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ALTERATIONS IN GABAERGIC NTS NEURON FUNCTION IN ASSOCIATION WITH TLE AND SUDEP

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ALTERATIONS IN GABAERGIC NTS NEURON FUNCTION IN ASSOCIATION WITH TLE AND SUDEP

DISSERTATION

A dissertation submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy in the College of Medicine at the University of Kentucky

By
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2018

ABSTRACT OF DISSERTATION

ALTERATIONS IN GABAERGIC NTS NEURON FUNCTION IN ASSOCIATION WITH TLE AND SUDEP

Epilepsy is a neurological disorder that is characterized by aberrant electrical activity in the brain resulting in at least two unprovoked seizures over a period longer than 24 hours. Approximately 60% of individuals with epilepsy are diagnosed with temporal lobe epilepsy (TLE) and about one third of those individuals do not respond well to anti-seizure medications. This places those individuals at high risk for sudden unexpected death in epilepsy (SUDEP). SUDEP is defined as when an individual with epilepsy, who is otherwise healthy, dies suddenly and unexpectedly for unknown reasons. SUDEP is one of the leading causes of death in individuals with acquired epilepsies (i.e. not due to genetic mutations), such as TLE. Previous studies utilizing genetic models of epilepsy have suggested that circuitry within the vagal complex of the brainstem may play a role in SUDEP risk. Gamma-aminobutyric acid (GABA) neurons of the nucleus tractus solitarius (NTS) within the vagal complex receive, filter, and modulate cardiorespiratory information from the vagus nerve. GABAergic NTS neurons then project to cardiac vagal motor neurons, eventually effecting parasympathetic output to the periphery. In this study, a mouse model of TLE was used to assess the effect of epileptogenesis on GABAergic NTS neuron function and determine if functional alterations in these neurons impact SUDEP risk. It was discovered that mice with TLE (i.e. TLE mice) have significantly increased mortality rates compared to control animals, suggesting that SUDEP occurs in this model. Using whole cell electrophysiology synaptic and intrinsic properties of GABAergic NTS neurons were investigated in TLE and control mice. Results suggest that during epileptogenesis, GABAergic NTS neurons become hyperexcitable, potentially due to a reduction in A-type potassium channel current and increased excitatory synaptic input. Increases in hyperexcitability have been shown to be associated with an increased risk of spreading depolarization and action potential inactivation leading to neuronal quiescence. This may lead to a decreased inhibition of parasympathetic tone, causing cardiorespiratory collapse and SUDEP in TLE.

KEYWORDS: temporal lobe epilepsy, sudden unexpected death in epilepsy, NTS, GABA

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Chapter 1 Introduction

1.1 Sudden unexpected death in epilepsy

1.1.1 History and Definitions

The International League Against Epilepsy defines epilepsy as when an individual has at least two unprovoked seizures occurring greater than 24 hours apart (Fisher et al., 2014). It has been well-established that death in individuals with epilepsy can be attributed to the epilepsy itself or to a seizure that is unable to stopped by medication (Mackenzie Bacon, 1868; Spratling, 1902; Munson, 1910; Hauser et al., 1980; Nashef and Brown, 1996; Nashef and Sander, 1996). According to the Centers for Disease Control, about 1.2% of the United States population have epilepsy, which amounts to about 3.4 million individuals nationwide. Individuals with epilepsy have a 2.5-fold increase in the risk for sudden death compared to a healthy individual (Shackleton et al., 1999). One of the leading causes of death in individuals with epilepsy is sudden unexpected death in epilepsy (SUDEP) (Nashef, 1997; Nashef et al., 1998; Kloster and Engelskjon, 1999; Lhatoo and Sander, 2005; Tomson et al., 2008; Tolstykh and Cavazos, 2013; Ellis and Szabo, 2018). Case studies of SUDEP first appear in the literature in the early 1970's via investigation of death certificates of individuals with epilepsy (Hirsch and Martin, 1971). Up until the use of translational animal models of epilepsy, SUDEP research focused on postmortem investigations and single-patient case studies (Terrence et al., 1975; Sarkioja and Hirvonen, 1984; Leestma et al., 1989; Dasheiff, 1991). By the mid-1990's SUDEP research experienced a resurgence, and several definitions of SUDEP were developed.

The definition of SUDEP, first published in 1997, is: “sudden, unexpected, unwitnessed or witnessed, non-traumatic and non-drowning death in patients with epilepsy, with or without status epilepticus, in which postmortem examination does not reveal a toxicologic or anatomic cause for death” (Nashef, 1997). More recently, the definition has been slightly modified to include that the death was not directly caused by a seizure (Annegers, 1997; Annegers and Coan, 1999). SUDEP cases are also divided into four categories: definite, probable, possible, and unlikely SUDEP (Annegers, 1997). Definite SUDEP meets all the definition criteria and has a description of the circumstances of death. Probable SUDEP meets the criteria but does not have postmortem information. Possible SUDEP includes cases where SUDEP cannot be ruled out, but also cannot be sufficiently confirmed based on medical records. Unlikely SUDEP includes deaths for which other causes are clearly established or circumstances make SUDEP improbable (Annegers, 1997). It can be difficult to ascertain if individuals with epilepsy have died from SUDEP because most individuals are not continuously monitored in the home setting. Presently, it is difficult to accurately identify individuals at risk for SUDEP and research has yet to identify underlying biomarkers for and mechanisms of SUDEP.

1.1.2 Patient Characteristics

The incidence of SUDEP in the United States is about 1.2 out of every 1000 individuals with epilepsy (Harden et al., 2017). Early studies examining individuals with epilepsy focused on potential cardiac and respiratory abnormalities. Individuals with epilepsy that are not previously diagnosed with a cardiac pathology

have an increased heart rate and electrocardiographic (ECG) abnormalities (e.g. QT lengthening) (Drake et al., 1993). Individuals with epilepsy were also shown to have apnea during a generalized tonic-clonic seizure (GTCS) (Fish, 1997). As early as 1987, it was hypothesized that autonomic nervous system imbalance, cardiac arrhythmia, and epilepsy may be linked (Lathers et al., 1987).

There have been many studies assessing potential risk factors for SUDEP in individuals with epilepsy. The risk for SUDEP has been well-established to increase with the duration of epilepsy, seizure severity, and seizure frequency (Ficker et al., 1998; Sperling, 2001; Devinsky, 2011), potentially because these individuals have a longer history of seizure events. It has been shown that the frequency of GTCS is strongly associated with SUDEP risk, regardless of the number of medications the individual has previously been prescribed (Lamberts et al., 2012). Therefore, it is crucial to decrease seizure frequency in order to attenuate SUDEP risk. However, not all individuals with epilepsy have seizures that are well-controlled with the first medication they are prescribed. Often, treatment with two or three medications is necessary before seizure frequency decreases. Even so, about 30% of individuals are unable to resolve their seizures by medication or surgery and have refractory epilepsy. This patient population is one of the highest at risk for SUDEP (Devinsky, 2011; Tolstykh and Cavazos, 2013; Thurman et al., 2014). Another patient risk factor is compliance with anti-epileptic medications (AEMs). It has been shown that if an individual with epilepsy does not follow their treatment regimen, their risk for seizures increases, thus increasing the risk for SUDEP. The environment in which a patient sleeps may also

play a role in increasing the likelihood of SUDEP. Patient studies have shown that individuals who have suffered from SUDEP, tend to have a lack of nighttime supervision and tend to be found in the prone position (Devinsky, 2011).

Autonomic imbalance relating to cardiorespiratory function in epilepsy patients may also be a risk factor for SUDEP. A study examining data from epilepsy monitoring units (EMUs) world-wide found that many individuals that suffered from SUDEP experienced respiratory abnormalities such as apnea (Ryvlin et al., 2013). During a seizure event, individuals with epilepsy have displayed cardiac arrhythmias, postictal bradycardia, and asystole (Surges and Sander, 2012). Additionally, some individuals have displayed lengthy postictal EEG suppression (PGES), characterized by a flat-lining of the electroencephalogram (EEG) after a seizure event. These individuals have a higher risk for SUDEP compared to those with shorter PGES (Lhatoo et al., 2010a; Surges et al., 2010). However, specific mechanisms that elucidate these risk factors or identify biomarkers for SUDEP remain to be established.

1.2 Suggested Mechanisms of SUDEP

In the past 5-10 years there has been an increased amount of research focused on potential mechanisms of SUDEP. Many of these studies utilize genetic mouse models of SUDEP, which will be further detailed in a following section. Dlouhy et al. hypothesized that a generalized tonic-clonic seizure (GTCS) spreads to three areas eventually resulting in SUDEP: the midbrain, respiratory centers and brainstem cardiovascular centers (Dlouhy et al., 2016).

