced C3 activation may alter hippocampal synaptic protein levels thereby promoting memory deficits.

To test the hypothesis, different groups of wild type (WT) or C3 deficient (C3KO) mice were injected with pilocarpine (350mg/kg) to induce SE or saline (controls): WT-C, WT-SE, C3KO-C, and C3KOSE. At two weeks after SE, mice were subjected to novel object recognition (NOR) to evaluate recognition memory, and Barnes maze (BM) to measure hippocampal-dependent spatial learning and memory. Following behavioral testing, mice were sacrificed and hippocampi collected at either 2 or 5 weeks after SE to measure changes in C3 protein levels and levels of synaptic proteins including PSD95, Vglut1, and Vgat. As a method of verifying our findings, we used a second model of

pilocarpine-induced SE in male Sprague Dawley rats. Starting at 7 days after SE, rats were treated with cobra venom factor (CVF; 100ng/g, i.p.) or vehicle (veh) every third day. On days 14-15 rats were subjected to open field and NOR to measure anxiety and recognition memory. On day 16, rats were sacrificed and hippocampi collected for western blotting.

WT and C3KO mice were able to reach stage 4.5-6 seizures after pilocarpine injections. In NOR trial 1, exploration time for both objects was similar in all groups ($p > .05$). In trial 2, WT-C and C3KO-C mice spent more time exploring the novel object than the familiar one ($p < .05$) while WT-SE mice explored both objects equally ($p > .05$). Interestingly, C3KO-SE mice spent more time with the novel object similar to controls ($p > .05$), suggesting that the deficit in object recognition memory induced by SE was attenuated in C3KO mice. Similarly, veh- and CVF-treated control rats spent more time exploring the novel object during trial 2 ($p < .05$). The veh-treated SE rats did not show significant preference for the novel object versus familiar ($p > .05$), whereas the CVF-treated SE rats explored the novel object significantly more than the familiar ($p < .05$). These findings support that C3 inhibition after SE prevents recognition memory deficits. Furthermore, there was a reduction in synaptic proteins PSD95 and Vgat in the SE-veh group compared to the C-veh group. This difference was not observed in the C-CVF and SE-CVF groups, suggesting that blocking C3 after SE is neuroprotective against hippocampal synaptic loss.

Taken together, these findings are the first to show an association between C3 activation and hippocampal and cognitive deficits in two rodent models of SE and acquired TLE. We found that depletion of C3 is sufficient to attenuate SE-induced

deficits in NOR-evaluated recognition memory and changes in the levels of an inhibitory synaptic protein. In conclusion, our data suggest that SE-induced complement C3 activation contributes to hippocampal synaptic remodeling and impairments in recognition memory, and that the complement C3 may be a potential therapeutic target for the memory comorbidities associated with SE. Future studies will determine the effect of C3 inhibition on spontaneous recurrent seizures, and whether C3-guided and microglial-dependent phagocytosis is an underlying mechanism for the SE-induced epileptogenic synaptic remodeling.

INTRODUCTION

Epilepsy

Epilepsy is a neurological disorder that affects over 70 million people world-wide (Thijs, Surges, O'Brien, & Sander, 2019). Temporal lobe epilepsy (TLE) is a common form of acquired focal epilepsy, and affects approximately 143,000–191,000 in the United States (Asadi-Pooya, Stewart, Abrams, & Sharan, 2017). Although epilepsy is mainly considered a disorder of seizures caused by an imbalance of excitation and inhibition in the brain (H.E. Scharfman, 2007), this pathology is merely the tip of the iceberg. There are a myriad of underlying mechanisms at play that may differ based on the cause or type of epilepsy, including genetic, metabolic, immune, or unidentified (idiopathic) mechanisms (Thijs et al., 2019). There are also underlying mechanisms that alter the brain to promote hyperexcitability that are not fully understood. Among these changes are aberrant dendritic morphology, cell death, synaptic loss, and neurogenesis (Casanova, Nishimura, Owens, & Swann, 2012; Swann, Al-Noori, Jiang, & Lee, 2000; Wong & Guo, 2013). These occur in parallel to neuroinflammation, microgliosis, astrogliosis, or immune complement activation (Brewster et al., 2013; Devinsky, Vezzani, Najjar, De Lanerolle, & Rogawski, 2013; Ravizza et al., 2005; Schartz et al., 2016; A. Vezzani, French, Bartfai, & Baram, 2011; Annamaria Vezzani, Friedman, & Dingledine, 2013; A. Vezzani & Granata, 2005). Thus, it is likely that neuro-immune interactions may play a role in the development of epilepsy (epileptogenesis). Seizures themselves are sufficient to illicit an inflammatory response, which can then alter the neuronal environment and promote further seizures (i.e. "seizures beget seizures") (Frigerio et al., 2018).

Status epilepticus (SE) is a medical emergency consisting of continuous seizure activity (Lawson & Yeager, 2016). SE was originally defined as a seizure that continues for "a sufficient length of time" or many seizures in succession without regaining consciousness in between episodes (Angeles, 1981; Betjemann & Lowenstein, 2015). Given that this definition is very ambiguous, a new definition of a seizure lasting a minimum of 60 minutes was adopted, and then refined to 30 minutes, which is the duration of seizure activity required to produce significant neuronal injury in humans (Betjemann & Lowenstein, 2015; Brophy et al., 2012; Lawson & Yeager, 2016). More recently, the clinical criteria for diagnosing SE has been decreased to 5 minutes due to the low likelihood that a seizure lasting longer than 5 minutes will self-terminate (Pichler & Hocker, 2017). In the United States, 18.3-41 per 100,000 individuals will develop SE (Lawson & Yeager, 2016), and it can have a mortality rate of 9-22% (Betjemann & Lowenstein, 2015; Pichler & Hocker, 2017). In adults, the most common causes of SE are traumatic brain injury, stroke, alcohol withdrawal, or brain tumors (Pichler & Hocker, 2017). Patients with SE have a higher risk of developing epilepsy, with 66.7% of those patients having acquired epilepsy at a follow-up appointment according to a 2015 study (Santamarina et al., 2015). The risk of a subsequent seizure after a single episode of SE is 3.3 times greater than the risk of another seizure following a short self-terminating seizure (Betjemann & Lowenstein, 2015). Furthermore, the risk of developing epilepsy after SE is greater when treatment is not applied immediately (Asadi-Pooya et al., 2017). SE is immediately followed by a seizure-free latent period, which can last for weeks, months, or even years in humans, and can lead to the onset of spontaneous recurrent seizures (SRS) (Curia, Longo, Biagini, Jones, & Avoli, 2008).

TLE is one of the most common forms of epilepsy that can result after SE and is often drug-resistant (Betjemann & Lowenstein, 2015). Because the origin of seizures in TLE is the temporal lobe, structures such as the hippocampus are vulnerable to injury thus resulting in hippocampal-dependent learning and memory deficits (Jiang et al., 2018; Lévesque, Avoli, & Bernard, 2015). Currently available anti-epileptic drugs (AEDs) only suppress seizures; they do not address the neuropathology or attenuate comorbid conditions. Furthermore, approximately 30% of individuals with TLE have intractable, or drug resistant seizures (Téllez-Zenteno & Hernández-Ronquillo, 2012). Because of the high rate of drug resistance and the additional risk of cognitive comorbid disorders in TLE, there is a critical need for the identification of novel therapeutic targets that may attenuate both SE-induced neuronal injury and cognitive comorbidities.

Behavioral Comorbidities

Aside from being a disorder of seizures, epilepsy is highly comorbid with other neurological conditions including cognitive deficits, psychiatric disorders such as anxiety or depression, and developmental disorders such as autism spectrum disorder (Keezer, Sisodiya, & Sander, 2016; LaFrance, Kanner, & Hermann, 2008; Stafstrom, 2014). Studies of children with epilepsy found a direct correlation between cognitive performance and seizure activity (Braakman et al., 2012; Ticci et al., 2019). Fifty percent of adults with epilepsy suffer from at least one comorbid condition (Keezer et al., 2016; Thijs et al., 2019). Approximately 15-24% of individuals with epilepsy also suffer from anxiety disorders, making it the second most common comorbid condition after depression. These comorbid conditions greatly affect the quality of life of epilepsy patients (Keezer et al., 2016).

The hippocampus is particularly vulnerable to seizure-induced injury in TLE because it is densely packed with excitatory synapses. Thus, memory deficits in TLE are common (Meisenhelter, Jobst, & Reports, 2018). In fact, the most common concern of epilepsy patients, behind unexpected seizures and concerns with driving, are memory problems (McAuley et al., 2010). Patients with epilepsy are more likely to have deficits in long-term memory (Breen, McCarthy, Blake, & Wroe, 2000), even with personal, emotionally salient information, which can have a profound effect on quality of life. There is evidence that memory deficits in patients with TLE correlate with psychiatric conditions such as depression (Demin, Berger, Holtkamp, & Bengner, 2018). Furthermore, anxiety can present itself as a symptom prior to the onset of seizures in TLE (LaFrance et al., 2008). Thus, understanding the comorbidities of epilepsy could contribute to decreasing the burden of the disorder.

To understand the underlying mechanisms that cause seizures and memory deficits, scientists use animal models of SE and acquired TLE. Common models include the use of chemoconvulsants such as pilocarpine or kainic acid, and electrical kindling (Kandratavicius et al., 2014; Lévesque et al., 2015). The role of the hippocampus in spatial and recognition memory has been verified by lesions studies. Rats with lesions in the dorsal hippocampus display a deficit in recognition memory, whereas rats with either dorsal or ventral lesions are slower to acquire spatial learning (Broadbent, Squire, & Clark, 2004). With this knowledge, researchers can correlate hippocampal damage and cognitive deficits in models of SE and TLE. In rat models, SE results in deficits of spatial and recognition memory at early and chronic time points (Pearson, Schulz, & Patel, 2014; Schipper et al., 2016), with evidence that memory deficits progress over the course of

epilepsy. The novel object recognition (NOR) test has been used widely in models of SE and acquired TLE, and has consistently revealed recognition memory deficits in rodents that undergo pilocarpine- or kainic acid-induced SE (Brewster et al., 2013; Oliveira et al., 2015; Pearson et al., 2014; Schartz, Wyatt-Johnson, Price, Colin, & Brewster, 2018). Rodent models of SE have also revealed deficits in spatial memory using a variety of tests, including the Morris water maze (Brewster et al., 2013), object location memory (Cho et al., 2015; Pearson et al., 2014), and Barnes maze (BM) (Schartz et al., 2018). These tests are valuable tools to understand the underlying pathology involved in epilepsy and cognitive deficits, and to identify potential therapeutic targets to treat the comorbidities of TLE.

Neuronal Injury

The cognitive comorbidities associated with TLE can in part be explained by neuronal injury that occurs in brain regions that mediate learning and memory. Hippocampal sclerosis is a common feature of focal TLE and consists of lesions, cell death, inflammation, and glial scarring, and is detectable with MRI scans (Curia et al., 2008). Hippocampal sclerosis is associated with deficits in working and spatial memory in patients with epilepsy (Abrahams et al., 1999). Reduced gray matter and functional connectivity in dorsal hippocampus (along with amygdala and thalamus) correlates with deficits in novel location and novel object memory tasks in a rat model of pilocarpine-induced SE and TLE (Jiang et al., 2018). In humans who died during SE, hippocampal, thalamic, and cortical cell loss and injury have been observed (Pichler & Hocker, 2017). Additionally, surgically resected brain tissue from patients with drug-resistant epilepsy displayed microlesions, characterized by decrease in dendritic marker

microtubule-associated protein 2 (Map2) staining and accompanied by markers of phagocytic microglia (Dachet et al., 2015).

Experimental models of SE and acquired TLE provide insight into the neuronal and molecular alterations that occur immediately following SE (acute phase), during epileptogenesis (latent period), and after the onset of SRS (chronic phase). Importantly, studying the events that occur during the latent period can provide insight into the mechanisms that contribute to the transformation of a normal brain to an epileptic brain. Hippocampal injury is a hallmark of experimental models of SE. Following SE, there is significant and profound loss of the dendritic structural protein Map2 in the CA1 region of the hippocampus (Brewster et al., 2013; Schartz et al., 2016). In addition, SE results in altered dendritic branching and dendritic spine density, as well as loss of dendritic spines and synaptic proteins (Bernard, 2012; Brewster et al., 2013; Schartz et al., 2016; Swann et al., 2000). SE also causes hippocampal neurogenesis and the formation of novel and aberrant networks (Botterill, Brymer, Caruncho, & Kalynchuk, 2015; Cho et al., 2015). This results in an imbalance of excitation and inhibition, and promotes an environment conducive to neuronal hyperexcitability that results in spontaneous seizures.

In parallel to neuropathological changes in the hippocampus, SE triggers activation of microglia, the resident immune cells and phagocytes of the brain (Brewster et al., 2013; Hiragi, Ikegaya, & Koyama, 2018; Schartz et al., 2016). Microglia survey their environment, respond to injury and inflammation, and phagocytose injured cells and cellular debris (Kettenmann, Hanisch, Noda, & Verkhratsky, 2011; Tremblay et al., 2011). Evidence for robust microgliosis has been observed in experimental models of SE and in human epileptic tissue (Brewster et al., 2013; Choi & Koh, 2008; Morin-Brureau

et al., 2018; Schartz et al., 2016; A. Vezzani & Granata, 2005; Wyatt, Witt, Barbaro, Cohen-Gadol, & Brewster, 2017). In animal models, microgliosis follows the same spatial and temporal pattern as the loss of neuronal and dendritic markers (Schartz et al., 2016), and microglia-dendritic contacts have been observed (Brewster et al., 2013). This suggests the possibility that microglia may be engulfing neuronal elements after SE. Further support for this hypothesis is that when a strong immunosuppressant is administered immediately following SE, microgliosis is suppressed and the loss of Map2 is attenuated (Brewster et al., 2013). After SE, microglia make multiple contacts with dendritic spines in the CA1 region of the hippocampus (Eyo, Murugan, & Wu, 2016; Eyo, Peng, et al., 2016), and may become aberrantly activated and phagocytose stressed but viable neurons after SE, thus promoting the formation of aberrant connections. There is strong evidence to support that immune activation plays a role in epileptogenesis. Treatment with immunomodulators (HMGB-1 or IL1B antagonists) reduces seizure frequency, seizure susceptibility, and improves learning and memory deficits in animal models of post-traumatic epilepsy (Semple et al., 2017; Zhao et al., 2018). Although evidence supports that microglia may prune synaptodendritic elements aberrantly after SE (Koenig & Dulla, 2018), the mechanisms that drive these neuro-immune interactions remains unknown.

The Immune Complement Cascade

The goal of this research is to identify potential mechanisms that drive neuroimmune interactions after SE. Although microglia are neuroprotective and anti-inflammatory under physiological conditions (Mirrione & Tsirka, 2011; Tremblay et al., 2011), over-activation can result in inflammation and cell death. One signaling

mechanism that promotes microglial activation and phagocytosis is the immune classical complement pathway.

The complement system is a part of innate immunity that promotes inflammation and tags target cells for phagocytosis (referred to as "find me" and "eat me" signaling, respectively) (Sarma & Ward, 2011). Complement activation consists of a set of proteolytic reactions that respond to infiltrating pathogens or damage associated molecular pattern molecules (DAMPs) to promote opsonization and phagocytosis and the downstream formation of the lytic membrane attack complex (MAC). Complement activation can occur through one of three pathways: classical, alternative, and mannose binding lectin pathway. The classical complement pathway has been implicated in development and maintenance of synaptic connections in the developing brain and aberrant activation of the classical pathway is associated with neurodegeneration (Hong et al., 2016; Schafer et al., 2012; Shi et al., 2015; Stephan, Barres, & Stevens, 2012; Stevens et al., 2007). C1q, the initiating protein of the classical complement pathway, tags cells and results in cleavage of C3 into C3a and C3b, which act as a chemoattractant and an opsonin, respectively (Stephan et al., 2012). C1q is a hexameric glycoprotein composed of the A, B, and C polypeptide chains, each repeated six times. These polypeptides are joined by sulfide-sulfide bonds to form six globular head regions connected to a fibril-like stalk domain. When the globular head region of C1q binds to surface proteins and the proteases C1r and C1s, the C1 complex is activated (Sarma & Ward, 2011). The activated C1 complex cleaves C2 and C4 into small a and larger b fragments, and C2a binds C4b to form the C3 convertase (C4bC2a) (Sahu & Lambris, 2001). Complement C3 is the central component of the three complement pathways. C3

is a large protein that must be cleaved in order to be activated. The major cleavage products are C3a and C3b. Once cleaved, C3a acts as a chemoattractant to recruit phagocytes and promote inflammation. C3b binds to the target cell surface and opsonizes to promote phagocytosis. and also contributes to the formation of MAC and cell perforation (Barnum, 2017). The alpha chain of C3b (C3bα) can be further cleaved by complement factors H or I into an inactive form of C3b (iC3b) which binds to cell surface proteins to promote phagocytosis (Sahu & Lambris, 2001). Once cleaved into iC3b, the protein cannot be cleaved further or contribute to the formation of MAC. C3 does not distinguish between self and non-self surface proteins, therefore regulation of C3 and other complement molecules is essential to avoid auto-immunity. C3 is an ideal target for regulating complement because its inhibition will prevent the formation of inflammatory C3a and C5a, as well as the downstream MAC (Sahu & Lambris, 2001).