1.2.1 The Midbrain

The midbrain is a portion of the central nervous system that is associated with vision, hearing, motor control, wakefulness, arousal, and temperature regulation. It contains the following structures: tectum, tegmentum, cerebral aqueduct, and peduncles. It adjoins to the pons, cerebellum, thalamus, and hypothalamus. Upon spread to the midbrain, seizures inhibit the ascending arousal system, located in the brainstem, which plays a role in sleep-wake consciousness (Dlouhy et al., 2016). Individuals with epilepsy that have suffered from SUDEP are often found lying in the prone position and researchers have hypothesized that this is due to dysfunction within the arousal system (Langan et al., 2000; Ryvlin et al., 2013; Sowers et al., 2013; Ellis and Szabo, 2018). The ascending arousal system, or ascending reticular activating system (ARAS), originates in the upper brainstem and is involved in regulating sleep-wake transitions via its interaction with the ventrolateral preoptic nucleus (VLPO). The primary neurotransmitter in the VLPO, γ-aminobutyric acid (GABA) inhibits ARAS during sleep. Interactions between the VLPO and ARAS maintains a sleep-wake switch (Edlow et al., 2012). In individuals with epilepsy this switch may malfunction, which could be an underlying factor in the correlation between nighttime seizures, inability to wake up at that time, and SUDEP (Lamberts et al., 2012; Sowers et al., 2013; Gumusyayla et al., 2016). Additionally, patients with epilepsy show dysfunction in the ARAS when measured via polysomnography and magnetic resonance imaging (MRI) (Gumusyayla et al., 2016; Englot et al., 2017b). Dysfunction within the arousal system does put

individuals with epilepsy at a higher risk for SUDEP but the neuronal mechanisms that contribute to this dysfunction remain to be elucidated.

1.2.2 Postictal EEG Suppression

PGES is defined as the absence of EEG activity (i.e. less than 10 μV in amplitude allowing for muscle movement, breathing, and electrode artifacts) (Lhatoo et al., 2010a). PGES is characterized by a short collapse of cardiac and respiratory rates post-seizure. There have been several studies examining patient incidence of PGES in association with seizures and SUDEP. An early study did a post-hoc examination of video EEG of individuals with epilepsy, some of whom went on to die of SUDEP (Lhatoo et al., 2010a). Fifty percent of patients had PGES following a seizure and its duration was significantly longer in the individuals that eventually died of SUDEP (Lhatoo et al., 2010a). Another study examined the association between PGES, seizure type, and SUDEP. While they found that PGES occurred postictally, neither the presence nor duration of PGES was significantly associated with SUDEP as an independent risk factor (Surges et al., 2011). Another study concluded that PGES is not related to seizure duration or severity, but that it is related to oxygen desaturation during a seizure (Seyal et al., 2012). Lee et al. did a retrospective analysis of heart rate and PGES in individuals with nocturnal seizures and also found that PGES did not impact SUDEP risk (Lee et al., 2013). Similarly, Lamberts et al., found that PGES was not significantly correlated with autonomic instability but did find an association with nocturnal seizures (Lamberts et al., 2013). Kuo et al., monitored patients with GTCS for PGES and found that it was not associated with seizure phase or duration (Kuo et

al., 2016). However, another study found that PGES duration was increased when seizure onset occurred during sleep (Peng et al., 2017). Contradictory to the previously mentioned studies, it was recently found that individuals that died of SUDEP had a shorter, not longer, PGES duration in association with SUDEP risk (Kang et al., 2017). Overall, the evidence regarding whether PGES is related to SUDEP remains inconclusive. However, alterations in both the arousal system and PGES in SUDEP have led researchers to postulate that the serotonergic system (5-HT), in addition to the respiratory centers may play a role.

1.2.3 Respiratory Centers

Recently respiratory dysfunction has been more closely examined as a potential mechanism for SUDEP because hypoxia, apnea, and other respiratory-related dysfunction have been shown to occur during seizures. (Watanabe et al., 1982; Coulter, 1984; James et al., 1991). Recently, respiratory dysfunction has been more closely examined as a potential mechanism for SUDEP. Nelson and Ray in 1968 published one of the first accounts of respiratory arrest after seizure but it was not until 1999 that parameters were published to monitor these changes in individuals with epilepsy (Fish, 1997). Apnea (i.e. temporary cessation of breathing) occurs in 100% of generalized seizures and since it is associated with hypoxia, researchers concluded that respiratory function should be monitored in epilepsy patients (Fish, 1997). In 1996, Nashef et al. showed that greater than 50% of patients had apnea in concert with seizures (Nashef et al., 1996). Additionally, these respiratory parameters are associated with impaired ventilation, O_2 desaturation, and hypoxia (Blum et al., 2000; Sowers et al., 2013). O_2 saturation

has been shown to drop to below 90% in some individuals and the longer a seizure lasts, the lower saturation becomes. In what is thought to be a seminal study by Ryvlin et al., respiratory and cardiac data from international EMUs was examined in cases where individuals died of SUDEP (Ryvlin et al., 2013). One of their major conclusions was that SUDEP was preceded by terminal apnea, followed by cardiac arrest and death (Ryvlin et al., 2013). This study is problematic for several reasons, one of which being that all respiratory measurements were done by visual inspection of EMU patient videos. No quantitative measurements were taken and no mention is made regarding the quality of the video or how visible the lack of respiratory movement was. Additionally, there was no effort made to resuscitate 5 out of the 16 patients that died of SUDEP. It was also not stated if individuals in this study had been previously diagnosed with any pulmonary dysfunction, which may have played a role in SUDEP.

Stimulation of higher brainstem centers to elicit seizures has been shown to be associated with respiratory depression. Stimulation in the orbital frontal cortex, temporal lobe, and amygdala can result in respiratory depression and central apnea (Kaada and Jasper, 1952; Bonvallet and Bobo, 1972). More recently, high resolution brain mapping has shown that seizures originating in the frontotemporal region, spread to the amygdala, eliciting central apnea (Dlouhy et al., 2015). Interestingly, during these procedures individuals were awake, but not aware that the apnea occurred. These data suggest that seizures may spread to and affect brain regions that are crucial for basic functions. There are other higher brain pathways that project downstream; for example, the temporal lobe connects

to respiratory centers (Bateman et al., 2010a; Faingold et al., 2010; Faingold, 2012). While these areas and the spread of seizures to respiratory nuclei may be involved in SUDEP, the neurons in these brain regions have yet to be directly examined in this context. In examining respiratory function and SUDEP, there are few studies that make direct measurements of breathing, so this potential mechanism remains poorly understood. One of the things that PGES and respiratory function have in common is signaling through serotonergic/5-hydroxytryptamine (5-HT) neurons (Ellis and Szabo, 2018).

1.2.4 5-HT Neurons

5-HT is a monoamine neurotransmitter that is primarily found within the raphe nuclei of the brainstem (Jacobs and Azmitia, 1992; Sowers et al., 2013). 5-HT neurons receive connections from a wide variety of regions, including the cortex, hypothalamus, and brainstem, and projects to many regions within the central nervous system (Sowers et al., 2013). 5-HT is involved in many brain functions, such as respiration, arousal, anxiety, depression, and central chemoreception (Sowers et al., 2013; Kennedy and Seyal, 2015). Since 5-HT neurons stimulate respiration and arousal, dysfunction in this system has been hypothesized to sudden death (Richerson and Buchanan, 2011).

The Pre-Bötzinger complex (BPC) in the brainstem is responsible for breathing, hypoxia, and gasping responses. The proper function of the BPC is dependent on 5-HT receptor activation by serotonin (Richerson, 2004; Hodges and Richerson, 2010; Massey et al., 2014). In mice where 5-HT receptors are deleted, they have apnea, increased mortality, and defects in arousal (Hodges et al., 2008;

Hodges et al., 2009; Buchanan et al., 2014). A separate study examined neuronal inhibition of 5-HT neurons with Designer Receptors Exclusively Activated by Designer Drugs (DREADDs) and found that ventilation was significantly blunted (Ray et al., 2011). Medullary 5-HT neurons act as chemoreceptors and respond to small changes in physiologic pH (Wang et al., 1998; Wang et al., 2001c; Richerson et al., 2005; Corcoran et al., 2009). Increased firing of 5-HT neurons increases respiratory output (Ptak et al., 2009). 5-HT neurons in the midbrain act as chemoreceptors by inducing arousal in response to changes in arterial carbon dioxide, which has been hypothesized to be defective in SUDEP. It is postulated that after a seizure, the individual would be unresponsive as the pillow blocks the airway and being unable to relieve the obstruction would result in hypoxia and death (Sowers et al., 2013). Earlier work with animals that lack the 5-HT_{2C} receptor, provides evidence of serotonin's involvement with SUDEP, as these mice suffer from respiratory depression and mortality (Brennan et al., 1997). In the DBA/2 mouse, administration of a selective serotonin reuptake inhibitor (SSRI) reduces respiratory depression and mortality (Tupal and Faingold, 2006). These mice also have abnormal 5-HT receptor expression in the brainstem (Uteshev et al., 2010). In some individuals with epilepsy, SSRIs reduced the incidence of seizure-induced apnea (Bateman et al., 2010b; Dlouhy et al., 2016). The SSRI fluoxetine has also been shown to prevent respiratory arrest in the DBA/2 and DBA/1 mouse models (Faingold et al., 2011b; Faingold et al., 2011a). There are several AEMs that also increase 5-HT extracellular concentrations as a by-product of their function (Bagdy et al., 2007). Previous studies have also shown that there is a decrease in 5-HT_{1A}

receptor binding in individuals with epilepsy (Toczek et al., 2003; Savic et al., 2004; Richerson and Buchanan, 2011). However, there is no evidence of 5-HT gene mutations in patients that are directly linked to epilepsy (Richerson and Buchanan, 2011). In 2016, Zhan et al. showed a decrease in 5-HT neuronal firing in the medullary raphe after an electrically induced seizure (Zhan et al., 2016). While this study was able to show a direct link between a single seizure and 5-HT function, individuals at risk for SUDEP have a history of seizures. This study does not address the potential effect multiple seizures, over a period of time, has on 5-HT function and subsequent respiratory output. Another study examined postictal serotonin levels in individuals with epilepsy. They found that serum serotonin was significantly increased post-GTCS compared to interictal levels, suggesting that 5-HT is acutely altered post-seizure. While it appears that 5-HT is generally involved in epilepsy, it is does not conclusively cause of SUDEP. Further work needs to be done in brainstem regions to provide direct evidence of 5-HT and SUDEP.