The role of complement activation in the brain has recently been under intense investigation. Specifically, C1q and C3b tag extranumerary synapses in the brain for microglia to phagocytose in the process of developmental pruning (Stevens et al., 2007). Complement signaling is also upregulated in models of neurodegenerative disorders such as Alzheimer's disease (AD) and stroke (Heydenreich et al., 2012; Hong et al., 2016; Stephan et al., 2012), and inhibiting complement in such models is neuroprotective, resulting in attenuated hippocampal synaptic loss and memory deficits (Hong et al., 2016). This suggests that complement activation may indeed play an important role in neurodegeneration. Interestingly, complement mRNA and protein is upregulated in human and experimental epilepsy (Aronica et al., 2007; Wyatt et al., 2017). While evidence suggests that C1q-C3 signaling play a role in seizures, the nature of complement

upregulation in epilepsy is unknown. C3-deficient mice are less susceptible to virus-induced seizures (Libbey, Kirkman, Wilcox, White, & Fujinami, 2010) and sequential administration of the components of MAC (C5-C9) produces seizures (Xiong, Qian, Suzuki, & McNamara, 2003). The purpose of the current study is to determine the role of complement C3 in the development of memory deficits and synaptodendritic changes in the pilocarpine model of SE and TLE.

Innovation

The majority of available treatments for epilepsy regulate ion channels or enhance inhibitory GABA activity (Rogawski & Loscher, 2004), meaning that they only stop the seizures, and do not treat the underlying causes epilepsy. Furthermore, these treatments do not attenuate symptoms other than seizures (i.e. memory deficits, psychiatric disorders, etc.), and in some cases, they exacerbate the comorbid conditions (de Kinderen et al., 2014; Perucca, 2005). In addition, only 70-80% of individuals with epilepsy respond to AEDs, leaving 20-30% of them with drug resistant seizures (Kwan & Brodie, 2006). A major goal of epilepsy research is to identify the underlying causes of hyperexcitability in the brain that result in spontaneous seizures. Targeting the underlying mechanisms of epileptogenesis could attenuate or even prevent the development of epilepsy, thereby offering disease-modifying interventions. The implication of microglia in synaptic remodeling following an initial insult (such as SE) has shifted the focus onto neuro-immune interactions following SE, during epileptogenesis, and in chronic TLE. Studies that block microglial activation before or immediately following SE have successfully attenuated hippocampal dendritic loss (Brewster et al., 2013), thus supporting the hypothesis that microglia contribute to the neuropathology of epilepsy. By

pinpointing the mechanism that directs unwanted synaptodendritic changes by microglia after SE, we may be able to attenuate SE-induced injury and pathology without unwanted side effects.

Complement activation in the brain drives activation of microglia and promotes elimination of tagged neuronal elements (Schafer et al., 2012). Complement inhibition has been used to reduce injury in ischemia (De Simoni et al., 2003), and to prevent dendritic and synaptic loss in neurodegenerative diseases such as AD (Hong et al., 2016). We and other groups have found that complement proteins are increased in experimental models of SE and TLE (Aronica et al., 2007; Kharatishvili et al., 2014; Schartz et al., 2018) and in tissue resected from humans with different epilepsies (Aronica et al., 2007; Wyatt et al., 2017). We previously reported that an episode of pilocarpine-induced SE results in increased protein levels of C1q and C3b in the hippocampus that correlate with a loss in dendritic protein markers such as Map2 and synaptic proteins. Higher C3b levels also correlate with the frequency of spontaneous seizures, and these are associated with learning and memory deficits (Schartz et al., 2018). However, the effects of complement _inhibition_ have not been determined in the context of epilepsy disorders. This work is the first to target complement activation in a model of SE and TLE to determine its role in seizure-associated pathology. Our study will contribute to the growing focus on complement C3 inhibition in neurodegenerative diseases and will open new possibilities for epilepsy treatment research.

Our _central hypothesis_ is that complement C3 activation contributes to hippocampal injury and cognitive deficits following an episode of SE. Our _rationale_ for this research is that identifying complement signaling as a regulator of SE-induced

pathology would provide justification of investigating complement inhibitors as a treatment for seizures and cognitive deficits. Using the pilocarpine model of SE with wild-type (WT) mice and mice deficient in C3 (C3KO), we addressed the following *Specific Aims*:

Specific Aim #1: Determine the role of C3 in SE-induced cognitive deficits. Our *working* hypothesis is that C3 activation promotes the development of hippocampal-dependent cognitive deficits that typically follow SE in this model. To test this hypothesis, we used two paradigms that have reliably demonstrated a deficit in rodents following an episode of SE. The novel object recognition (NOR) test and Barnes maze (BM) measure recognition and spatial memory, respectively. Mice were tested between 2 and 3 weeks after SE, the time at which multiple studies have reported deficits in these tasks.

Specific Aim #2: D*etermine the role of C3 in SE-induced loss of synaptic proteins*. Prolonged seizures trigger activation of the classical complement pathway in the hippocampus, demonstrated by cleavage of complement C3b. C3b binds to cellular surfaces and primes for engulfment by microglia. Two weeks following SE, there is a loss of hippocampal synaptic proteins, and levels of these proteins are negatively correlated with levels of C3b. Therefore, our *working hypothesis* is that eliminating C3 will prevent loss of synaptic elements after SE. To address this hypothesis, we induced SE in WT and C3 KO mice and determined

the extent of loss of synaptic proteins PSD95, Vgat, and Vglut1 in the

hippocampus using Western blotting.

At the completion of this research, our *expectation* is to have identified

complement signaling as a novel mechanism that contributes to loss of hippocampal

dendritic structural integrity and thus promotes synaptic remodeling and

hyperexcitability. This work will have a *positive impact* by identifying a potential

therapeutic target that may prevent hippocampal synaptic loss and memory deficits after a

severe injury such as SE.

METHODS

Animals

Mice homozygous for the C3 ligand knockout targeted mutation were developed on a C57BL/6 background by Michael Carroll at the Center for Blood Research. The C3 gene is located on chromosome 17 in the mouse (Janssen et al., 2005). Approximately 600 nt of the C3 gene were deleted with PGK/Neo cassette (this deleted the C-terminal region of the beta chain and N-terminal region of the alpha chain). The cassette was transfected into J1 ES cells, injected into 3.5-day-old C57BL/6 blastocysts, and then implanted into C57BL/6 pseudopregnant females. C3KO mice were bred on a C57BL/6 background and purchased from Jackson Labs (JAX 003641). C57BL/6 WT mice were also purchased from Jackson labs (JAX 000664). C57BL/6 WT mice were used as controls. A breeding colony was established with homozygous pairing to produce experimental mice. Experiments were performed on male C3KO and WT mice beginning at 6-8 weeks of age. Due to time restraints, only male mice were used in the present study. However, future studies in Dr. Brewster's laboratory that are related to this project will include both males and females. Mice were housed in groups of 3-4 in ambient temperature with free access to food and water. The room was on a 12-hr light-dark cycle (6:00-18:00).

Pilocarpine Dose Response

As part of our viability testing, we tried the efficacy of pilocarpine (Cat# P6503-10G, Sigma-Aldrich, St Louis, MO) by using three different doses on WT and C3KO mice (300 mg/kg, 325 mg/kg, and 350 mg/kg). Intraperitoneal (i.p.) injections of scopolamine methyl bromide (1 mg/kg; Cat# S8501-1G, Sigma-Aldrich) were given

thirty minutes prior to pilocarpine injections (300-350 mg/kg, i.p.). The range or doses

used was selected based on previous studies of SE in mouse models (Hosford, Liska, &

Danzer, 2016; Turski et al., 1984). Mice were monitored continuously after pilocarpine

injection and seizure severity was determined behaviorally according to the Racine scale

(Racine, 1972). After one hour of seizure activity class 4.5-6 in the Racine scale, mice

were euthanized with CO_2 and decapitated. Brains were quickly removed and hippocampi

were sub-dissected and immediately frozen at -80°C. Blood samples were also collected

and serum was isolated and stored at -20°.

Pilocarpine-Induced Status Epilepticus (SE)

SE was induced in WT and C3KO mice aged 6-8 weeks. Mice received

scopolamine methyl bromide injections (1 mg/kg, i.p.). After thirty minutes, mice were

injected with pilocarpine (350 mg/kg, i.p.) or equal volume of 0.9% saline (sham

controls). Mice were monitored and behavioral seizure severity was recorded in

accordance with the Racine scale. Diazepam (10 mg/kg, i.p; Hospira, Inc., Lake Forest,

IL) was injected approximately one hour after pilocarpine. Mice that did not reach a stage

5 or 6 seizure were categorized as "non-SE." Following Diazepam injections, mice were

given about 0.2 mL of 0.9% saline injections (i.p) for hydration. Subsequent saline

injections were administered as needed, along with supplementary diet of chocolate

Ensure to help recovery. Mice were monitored daily after SE for weight gain and

hydration. We injected 41 WT and 27 C3KO mice with pilocarpine and 12 WT and 12

C3KO mice with saline. Out of those injected with pilocarpine, 17 (41.4%) WT and 11

(40.7%) C3KO mice died from SE or SE-related complications. Sixteen (39.02%) WT

and 12 (44.44%) C3KO mice developed SE and survived to be used in subsequent experiments.

Open Field (OF)

Behavioral testing began two to three weeks following SE. Cage cards were covered and coded to ensure blinding the groups from the experimenter during testing. On the days of behavioral testing, mice were temporarily single housed from acclimation until the end of behavioral testing. They were allowed to acclimate to the central testing suite for a minimum of 30 minutes each day before testing began. Twenty-four hours before NOR, mice were habituated to the empty testing arena for 10 minutes. We recorded this habituation to measure distance travelled, average speed, time freezing, and time spent in the inner and outer zones using Any-Maze. This measure was to determine if there are any general locomotion or anxiety-like differences between treatment groups that may influence their exploratory behavior on the NOR and BM tests.

Novel Object Recognition (NOR)

The NOR test was performed following previously described protocols with some modifications (Bevins & Besheer, 2006; Brewster et al., 2013; Hodges et al., 2018; Schartz et al., 2018). Trial 1 consisted of a familiarization phase, during which each mouse was allowed to explore two similar objects inside the testing apparatus (40 x 40 x 30 cm) for 10 minutes. The objects were placed in opposite and symmetrical positions in the center of the apparatus. The second phase, trial 2, took place 5 days after familiarization (the inter-trial interval was determined after testing multiple time points, data not shown). During the test phase, one of the objects was replaced with a novel object and the mice were allowed to explore for 45 minutes. Any-maze video tracking

system V4.99 (Wood Dale, IL) was used to determine the seconds each mouse spent exploring the object (when the nose of the mouse was < 2 cm from the object). This was verified with hand scoring by multiple investigators blinded to treatment group. Location of the novel object was counterbalanced to control for possible side preference. For each trial, a discrimination index was calculated [(Novel-Familiar)/(Novel+Familiar)]. Mice that had a discrimination index greater than 0.25 during the familiarization phase were excluded due to an innate preference to one of the two similar objects. The testing arena was thoroughly cleaned with 70% ethanol after each trial.

Barnes Maze (BM)

BM training began the day after NOR is completed. The BM is a circular platform, 92 cm in diameter, elevated 95 cm from the ground purchased from Noldus (Leesburg, VA). There are 20 circular holes around the outer edge of the platform that are 5 cm in diameter. Under one of the holes, there is a black removable escape box. The BM protocol was completed as previously described (Levin, Serrano, & Dingledine, 2012; Schartz et al., 2018). During the first 4 days, mice were trained to find the escape box that is under the BM. The following training trials were repeated 4 times each day, separated by 15-20 minutes. Each mouse was brought into the testing room in the dark and placed under an opaque start box in the center of the platform. Following a 10 second delay, bright lights were turned on and the start box lifted. The mouse had up to 3 minutes to explore the BM and find the escape box using spatial cues placed on the walls. If it failed to enter the escape box after 3 minutes, it was gently guided into it and allowed to remain in the box for 1 minute. The latency to enter the escape box for each trial was recorded. All trials were recorded from above and tracked with Any-maze software. On day 5, mice

were tested with a probe trial, in which the escape box was removed. Mice were placed under the start box before bright lights were turned on, and they had 90 seconds to explore the maze. Time spent over the target (i.e. the location where the box was during training) was measured with the Any-maze software. The maze was thoroughly cleaned with 70% ethanol after each trial, and the platform and escape box were rotated so that the box remained in the same spatial location for every trial.

Tissue Preparation for Western Blot

Mice were given a lethal dose of Beuthenasia (200 mg/kg i.p.) and transcardially perfused with ice-cold 1 X phosphate buffered saline (PBS; 137 mM NaCl, 2.7 mM KCl, 4.3 mM Na_2HPO_4, 1.47 KH_2PO_4, pH 7.4) for approximately five minutes. Brains were immediately removed and hippocampi and cortices were sub-dissected and stored in -80°C until ready to be used. The different brain regions were then homogenized in 1X PBS with 10% protease inhibitor cocktail (Cat# P2714-1BTL, Sigma-Aldrich). Protein concentrations were measured and then samples diluted to a final concentration of 1 µg of protein per 1 µL of sample in 200 µL total volume. Samples were diluted in 1X PBS and 4X Laemmli buffer (40% Glycerol, 240 mM Tris/HcL pH6.8, 8% Sodium Dodecyl Sulfate, 40% Bromophenol blue, 8% βmercaptoethanol, in distilled H_2O). Diluted samples were stored at -20°C until ready to use for sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE).

SDS-PAGE

Samples were loaded onto 10% separating gels (buffer, 30% acrylamide, 10% SDS, 10% ammonium persulfate, 10 µl TEMED) or 4-12% pre-cast gradient gels (Cat# 12001-068, VWR, Radnor, PA) while submerged in running buffer inside the

SDS-PAGE mini protean 3 cell (Cat# 525BR 010890, Biorad, Hercules, CA). Seven

microliters of the Amersham full-range rainbow molecular weight marker (Cat#

RPN800E, GE Life Sciences, Pittsburgh, PA) were loaded onto one well in every gel. Gel

electrophoresis was run at 100V for approximately 2 hours using a power supply (VWR

Scientific Products, ACCU Power, model 300). Gels were removed from the glass and

placed in a mini trans-blot cell (Cat# 153 BR102550, Biorad) between sponges and filter

paper and laid next to polyvinylidene difluoride (PVDF) (Cat# 88518, Thermo Scientific,

Rockville, IL) membrane. Protein transfer from the gel to the PVDF membrane was run

at 100V for 1 hour on ice.

Western Blot (WB)

Immediately following transfer, PVDF membranes were blocked with 5% milk in

1X Tris buffered saline (50mM Tris-HCl, pH 7.4, 150mM NACl) with 0.1% tween (0.1%

1X TBST) for 1-2 hours at room temperature (RT). Then membranes were incubated

with primary antibody diluted in 5% milk overnight at 4°C or for 2 hours at room

temperature. The antibodies used include goat anti-mouse C3 (1:500; Cat# SKU

0855444, MP Biomedical, Solon, OH) mouse anti-PSD-95 (1:50K; Cat# 75-028,

Neuromab, Davis, CA), mouse anti-Vglut1 (1:250; Cat# 75-066, Neuromab), mouse anti-

Vgat (1:100; Cat# 73-457, Neuromab), and rabbit anti-actin (1:5K; Cat# ab198991,

Abcam, Cambridge, United Kingdom) was used as a loading control. Following

incubation with primary antibodies, membranes were washed with 0.1% 1X TBST and

incubated with horseradish peroxidase (HRP)-labeled secondary antibodies anti-mouse

(1:1K, Cat# 7076S, Cell Signaling, Danvers, MA), anti-rabbit (1:1K, Cat# 7074S, Cell

Signaling), and donkey anti-goat (3:5K, Cat# AP180P, Millipore, Burlington, MA) for 1

hour at RT. Then, membranes were washed, incubated with enhanced

chemiluminescence WB detection substrate (Cat# 32106, Thermo Scientific), and

developed on radiography film (MIDSCI, St. Louis, MO). Membranes were stripped with

stripping buffer (25 mM glycine, pH 2.0, 10% SDS) and reblotted for the different

antibodies.

Densitometry Analysis for Western Blot

Developed films were scanned and pixel intensity was measured using Image J

software V1.49 (NIH; Bethesda, MD). First, images were inverted and a measure of the

background was taken. Then, the mean pixel intensity of the immunoreactive bands were

measured. The size of the selected area to be measured remained constant within each

film. All acquired measures were subtracted from the background and normalized to the

actin bands.

RAT COBRA VENOM FACTOR (CVF) STUDY

As outlined in the alternative methods, CVF was used as an additional tool to block C3 after SE in the rat model of SE and acquired epilepsy.

Animals

Male Sprague Dawley rats (175-200 g) were purchased from Envigo (Indianapolis, IN). Rats were given at least one week between delivery and the start of experiments. All procedures began at 6-8 weeks of age. Rats were housed in pairs in ambient temperature with free access to food and water. The room was on a 12-hr light-dark cycle (6:00-18:00).

CVF Dose Response

Many studies have demonstrated that CVF depletes C3 in the serum of mice. To verify the duration of the effects of CVF in the blood and the brain, we injected 100 ng/g of CVF or equal volume of 0.9% saline (i.p.) in WT mice and collected the hippocampi and blood at 1, 3, and 6 days after injection. We used WB to measure levels of C3bα and iC3b in the hippocampus relative to the saline treated mice, and ELISA to measure levels in the serum.

The remainder of CVF experiments were done in Sprague Dawley rats because of the thorough characterization of hippocampal injury and complement activation done in this model by our lab (Schartz et al., 2016; Schartz et al., 2018). Previous studies demonstrated that i.p. injections of CVF deplete C3 levels in serum and brain tissue of rats for several days after treatment (Cowell, Plane, & Silverstein, 2003; Jha, Banda, Tytarenko, Bora, & Bora, 2011). We tested a high (500 ng/g) and low (100 ng/g) dose of CVF based on studies that determined a safe range of doses (Huang et al., 2018; Vasthare

et al., 1998), then collected blood and perfused hippocampi at 1 and 3 days after injection. A set of vehicle rats were injected i.p. with equal volumes of saline.

Enzyme-Linked Immunosorbent Assay (ELISA)

Blood samples were left at room temperature for at least one hour after collection. Then, samples were centrifuged at 1,000 rpm for 10 minutes at room temperature. Serum was collected and the pellet was discarded. Serum was stored at -20°C until ready to use. ELISA experiments were completed following kit instructions to measure serum levels of rat C3 (Abcam ab157731, Abcam) or mouse C3 (Cat# ab157711, Abcam). Absorbance of signal was measured using a Chromate plate reader (Awareness Technology Inc., Palm City, FL) and C3 levels were interpolated using a standard curve and GraphPad Prism software. C3 levels were compared between the four groups (Control-veh, SE-veh, Control-CVF, SE-CVF) using a two-way ANOVA with Tukey's post-hoc test.

Pilocarpine-Induced Status Epilepticus (SE)

SE was induced in male Sprague Dawley rats aged 6-8 weeks. Rats received scopolamine methyl bromide injections (1mg/kg, i.p.). After thirty minutes, rats were given pilocarpine injections (300 mg/kg, i.p.). Rats were monitored and behavioral seizure severity was recorded in accordance with the Racine scale. Diazepam (10mg/kg, i.p.) was injected 45 minutes after onset of SE stage 5-6. Rats that did not reach a stage 5 or 6 seizure were categorized as "non-SE" and excluded from further investigations. Following Diazepam injections, rats were given 1.5 mL of 0.9% saline injections (i.p) for hydration. Subsequent saline injections were administered as needed, along with supplementary diet of chocolate Ensure and Kellogg's Fruit Loops to help recovery. Rats were monitored daily after SE for weight gain and hydration. Twenty-eight rats were

injected with pilocarpine. Out of those, 8 (28.5%) did not develop SE, 4 (14.3%) died

from SE or SE-related complications, and 16 (57.1%) developed SE and survived to be

included in subsequent experiments. Nine of the SE rats were used for CVF treatment

and 7 for vehicle treatment. Eighteen sham-treated controls were included of which 9

were also treated with CVF and the other 9 were treated with vehicle.

CVF Treatment

In previous studies, it was determined that complement levels were elevated in the

rat hippocampus starting at 2 weeks after SE (Schartz et al., 2018). Therefore, to inhibit

complement C3 before the increase was apparent, rats were treated with CVF starting at

seven days after SE. Control and SE rats were given a dose of CVF (100 ng/g; i.p.) or

equal volume of vehicle (saline) at seven, ten, and thirteen days after pilocarpine

treatment.

For a pilot study, 8 SE rats and 6 controls were injected with either CVF (100

ng/g, i.p.) or an equal volume of vehicle. Blood was collected from the lateral saphenous

vein in the leg prior to each CVF or saline (veh) injection on days 7, 10, 13 after SE, as

well as prior to perfusion on day 20 (Figure 9). This was to ensure that after multiple

doses, C3 levels are still reduced in serum up to 3 days after injection.

Behavioral Testing

Behavioral testing began 14 days after SE. Prior to behavioral testing, all

identification information on cage cards was covered and rats were randomly assigned

new identification numbers to blind the investigator and prevent bias. On testing days,

rats were placed in single holding cages and allowed to acclimate to the central testing

suite in the dark for 30 minutes each day before testing began. All behavioral testing was

completed under red light conditions. The behavior protocols were similar to the

protocols used in mice, with the following exceptions.

OF

OF was completed twenty-four hours before NOR. Rats were placed inside the

square testing arena for 10 minutes under red light conditions. The sessions were

recorded from above and distance travelled, average speed, time freezing, and time spent

in the inner and outer zones were tracked using Any-Maze video tracking system.

NOR

NOR took place on day 15 after SE. After acclimation, rats were placed in the

NOR testing apparatus (62 X 62 X 46 cm) for 5 minutes and allowed to explore two

similar objects (familiarization trial). After a two-hour delay rats were returned to the

testing arena with one of the objects replaced by a novel one and again were allowed to

explore for 5 minutes (test trial). Exploration of each object was measured with Any-

Maze and verified with hand-scoring by blinded investigators. Location of the novel

object was counterbalanced to control for possible side preference. The testing arena was

thoroughly cleaned with 70% ethanol after each trial.

Tissue Preparation

For tissue collection, rats were deeply anesthetized with Beuthanasia (200 mg/kg).

Once all reflexes were unresponsive, rats were transcardially perfused with ice cold 1 X

PBS for approximately five minutes. Before 1 X PBS perfusion began, blood was

collected from the right atrium for isolation of serum for enzyme-linked immunosorbent

assays (ELISA). After the perfusion was complete, brains were removed and divided in

half along the midline. One hemisphere was subdissected to remove the cortex and

hippocampus for western blotting. These samples were stored in -80°C until ready for testing. The other hemisphere was post-fixed in 4% paraformaldehyde (PFA) for 24 hours, cryoprotected in 30% sucrose made in 1 X PBS, and then frozen to be used for immunohistochemistry in future studies. The hemispheres used for western blotting or immunohistochemistry were counter-balanced.

SDS-PAGE and Western Blot (WB)

The SDS-PAGE and western blotting protocols followed were identical to those used for mouse tissue. The antibodies used in the rat tissues were goat anti-rat C3 (1:500; Cat# SKU 0855730, MP Biomedical) mouse anti-PSD-95 (1:50K) mouse anti-Vgat (1:100), mouse anti-Vglut1 (1:250), and rabbit anti-actin (1:5K) as a loading control.

Statistical Analyses

All analyses were completed using GraphPad Prism Software version 8.0.1. Sample sizes were determined with power calculations using G*Power with previously gathered data. A post hoc power analysis was also performed to ensure that sufficient power was achieved at the end of the studies. Data points that deviated more than ± 2 standard deviations from the mean were excluded as outliers. The appropriate statistical analysis and post hoc test were used for each experiment. **(1) Pilocarpine dose response**: To compare the behavioral dose response to different pilocarpine doses over time, a repeated measures ANOVA was used with Sidak's post hoc test for multiple comparisons. Sidak's test is ideal when not comparing every mean with every other mean, and produces an adjusted p value with more power. **(2) NOR**: Preference of objects (left vs right; familiar vs novel) was compared using paired t-tests. The discrimination index was calculated ([novel-familiar]/[novel+familiar]) to compare

discrimination of the novel object between treatment groups using using a Two-way ANOVA (genotype/drug X treatment) with Tukey's post hoc test. To determine if the DI's were significantly different from zero (i.e. no discrimination) a one-sample t-test was used. Total exploration time of objects during the first and second trials was compared between treatment groups (WT C, WT SE, C3KO C, C3KO SE or C-veh, SE-veh, C-CVF, SE-CVF) to determine if there was a difference in overall exploratory behaviors between the groups using two-way ANOVA (genotype/drug X treatment) with Tukey's post hoc test. **(3) BM**: The escape latency for BM was averaged for the 4 trials of each day. Three-way repeated measures ANOVA (genotype X treatment X training day) with Tukey's post hoc test was used to compare escape latency between each of the 4 groups and over the 4 days. To compare the time spent over the target and latency to reach the target during the probe trial (day 5), a two-way ANOVA (genotype X treatment) was used with Tukey's post hoc test. **(4) OF**: Behaviors recorded during OF were compared using a two-way ANOVA (genotype X treatment) with Tukey's post hoc test to determine effects of genotype/drug and of treatment. The behaviors analyzed were (1) time freezing, (2) inner duration, (3) distance travelled, and (4) average speed. **(5) WB**: For all WB experiments, the bands of interest were normalized to the loading control (actin). WB densitometry was analyzed using two-way ANOVA to compare between genotype (WT vs. C3KO) and treatment (control vs. SE) or drug (CVF vs. vehicle) and treatment (control vs. SE) and Tukey's post hoc test was used to determine individual differences for each antibody used. **(6) ELISA:** ELISA standard absorbance and protein values were entered into a prism file with the absorbance of samples. The standard curve was extrapolated to determine the protein levels of the samples. Once

calculated, the values were compared using two-way ANOVA with Tukey's post hoc

test.

RESULTS

Dose Response: WT and C3KO Mice Develop Seizures After Pilocarpine Injections

To determine the potential role of C3 in the cognitive and synaptic deficits provoked by a single episode of SE, we first investigated whether WT and C3KO mice responded similarly to pilocarpine-induced SE. We performed a pilocarpine dose response test in which WT and C3KO mice were injected with either 325 mg/kg or 350 mg/kg of pilocarpine and observed for one hour (Figure 1). Behavioral seizure activity was recorded from 5 minutes to 80 minutes after pilocarpine injection and compared between WT and C3KO mice according to a modified Racine scale (1 = rigid posture, mouth moving; 2 = tail clonus; 3 = partial body clonus, head bobbing; 4 = rearing; 4.5 = severe whole body clonic seizures while retaining posture; 5 = rearing and falling; 6 = tonic-clonic seizure with jumping or loss of posture) (Racine, 1972) (Figure 1A-B). There were no genotype-dependent differences in the behavioral response to the lower dose of pilocarpine 325 mg/kg [$F(1, 9) = 0.06050$, $p = 0.8112$]. The higher dose (350 mg/kg) also did not yield genotype-dependent differences in seizure severity [$F(1, 11) = 1.843$, $p = 0.2018$]. However, there was an effect of time [$F(4.907, 41.22) = 7.340$, $p = 0.0016$], with both the WT and C3KO mice having higher seizure scores representative of SE (4.5-6) between 20 to 45 minutes after pilocarpine injection. At least one hour after the onset of seizure activity, mice were euthanized with CO_2 and hippocampi were dissected for western blotting. We determined levels of phosphorylated S6 in relation to total levels of S6, a ribosomal protein that is activated downstream of the mechanistic target of rapamycin (mTOR) pathway, and is a reliable marker of neuronal hyperactivity and thereby seizure activity (Figure 1C-D) (Holley et al., 2018; Liu et al., 2014; Zeng,

Rensing, & Wong, 2009). There was a significant effect of pilocarpine dose on phospho-S6 levels [$F(2, 26) = 10.17$, $p = 0.0005$] but no effect of genotype [$F(1, 26) = 3.954$, $p = 0.0574$], and no interaction of dose and genotype [$F(2, 26) = 0.4976$, $p = 0.6137$]. There was a main effect of dose, with higher doses resulting in higher levels of phopho-S6. Therefore, for the remainder of our study, we used the 350 mg/kg dose of pilocarpine to induce SE.

C3KO Mice are Protected Against SE-Induced Recognition Memory Deficits in NOR Test

Previous studies have shown that SE results in hippocampal dependent spatial memory deficits and recognition memory deficits (Brewster et al., 2013; Schartz et al., 2018; Schipper et al., 2016). In order to test if our WT-SE mice had these deficits, and if the C3KO mice were protected from SE-induced cognitive deficits, we used the NOR test and BM. NOR was used as a test for recognition memory (Figure 2). During the familiarization trial, mice were exposed to two similar object for 10 minutes, and exploration of each object was recorded and compared (Figure 2A). As expected, during familiarization, no groups showed preferential exploration of the right or left object (WT-C, [$t(13) = 0.2855$, $p = 0.7798$]; WT-SE, [$t(10) = 0.1998$, $p = 0.0736$]; C3KO-C, [$t(15) = 0.0811$, $p = 0.9364$]; C3KO-SE, [$t(10) = 0.0839$, $p = 0.9348$]). We calculated the discrimination index (DI; [(left – right)/total*100]) for trial one (Figure 2B), and any mouse that had a discrimination greater than ± 25 was removed from further NOR analysis. This resulted in the removal of one C3KO-SE mouse. We also compared the DI between groups and found no statistically significant difference between genotypes [$F(1, 48) = 0.7593$, $p = 0.3879$], treatment groups [$F(1, 48) = 0.3853$, $p = 0.5377$], or an

interaction [$F(1, 48) = 0.3450$, $p = 0.5597$], and using a one-sample t-test revealed that all of the DI's were not different from zero (WT-C, [$t (13) = 0.2858$, $p = 0.7769$]; WT-SE, [$t(10) = 0.1.998$, $p = 0.0736$]; C3KO-C, [$t(15) = 0.08176$, $p = 0.9359$]; C3KO-SE, [$t(10) = 0.08361$, $p = 0.9350$]), suggesting that the mice did not discriminate between the left and right objects. We also measured the total time spent exploring objects during the familiarization trial (normalized to the duration of the trial) and found a significant group effect of treatment [$F(1, 48) = 8.057$, $p = 0.0066$], and genotype [$F(1, 48) = 10.46$, p = 0.0022], but no interaction of treatment and genotype [$F(1, 48) = 1.396$, $p = 0.2431$] (Figure 2C).

The test trial occurred 5 days after familiarization (Figure 2D-F). We selected the 5 day inter-trial interval after a pilot study that tested recognition at 2 hours, 1 day, and 5 days after familiarization. The earlier time-points did not result in any recognition memory deficits. During the test trial, one of the previously explored objects was replaced by a novel object and mice were placed back in the apparatus and allowed to explore for 5 minutes. First, we compared the percent exploration time of the novel vs. familiar objects for each group (Figure 2D). As expected, the WT-C mice spent more time exploring the novel object compared to the familiar object [$t(13) = 6.643$, $p < 0.0001$], as did the C3KO-C [$t(15) = 5.085$, $p = 0.0001$]. The WT-SE mice did not show exploratory preference for either object [$t(9) = 1.801$, $p = 0.1052$] suggesting this group did not remember the familiar object from trial 1. Interestingly, the C3KO-SE mice performed similarly to the controls, exploring the novel object significantly more than the familiar [$t(10) = 2.279$, $p = 0.0458$]. For further understanding of the differences in recognition memory, we calculated the DI for the test trial [(novel-familiar)/total*100]

(Figure 2E). There was a significant effect of treatment on DI [$F(1, 47) = 5.545$, $p = 0.0228$], but no effect of genotype [$F(1, 47) = 0.4287$, $p = 0.5158$] and no interaction between treatment and genotype [$F(1, 47) = 0.4823$, $p = 0.4908$]. Interestingly, one-sample t-test determined that the DI's of the WT-C [$t(13) = 6.644$, $p < 0.0001$], C3KO-C [$t(15) = 5.086$, $p < 0.0001$], and C3KO-SE [$t(10) = 2.280$, $p = 0.0458$] were all significantly different from zero. The DI of the WT-SE group, however, was not different from zero [$t(9) = 2.095$, $p = 0.0626$], suggesting that this group did not recognize the familiar object. Finally, we observed an effect of genotype on total exploration of both objects during trial 2 [$F(1, 47) = 12.43$, $p = 0.001$] (Figure 2F). Similarly to the open field results, the C3KO controls showed more exploration than the WT-SE group ($p = 0.0128$). These findings suggest that the WT-SE group did not discriminate between the two objects whereas the WT-C, C3KO-C, and C3KO-SE groups did so. These data indicate that while WT mice that undergo SE experience recognition memory deficits, the recognition memory of C3KO remains intact, thereby suggesting a role for C3 in this pathophysiology.

Since the placement of the novel object was counterbalanced, we compared exploration of the novel object based on its placement. We compared percent exploration of the novel object, discrimination index, and total exploration time (Figure 4). Mice showed no difference in novel object exploration (WT-C, $p = 0.9337$; WT-SE, $p = 0.9484$; C3KO-C, $p = 0.6010$; C3KO-SE, $p = 0.1658$). Furthermore, the DI and total object exploration were not different based on placement of the novel object (DI: WT-C, $p = 0.9340$; WT-SE, $p = 0.9485$; C3KO-C, $p = 0.6011$; C3KO-SE, $p = 0.1659$; Total exploration: WT-C, $p = 0.3585$; WT-SE, $p = 0.1096$; C3KO-C, $p = 0.5578$; C3KO-SE, p

= 0.9258). Therefore, these data support that the preference of the novel object during trial two was not based on a side preference.

SE Does not Cause Spatial Learning and Memory Deficits in WT or C3KO Mice in the BM Test

We and others have shown that an episode of SE results in deficits in spatial learning and memory (Brewster et al., 2013; Schartz et al., 2018). To determine if blocking C3 can attenuate SE-induced spatial memory deficits, we used the BM test. Mice were trained on a Barnes maze over 4 days to find a hidden escape box using spatial cues placed on the walls of the testing room (Figure 4). Escape latency for each treatment group was recorded and compared over time (Figure 4A). Using a three-way ANOVA (training day X genotype X treatment), we found an effect of training day [$F(3, 60) = 40.27, p < 0.0001$] and genotype [$F(1, 52) = 8.379, p = 0.0055$], but no effect of treatment [$F(1, 20) = 0.3163, p = 0.5801$]. There was also no significant interaction between training day and genotype [$F(3, 52) = 1.693, p = 0.1798$], training day and treatment [$F(3, 52) = 0.8483, p = 0.4738$], genotype and treatment [$F(1, 52) = 0.8119, p = 0.3717$], or three-way interactions [$F(3, 52) = 0.9363, p = 0.4298$]. As expected, there was a main effect of training day, with the later training days resulting in shorter escape latency. Interestingly, there was also a main effect of genotype, with the escape latency of WT being faster than C3KO. These data suggest that while all groups are able to acquire spatial information in the BM, it is possible that the C3KO group might require more training to learn spatial cues compared to WT mice.