1.2.5 Cardiac and Autonomic Dysfunction

Another focus of potential SUDEP mechanisms are cardiac and autonomic changes in relation to epilepsy that may contribute to sudden death. Peri-ictal autonomic dysregulation in individuals with epilepsy has been published as early as 1972 by Gastaut (Gastaut, 1972). The autonomic nervous system is responsible for regulating sympathetic and parasympathetic output to the periphery, including partially modulating cardiac activity. Information regarding cardiac function and blood pressure is relayed to the brainstem via the afferent fibers of the vagus nerve. Blood pressure changes are influenced primarily by the baroreceptor reflex,

which involves baroreceptors located on the carotid sinus and aortic arch. The baroreceptors travel up the afferent fibers of the vagus nerve, directly to neurons within the brainstem, in a region called the nucleus tractus solitarius (NTS). Excitatory NTS neurons synapse to the caudal ventrolateral medulla (CVLM), which projects to the rostral ventrolateral medulla (RVLM), inhibiting those neurons. As the primary regulator of sympathetic output to the periphery, the RVLM projects glutamatergic fibers to sympathetic preganglionic neurons in the spinal cord. When blood pressure increases, the baroreceptors are activated and the NTS increases excitatory neurotransmission to the CVLM, inhibiting the RVLM, decreasing excitatory neurotransmission to the heart and decreasing blood pressure. When blood pressure is low, the baroreceptors receive less tonic activation and the sympathetic output increases to maintain a steady blood pressure. GABAergic NTS neurons synapse to cardiac vagal motor neurons (CVMNs) in the nucleus ambiguus (NA) and the dorsal motor nucleus of the vagus (DMV) to regulate vagal tone, slowing heart rate. This is further increased when blood pressure is high. It is important to note that the circuitry relating to the parasympathetic nervous system is primarily directed toward regulating heart rate (Gordan et al., 2015).

With regard to cardiac function, the sympathetic nervous system serves to increase heart rate and myocardial contractility under various pathological conditions. The parasympathetic nervous system serves to reduce heart rate and functions primarily during sleep or resting conditions. An additional factor in heart rate that is influenced by the autonomic nervous system is heart rate variability

(HRV), which is defined as the variation in the time between each heartbeat. Clinically, two measures of HRV are the standard deviation between the R-R intervals (SDNN) and the root mean squared of the R-R interval (RMSSD) (Stein and Kleiger, 1999). The SDNN is interpreted as the general HRV and the RMSSD as the parasympathetic or vagal tone component of HRV (Shaffer and Ginsberg, 2017). Studies examining individuals with epilepsy in association with cardiac function have focused primarily on changes in blood pressure, heart rhythms, and HRV as potential mechanisms of SUDEP. There have been a paucity of studies investigating blood pressure changes as a potential mechanism for SUDEP. The first paper was a patient case study which examined an individual with epilepsy and measured systolic, diastolic, and mean arterial blood pressure. In this case, a GTCS during sleep increased blood pressure measurements, and the patient showed a period of post-ictal hypotension that concluded with PGES (Bozorgi et al., 2013). Since this patient had no prior history of cardiac dysfunction, this study suggests that the seizure had an impact on autonomic control of blood pressure. It would have been more impactful to measure interictal blood pressure to see if it is altered without a seizure event as a measure of autonomic dysfunction. Another study compared awake interictal blood pressure and heart rate in individuals that eventually died of SUDEP to refractory and AEM controlled individuals (Nei et al., 2016). Their retrospective analysis showed that there was a trend towards an increase in diastolic blood pressure in individuals who eventually died of SUDEP (Nei et al., 2016). However, the authors only used heart rate and blood pressure measurements from the three most recent outpatient visits and available peri-

mortem data was not reported. Another study measured mean arterial blood pressure, heart rate, and O_2 saturation in individuals with focal and bilateral seizures (Hampel et al., 2016). This study found no changes in O_2 saturation but mean arterial blood pressure was increased (Hampel et al 2016). However, they did not state if this change was post-seizure, and therefore cannot make conclusions regarding autonomic changes related to seizure activity. Conflictingly, another study by the same group found that there were no alterations in the baroreflex response in individuals with focal seizures (Hampel et al., 2017). While the data suggest that blood pressure and baroreflex sensitivity is altered in individuals with epilepsy, it has not been shown that these changes cause SUDEP. More work needs to be done where blood pressure and the baroreflex response are consistently monitored in individuals with epilepsy over time.

Epilepsy researchers have also hypothesized that autonomic dysfunction manifesting itself as a change in HRV, arrhythmia, tachycardia, asystole, bradycardia, and QT lengthening, occurring pre-ictally, ictally, post-ictally, and peri-ictally contribute to SUDEP (Stollberger and Finsterer, 2004; Massey et al., 2014; Bermeo-Ovalle et al., 2015). However, in many of these patients, information regarding prior cardiovascular disease diagnoses or medications is often lacking, making it difficult to account for confounding variables.

Several forebrain structures including the hippocampus, medial frontal cortex, and hypothalamus are involved in seizure networks and autonomic output (Cechetto and Chen, 1990). These areas project to the brainstem, regulating sympathetic and parasympathetic function. HRV is primarily dependent on vagal

tone and modulates heart rate in response to breathing, activity, wakefulness, and sleep (Hartikainen et al., 1996; Stein and Kleiger, 1999; Gordan et al., 2015; Shaffer and Ginsberg, 2017). In addition to the SDNN and RMSDD (i.e. time domain parameters), HRV can be analyzed via frequency domain parameters (i.e. low frequency, high frequency) (Stein and Kleiger, 1999). The frequency domain provides information regarding the amount of variance in heart rhythms explained by oscillations in heart rate at various frequencies allowing for the interpretation of autonomic balance (Stein and Kleiger, 1999). High frequency (HF) power is mediated by vagal tone and respiratory sinus arrhythmia, while low frequency (LF) power is mediated by a combination of parasympathetic tone, sympathetic tone, and the baroreflex response (Hyndman et al., 1971; Pomeranz et al., 1985; Fallen et al., 1988; Stein and Kleiger, 1999; Jansen and Lagae, 2010). There have been several studies that found alterations in measurements of HRV in individuals with epilepsy and concluded that it may be associated with SUDEP (Harnod et al., 2008; DeGiorgio and DeGiorgio, 2010; Toth et al., 2010; Lotufo et al., 2012; Sarkis et al., 2015; Sivakumar et al., 2016; DeGiorgio et al., 2017; Stollberger and Finsterer, 2017). The majority of these studies have found either a decrease in vagal tone with an increase in sympathetic drive after a GTCS (Sarkis et al., 2015; Sivakumar et al., 2016), with one study finding that this persisted 5-6 hours post-seizure (Toth et al., 2010). When examining inter-ictal HRV, the research shows that time and frequency domain measures are lower, suggestive of a reduction in vagal tone and long-term autonomic imbalance in individuals with epilepsy (Ansakorpi et al., 2000; Ansakorpi et al., 2002; Harnod et al., 2008). These data

also suggest that epilepsy impacts autonomic function outside of the pre-ictal, ictal, and post-ictal periods. There have also been a few studies stating that there is no association between autonomic dysfunction, epilepsy, and SUDEP (Persson et al., 2007; Lee et al., 2013). One study found that while there was a decrease in heart rate just prior to a nighttime seizure, there were no post-ictal changes in heart rate, leading to the conclusion that there was no evidence that SUDEP is associated with postictal autonomic dysfunction (Lee et al 2013). Additionally, an older study found that there were no changes in time or frequency measurements in individuals with untreated epilepsy (Persson et al., 2007). There was another study that examined respiratory sinus arrhythmia (RSA) and heart rate in adolescents with epilepsy and found that in 5 of the non-epileptic individuals had increased RSA and decreased heart rate, suggestive of autonomic imbalance and were later diagnosed with epilepsy (Sivakumar et al., 2016). This suggests that autonomic dysfunction may precede the onset of epilepsy and may be a useful predictive marker for SUDEP. Many studies have also found changes in cardiac function in individuals with epilepsy who have not been diagnosed with any prior cardiac dysfunction (Surges et al., 2010; Velagapudi et al., 2012; Massey et al., 2014; Dlouhy et al., 2016). While overall, the data suggest that autonomic and cardiac dysfunction occurs in individuals with epilepsy, alterations in the function of brainstem neurons that regulate autonomic output should be investigated to determine if changes in their function are associated with autonomic imbalance and SUDEP.