Twenty-four hours after the last training day, mice were tested with a probe trial, during which the escape box was removed and time spent around or directly on top of the

target (where the escape box was during training) was measured (Figure 5B-C). There was no effect of treatment on time spent directly over the target [$F(1, 39) = 0.4269$, $p = 0.5173$]. However, there was an effect of genotype [$F(1, 39) = 4.676$, $p = 0.0368$], and no interaction [$F(1, 39) = 0.008145$, $p = 0.9286$]. There was also an effect of genotype on the latency to find the target during the probe trial [$F(1, 39) = 10.96$, $p = 0.002$], but none of treatment [$F(1, 39) = 1.808\text{e-}005$ $p = 0.9966$] and no interaction [$F(1, 39) = 0.1415$, $p = 0.7088$].

SE Does not Alter Locomotion or Anxiety-Like Behaviors in WT and C3KO Mice

Before the NOR test, mice were habituated to the testing apparatus and this doubled as an OF test for measures of locomotion and anxiety-like behaviors to control for potential confounds of exploratory behavior during NOR and BM. During OF, mice were allowed to freely explore the testing apparatus for 10 minutes while being recorded from overhead. The behaviors that were recorded and analyzed included total distance travelled (Figure 5A), average speed (Figure 5B), time freezing (Figure 5C), and percent of time spent around the outer perimeters of the arena near the walls (Figure 5D). We found an overall effect of treatment on distance travelled [$F(1, 48) = 7.409$, $p = 0.009$], average speed [$F(1, 48) = 7.604$, $p = 0.0082$], and time freezing [$F(1, 48) = 4.606$, $p = 0.0369$]. However there was no treatment effect on outer duration [$F(1, 48) = 0.2906$, $p = 0.5923$]. In contrast, there was an overall genotype effect on outer duration [$F(1, 48) = 7.636$, $p = 0.0081$] but no effect of genotype on the other measures (distance [$F(1, 48) = 2.653$, $p = 0.1099$]; speed [$F(1, 48) = 2.712$, $p = 0.1061$]; freezing [$F(1, 48) = 0.2001$, $p = 0.6567$]). Furthermore, there was no significant interaction of genotype and treatment (distance [$F(1, 48) = 0.2643$, $p = 0.6095$]; speed [$F(1, 48) = 0.2831$, $p = 0.5972$]; freezing

$[F(1, 48) = 0.4606, p = 0.5006]$; outer duration $[F(1, 48) = 0.4566, p = 0.5024]$). In summary, SE did not provoke changes in locomotion or anxiety-like behaviors in either WT or C3KO mice.

Levels of Pre-Synaptic Excitatory Proteins in the Hippocampus Correlate With Levels of C3bα and iC3b in the Chronic Epilepsy Phase

SE is widely known to cause damage to hippocampal structural and functional proteins. To determine if blocking C3 can attenuate SE-induced hippocampal synaptic protein loss, we used western blotting to measure hippocampal levels of post synaptic marker PSD95, and the presynaptic excitatory and inhibitory markers, Vglut1 and Vgat, respectively. We chose two time points to examine: 2 weeks after SE, which represents the end of the latent period and is the time point during which we have observed the most extensive damage in the rat model (Figure 6); and 5 weeks after SE, which represents the chronic epilepsy phase, during which there is not a robust immune response, but it is the time during which spontaneous seizures are expected to occur (Figure 7) (Schartz et al., 2016; Schartz et al., 2018).

As expected, at 2 weeks after SE, there was a difference in C3bα and iC3b levels based on the genotype (C3bα, $[F(1, 18) = 93.81, p < 0.0001]$, iC3b $[F(1, 18) = 42.30, p < 0.0001]$). This difference was driven by the lack of C3 protein in the C3KO mice (Figure 1C). However, there was no effect of treatment (C3bα, $[F(1, 18) = 3.296, p = 0.0862]$, iC3b $[F(1, 18) = 0.2390, p = 0.6308]$), and no interaction of treatment and genotype (C3bα, $[F(1, 18) = 0.0441, p = 0.8359]$, iC3b $[F(1, 18) = 1.303, p = 0.2686]$) (Figure 6A-C). This would suggest that either C3 is not elevated in the mouse at 2 weeks after SE, or that pilocarpine-induced SE did not produce a sufficient injury to trigger activation of the

complement pathway. This could also be due to a different time-course of complement activation after SE differs from the rat model, and perhaps we missed a time window of transient complement activation in the mouse. We also measured levels of hippocampal synaptic proteins 2 weeks after SE. There was no effect on the levels of PSD95 by genotype [$F(1, 20) = 0.2182$, $p = 0.6455$], treatment [$F(1, 20) = 4.249$, $p = 0.0525$], and no interaction of genotype and treatment [$F(1, 20) = 0.07163$, $p = 0.7917$] (Figure 6D). Similarly, when we measured the excitatory vesicular protein Vglut1 at 2 weeks after SE, we did not observe an effect of genotype [$F(1, 19) = 0.5112$, $p = 0.6455$], treatment [$F(1, 19) = 1.190$, $p = 0.2890$], and no interaction [$F(1, 19) = 1.154$, $p = 0.2962$] (Figure 6E). We also did not observe any effects on the inhibitory GABA transporter Vgat by genotype [$F(1, 20) = 3.712$, $p = 0.0684$], treatment [$F(1, 20) = 0.4248$, $p = 0.5220$], and no significant interaction of treatment and genotype [$F(1, 20) = 0.5481$, $p = 0.4677$] (Figure 6F). Furthermore, among the WT mice, there was no correlation between the signal intensity of C3bα and PSD95 within the same samples ($r = -0.2446$, $p = 0.4685$) or Vglut1 ($r = -0.1673$, $p = 0.6229$). There was, however a significant positive correlation between C3bα and the inhibitory synaptic marker Vgat ($r = 0.3901$, $p = 0.2356$) (Figure 6G). There was no correlation between iC3b and PSD95 ($r = -0.2387$, $p = 0.5067$), Vglut1 ($r = -0.0714$, $p = 0.98058445$ or Vgat ($r = -0.0951$, $p = 0.7939$) (Figure 6H).

At 5 weeks after SE, the difference in C3bα and iC3b levels by genotype was still significant (C3bα, [$F(1, 17) = 5.988$, $p = 0.0256$]; iC3b, [$F(1, 17) = 4.647$, $p = 0.0457$]). There was however, no effect of treatment (C3bα, [$F(1, 17) = 0.9914$, $p = 0.3334$]; iC3b, [$F(1, 17) = 0.3285$, $p = 0.5741$]) and no interaction of genotype by treatment (C3bα, [$F(1, 17) = 0.0002$, $p = 0.9888$]; iC3b, [$F(1, 17) = 0.4341$, $p = 0.5188$]). The WT-C and

WT-SE mice did not have different levels of C3bα ($p = 0.6313$) or iC3b ($p = 0.9996$)

protein levels in the hippocampus (Figure 7A-C). Furthermore, when we measured

hippocampal synaptic protein levels at 5 weeks after SE, we did not see an effect on

PSD95 by genotype [$F(1, 24) = 1.749$, $p = 0.1985$], treatment [$F(1, 24) = 0.5759$, $p =$

0.4553], and no interaction [$F(1, 24) = 2.026$, $p = 0.1675$] (Figure 7D). Similarly, when

we measured the excitatory vesicular protein Vglut1 at 2 weeks after SE, we did not

observe an effect of treatment [$F(1, 23) = 0.0456$, $p = 0.8328$], or an interaction

interaction [$F(1, 23) = 0.4628$, $p = 0.5031$]. There was, however, a significant effect of

genotype on Vglut1 levels at 5 weeks post-SE [$F(1, 23) = 5.140$, $p = 0.0331$] (Figure 7E).

There was a significant effect of treatment on Vgat levels [$F(1, 11) = 6.174$, $p = 0.0303$],

but no effect of genotype [$F(1, 11) = 0.1408$, $p = 0.7146$] and no interaction [$F(1, 11) =$

0.7985, $p = 0.3907$]. (Figure 7F).

Because complement activation can promote the elimination of synaptic proteins

(Schafer et al., 2012; Stevens et al., 2007; Vasek et al., 2016), we measured the

correlation between C3bα and iC3b and synaptic proteins at 5 weeks after SE. There was

no correlation between C3bα and PSD95 ($r = -0.4306$, $p = 0.2142$). There was a

significant positive correlation between C3bα and Vglut1 ($r = 0.7012$, $p = 0.0239$) and

Vgat ($r = 0.8175$, $p = 0.0469$) protein levels in the hippocampus within the same mouse

(Figure 7G). Similarly, there was no correlation between iC3b and PSD95 ($r = -0.3357$, p

$= 0.3429$). There was, however, a significant positive correlation between iC3b and

Vglut1 ($r = 0.7732$, $p = 0.0087$), but not with Vgat ($r = 0.5083$, $p = 0.3032$) (Figure 7H).

These data suggest that in the chronic epilepsy phase in mice, C3 activation may promote

the survival of excitatory synapses over inhibitory ones, thus promoting a hyperexcitable

network.

RAT EXPERIMENTS

Dose Response: CVF Reduces Levels of C3 Protein Levels in the Mouse and Rat Hippocampus

The C3KO mice serve as an important tool to determine the role of complement activation in SE-induced memory deficits and hippocampal injury. However, since the mice lack C3 from conception, there may be developmental compensatory mechanisms that we are not aware of that may result in differences in neuronal connectivity. To address this limitation, we used an alternative method to blocking C3. We used cobra venom factor (CVF) injected systemically (i.p.) in controls or after SE (Figure 8-9). CVF is a glycoprotein found in cobra venom that cleaves the complement C3 protein. CVF is a functional homolog of C3b, which binds factor B to create a C3 convertase (Vogel, Fritzinger, Hew, Thorne, & Bammert, 2004). In contrast to naturally occurring C3 convertase CVF bound to factor B has a very long half-life and is resistant to regulatory complement factors, thus resulting the cleavage of the C3 protein until it is depleted (Vogel & Fritzinger, 2010).

The objective of our first experiment was to confirm that CVF blocks C3 in the mouse brain, and determine the longevity of the effects in the brain and blood of mice (Figure 8A-C). We injected 100 ng/g of CVF or equal volume of 0.9% saline (i.p.) in mice and collected hippocampi and blood samples at 1, 3, and 6 days after injection to measure C3 levels in the brain with western blot and serum with ELISA. There was a significant effect on C3bα of treatment (CVF vs vehicle) [$F(1, 6) = 124.8, p < 0.0001$], day [$F(2, 6) = 244.5, p < 0.0001$], and a significant interaction [$F(2, 6) = 244.5, p < 0.0001$]. There was also an effect on iC3b by treatment [$F(1, 6) = 59.78, p = 0.0002$] and

day [$F(2, 6) = 21.76$, $p = 0.0018$], and a significant interaction [$F(2, 6) = 21.77$, $p = 0.0018$]. Using ELISA we observed a significant effect of treatment on levels of serum C3 [$F(1, 16) = 30.83$, $p < 0.0001$], but no effect of day [$F(2, 16) = 2.824$, $p = 0.0890$]. However, there was a significant interaction between treatment and day of serum collection [$F(2, 16) = 9.257$, $p = 0.0021$]. Both the CVF samples collected at 1 day and at 3 days had decreased levels of C3 compared to their corresponding controls (1 day: $p = 0.0002$; 3 days: $p = 0.0096$). However, the serum C3 levels of the samples collected at 6 days after CVF injection did not differ from the controls ($p > 0.9999$), suggesting that the effect of CVF wears off by this time point.

Most of the preliminary data used for characterizing behavioral deficits and complement activation after SE was collected from the rat model. Therefore, we decided to verify if CVF could also block C3 in these rats using two previously reported doses (100 ng/g and 500 ng/g) (Figueroa, Gordon, Feldhoff, & Lassiter, 2005; Vasthare et al., 1998). We verified that C3bα and iC3b levels were decreased in the hippocampus at 1 and 3 days after CVF injection. There was a significant effect of dose [$F(2, 12) = 18.49$, $p = 0.0002$] but no effect of day [$F(1, 12) = 1.504$, $p = 0.2436$] and no interaction of dose and day [$F(2, 12) = 3.731$, $p = 0.0549$] on C3bα protein levels. Similarly, levels of iC3b were affected by dose [$F(2, 12) = 7.098$, $p = 0.0092$], but not day [$F(1, 12) = 2.224$, $p = 0.1617$], and no interaction [$F(2, 12) = 1.250$, $p = 0.3214$]. There was a main effect of dose, with the higher CVF doses resulting in lower C3 levels. In summary, there was a 45% reduction in C3b levels one day after 100ng/g of CVF and 34% reduction by day 3, compared to 80% and 45% reduction, respectively, with the 500ng/g dose. We used an ELISA kit to measure circulating levels of C3 in the serum. There was a significant effect

of dose [$F(2, 11) = 506.1$, $p < 0.0001$] on serum C3 levels. Both doses were sufficient to reduce C3 levels at 1 day after injection (100 ng/g: $p < 0.0001$; 500 ng/g: $p < 0.0001$) and at 3 days after injection (100 ng/g: $p < 0.0001$; 500 ng/g: $p < 0.0001$) compared to the controls. However, there was no significant effect of the day at blood collection [$F(1, 11) = 4.820$, $p = 0.0505$] and no interaction of dose and day [$F(2, 11) = 3.259$, $p = 0.0774$]. Because the lower dose was sufficient to reduce C3b levels in the hippocampus, for the remainder of the experiments we used 100 ng/g of CVF.

The objective of the next study was to determine if CVF can alter C3 levels after SE and if C3 levels remain low after multiple injections (Figure 9). Our preliminary study determined that C3 levels remain low up to 3 days after CVF injection (Figure 8), so we decided to inject CVF every third day. Each day before injecting CVF or vehicle, we took a blood sample to determine baseline C3 levels. We found that C3 levels were reduced in the blood at each time-point after CVF injection, whereas there was not a significant change in serum C3 levels over time in the veh-treated rats (Figure 9B). There was a significant effect of the day the serum was collected [$F(3, 18) = 14.55$, $p < 0.0001$], as well as an effect of CVF treatment [$F(1, 10) = 17.66$, $p = 0.0018$], but no effect of the group (C vs SE) [$F(1, 6) = 0.5550$, $p = 0.4844$]. Furthermore, there was an interaction between the day and drug (veh vs. CVF) [$F(3, 10) = 8.401$, $p = 0.0044$], There was no interaction between day and group [$F(3, 10) = 0.8145$, $p = 0.5146$] or group and drug [$F(1, 10) = 0.0007$, $p = 0.9794$] and no three-way interaction [$F(3, 10) = 1.075$, $p = 0.4030$]. As expected, serum levels of C3 did not significantly change over time in the control-veh group when comparing levels from day 7 to day 10 ($p > 0.9999$), 13 ($p = 0.9971$), or day 20 ($p = 0.7972$). This was the same for the SE group treated with vehicle

(day 7 vs 10 $p > 0.9999$; 7 vs 13 $p = 0.9791$; 7 vs 20 $p = 0.4711$). Control-CVF rats had

reduced C3 levels when comparing serum from day 7 (before any CVF injection) to day

10 ($p = 0.0387$), to day 13 ($p = 0.0359$) but not to day 20 ($p = 0.1976$), consistent with

our previous findings in mice that the effects of CVF are no longer evident after 6 days.

Interestingly, in this cohort, there were no changes in C3 serum levels in the SE-CVF

group when compared to the baseline on day 7 (day 7 vs 10 $p = 0.3949$; 7 vs 13 $p = $

0.0960; 7 vs 20 $p = 0.5388$). This might be due to the high variance of the C3 levels on

days 10 and 13, which may be indicative of an antibody response to multiple CVF

injections (Ing et al., 2018).

Rats Treated With CVF Following SE do not Display SE-Induced Recognition

Memory Deficits

For the remainder of our experiments, rats were treated with either vehicle or

CVF on days 7, 10, and 13 after pilocarpine or saline treatment. On days 14 and 15, rats

were challenged with open field and novel object recognition. To determine if CVF

treatment had an effect on SE-induced recognition memory deficits, rats were tested with

NOR (Figure 10). During the familiarization trial, rats were exposed to two similar object

for 5 minutes, and exploration of each object was recorded and compared (Figure 10A).

As expected, during familiarization, no groups showed preferential exploration of the

right or left object (control-veh, [$t(9) = 0.8749$, $p = 0.4043$]; SE-veh, [$t(10) = 0.8627$, $p = $

0.4085]; control-CVF, [$t(11) = 0.0086$, $p = 0.9933$]; SE-CVF, [$t(6) = 1.026$, $p = 0.3443$]).

We also compared the DI between groups (Figure 10B) and found no statistically

significant difference between treatment group [$F(1, 37) = 0.1303$, $p = 0.7202$], drug

[$F(1, 37) = 1.751$, $p = 0.1939$], and no significant interaction [$F(1, 37) = 0.6332$, $p = $

0.4313], and using a one-sample t-test revealed that all of the DI's were not different from zero (control-Veh, [$t(9) = 0.8404$, $p = 0.4224$]; SE-Veh, [$t(10) = 0.8630$, $p = 0.4084$]; control-CVF, [$t(11) = 0.0081$, $p = 0.9937$]; SE-CVF, [$t(7) = 1.1.336$, $p = 0.2142$]), suggesting that there was no discrimination by any group of the two similar objects. We also measured the total time spent exploring objects during the familiarization trial (Figure 10C) and found no effect of treatment [$F(1, 37) = 1.492$, $p = 0.2297$], or drug [$F(1, 37) = 0.04358$, $p = 0.8358$], but we did observe a significant interaction of treatment group and drug [$F(1, 37) = 4.800$, $p = 0.0348$]. Individual rats who showed a preference to one object during trial one were removed from further analyses in NOR. This resulted in the exclusion of two vehicle-treated controls and three CVF-treated SE rats.