1.3 Genetic Models of SUDEP

1.3.1 Dravet's Syndrome

Developing genetic models of epilepsy where the animals suffer from SUDEP has been popular due to the ease of gene deletion that leads to a seizure phenotype. Two of the most well-established models are the Dravet's syndrome (DS) and the *KCNA1*-null mouse. DS is an epilepsy channelopathy characterized by mutations in the voltage-gated Na^+ channel, Nav1.1, which is encoded by the *SCN1A* gene. There are several different mutations in the *SCN1A* gene can that lead to altered function in Nav1.1 and epilepsy. These mutations can occur in the regions of the gene that encode for any part of the six transmembrane spanning unit that comprises the channel and/or on the C- and N-termini (Catterall et al., 2010; Catterall, 2012). Additionally, complete deletion of *SCN1A* in humans and mice is embryonically lethal (Catterall et al., 2010). These mutations can be responsible for various forms of inherited epilepsies, including DS (Escayg et al., 2000; Catterall et al., 2010). Individuals with DS suffer from SUDEP and display depressed HRV and abnormal cardiac rhythms (Le Gal et al., 2010; Delogu et al., 2011; Sakauchi et al., 2011; Goldman et al., 2016). In mouse models, the mutation known to cause Dravet's Syndrome in humans was introduced into the *SCN1A* gene to create reduced Nav1.1 channel function, subsequently leading to a spontaneous seizure phenotype (Yu et al., 2006; Oakley et al., 2009; Oakley et al., 2011; Cheah et al., 2012; Auerbach et al., 2013; Kalume et al., 2013; Ogiwara et al., 2013; Aiba and Noebels, 2015). In this model, mice begin to have spontaneous seizures by postnatal day 21 and sudden death begins to occur around postnatal

day 23 (Yu et al., 2006; Ogiwara et al., 2007; Cheah et al., 2012; Kalume et al., 2013). DS mice have a decrease in the density of GABA interneurons, reduced Na^+ current, and action potential firing without any changes in excitatory neurons (Yu et al., 2006; Cheah et al., 2013). The imbalance between inhibition and excitation may be responsible for the seizure phenotype. Additionally, these mice display altered cardiorespiratory function, such as QT prolongation, bradycardia, suppressed inter-ictal HRV, and gasping (Kalume et al., 2013; Aiba and Noebels, 2015). It has been hypothesized that significant post-ictal bradycardia due to increased vagal tone contributes to SUDEP in DS (Kalume et al., 2013). This model implicates the involvement of central autonomic neurons, because animals have cardiac dysfunction, even in a GABA forebrain neuron specific knockout (Kalume et al., 2013). Additionally, causing seizures to spread from the cortex to brainstem leads to cardiorespiratory collapse and sudden death (Aiba and Noebels, 2015). This model has provided significant insight into potential mechanisms for SUDEP, but it only affects a small percent of the total epilepsy population.

1.3.2 Potassium Channel Mutations

Another common genetic model of epilepsy is the *KCNA1*-null mouse model. This involves the deletion of the *KCNA1* gene that encodes the Kv1.1 voltage-gated K^+ channel (Glasscock et al., 2010; Moore et al., 2014; Gautier and Glasscock, 2015; Simeone et al., 2018). Kv1.1 is expressed in the brain and the vagus nerve (Glasscock et al., 2010; Glasscock et al., 2012; Glasscock, 2013; Goldman et al., 2016). In addition to displaying an epilepsy phenotype, Kv1.1

knockout mice die suddenly and prematurely (Smart et al., 1998; Glasscock et al., 2007; Glasscock et al., 2010). They also have a high frequency of atrio-ventricular (AV) blocks that can be prevented by atropine administration, suggestive of autonomic imbalance (Glasscock et al., 2010). Additionally, Kv1.1 mice display hyperexcitability in axons from the mouse vagus nerve, indicative of increased excitatory transmission to central autonomic neurons (Glasscock et al., 2012). These mice also have increased respiratory drive and apnea (Simeone et al., 2018). Although this study showed that respiratory dysfunction occurs in Kv1.1 knockout mice, the authors did not show that sudden death occurred in concert with respiratory dysfunction. Researchers have also shown that increased activation of *KCNQ2*, which encodes the Kv7.2 potassium channel, in vagal nerve fibers rescues hyperexcitability seen in Kv1.1 knockout mice (Glasscock et al., 2012) and that *SCN2A* deletion improves survival rates in Kv1.1 knockout mice (Mishra et al., 2017).

Another genetic epilepsy model for SUDEP introduces a mutation into *KCNQ1* gene that encodes the Kv7.1 channel, one of the most common genes for long-QT syndrome in humans. Individuals with long-QT are at high risk for sudden death due to their propensity to have fatal ventricular arrhythmias (Goldman et al., 2016). When a dominant point mutation is introduced into the gene, it reproduces the human phenotype and these mice display dysfunctional neuronal repolarization, seizures, and sudden death (Goldman et al., 2009). The examination of genes encoding voltage-gated K^+ channels in the brain and the heart has provided valuable insight into how their dysfunction contributes to

epileptogenesis, autonomic dysfunction and SUDEP, however it is difficult to separate the genetic mutation from the seizures and sudden death.

1.3.3 Audiogenic Seizure Models

Researchers have used DBA/1 and DBA/2 mice to study mechanisms of SUDEP because they are susceptible to seizures when exposed to high-intensity acoustic stimulation (i.e. audiogenic seizures) (Faingold et al., 2010). Faingold et al. has shown that an audiogenic seizure leads to respiratory arrest in 30% of these mice (Faingold et al., 2010). However, this study did not complete any quantitative respiratory measures and only visual assessment of the chest rising and falling was done. Administration of SSRIs in these mice reduced the incidence of seizure-induced respiratory arrest (Tupal and Faingold, 2006; Zeng et al., 2015; Faingold et al., 2016). These mice also displayed altered expression of 5-HT receptors in the caudal brainstem (Uteshev et al., 2010). However, there are several pitfalls with this model: 1) they do not develop spontaneous seizures; 2) mice sometimes die after the first seizure, which does not meet the criteria for SUDEP; 3) It does not appear that autonomic dysfunction has been examined in this model. While genetic models of epilepsy have been informative to the field of SUDEP, they are lacking in that they only apply to a very small percent of individuals with epilepsy. Acquired epilepsies, (i.e. not caused by genetic mutations), affect a much larger percentage of individuals with epilepsy who are at risk for SUDEP and acquired epilepsy mouse models need to be utilized to determine biomarkers and preventative options for these individuals as well.

1.4 Acquired Epilepsies

1.4.1 Definitions and Epidemiology

Acquired epilepsies are broadly defined as a neurological disorder that is initiated by some type of insult to the brain (Delorenzo et al., 2005; Dudek and Staley, 2012). While epilepsy affects 1-2% of the population, about half of those individuals have acquired epilepsy (Delorenzo et al., 2005; McNamara and Scharfman, 2012). The other 50% of epilepsies are either idiopathic (i.e. no known cause in the absence of brain abnormalities) or genetic (Delorenzo et al., 2005). In acquired epilepsy, there is typically a known cause of the neurological insult that induces a permanent change in a previously normal brain leading to the development of epilepsy (Delorenzo et al., 2005). This occurs in 3 phases: the initial insult, the latent period of epileptogenesis, and spontaneous recurrent seizures (Delorenzo et al., 2005).

1.4.2 Temporal Lobe Epilepsy

The most common type of acquired epilepsy is TLE which affects 60% of all individuals with epilepsy according to the Epilepsy Foundation. TLE involves an imbalance in the excitatory and inhibitory networks within the temporal lobe. This includes the hippocampus, amygdala, auditory cortex, and the entorhinal cortex. TLE is form of partial or focal epilepsy that can be further split into 2 types: medial TLE and neocortical TLE. Medial TLE is the most common of the two (Tellez-Zenteno et al., 2005; McNamara and Scharfman, 2012). While there is little research done on the epidemiology of TLE, the reported incidence is 10.4 per 100,000 individuals (Tellez-Zenteno and Hernandez-Ronquillo, 2012).

1.4.3 Pathophysiology of TLE

As in acquired epilepsy, an initial insult followed by a latent period culminates in recurrent seizure pathology (White, 2012). The pathogenesis of TLE is progressive in nature and it is hypothesized that synaptic reorganization and a disruption in the balance of excitation and inhibition play key roles in the development of spontaneous seizures (Dudek and Staley, 2012). The death of GABAergic interneurons directly following the insult and during the latent phase is hypothesized to be responsible for decreases in GABA-mediated inhibition, allowing for increases in network excitation (Kobayashi and Buckmaster, 2003; Ben-Ari and Dudek, 2010; Dudek and Staley, 2012). Axon sprouting also contributes to the development of increased excitation in TLE (de Lanerolle et al., 1989; Sutula et al., 1989; Dudek and Staley, 2012), as the majority of these nerve connections are excitatory.

1.5 Animal Models of TLE

1.5.1 Electrical Kindling

Kindling is a process that consists of daily electrical stimuli delivered to brain structures such as the amygdala or hippocampus. When repeated over time, this induces a generalized convulsive seizure and mice eventually develop a shorter latency to seize as a response to stimulation. However, it is still debated if kindling accurately reflects the human condition since rodents do not develop recurrent spontaneous seizures (Loscher and Brandt, 2010). In terms of changes to cellular pathology, increased recurrent excitation and altered GABA interneuron

dysfunction does occur in this model (Lynch and Sutula, 2000; Sutula and Dudek, 2007; White, 2012).