The test trial occurred 2 hours after familiarization (Figure 10D-F), based on the timeline established by previous studies (Bevins & Besheer, 2006; Schartz et al., 2018). During the test trial, one of the previously explored objects was replaced by a novel object and rats were placed back in the apparatus and allowed to explore for 5 minutes. First, we compared the percent exploration time of the novel vs. familiar objects for each group (Figure 10D). During trial 2, the vehicle-treated controls spent more time exploring the novel object [$t(9) = 2.456$, $p = 0.0364$] compared to the familiar object. As reported in previous studies, the SE rats that were treated with vehicle did not show a significant preference for the novel object [$t(10) = 1.889$, $p = 0.0883$]. Similarly to the veh-treated controls, the CVF-treated controls showed a significant preference for the novel object over the familiar object [$t(11) = 2.926$, $p = 0.0138$], suggesting that their recognition memory is not altered by CVF injections. Interestingly, the SE rats that were treated with

CVF also showed a significant preference for exploration of the novel object during trial 2 [$t(67) = 5.981$, $p = 0.001$]. When comparing the DI, there was no effect of treatment [$F(1, 37) = 0.0992$, $p = 0.7545$], drug [$F(1, 37) = 0.5661$, $p = 0.4566$], and no significant interaction [$F(1, 37) = 0.0866$, $p = 0.7702$]. One sample t-test showed that all the DI's were different from zero except the SE-veh group (control-veh, [$t(9) = 2.490$, $p = 0.0344$]; SE-veh, [$t(10) = 1.768$, $p = 0.1075$]; control-CVF, [$t(11) = 2.296$, $p = 0.0138$]; SE-CVF, [$t(7) = 2.327$, $p = 0.0528$]). Finally, there was no effect of treatment [$F(1, 37) = 2.227$, $p = 0.1441$], drug [$F(1, 37) = 1.205$, $p = 0.2794$], and no significant interaction [$F(1, 37) = 0.0916$, $p = 0.7639$] on the total time spent exploring both the novel and familiar object during the test trial.

SE Does not Alter locomotion or Anxiety-Like Behaviors in Rats After Two Weeks

The OF trial served both as a habituation to the NOR apparatus and a control for locomotion and anxiety-like behaviors (Figure 11). Rats were placed in the NOR chamber 24 hours before NOR for 10 minutes under red light and were recorded from above. Any-maze software was used to measure distance travelled (Figure 11A), average speed (Figure 11B), time spent freezing (Figure 11C), and the percent of time spent in the outer perimeter of the arena (Figure 11D). There was no effect of treatment group (control vs SE) [$F(1, 42) = 0.6336$, $p = 0.4305$], drug (vehicle vs CVF) [$F(1, 42) = 1.027$, $p = 0.3166$], and no interaction [$F(1, 42) = 0.3767$, $p = 0.5427$] on distance travelled. There were also no changes in average speed (treatment: [$F(1, 42) = 0.5253$, $p = 0.4726$]; drug: [$F 1, 42) = 1.045$, $p = 0.3125$]; interaction: [$F(1, 42) = 0.4572$, $p = 0.5026$]) or freezing time (treatment: [$F(1, 42) = 0.8324$, $p = 0.3668$]; drug: [$F(1, 42) = 2.064$, $p = 0.1583$]; interaction: [$F(1, 42) = 0.001485$, $p = 0.9694$]). However, there was an effect of

treatment on the time spent in the outer perimeter [$F(1, 42) = 4.752$, $p = 0.0349$], with no effect of drug [$F(1, 42) = 2.326$, $p = 0.1347$] and no significant interaction [$F(1, 42) = 2.690$, $p = 0.1085$].

CVF Prevents Loss of Hippocampal Synaptic Proteins After SE

To determine the effects of CVF on hippocampal synaptic protein levels after SE, we used western blotting (Figure 12). We measured the protein levels of C3bα and iC3b along with post-synaptic protein PSD95 and the inhibitory and excitatory pre-synaptic proteins Vgat and Vglut, respectively (Figure 12A). When we measured levels of C3bα and iC3b in the hippocampus, we did not observe an effect of the CVF treatment (C3bα, [$F(1, 29) = 0.1037$, $p = 0.7497$] iC3b, [$F(1, 29) = 0.7216$, $p = 0.4026$]) (Figure 12B-C). There was, however, an effect of treatment on levels of C3bα [$F(1, 29) = 22.45$, $p < 0.0001$] and iC3b [$F(1, 29) = 23.67$, $p < 0.0001$]. However, there was no significant interaction between CVF treatment and SE on C3bα [$F(1, 29) = 0.063$, $p = 0.8036$] or iC3b [$F(1, 29) = 0.4145$, $p = 0.5248$] levels. There was a main effect of pilocarpine treatment, with the SE rats having higher C3bα and iC3b levels compared to sham-treated controls.

SE and epilepsy can alter synaptic spine morphology and result in loss of synaptic proteins to promote synaptic dysfunction (González, Cruz Del Angel, & Brooks-Kayal, 2013; Wong & Guo, 2013). PSD95 was significantly reduced in veh-treated SE rats compared to veh-treated controls ($p = 0.0045$), and reduction was not observed in CVF-treated SE rats compared to CVF-treated controls ($p = 0.3370$) (Figure 12D). We found an effect on PSD95 of treatment (control or SE) [$F(1, 28) = 6.569$, $p = 0.0160$], but no effect of drug used (vehicle or CVF) [$F(1, 28) = 0.6074$, $p = 0.4423$] and no interaction

$[F(1, 28) = 0.7261, p = 0.4014]$. When we measured Vglut1 levels, there appeared to be a decrease in Vglut1 protein levels in the hippocampus of veh-treated SE rats compared to veh-treated controls. However, there was not a significant effect on Vglut1 based on treatment $[F(1, 28) = 1.790, p = 0.1916]$, drug $[F(1, 28) = 1.287, p = 0.2663]$, and no interaction $[F(1, 28) = 2.402, p = 0.1324]$ (Figure 12E). These findings suggest that the excitatory protein Vglut1 is not altered after SE in this study. Interestingly, after SE, there was a significant reduction in the protein levels of the inhibitory synaptic element Vgat in the hippocampus of veh-treated SE rats compared to veh-treated controls ($p = 0.0475$). This difference was not observed in CVF-treated SE and control rats ($p = 0.6686$) (Figure 12F). Overall, there was a significant effect of treatment $[F(1, 28) = 7.823, p = 0.0092]$, but no effect of drug $[F(1, 28) = 0.4641, p = 0.5013]$ and no interaction $[F(1, 28) = 1.567, p = 0.2210]$. These findings support that CVF treatment is protective and prevents SE-induced decreases in the levels of PSD95 and Vgat. Our findings suggest that some synaptic proteins may be more vulnerable to SE-induced decline, but treatment with CVF can prevent such loss.

Finally, because complement activation can mediate the elimination of synaptic elements, we determined the correlation between C3bα and iC3b levels with levels of PSD95, Vgat, and Vglut1 (Figure 12G-J). With all the samples grouped, there was no correlation between C3bα and PSD95 ($r = -0.3481, p = 0.0550$) or Vglut1 ($r = 0.0526, p = 0.7788$), but there was a significant inverse correlation between C3bα and Vgat ($r = -0.5461, p = 0.0015$). We then teased apart the treatment groups to determine if one particular treatment was influencing this correlation. When grouped only by vehicle or CVF treatment, the veh-treated rats had a significant negative correlation of C3bα and

PSD95 ($r = -0.5669$, $p = 0.0220$) and Vgat ($r = -0.6357$, $p = 0.0081$), but no correlation between C3bα and Vglut1 ($r = -0.2995$, $p = 0.2598$) (Figure 12G). The CVF-treated rats had a significant inverse correlation of C3bα and Vgat (r = -0.5765, $p = 0.0309$), but no correlation between C3bα and PSD95 (r = -0.1213, $p = 0.6796$), or Vglut1 (r = 0.4423, $p = 0.1133$), (Figure 12H).

When we analyzed the correlation of iC3b with synaptic proteins in all samples, we observed a significant inverse correlation between iC3b and Vgat ($r = -0.5079$, $p = 0.0035$), but no correlation with PSD95 ($r = -0.3134$, $p = 0.0861$) or Vglut1 ($r = 0.0294$, $p = 0.8751$). Similarly, there was no correlation between iC3b and PSD95 ($r = -0.3134$, $p = 0.0861$) or Vglut1 ($r = 0.0294$, $p = 0.8751$) in the hippocampi of veh-treated rats (Figure 12I). However there was a significant inverse correlation of iC3b and Vgat ($r = -0.5079$, $p = 0.0035$). The CVF-treated rats did not show a correlation between iC3b and PSD95 ($r = -0.4305$, $p = 0.0960$) or Vglut1 ($r = -0.1764$, $p = 0.5133$), but did have a significant inverse correlation between iC3b and Vgat ($r = -0.6677$, $p = 0.0047$) (Figure 12J). These data suggest that that there is a relationship between C3 levels and loss of inhibitory synaptic markers such as Vgat, but with CVF treatment, this relationship is no longer present. This suggests that in the rat model of pilocarpine-induced SE, C3 activation may be promoting the loss of inhibitory synaptic transporters, and blocking C3 activation with CVF prevents the targeted elimination of Vgat.

DISCUSSION

One of the greatest concerns of individuals with epilepsy are cognitive deficits that often accompany the disorder (Demin et al., 2018; Paudel, Shaikh, Shah, Kumari, & Othman, 2018). However, currently available antiepileptic drugs do little to address these concerns and several AEDs have side-effects that exacerbate learning and memory disturbances (Eddy, Rickards, & Cavanna, 2011). This highlights a need for the discovery of novel therapeutic targets for epilepsy that can attenuate both seizures and cognitive deficits. The present study provides evidence that increased signaling through the central component of the complement pathway (C3) contributes to spatial learning and memory deficit associated with SE. We found that inhibition of C3 is protective against memory deficits in two different rodent models of SE. Using the pilocarpine model of SE and acquired TLE in rats and mice, we targeted the latent period that follows SE to determine if blocking complement activation could prevent the associated hippocampal injury and memory deficits. First, we used a genetic model of C3KO mice which are commercially available through Jackson Labs. We found that wild-type mice developed recognition memory deficits following an episode of SE, whereas mice lacking C3 protein did not develop this deficit (Figure 2). These data suggest that activation of the C3 signaling cascade participates in the mechanisms underlying memory in the NOR test in mice. In our experiments, hippocampal levels of excitatory synaptic protein Vglut1 had a positive correlation with C3bα and iC3b protein levels. Thus, it is possible that complement activation in WT mice promotes increased excitatory synaptic transmission. However, since these mice are constitutive knockouts, it is also possible that the observed effect is due to some undetermined compensatory mechanism. Therefore, to confirm our findings

we used alternative pharmacological approach to reduce C3 signaling with the C3

inhibitor CVF. CVF was used in the rat model because our previous studies thoroughly

characterized SE-induced hippocampal changes such as neuronal injury, microgliosis,

dendritic protein loss, synaptic protein loss, and complement activation in male Sprague

Dawley rats (Schartz et al., 2016; Schartz et al., 2018). Using the established rat

pilocarpine model, we obtained evidence of a potential role for C3 in the associated

cognitive pathophysiology. We found that rats treated with CVF after SE did not have

recognition memory deficits (Figure 10). We also determined that hippocampal synaptic

protein levels correlate with increased C3 protein levels. Specifically, in the mouse, C3

was positively correlated with excitatory Vglut1 (Figure 7), and in the rat, increased C3

levels were negatively correlated with the inhibitory protein Vgat (Figure 12). Both of

these findings support that seizure-induced C3 activation is associated with the

modulation of hippocampal synaptic proteins, and may either increase excitation by

promoting survival of excitatory synaptic structures, or by promoting the elimination of

inhibitory synaptic proteins. Taken together, these findings are the first evidence that

identifies a strong association between complement C3 activation and memory deficits as

well as hippocampal synaptic protein loss in a model of SE and acquired TLE.

Identifying the underlying mechanisms that results in seizure-induced cognitive

deficits and synaptic remodeling is a major focus of epilepsy research. One candidate

mechanism is the classical complement pathway. Recent studies support that the

complement proteins C1q and C3 play a role in microglial-mediated synaptic refinement

and elimination during development and in models of neurodegenerative disorders (Hong

et al., 2016; Schafer et al., 2012; Stevens et al., 2007; Vasek et al., 2016). In the mouse

developing visual system C1q and C3 complement proteins are linked to the microglia-mediated phagocytosis of synapses (Schafer et al., 2012; Schafer, Lehrman, & Stevens, 2013; Stephan et al., 2012; Stevens et al., 2007). Elimination of C3 or the C3 receptor in microglia results in inefficient pruning during development and inhibits synaptic refinement that is necessary for normal function (Schafer et al., 2012; Stevens et al., 2007). In contrast, higher levels of C3 protein are associated with exacerbated losses of hippocampal synapses in mouse models of AD (Hong et al., 2016) and West Nile virus neuroinvasive disease (Vasek et al., 2016). Blocking complement C3 in these models, either by genetic manipulation or pharmacologically, results in the protection of synaptic proteins and cognitive function (Hong et al., 2016; Shi et al., 2015; Vasek et al., 2016). In addition, C3 protein is associated with protection against age-related cognitive decline in mice (Perez-Alcazar et al., 2014). Because increased C1q-C3 complement activation is linked to deficits in spatial memory, cognitive flexibility (reversal learning), recognition memory (NOR), and age-dependent memory decline in rodent models of neurodegeneration (Hernandez et al., 2017; Shi et al., 2015), we hypothesized that eliminating C3 in the pilocarpine model of SE would attenuate memory deficits.

The observation that SE results in recognition memory deficits have been widely replicated in the epilepsy field (Brewster et al., 2013; Oliveira et al., 2015; Pearson et al., 2014; Schartz et al., 2018). Lesion studies demonstrated that damage to the dorsal hippocampus alone is sufficient to produce a deficit in recognition memory (Broadbent et al., 2004). A single, brief flurothyl-induced seizure can produce acute recognition memory deficits (Holley et al., 2018). Recognition memory deficit after SE is associated with hippocampal injury including gliosis and cell loss. Reinstating hippocampal cells

using neural stem cell grafts is sufficient to attenuate memory deficits in the NOR test (Hattiangady & Shetty, 2012), suggesting that hippocampal injury precedes learning and memory deficits. Given that it is possible to attenuate hippocampal injury after SE and in epilepsy, perhaps the associated memory deficits can also be reversed. If this is the case, delayed treatment with a complement inhibitor can still be effective. In the present study, we observed SE-induced deficits in the NOR test that were accompanied by hippocampal decline of synaptic proteins (Figures 2, 6-8, 12). However, elimination of C3 either via genetic knockout or CVF-induced depletion prevented the memory deficits and the loss of some hippocampal synaptic proteins in animals that sustained SE. Our findings are supported by a previous report showing that that the attenuation of SE-induced hippocampal synaptodendritic injury paralleled significant improvements in the associated memory deficits (Brewster et al., 2013).

Although the NOR test showed SE-induced memory deficits in both rats and mice, we did not observe a deficit in spatial memory with the Barnes maze in mice. All mice from every experimental group were able to learn to navigate the BM to find the escape box faster over time (Figure 4).We did not observe an effect of SE on BM escape latency. The BM is a modified version of the Morris water maze (MWM), which is more widely used in the field of epilepsy (Hattiangady & Shetty, 2012; Shetty, 2014). We decided to use the BM instead of MWM because of the potential for added stress to mice that may experience spontaneous seizures and the risk of drowning. We previously used BM to measure spatial memory in rats after SE and were able to detect a significant deficit between SE and control rats (Schartz et al., 2018). In fact, the objective to test hippocampal-dependent spatial learning and memory for the BM is not very different

from the MWM. The difference is that BM uses a flat table with a hidden box instead of a small pool of opaque water with a hidden platform as the MWM. The MWM, however, elicits a stronger stress response from rodents, thus it is possible that the BM test was not stressful enough for our mice to drive them to seek shelter from bright lights in the escape box. Since the MWM has been used to identify hippocampal-dependent spatial learning and memory deficits in mice and rats after SE (Brewster et al., 2013; Gröticke, Hoffmann, & Löscher, 2008; Murphy, 2013; Patil, Sunyer, Hoger, & Lubec, 2009), it is possible that the BM is not the appropriate test to use in epileptic mice.

Spatial cognitive deficits are correlated with decreased hippocampal cell density (Edalatmanesh, Nikfarjam, Vafaee, & Moghadas, 2013; Schipper et al., 2016). We did not measure cell loss in mice and thereby it is possible that mice don't have as much SE-induced cell loss as that observed in rats (Schartz et al., 2016). In our earlier studies with rats and BM, rats that sustained SE had decreased Map2-stained dendrites in the dorso-ventral CA1 hippocampal region and this was associated with spatial learning and memory deficits in the BM. This could be one additional potential reason for the deficit being present in rats but not mice. In future studies, we will determine if there are SE-induced changes in the spatiotemporal distribution of Map2 as well as other synaptic and neuronal markers in mouse models of acquired epilepsy.

Studies of human resected epileptic brain tissues have found a decrease in neuronal and dendritic markers (Dachet et al., 2015; Morin-Brureau et al., 2018; Wyatt et al., 2017), as well as specific synaptic markers such as synaptic vesicle protein 2 (SV2) (Crèvecœur et al., 2014). These neuronal alterations parallel an increase in markers of reactive microglia (Dachet et al., 2015; Morin-Brureau et al., 2018). Similarly, SE results

in changes to hippocampal structural proteins in rodent models. SE results in decreased

synaptic density and morphological changes to dendrites and dendritic spines (Brewster

et al., 2013; Schartz et al., 2016; Swann et al., 2000; Wong, 2005; Wong & Guo, 2013).