1.5.2 Kainic-Acid Model

Kainic acid (KA) is an excitatory neurotransmitter agonist that activates glutamate receptors and produces cell death via excitotoxicity when administered to rodents (Cronin and Dudek, 1988). A single injection of KA leads to seizure development in 50% of rats (Cronin and Dudek, 1988) and multiple low-dose injections of KA leads to SE and subsequent TLE in 75-90% of rats (Hellier et al., 1998; Hellier and Dudek, 2005; Williams et al., 2009). Recurrent neuronal excitation and synaptic reorganization also occurs in this model. However, some mouse strains, such as C57BL/6 and BALB/c mice, are more resistant to KA-induced cell death and degeneration at single and multiple injections (Schauwecker and Steward, 1997; McKhann et al., 2003). Recurrent seizure activity can reliably occur when KA is injected directly into the hippocampus (Bouilleret et al., 1999), although this technique is more invasive than intraperitoneal injections.

1.5.3 The Pilocarpine-Induced Status Epilepticus Model of TLE

The pilocarpine-induced status epilepticus (pilo-SE) model of TLE was discovered to cause an initial period of severe seizures by Turski et al. in the early 1980's. They observed that administration of pilocarpine results in a 1-2 hour period of behavioral limbic seizures (Turski et al., 1983; Turski et al., 1984). It was later found that following a latent period of 4-6 weeks, mice that developed SE, also developed spontaneous seizures (Turski et al., 1989; Cavalheiro et al., 1994;

Cavalheiro et al., 1996; Shibley and Smith, 2002; Winokur et al., 2004). About 30-40% of mice develop SE from the pilocarpine injection, there is an ~30% mortality rate, and the rest of the mice that do not have SE do not develop spontaneous seizures (Shibley and Smith, 2002).

The pilo-SE model of TLE will be used in this dissertation because it more accurately models the human form of TLE. Additionally, various mouse strains reliably develop spontaneous seizures, whereas other models vary across strain in terms of TLE development. It follows the previously set forth hypothesis of an initial insult, followed by a latent period, and then spontaneous seizures. Pilocarpine has been shown to induce cell death within a few days of administration (Kobayashi and Buckmaster, 2003). Research has suggested eventual development of recurrent seizures in this model is associated with synaptic reorganization and aberrant sprouting in the hippocampus (Dudek and Spitz, 1997; Dudek, 2002). Additionally, pilocarpine causes an insult in the same structures within the temporal lobe that are affected in individuals with TLE. There are mouse strain differences in the mortality and cell death in the hippocampus following the pilocarpine treatment (Schauwecker, 2012). For the purposes of this dissertation, mouse strains were used that have been well-established to develop chronic spontaneous seizures or were monitored for seizure activity (Shibley and Smith, 2002; Winokur et al., 2004; Bhaskaran and Smith, 2010b).

1.6 Significance of SUDEP in Individuals with TLE

About 60% of all individuals with epilepsy have TLE and about half of those individuals have refractory TLE. Refractory TLE is defined by the Epilepsy

Foundation as seizures that are not controlled by medications. In this case, the individual has failed to become and stay seizure free with two or more trials of AEMs. In fact, refractory epilepsy is one of the biggest SUDEP risk factors (Tomson et al., 2008; Lhatoo et al., 2010b). Additionally, individuals with refractory TLE have a higher frequency of seizures compared to those on AEMs, placing them at an even higher risk for sudden death (Devinsky, 2011; Lamberts et al., 2012; Tolstykh and Cavazos, 2013; Thurman et al., 2014).

There have been several studies showing that individuals with TLE have cardiac and autonomic dysfunction. Increases in heart rate can occur in children and adolescents with TLE (Mayer et al., 2004) and in newly diagnosed individuals, who show ictal sympathetic overdrive, indicative of autonomic imbalance (Romigi et al., 2016). While the evidence in patient studies is suggestive of autonomic and cardiac dysfunction, it is conflicting as to whether this is associated with an increase in sympathetic drive, a decrease in vagal tone or a combination of both leads to autonomic imbalance. Many studies have found there to be an increase in the time and frequency measurements of HRV, including impaired baroreflex sensitivity potentially contributing to SUDEP risk (Dutsch et al., 2006; Romigi et al., 2016). Others have observed a decrease in HRV, indicative of a decrease in vagal tone (Massetani et al., 1997; Tomson et al., 1998; Ansakorpi et al., 2002; Ronkainen et al., 2005; Jansen and Lagae, 2010; Surges et al., 2010; Suorsa et al., 2011). HRV measurements seem to improve if TLE is controlled by medication or surgery (Tomson et al., 1998; Hilz et al., 2002; Suorsa et al., 2011). Suorsa et al. measured heart rate and HRV in individuals with TLE and in a follow-up study

6 years later found that individuals with refractory TLE had decreased HRV over time (Suorsa et al., 2011). This suggests that recurrent seizure activity has a progressive effect on worsening autonomic imbalance. Many studies have also found that individuals with TLE have ictal tachycardia, increased ictal and post-ictal heart rate, arrhythmia, and pathologic repolarization, suggestive of cardiac dysfunction (Massetani et al., 1997; Jansen and Lagae, 2010; Surges et al., 2010). HRV and vagal suppression may also have a circadian component, as individuals with TLE have more pronounced HRV suppression at night compared to healthy individuals (Ronkainen et al., 2005). Interestingly, no respiratory abnormalities were found in individuals with TLE, but respiration was only measured in the inter-ictal periods (Scorza et al., 2007). Additional evidence linking TLE to brainstem dysfunction regulating autonomic output comes via functional MRI studies. There was found to be lower connectivity from the temporal lobe to the ARAS and brainstem in TLE individuals at high risk for SUDEP (Allen et al., 2017; Englot et al., 2017a). Studies examining pilocarpine-induced SE in rodents also suggest cardiac and autonomic imbalance occurs. In acute pilocarpine-induced SE, there is an increase in heart rate and blood pressure in the hour following SE (Metcalf et al., 2009b; Metcalf et al., 2009a). This suggests that an increase in sympathetic drive occurs in the weeks following pilo-SE. Another study found altered autonomic control of heart rate in rats 1-2 weeks after pilo-SE (Bealer et al., 2010). This alteration in sympathovagal balance may increase the risk for arrhythmia and sudden death. However, there has been a paucity of studies examining the effect of spontaneous recurrent seizures in the pilocarpine-induced SE (i.e. pilo-SE)

model of TLE in relation to SUDEP. The focus of this dissertation will be on the association between the pilo-SE model of TLE, central autonomic dysfunction, and SUDEP.

1.7 The Vagal Complex

1.7.1 Vagal Complex Anatomy

The vagal complex is located in the caudal brainstem and is a critical central regulator of autonomic function. Viscerosensory inputs from the afferent fibers of the vagus, glossopharyngeal, and facial nerves end in a bundle of primary sensory fibers called the solitary tract (ST). The ST also conveys information from the baroreceptors and chemoreceptors in the cardiovascular and respiratory systems. The terminals of the ST release glutamate onto neurons within the nucleus tractus solitarius (NTS). The NTS contains a heterogeneous population of both GABAergic and glutamatergic neurons. These neurons serve as critical filters, integrators, and modulators of sensory afferent information in order to regulate sympathetic and parasympathetic output to the periphery. NTS neurons project their axons to preganglionic parasympathetic motor neurons in the DMV and NA (Andresen and Kunze, 1994; Doyle and Andresen, 2001; Wang et al., 2001a; Travagli et al., 2006; Bailey et al., 2008). Glutamatergic neurons are responsible for the regulation of sympathetic output of cardiac activity. Their axons project and synapse to inhibitory neurons in the CVLM. CVLM neurons project to the RVLM, which send glutamatergic fibers to preganglionic neurons in the spinal cord (Gordan et al., 2015). Glutamatergic NTS neurons also regulate the DMV by providing phasic excitation (Travagli et al., 1991; Gao and Smith, 2010).

GABAergic NTS neurons maintain tonic inhibition that aids in the regulation of vagal outflow (Davis et al., 2004; Travagli et al., 2006). About 20% of neurons in the DMV influence the regulation of vagal tone to the periphery. The rest of the GABA NTS neurons project to cardiac vagal motor neurons (CVMNs) in the nucleus ambiguus (NA). CVMNs are motor neurons that synapse onto postganglionic neurons in the cardiac ganglia at the base of the heart (Kunze and Ritchie, 1990; Dergacheva et al., 2013). Vagal circuit plasticity has been shown to occur in other disease states that affect autonomic regulation of homeostasis (Mei et al., 2003; Bach et al., 2015; Boychuk et al., 2015a). It therefore stands to reason that vagal complex neurons may have altered function in response to epileptogenesis, especially since patient data suggests altered autonomic function in TLE (Ronkainen et al., 2005; Romigi et al., 2016).

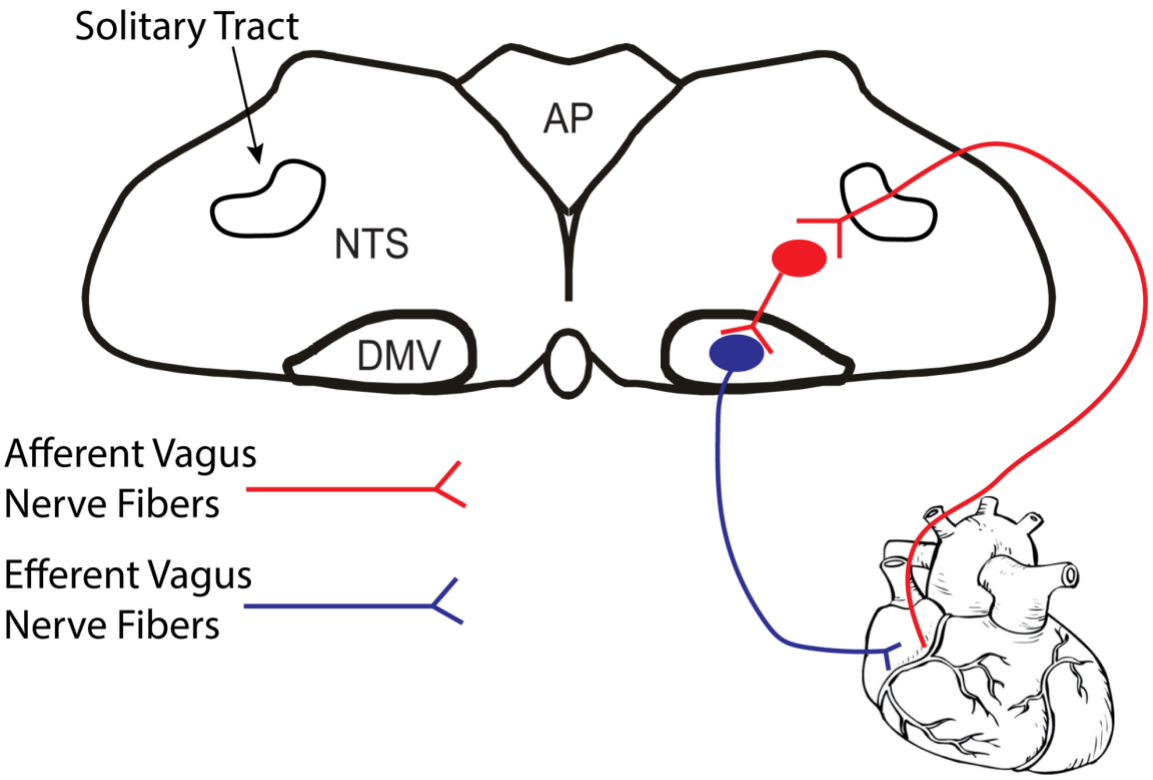

Figure 1.1 Schematic of vagal complex circuitry.
NTS = nucleus tractus solitarius; DMV = dorsal motor nucleus of the vagus

1.7.2 Characteristics of GABA NTS Neurons

GABAergic neurons make up a large proportion of NTS neurons and are interspersed throughout the lateral and medial NTS (Blessing, 1990; Minson et al., 1997; Fong et al., 2005). While GABA NTS neurons project to the DMV and NA, they can also act as local interneurons, inhibiting NTS signaling (Davies et al., 1987; Ezure and Tanaka, 1996; Davis et al., 2004; Kubin et al., 2006; Travagli et al., 2006). The medial NTS tends to receive major input from arterial baroreceptors (Andresen and Kunze, 1994; Andresen and Yang, 1995). Lateral NTS neurons also play a role in respiratory regulation (Wasserman et al., 2002).

GABA is synthesized from glutamate via the enzyme glutamate decarboxylase (GAD). GAD is present only in cells that use GABA as a neurotransmitter (Simon et al., 1985; Erlander and Tobin, 1991; Roth and Draguhn, 2012). Cytosolic GABA is shuttled into vesicles by vesicular GABA transporter (VGAT), which is highly concentrated in presynaptic terminals (Chaudhry et al., 1998; Jin et al., 2003). When a depolarization of the presynaptic neuron prompts the release of the GABA into the synaptic cleft, GABA binds to postsynaptic receptors, hyperpolarizing the postsynaptic neuron. GABA's action is terminated via reuptake into the presynaptic terminals and/or nearby glial cells. Then GABA can be degraded by GABA transaminase into glutamate and succinic semialdehyde (Roth and Draguhn, 2012).

GABA NTS neurons are important to many processes for autonomic homeostasis as they are central integrators of peripheral cardiovascular, respiratory, and gastrointestinal systems. GABA NTS neurons are able to directly

modulate heart rate, as microinjection of GABA antagonists into the NA decreases heart rate (DiMicco et al., 1979; Wang et al., 2001a). GABA NTS neurons also play a role in hypoxia and respiration (Accorsi-Mendonca et al., 2015), gastrointestinal regulation, and glucose sensing (Browning et al., 2002; Bach and Smith, 2012; Boychuk et al., 2015b; Boychuk et al., 2017).

1.8 Voltage-Gated K^+ Channels

1.8.1 Structure and Function of Voltage-Gated K^+ Channels

Voltage-gated K^+ (Kv) channels are widely expressed in the brain and throughout the central and peripheral nervous system (Coetzee et al., 1999; Birnbaum et al., 2004; Villa and Combi, 2016). The human genome contains at least 100 different Kv channel subunits (Cooper, 2012). All Kv channels and their variants are built similarly with six transmembrane spanning (TM) domains. The ion conducting pore is made up of four polypeptide domains arranged around a central water-filled transmembrane region. There are four pore-forming segments with intracellular N- and C-termini, 2 transmembrane segments, and an extracellular loop that partially dips into the pore acting as a K^+ selectivity filter. There is also a very short, highly conserved length of polypeptide that allows for the movement of the voltage-sensor by pulling open the pore during depolarization, and pushing it closed at rest (Cooper, 2012). Humans have 36 6TM Kv channel subunit genes that vary in their biophysical properties (Coetzee et al., 1999; Cooper, 2012).

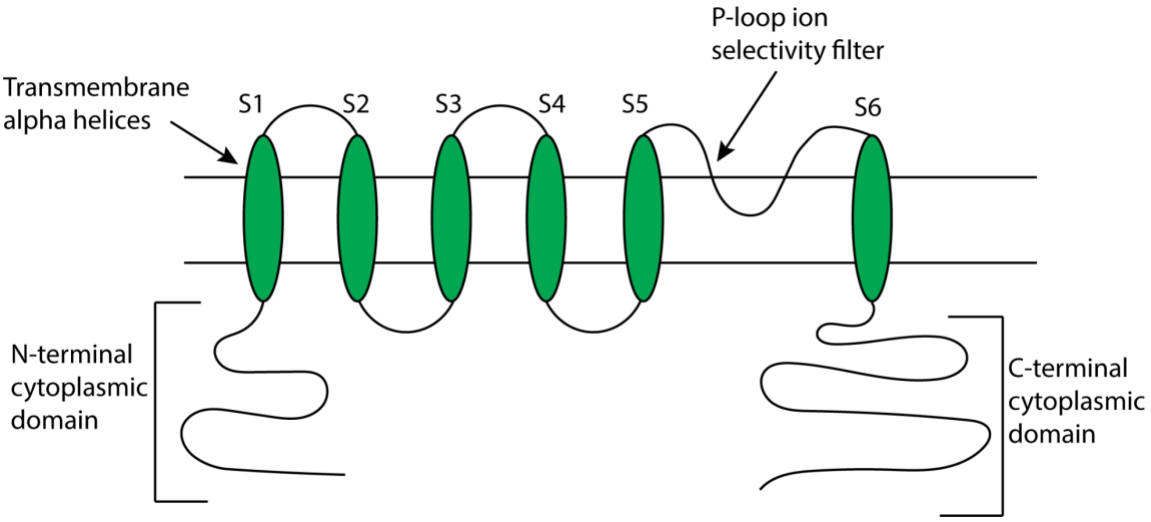

Figure 1.2 Schematic of Kv4 ion channel

Kv4 channels are one subfamily of the voltage-gated K^+ channels that are in both the cortex and the NTS. There are 3 different Kv4 subtypes: Kv4.1, Kv4.2, and Kv4.3 (Birnbaum et al., 2004). These channels influence neuronal firing properties and cellular excitability (Jerng et al., 2004; Covarrubias et al., 2008; Strube et al., 2015). Various K^+ channel subunits have been found to modulate Kv4 activity. The most well-characterized of those subunits are known as K^+ channel interacting proteins (KChIPs). KChIPs are neuronal calcium sensors that have a variable amino acid terminal domain and a conserved carboxyl terminal with calcium binding motifs (Birnbaum et al., 2004). There are 4 different types of KChIPs (KChIP1, KChIP2, KChIP3, KChIP4) that are found in the brain and colocalize with the Kv4 family of channels (Birnbaum et al., 2004; Jerng et al., 2004). KChIP1-4 have been shown to colocalize with all members of the Kv4 family in a nonspecific manner (An et al., 2000; Bahring et al., 2001; Birnbaum et al., 2004; Covarrubias et al., 2008; Cheng et al., 2016). When co-expressed with Kv4 channels, KChIPs increase current density by increasing the surface expression Kv4 channels (Birnbaum et al., 2004; Jerng et al., 2004; Covarrubias et al., 2008; Schwenk et al., 2008). Additionally, KChIPs can alter the kinetics of activation and inactivation of Kv4 channels, such as hyperpolarizing the activation curve, quicken recovery from inactivation, and slowing the rate of inactivation (Birnbaum et al., 2004; Covarrubias et al., 2008). KChIPs can also promote channel trafficking to the membrane (An et al., 2000; Jerng et al., 2004; Covarrubias et al., 2008).