Previous studies of pilocarpine-induced SE and acquired epilepsy reported a significant

reduction in GABA associated proteins in the CA1 region of the hippocampus, resulting

in impaired inhibitory neurotransmission (González et al., 2013). Interestingly, in this

study the reduction of GABAergic proteins does not correlate with neuronal loss,

suggesting that mechanisms other than cell death are responsible for the synaptic

modulation (González et al., 2013).

Similarly, we found that SE leads to a significant decrease in the inhibitory

vesicle transport protein Vgat that correlates with increased levels of C3, and that

treatment with CVF is sufficient to prevent this loss (Figure 12). Interestingly, previous

studies have found evidence that lipopolysaccharide (LPS)-triggered inflammation results

in activation of microglia and increased number of microglial-neuronal contacts that was

associated with a decrease in inhibitory synaptic proteins (Chen et al., 2014). In this case,

it is possible that SE prompts C3 proteins to tag inhibitory synapses for elimination by

microglia thus resulting in an imbalance of inhibition and excitation that may result in

neuronal hyperexcitability. The attenuation in the SE-induced loss of inhibitory synaptic

proteins such as Vgat in the CVF-treated SE rats suggests that C3b activation contributes

to the mechanisms underlying the memory deficits.

Neurotransmitter release is dependent on presynaptic transport proteins such as

Vgat and Vglut that load neurotransmitters into vesicles. The amount of transporters

influences the amount of neurotransmitters loaded, and thus directly effects the efficacy

of neurotransmission (Wojcik et al., 2004). In the adult rodent brain, Vglut1 is predominately distributed in the cerebellar cortices and the hippocampus, and elimination of Vglut1 results in significantly reduced EPSC amplitudes (Wojcik et al., 2004). Similarly, overexpression of Vglut1 results in increased EPSC amplitude (Liguz-Lecznar & Skangiel-Kramska, 2007). GABAergic transmission plays critical roles in regulating network activity, cognition, and movement (Saito et al., 2010). In a model of post-traumatic epilepsy, injury to cortical structures results in reduction in levels of the GABA precursor GAD65 as well as the vesicular GABAergic transporter Vgat (Gu et al., 2017). This model also results in structural changes to the axonal terminals and dendrites, without a significant loss of cell numbers, supporting that post-traumatic synaptic remodeling can promote epileptogenesis. Therefore, in the rat model of pilocarpine-induced SE, significant loss of Vgat (Fig. 12) may promote hyperexcitability by downregulating inhibition and lead to cognitive deficits.

We previously reported that higher seizure frequency is significantly correlated with increased hippocampal protein levels of iC3b (Schartz et al., 2018). The findings by Kharatishvili et al (2014) further support that increased levels of C3 in the hippocampus correlates with increased spike frequency in epileptic mice (Kharatishvili et al., 2014) In addition, mice that lack the complement proteins C3 are resistant to developing seizures when challenged with viral infections (Libbey et al., 2010). Similar observations have been reported when blocking C5 activation with pharmacogenetic approaches. For example, C5KO mice are resistant to seizures provoked by cerebral malaria (Buckingham, Ramos, & Barnum, 2014), and inhibition of C5a receptor (C5ar1) decreases the severity of seizures in a rodent model of acquired epilepsy (Benson et al.,

2015). These data support that aberrant signaling of the classical complement pathway may play a role epileptogenic mechanisms.

Complement activation has been observed in many models of brain injury or neurodegenerative diseases. The hypothesis that complement contributes to neuronal injury has been tested in several disease models. One effective tool to determine the role of complement activation in neuronal injury is CVF. CVF is a peptide that depletes complement C3/C5 when administered systemically (Vasthare et al., 1998; Vogel & Fritzinger, 2010; Vogel et al., 2004; Xi, Hua, Keep, Younger, & Hoff, 2001). CVF is composed of three protein chains and resembles the complement component C3c (Vogel & Fritzinger, 2010). CVF binds to factor B of the alternative pathway to form CVF,Bb and thus acts as a C3 and C5 convertase. However, CVF,Bb has different properties from naturally occurring C3 convertases, which makes it more efficient at depleting C3. First, naturally occurring C3 convertases, such as C3bBb have very short half-lives, making them active for a limited time. CVF,Bb has a half-live of about 7 hours, so it can cleave C3 and C5 for a longer time. Second, CVF,Bb is insensitive to complement inhibitors such as factor H and I. Lastly, CVF can cleave C5 in independently of C3, making it more efficient at cleaving complement C5. These properties make CVF efficient at completely depleting complement proteins in serum (Vogel et al., 2004). In a model of post-ischemic cerebral injury, pre-treatment with CVF resulted in a reduction of cerebral infarct volume and preservation of the cerebral parenchyma of rats that were subjected to occlusion of the middle cerebral artery (Figueroa et al., 2005). CVF treatment also improves post-ischemic blood flow (Vasthare et al., 1998). Furthermore, in a model of brain edema, treatment with CVF attenuated the recruitment of inflammatory cytokines

such as tumor necrosis factor α (TNF-α) (Xi et al., 2001). Interestingly, none of the previous studies that used CVF to determine the effects of complement on neuronal injury have assessed cognitive or behavioral outcomes. The present study is the first to provide evidence supporting that complement inhibition with CVF can attenuate memory deficits in an injury model (in this case SE).

Interestingly, in the current study, we observed that repeated CVF injections after SE does not deplete C3 levels in the brain. Although many studies that have used CVF report a decrease in injury outcomes (Cowell et al., 2003; Figueroa et al., 2005; Vasthare et al., 1998), only one measured levels of C3 (Cowell et al., 2003). Cowell et al reported that a single injection of CVF depletes C3 in the control neonatal brain one day after injection, but in the injured brain, this is not the case. It is possible that the bimodal effects of CVF treatment following brain injury may be due to restoration of blood-brain barrier (BBB) integrity. Peripherally-derived complement proteins such as C3 do not penetrate the into the brain parenchyma unless injury causes the BBB to become permeable (Alexander, 2018; A. Jacob & J. J. Alexander, 2014). It is known that seizures and SE can compromise BBB integrity, and this may allow peripherally-derived complement proteins to cross into the brain (Broekaart et al., 2018). Furthermore, aberrant complement activation is associated with damage to the BBB (reference?), thus higher levels of C3 after SE may exacerbate BBB damage.. Furthermore, blocking complement activation is associated with repair to BBB integrity (A. Jacob & J.J. Alexander, 2014). Thus, it is possible that acute CVF treatment after SE repairs the BBB, and does not allow subsequent administration of CVF to cross into the brain. This would suggest that increase in locally produced C3 in the brain may be neuroprotective, and that

C3 crossing the BBB after SE is detrimental. This would explain why we see an attenuation of synaptic protein loss and better cognitive performance in the CVF-treated SE rats even though there is no reduction of C3 levels. Future studies could implement a simple test of BBB integrity using Evan's Blue dye.

In addition to the BBB response, another factor that may influence the efficacy of CVF treatment is an immune response. Although CVF is extremely efficient at activating C3 until it is depleted, it is also capable of eliciting an antibody response. After a single injection of CVF, neutralizing antibodies are formed, rendering subsequent doses of CVF unreliable at depleting C3 (Ing et al., 2018). However, this limitation can be overcome with the use of humanized CVF. Humanized CVF was formed by replacing the C-terminal of C3 with the homologous sequence of CVF, resulting in a protein that cleaves C3 until depleted but does not result in an antibody response (Ing et al., 2018). Although using humanized CVF is a potentially viable alternative to natural CVF, it is not clear that it would be necessary. We did not successfully reduce C3 levels after SE, but we did reduce the negative outcomes of SE. Perhaps a more valuable alternative would be to search for the ideal time-point after SE to give a single dose of CVF to maximize its protective properties.

A major limitation of the present study is that we did not examine potential sex differences in SE-induced deficits. Epilepsy affects both men and women, but not equally. Women are more likely to have idiopathic epilepsy while men are more likely to have focal epilepsy (Doodipala Samba Reddy, 2017; Helen E. Scharfman & MacLusky, 2014). Furthermore, the hormonal fluctuations associated with the menstrual cycle can increase the risk of seizures in women with epilepsy (Doodipala Samba Reddy, 2017). In

animal models, male rodents have a lower seizure threshold than females (D. S. Reddy, 2009). In addition, there is a sexual dimorphic neuronal response to seizures. After pilocarpine-induced SE, females have regional loss of neurons, whereas neuronal loss is not evident in mice (Helen E. Scharfman & MacLusky, 2014). A study of male and female mice reported that female mice have similar sensitivity to pilocarpine and develop recognition memory deficits at a similar rate than males (Oliveira et al., 2015). However, the immune response can differ between males and females. For instance, in an animal model of obesity, both males and females had deficient macrophage responses, but males had lower phagocytic ability than females (Hunsche, de Toda, & De la Fuente, 2019). Similarly, a clinical study of prenatal stress exposure reported an inverse correlation between stress exposure and inflammatory cytokines during infection in boys but not girls, suggesting that the inflammatory response can be influenced by both early-life experiences and sex (Brunwasser et al., 2019). These findings provide a rationale to continue investigating the effects of complement inhibition after SE on females, as their response may be different than what we observed in males. Another limitation of this study is that we used homozygous pairing for breeding, creating homozygous WT and C3KO mice that are from independent lines. This can have developmental, environmental, and compensatory effects that are directly associated to their genetic backgrounds. Thus, future studies will crossbreed WT and C3KO mice to create a colony of animals from which we could use WT, KO, and heterozygous mice that are littermates and thereby reduce the genetic variability that can impact the pathophysiological responses to SE. Cognitive deficits are only a subset of comorbid conditions that can occur with epilepsy. Epilepsy is associated with psychiatric disorders such as anxiety and

depression and with autism spectrum disorders (ASD) (Ertem et al., 2017; Paudel et al., 2018). Approximately 30% of individuals with epilepsy also have ASD, making it a more common comorbidity than cognitive deficits (Buckley & Holmes, 2016; Jeste & Tuchman, 2015). The focus of our behavioral study was on learning and memory, however, due to our promising findings that C3 inhibition can protect against these deficits in rodent models of acquired epilepsy, we can now widen the scope to examine psychiatric and social behaviors. ASD and epilepsy have similar underlying pathologies, including hyperexcitability, altered synaptic spine morphology, and altered microglia, suggesting that they may be different sides of a spectrum of the same disease (Mazarati, Lewis, & Pittman, 2017). ASD, broadly defined, is a developmental disorder potentially caused by interruptions in synaptic refinement (Thomas, Davis, Karmiloff-Smith, Knowland, & Charman, 2016). Although a link between ASD and complement activation is not clear, there is evidence of increased circulating complement proteins in children with severe autism (Corbett et al., 2006).

Although the hippocampus is most vulnerable to seizure-induced injury, seizures can also lead to hippocampal-related network dysfunction. In the rat model, SE results in structural and functional deficits in the amygdala, thalamus, entorhinal cortex, perirhinal cortex, piriform and somatosensory cortex (Jiang et al., 2018; Schipper et al., 2016; Wang et al., 2017). These structures regulate memory, emotion, and executive functions. The perirhinal cortex, along with the hippocampus, is necessary for object recognition memory (Kesner, Ravindranathan, Jackson, Giles, & Chiba, 2001; Schipper et al., 2016). Memory deficits are not the only comorbid condition associated with epilepsy. Depression and anxiety are among the most common comorbidities of epilepsy, which

can affect the quality of life of epilepsy patients (Alonso-Vanegas et al., 2013; Stafstrom, 2014; Steiger & Jokeit, 2017). Therefore, examining brain structures that functionally associate with the hippocampus could give more insight to the pathology. Along with examining other behaviors that are not learning and memory related, such as social interaction or anxiety or depressive behaviors, it would be pertinent to examine the underlying structures that mediate such behaviors. In our previous studies, we observed increased immunoreactivity of C1q and IBA1 antibodies in the thalamus and amygdala, although we did not analyze these regions for statistical comparisons.

Finally, it is still unknown if inhibiting complement signaling can have an effect on the development of spontaneous seizures after an episode of SE. We were able to induce SE in the C3KO mice, suggesting that pilocarpine does not act through C3-dependent pathways to induce seizures. Due to the scope of the present study, we did not monitor our animals for spontaneous behavioral seizures. However, the increase in hippocampal C3 levels correlates with the number of spontaneous seizures that follow SE (Kharatishvili et al., 2014; Schartz et al., 2018), suggesting that there may be an interplay between seizure severity and complement activation. In addition, sequential administration of the components of MAC (C5-C9) is sufficient to induce seizures (Xiong et al., 2003), suggesting that activation of signals downstream of C3 are required for seizure development. Future studies will continue to investigate the potential of C3 inhibition in epilepsy, by testing with different pharmacological approaches with varying doses, timing (right after SE, before SE, or in the chronic epilepsy phase), and administration route of the treatments. We will also use long-term video monitoring and

electroencephalogram to detect and quantify spontaneous behavioral and electrographic seizures.

Taken together, these findings are the first to show an association between C3 activation and hippocampal and cognitive deficits in two rodent models of SE and acquired TLE. We found that depletion of C3 is sufficient to attenuate SE-induced deficits in NOR-evaluated recognition memory and changes in inhibitory synaptic proteins. In conclusion, our data strongly support that SE-induced complement C3 activation contributes to hippocampal synaptic remodeling and impairments in recognition memory, and suggest that the complement C3 may be a potential therapeutic target for the memory comorbidities in epilepsy. Future studies will determine the effect of C3 inhibition on spontaneous recurrent seizures, and whether C3-guided and microglial-dependent phagocytosis is an underlying mechanism for the SE-induced epileptogenic synaptic remodeling.

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APPENDIX A
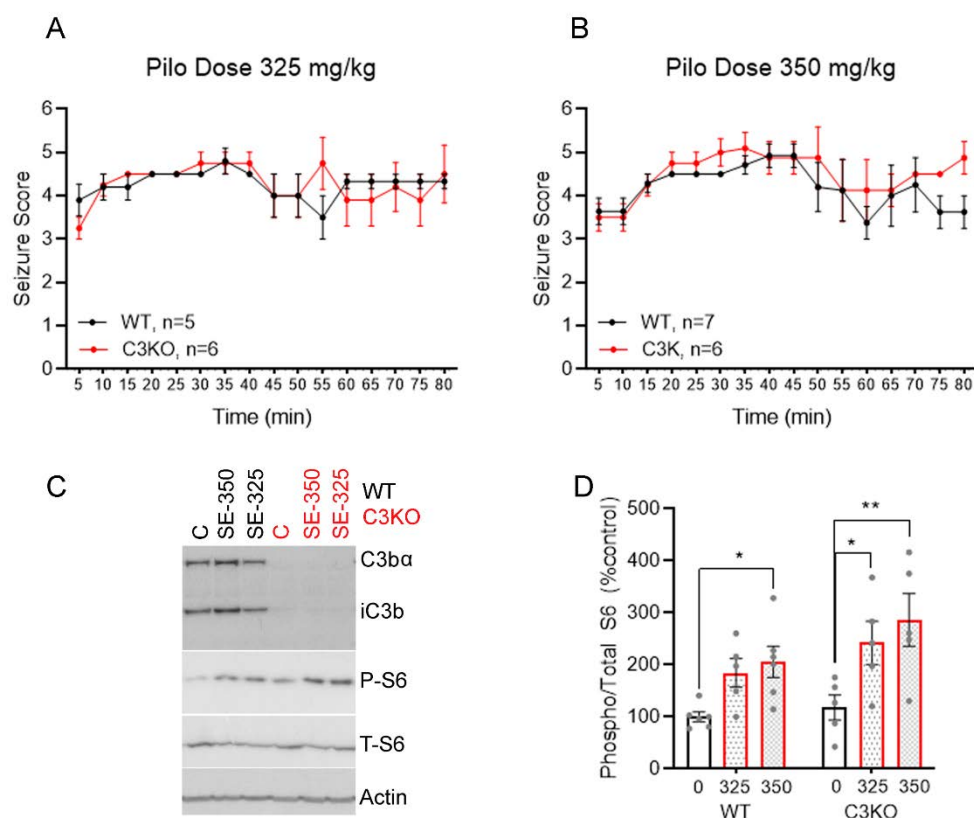
Note. WT and C3KO mice were injected with either 325 mg/kg (A) or 350 mg/kg (B) of pilocarpine and observed for behavioral seizures according to the Racine scale (1 = rigid posture, mouth moving; 2 = tail clonus; 3 = partial body clonus, head bobbing; 4 = rearing; 4.5 = severe whole body clonic seizures while retaining posture; 5 = rearing and falling; 6 = tonic-clonic seizure with jumping or loss of posture). Repeated measures ANOVA with Sidak's multiple comparisons test was used to compare seizure severity between genotype at each time-point. WB was used to compare hippocampal levels of phosphorylated S6 at 1 hour after SE (C-D). Two-way ANOVA with Tukey's multiple comparisons test. Data shown as mean ± SEM, *n* = 5 (WT-C), 5 (WT-325), 6 (WT-350), 5 (C3KO-C), and 5 (C3KO-325), 5 (C3KO-350).

p < 0.05. **p* < 0.01.

*Figure 1*. WT and C3KO mice have similar behavioral response to pilocarpine injection.