1.8.2 Association with TLE

While there are many Kv subtypes that have been associated with epilepsy in humans and animals, this dissertation places particular focus on the Kv4 subfamily (D'Adamo et al., 2013; Villa and Combi, 2016). The Kv4 subfamily has been shown to be associated with human epilepsies and it is also expressed in the vagal complex (Singh et al., 2006; Smets et al., 2015; Villa and Combi, 2016). Voltage-gated K^+ (Kv) channels have been shown to be altered in individuals with epilepsy with TLE (Singh et al., 2006; Villa and Combi, 2016). Kv channels play a major role in neuronal excitability and K^+ channelopathies can contribute to seizures (Brenner and Wilcox, 2012; D'Adamo et al., 2013; Villa and Combi, 2016). A truncated Kv4.2 subunit has been found to cause seizures in a patient case study (Singh et al., 2006) and other gain of function mutations in Kv4.2 were found in intractable epilepsy (Smets et al., 2015).

Kv4 channels are essential for the regulation of neuronal excitability, specifically modulating the A-type current (I_A). The modulation of I_A is crucial in the repolarization phase of the action potential and prevents action potential back propagation. Bernard et al. has shown hippocampal neurons from pilo-SE mice have decreased Kv4.2 subunit expression and altered I_A (Bernard et al., 2004). However, in this study, there was no mention of how long the mice had spontaneous seizures prior to being used in their experiments. This is crucial as many rodents develop spontaneous seizures at different time points (Shibley and Smith, 2002), which may have an effect on Kv4.2 expression. A separate study found a decrease in Kv4.2 and KChIP2 expression in the hippocampus 4 and 12

weeks after pilo-SE treatment (Monaghan et al., 2008). Another study measured Kv4.2 expression in the chronic spontaneous seizure phase and found that there was a decrease in Kv4.2 and KChIP1 protein levels in the hippocampus (Su et al., 2008). Alterations in these channels have yet to be investigated in brain regions outside of the hippocampus in a mouse model of TLE.

Kv4 subtypes and I_A have been shown to be expressed in the NTS and be altered in pathological states. I_A modulates synaptic transmission to other vagal complex pathways, current amplitude, activation kinetics, and protects against excitotoxicity (Bailey et al., 2002; Yang et al., 2005; Bailey et al., 2007; Covarrubias et al., 2008; Strube et al., 2015). In pathological states, I_A has been shown to be altered in hypertension (Sundaram et al., 1997; Belugin and Mifflin, 2005). Additionally, pharmacologic blockade of I_A modulates electrophysiological properties of NTS neurons and attenuates baroreflex regulation of heart rate (Butcher and Paton, 1998). Therefore, if the NTS altered during TLE, I_A may also have altered function as well.

1.9 Study Aims and Significance

This study will focus on the association between the pilo-SE induced model of TLE, central control of autonomic function, and SUDEP. This study will further focus on alterations in GABA NTS neuron function during epileptogenesis. Other studies examined cardiac function acutely following pilocarpine; therefore, this study will have longer time points allowing for the investigation of long-term effects of epilepsy development with chronic spontaneous seizures on cardiac and GABA NTS function. The investigation into mechanisms of SUDEP in TLE could lead to

the development of SUDEP biomarkers or preventative treatments in an under-researched population that is at high risk for SUDEP. The hypothesis that the pilocarpine-induced SE model of TLE is associated with SUDEP, altered cardiac, and alterations in the synaptic and functional properties of GABAergic NTS neurons was tested.

The specific aims of this project are as follows:

1) **Determine if pilo-SE mice suffer from SUDEP with related changes in cardiac and GABA NTS neuron function.** Because the pilocarpine-induced SE model of TLE has not been used to study SUDEP, it was first determined if pilo-SE mice die suddenly in the weeks following survival from the initial pilocarpine-induced SE. Cardiac and autonomic dysfunction occurs in individuals with TLE, thus ECG was used to investigate these factors in the weeks following pilocarpine-induced SE. Lastly, as GABAergic NTS neurons directly modulate autonomic output to the periphery, electrophysiology was used to assess their function 1, 6, and 12 weeks after pilocarpine-induced SE.
2) **Determine if Kv4 subunits and I_A are altered in the pilo-SE model of TLE in GABA NTS neurons.** Immunohistochemistry and western blot have shown that Kv4 subunits are downregulated in the hippocampus following pilocarpine-induced SE. No studies have assessed alterations in Kv4 expression or I_A in the context of TLE within vagal complex. Whole-cell patch-clamp electrophysiology was used to measure action potential

frequency and half-width, and to isolate I_A density. Differential expression of Kv4.1, Kv4.2, Kv4.3 and KChIPs1,3, and 4 was also measured.

3) **Utilize chemogenetic manipulation of GABA NTS neurons to determine if activation of these neurons can alter seizure threshold**. Designer receptors exclusively activated by designer drugs (DREADDs) have been shown to selectively excite or inhibit specific neuronal subtypes in order to modulate both animal behavior and neuronal function. The excitatory DREADD, pAAV8-hSyn-DIO-hM3D(Gq)-mCherry, was used to selectively excite GABAergic NTS neurons in pilo-SE mice. *In vivo* studies were done to determine if behavioral seizures could be elicited via DREADD activation or if seizure latency could be modulated. Electrophysiology was used to determine the effect of DREADD activation on firing properties of GABA NTS neurons and synaptic properties of local DMV neurons.

Chapter 2 Materials and Methods

2.1 Mice

For the survival study and in vivo electrocardiography (ECG) in Chapter 3, 5 week old male CD-1 mice (Harlan Laboratories were used). For electrophysiology studies in Chapters 3 and 4, 4-6 week old male Green Inhibitory Neuron (i.e. GIN; FVB-Tg (GADGFP) 4570Swn/J; The Jackson Laboratory) was used. GIN mice utilize GFP as a fluorescent reporter for somatostatin expressing GABAergic neurons within the NTS, which allows for the targeted recording of these neurons (Oliva A.A. et al., 2000). For chemogenetic studies in Chapter 5, 4-6 week old male Slc32a1$^{tm2(cre)Lowl}$/J (i.e. Vgat-ires-cre; The Jackson Laboratory) were used. Vgat-ires-cre mice express cre recombinase activity in GABAergic neurons without interrupting endogenous vesicular GABA transport activity. All procedures were approved by the University of Kentucky Animal Care and Use Committee and NIH guidelines were adhered to for the care and use of animals. All animals were housed in Association for Assessment and Accreditation of Laboratory Animal Care approved facilities, on a 14:10 light:dark cycle with food and water available *ad libitum*.

2.2 The Pilocarpine-Induced Model of TLE

TLE was induced in all experimental mice following procedures similar to that of Shibley and Smith 2002 (Shibley and Smith, 2002). First, all mice were given an intraperitoneal (i.p.) injection of methylscopolamine (1 mg/kg in 0.9% saline). Methylscopolamine is a muscarinic antagonist that does not cross the blood brain barrier and its administration serves to reduce the peripheral

convulsant effects of pilocarpine. After 20 minutes, mice were given either an i.p. injection of pilocarpine (280-285 mg/kg in 0.9% saline) or vehicle (0.9% saline). Pilocarpine is a tertiary amine that readily crosses the blood brain barrier and acts as a nonselective muscarinic receptor agonist (Geller, 1984). Twenty to 30 minutes after pilocarpine injection, mice began to display behavioral seizures which are rated on based on a modified Racine scale, from category 1-5, with 5 being the most severe (Racine, 1972). All mice were monitored for behavioral seizures for 2 hours post-injection. Category 1 and 2 consist of tail-stiffening, freezing, wet dog shakes, and facial automatisms. All mice exhibited some or all of these behaviors. Category 3 (i.e. unilateral forelimb myoclonus), category 4 (i.e. bilateral forelimb myoclonus and rearing), and category 5 (i.e. bilateral fore- and hindlimb myoclonus and transient loss of postural control) were considered to be generalized convulsive seizures. Seizures were typically 30-90 seconds in duration and separated by periods of relative inactivity. Mice that displayed a minimum of 3 category 3-5 seizures were considered to have undergone status epilepticus (SE) and were subsequently classified as pilo-SE mice. Greater than 90% of pilo-SE mice went on to develop spontaneous seizure activity 4-6 weeks post-injection (Shibley and Smith, 2002; Winokur et al., 2004; Bhaskaran and Smith, 2010b). No seizure activity was observed in control mice at any time post-injection.

In addition to standard diet, mice were given water-moistened food and a 5% glucose solution in their cage for 4 days post-treatment. Animals were also given diet gel to supplement their standard chow and aid in avoiding dehydration. Animals were weighed twice a day for 4 days following SE. If an animals weight

decreased by more than 20% following SE and they did not recuperate following subcutaneous ringer solution injections, they were euthanized.