NOR Trial 1: Familiarization

NOR Trial 2: Test Trial
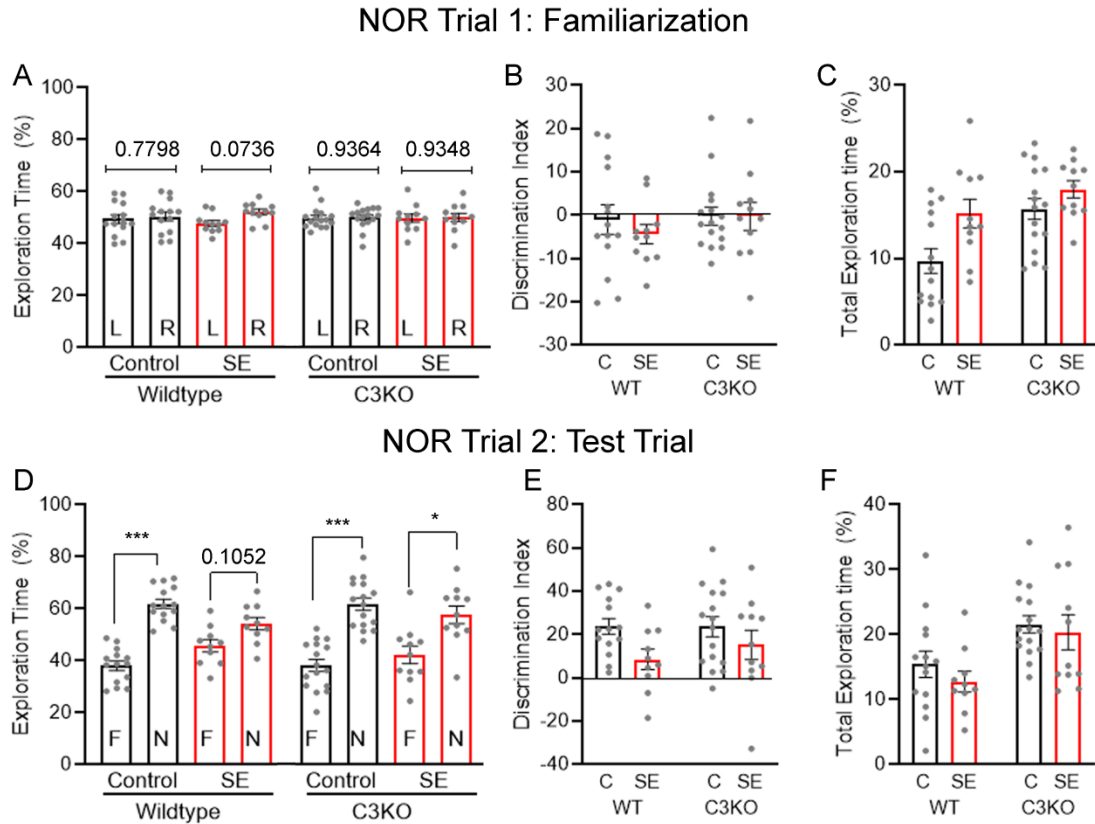
Note. Mice were familiarized with two similar objects for 10 minutes during trial 1 and the percent exploration time of the left and right objects were calculated and compared (A). The test trial occurred 5 days after familiarization and the percent exploration of the familiar and novel objects were calculated and compared (D). (B and E) Discrimination index (DI) was calculated for each trial. (C and F) The total object exploration time was normalized to the duration of the trial and compared between groups. Comparison of left vs. right or familiar vs novel objects was done with paired t-tests. All other analyses used two-way ANOVA with Tukey's post hoc test. Data shown as mean ± SEM, *n* = 14 (WT-C), 11 (WT-SE), 16 (C3KO-C), and 11 (C3KO-SE).

*p < 0.05. **p < 0.01. ***p < 0.001. ****p < 0.0001.

*Figure 2*. SE does not result in recognition memory deficits in C3KO mice.
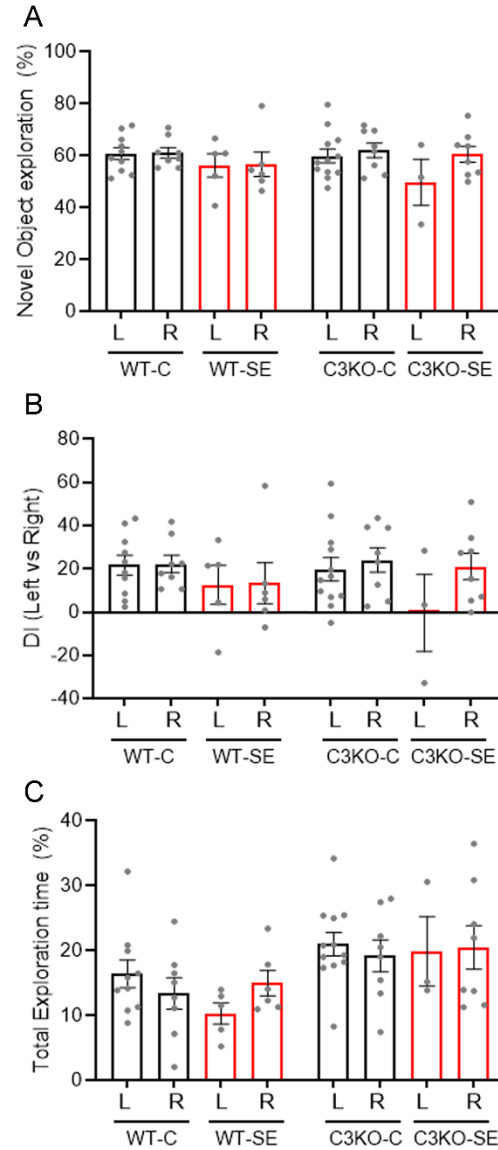
Note. (A) Percent exploration of the novel object during novel object recognition (NOR) trial 2 was compared for objects placed on the left versus right side of the apparatus. (B) The discrimination index was compared for NOR trial 2 for novel objects placed on the left versus right side. (C) Total exploration time of both objects during trial 2 was compared based on the placement of the novel object. Student's t-test. Data shown as mean ± SEM, *n* = 3-11 per group.

Figure 3. Location of novel object placement does not influence exploration preference

to novel object.
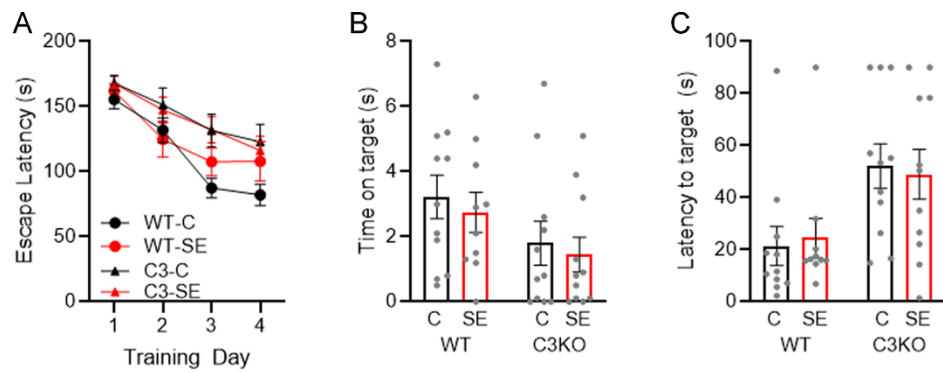
Note. Mice were trained to navigate a circular maze to find a hidden box over 4 days and latency to reach the box was recorded each day (A). On the fifth day, the escape box was removed and the time spent over the target (i.e. the location where the box was during training; B) and the latency to reach the target (C) were measured. Three-way repeated measures ANOVA was used for BM training (day X genotype X treatment). Two-way ANOVA with Tukey's post hoc test. Data shown as mean ± SEM, *n* = 11 (WT-C), 10 (WT-SE), 11 (C3KO-C), and 10 (C3KO-SE).

Figure 4. SE does not alter spatial learning and memory in the Barnes maze test.
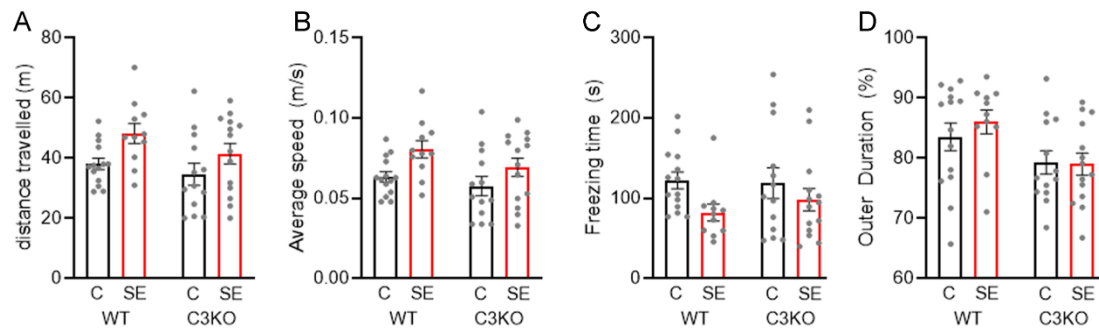
Note. Mice were habituated to the novel object recognition (NOR) testing arena 24 hours before NOR for ten minutes and their activity was tracked for measures of distance (A), average speed (B), freezing time (C), and the percent time spent around the outer perimeter of the testing apparatus (D). Two-way ANOVA with Tukey's multiple comparisons test. Data shown as mean ± SEM, n = 14 (WT-C), 11 (WT-SE), 13 (C3KO-C), and 14 (C3KO-SE).
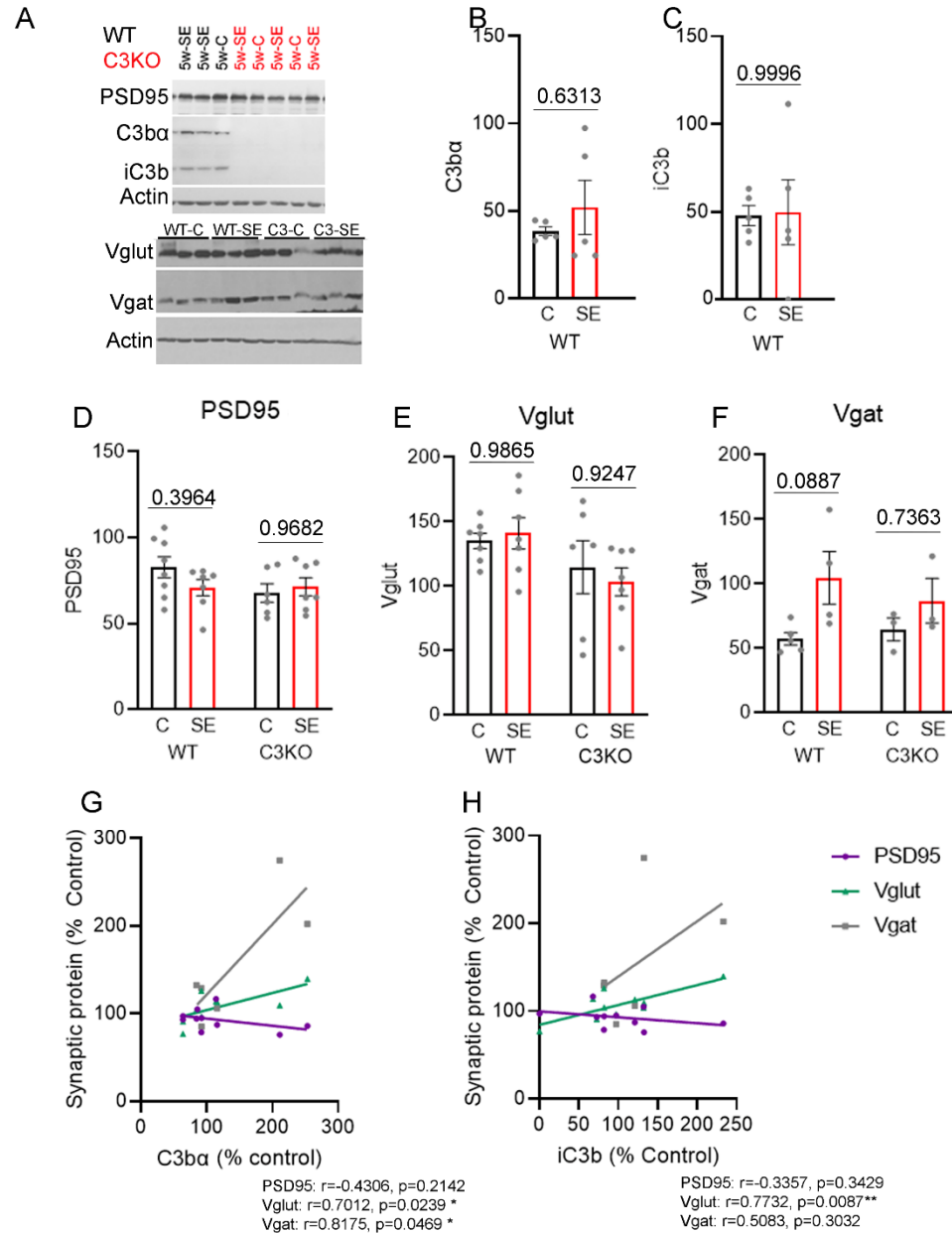
Figure 5. SE does not promote changes in locomotion or anxiety-like behaviors in either
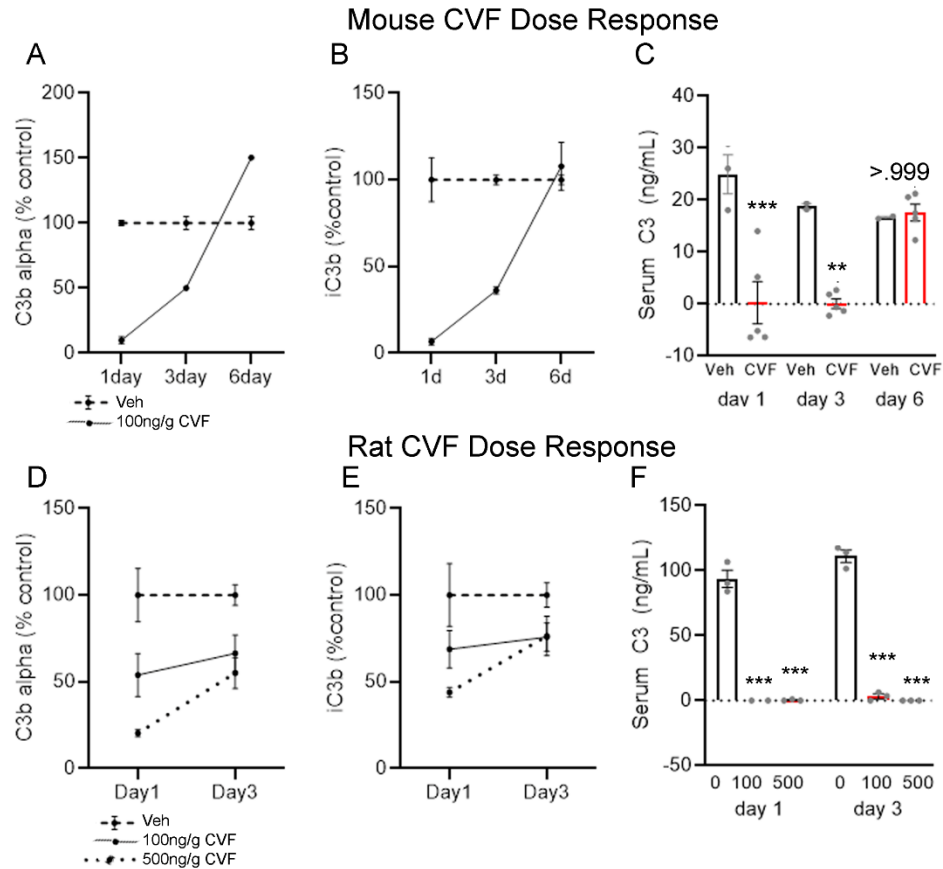
WT or C3KO mice.
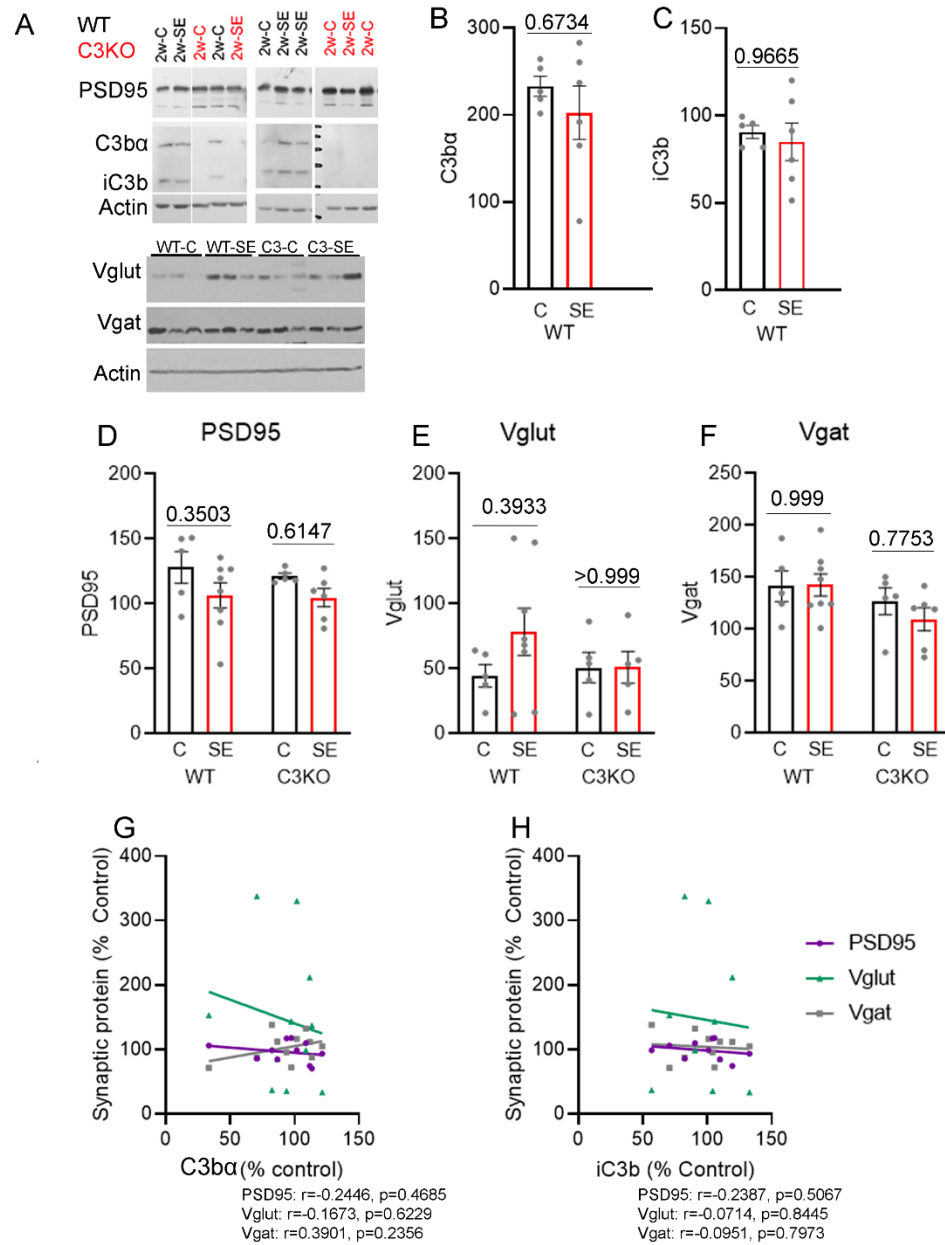
Note. A cohort of mice were sacrificed two weeks after SE and hippocampi were collected for western blotting (A). We measured the intensity of the immunoreactive bands corresponding to antibodies against C3 (B-C), PSD95 (D), Vglut (E), and Vglut (F). The proteins levels of C3bα (G) and iC3b (H) were compared in WT-C and WT-SE mice to levels of PSD95, Vglut, and Vgat within the same animal using correlation analysis. Two-way ANOVA with Tukey's multiple comparisons test. Data shown as mean ± SEM, *n* = 5 (WT-C), 8 (WT-SE), 5 (C3KO-C), and 6 (C3KO-SE).

Figure 6. C3 protein levels do not correlate with synaptic proteins in the hippocampus at

two weeks after SE.

Note. A cohort of mice were sacrificed five weeks after SE and hippocampi were collected for western blotting (A). We measured the intensity of the immunoreactive bands corresponding to antibodies against C3 (B-C), PSD95 (D), Vglut (E), and Vglut (F). The proteins levels of C3bα (G) and iC3b (H) were compared in WT-C and WT-SE mice to levels of PSD95, Vglut, and Vgat within the same animal using correlation analysis. Two-way ANOVA with Tukey's multiple comparisons test. Data shown as mean ± SEM, n = 7 (WT-C), 7 (WT-SE), 6 (C3KO-C), and 7 (C3KO-SE).

$*p < 0.05. **p < 0.01.$

Figure 7. Synaptic proteins Vgat and Vglut correlate with C3bα and iC3b levels in the

hippocampus at five weeks after SE.

Note. Mice were injected with CVF (i.p.) or saline and sacrificed 1, 3, and 6 days later to measure levels of C3b alpha (A) and iC3b (B) in the hippocampus. Blood was also collected to determine serum levels of C3 with ELISA (C). Rats received either a low (100 ng/g) or high (500 ng/g) dose of CVF i.p. Hippocampi were collected for WB to measure C3b alpha (D) and iC3b (E) protein levels at 1 and 3 days after injection. Blood was also collected to determine C3 levels in serum with ELISA (F). Two-way ANOVA (for WB) and one-way ANOVA (ELISA) with Tukey's post hoc test. Data shown as mean ± SEM, *n* = 3 per group.

**$p < 0.01$. ***$p < 0.001$.

Figure 8. Cobra Venom Factor (CVF) reduces C3b levels in the hippocampus and blood

of mice and rats.
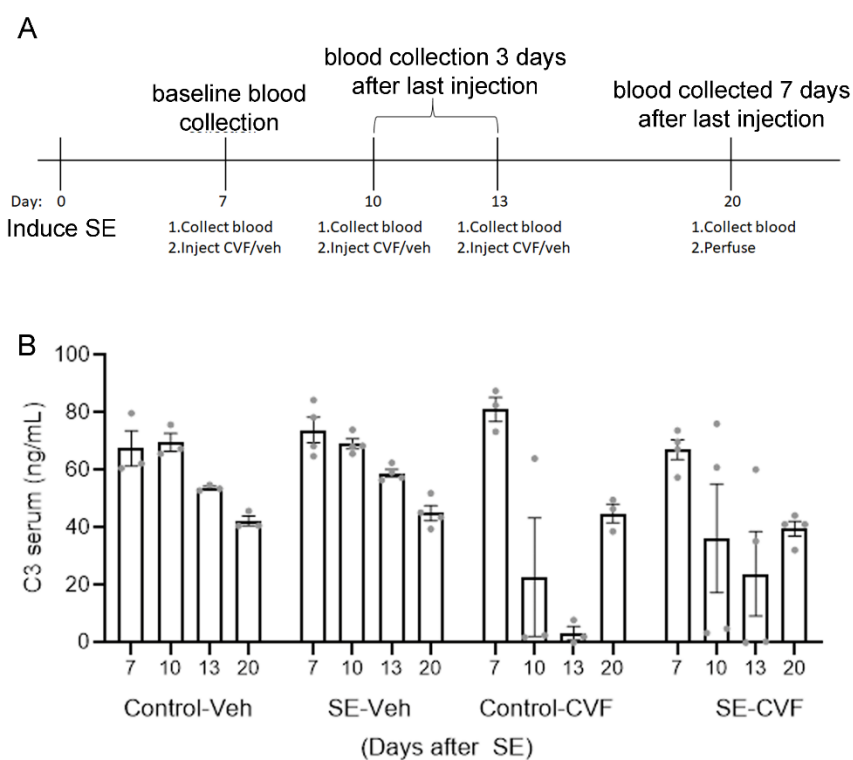
Note. One cohort of rats was injected with saline or pilocarpine to induce SE (A). They were treated with Vehicle or CVF (100 ng/g) on days 7, 10, and 13 after SE. Prior to each injection, blood samples were collected to measure levels of C3 protein in the serum. Rats were sacrificed on day 20 and blood collected for analysis with ELISA. Using ELISA, serum levels of C3 were compared between Controls (vehicle and CVF treated) and SE rats (vehicle and CVF treated) and over time (B). Repeated 3-way ANOVA (time X drug [cvf or veh] X group [c or SE]) with Tukey's post hoc test. Data shown as mean ± SEM, $n = 3$ (C-Veh), 4 (SE-Veh), 3 (C-CVF), and 4 (SE-CVF).

Figure 9. Multiple treatments with CVF keep serum C3 levels reduced.

NOR Trial 1: Familiarization

NOR Trial 2: Test Trial
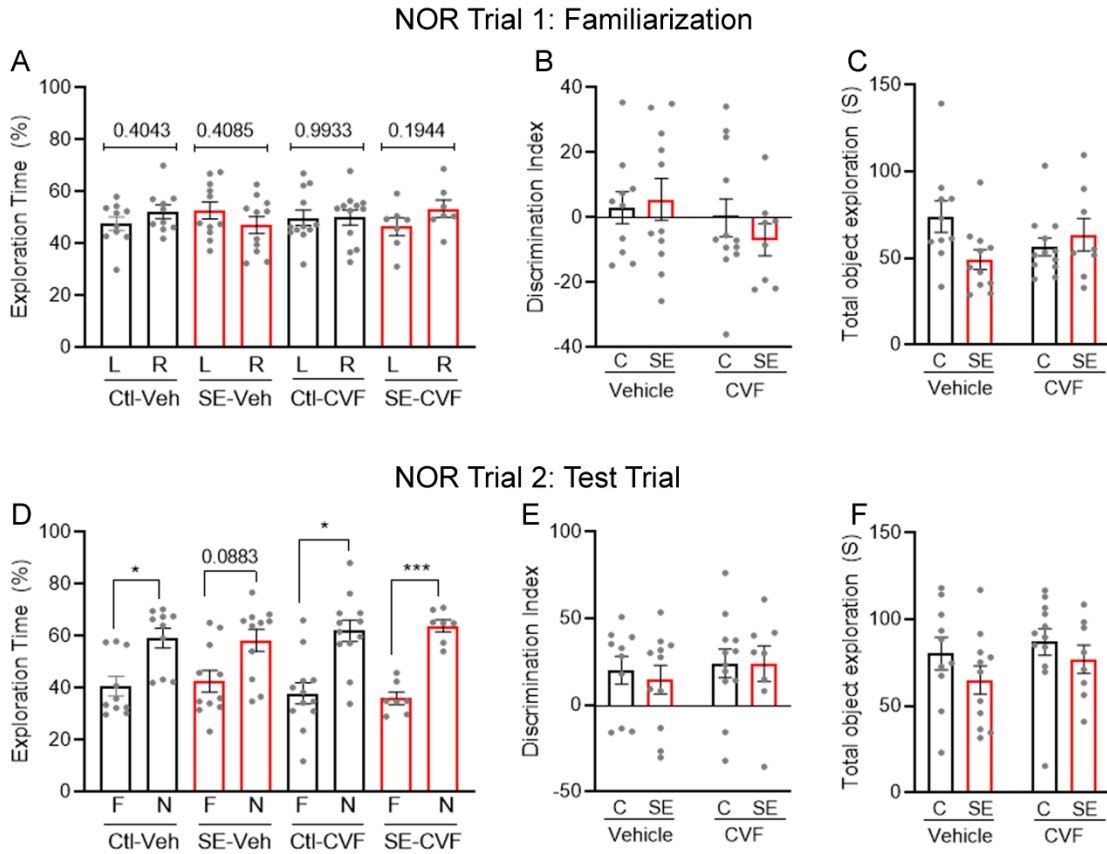
Note. Rats were familiarized with two similar objects for 5 minutes during trial 1 and the percent exploration time of the left and right objects were calculated and compared (A). The test trial occurred 2 hours after familiarization and the percent exploration of the familiar and novel objects were calculated and compared (D). (B and E) Discrimination index (DI) was calculated for each trial. (C and F) The total object exploration time was normalized to the duration of the trial and compared between groups. Comparison of left vs. right or familiar vs novel objects was done with paired t-tests. All other analyses used two-way ANOVA with Tukey's post hoc test. Data shown as mean ± SEM, n = 9 (C-Veh), 11 (SE-Veh), 12 (C-CVF), and 7 (SE-CVF).

$*p < 0.05. **p < 0.001.$

Figure 10. SE does not result in recognition memory deficits in CVF-treated rats.
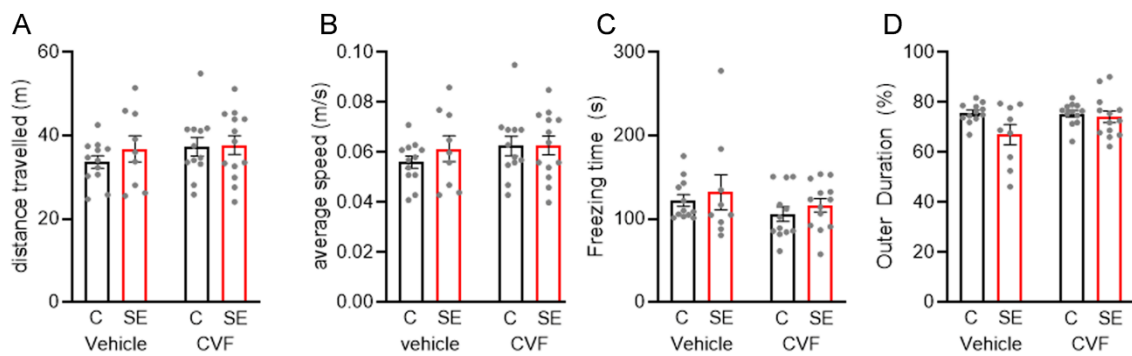
Note. Rats were habituated to the novel object recognition (NOR) testing arena 24 hours before NOR for ten minutes and their activity was tracked for measures of distance (A), average speed (B), freezing time (C), and the percent time spent around the outer perimeter of the testing apparatus (D). Two-way ANOVA with Tukey's multiple comparisons test. Data shown as mean ± SEM, n = 12 (C-veh), 9 (SE-veh), 12 (C-CVF), and 13 (SE-CVF).

Figure 11. SE does not promote changes in locomotion or anxiety-like behaviors in rats treated with vehicle or Cobra Venom Factor.
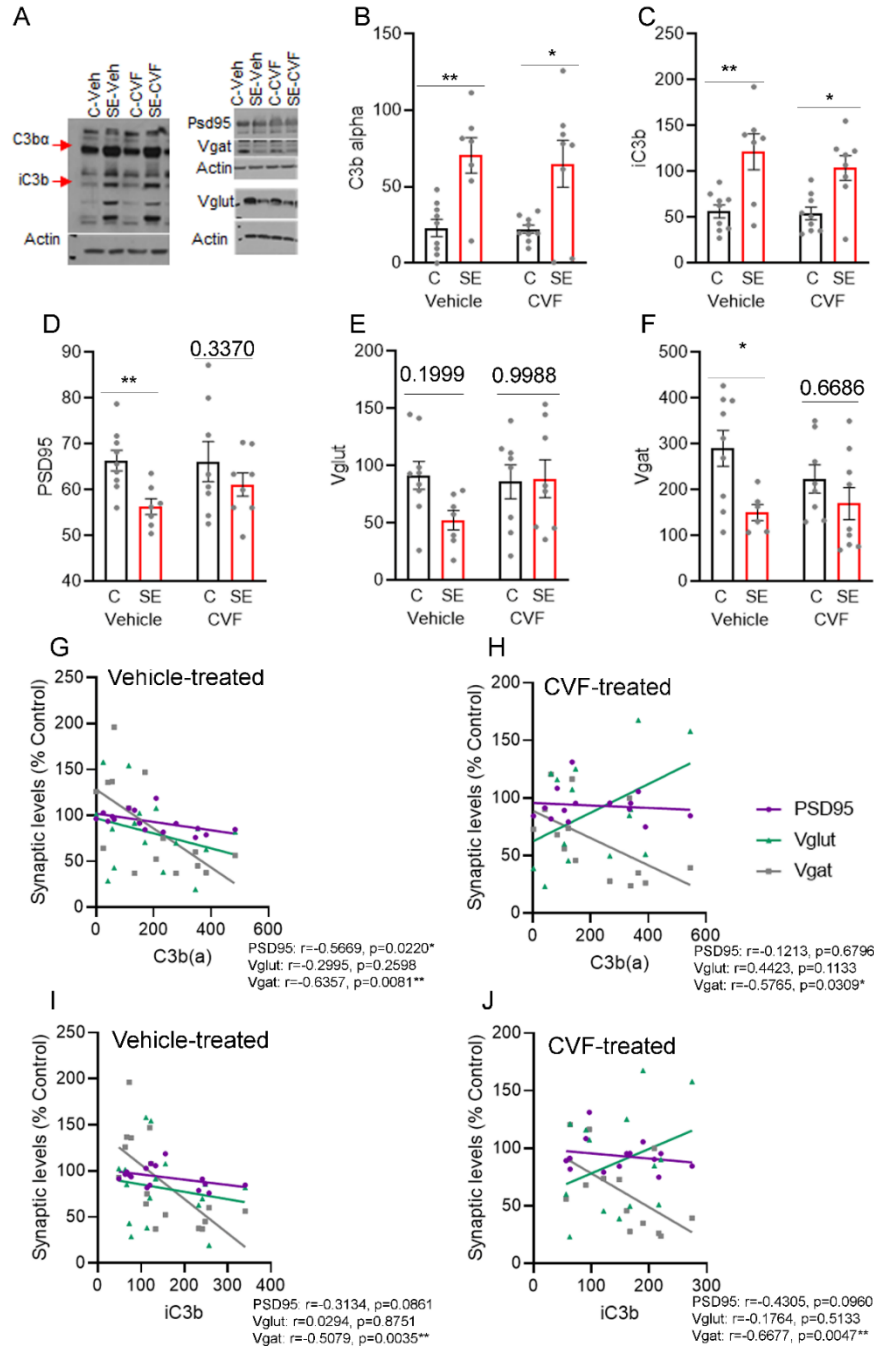
Note. Rats were perfused and tissue collected at 16 days after SE for western blotting (A). Hippocampal levels of C3bα (B), iC3b (C), PSD95 (D), Vglut (E), and Vgat (F) proteins were measured. The densitometry data was separated out based on treatment with vehicle (G and I) or CVF (H and J) to compare levels of C3bα and iC3b to synaptic protein levels using correlation analysis. Two-way ANOVA with Tukey's multiple comparisons test. Data shown as mean ± SEM, n = 9 (C-veh), 6 (SE-veh), 8 (C-CVF), and 9 (SE-CVF).

*p < 0.05. **, p < 0.01.

Figure 12. CVF treatment prevents synaptic protein loss in the hippocampus after SE.