A small cohort of GIN mice were observed two weeks prior to their use in electrophysiology experiments for behavioral seizure activity. Mice were observed for 2 hours, 3 times per week (Shibley and Smith, 2002; Winokur et al., 2004; Hunt et al., 2013). Using this same protocol, behavioral observation for seizures were conducted in a small cohort of Vgat-ires-cre animals in order to establish that they developed chronic spontaneous seizures, as some mouse strains are resistant to seizure morphology (Schauwecker, 2012).

2.3 Brainstem Slice Preparation

Patch-clamp recordings were made in brainstem slices from mice. Mice were deeply anesthetized via inhalation of isofluorane to effect (i.e. lack of response to toe-pinch) and decapitated. The brain was removed and placed into ice-cold (2-4 °C) artificial cerebrospinal fluid (ACSF) containing (in mM): 124 NaCl, 3 KCl, 2 $CaCl_2$, 1.3 $MgCl_2$, 1.4 NaH_2PO_4, 26 $NaHCO_3$, and 11 glucose (pH 7.2-7.4). The brain was blocked on an ice-cold stand and glued on a platform for sectioning in the coronal plane. Transverse 300 μm thick sections from the caudal brainstem were made in cold ACSF using a vibrating microtome (Vibratome series 1000; Technical Products International) and transferred to a holding chamber containing warmed (30-32°C) oxygenated ACSF. Slices were incubated for at least one hour in the holding chamber prior to use in electrophysiological experiments. These brainstem slices contain the NTS and DMV and preserve primary afferent connections in the solitary tract.

2.4 Electrophysiological Recordings

Brainstem slices were transferred to a chamber mounted on a fixed stage under an upright microscope (BX51WI; Olympus, Center Valley, PA), where it was superfused with continuously warmed (30-32°C) oxygenated ACSF. Somatostatin containing GABAergic EGFP neurons were targeted under epifluorescence. Patch pipettes for recordings were pulled from borosilicate glass (open tip resistance 3-5 MΩ; King Precision Glass Co, Claremont, CA). The pipette solution contained (in mM): 130 K^+ gluconate, 1 NaCl, 5 EGTA, 10 HEPES, 1 $MgCl_2$, 1 $CaCl_2$, 3 KOH, and 2 ATP. Recordings were obtained using an Axon 700B amplifier (Molecular Devices, San Jose, CA), low pass filtered at 2-3 kHz, digitized at 20 kHz, and recorded onto a computer (Digidata 1440A, Molecular Devices). Seal resistance was between 2 and 5 GΩ for on-cell and whole-cell recordings.

On-cell patch-clamp recordings of spontaneous action potentials were recorded in voltage clamp mode at resting membrane potential in GABAergic NTS neurons. For whole-cell patch-clamp recordings, cells were voltage-clamped at -70 mV and allowed to equilibrate with the pipette recording solution for approximately five minutes. Spontaneous and miniature excitatory postsynaptic currents (sEPSCs and mEPSCs) were recorded in voltage-clamp mode at a holding potential of -70 mV. Resting membrane potential was recorded in I=0 mode, where no holding current was applied. Occasionally, spontaneous firing activity was seen in I=0, in which case the resting membrane potential was calculated by averaging regions where there was not firing activity. Spontaneous action potentials and current-voltage (I-V) curves were recorded in current-clamp

mode at resting membrane potential (i.e. I=0 pA). I-V curves were completed to measure GABAergic NTS neuron input resistance (R_{in}) and consisted of 6 current injections (-20 pA to +30 pA, 10 pA increments, 500 ms duration) with the resulting change in membrane potential being measured. R_{in} was calculated by plotting the linear portion of the I-V curve and calculating the slope. Series resistance was less than 25 MΩ and monitored periodically throughout the recordings. If the series resistance changed by more than 20% over the course of the experiment, the recording was discarded.

2.5 Drugs Used for Electrophysiological Recordings

The drugs and their respective concentrations used for experiments were: Tetrodotoxin (TTX; 1 μM; Alomone Labs), 4-Aminopyridine (4-AP; 5 mM; Sigma-Aldrich), (-)-Bicuculline Methiodide (BIC; 30 μM; Tocris Biosciences), Kynurenic Acid (KYN; 1 mM; Sigma-Aldrich); Tetraethylammonium Chloride (TEA-Cl; 10 mM; Sigma-Aldrich).

2.6 Statistical Analyses

For the survival study, a Kaplan-Meier survival curve was generated to assess mortality differences between control and pilo-SE mice. A log-rank (Mantel-Cox) test was used to assess statistical significance. This is a nonparametric test used to compare the survival of two samples. Data analysis was performed using GraphPad Prism (La Jolla, CA).

Electrophysiology measurements included sEPSC frequency, sEPSC amplitude, mEPSC frequency, mEPSC amplitude, spontaneous action potential frequency (on-cell and whole-cell), paired-pulse ratio (PPr), frequency dependent

depression, spontaneous action potential half-width, A-type K^+ current amplitude, sIPSC frequency, sIPSC amplitude, resting membrane potential and R_{in}. Analysis of electrophysiology measurements was performed using pClamp 10.2, MiniAnalysis (version 6.0.7, Synaptosoft; Decatur, GA) and GraphPad (La Jolla, CA) software programs. To measure sEPSC, mEPSC, whole and on-cell spontaneous action potential firing frequencies a 2-minute segment of continuous firing activity was used. For sEPSCs and mEPSCs only events with five times the root mean squared baseline noise were included. Events characterized by a fast rise phase and exponential decay were automatically detected and then manually verified by MiniAnalysis. An unpaired t-test was used to compare on and whole-cell action potential frequency, sEPSC frequency, sEPSC amplitude, mEPSC frequency, mEPSC amplitude, PPr, frequency dependent depression, and action potential half-width between control and pilo-SE mice. A two-way ANOVA with a Sidak's *post hoc* was used to compare the peak transient outward K^+ current at each voltage step between control and pilo-SE mice in normal ACSF and before and after the application of 4-AP. A 2-way ANOVA (Tukey's *post* hoc) was done to test for differences in the voltage dependence of activation and inactivation between control and pilo-SE mice. A 2-way ANOVA (Tukey's *post* hoc) was completed to assess differences in steady state values between control and pilo-SE mice. All data are presented as mean ± SEM and statistical significance was set at $p < 0.05$.

Chapter 3 Functional neuroplasticity in the nucleus tractus solitarius and increased risk of sudden unexpected death in mice with acquired temporal lobe epilepsy

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3.1 Introduction

Sudden unexpected death in epilepsy (SUDEP) occurs when an individual with epilepsy who is otherwise healthy dies suddenly for unknown reasons (Annegers, 1997; Nashef, 1997; Nashef et al., 2012; Tolstykh and Cavazos, 2013). For epilepsy patients, the risk of sudden unexpected death is >20-fold higher than in the general population and accounts for ~17% of epilepsy-related deaths (Nashef, 1997; Kalume et al., 2013; Tolstykh and Cavazos, 2013), so it is imperative to elucidate its underlying mechanisms. Patients with longstanding epilepsy characterized by frequent generalized tonic-clonic seizures that are relatively poorly controlled are at highest risk (Tolstykh and Cavazos, 2013; Thurman et al., 2014). Patients with TLE represent ~60% of all epilepsies, and seizures are medically intractable in about 30% of these patients, making this the largest population at risk of SUDEP, yet mechanisms underlying increased SUDEP risk have not been identified in animal models of acquired TLE. Peri-ictal, centrally originating or peripheral autonomic irregularities leading to cardiorespiratory collapse may be the immediate cause of death in SUDEP (Ryvlin et al., 2013), but few studies have been aimed at identifying mechanisms of autonomic failure.

Seizures can increase activity of neurons in brainstem autonomic areas, independently of physical activity or peripheral metabolic influences (Kanter et al., 1995; Takakura et al., 2011) and autonomic irregularities often develop over time in individuals with epilepsy, implicating central or peripheral autonomic reactive neuroplasticity as a potential driver of increased SUDEP risk in patients and rodent epilepsy models (Glasscock et al., 2010; Massey et al., 2014; Biet et al., 2015). Thus, recurrent seizures might induce changes in central or system physiologic functions that increase the risk for sudden death.

The brainstem vagal complex is the principal neural center mediating parasympathetic visceral regulation. Within the vagal complex, neurons of the nucleus tractus solitarius (NTS) receive viscerosensory information via vagal afferents and project their axons to preganglionic parasympathetic motor neurons in the dorsal motor nucleus of the vagus (DMV) and the nucleus ambiguus (NA; (Andresen and Kunze, 1994; Doyle and Andresen, 2001; Wang et al., 2001a; Wang et al., 2001b; Davis et al., 2004; Travagli et al., 2006; Glatzer et al., 2007; Bailey et al., 2008) as well as to brainstem and hypothalamic areas responsible for premotor sympathetic regulation and respiratory reflexes (Takenaka et al., 1995; Fontes et al., 2001; Irnaten et al., 2001; Bonham et al., 2006; Affleck et al., 2012; Zoccal et al., 2014). Inhibitory GABAergic NTS neurons participate in vagal reflexes and prominently regulate parasympathetic output (Davis et al., 2004; Travagli et al., 2006). Evidence from genetic epilepsy models suggests that epilepsy-related alterations in peripheral or central vagal function contribute to cardiorespiratory collapse and SUDEP (Glasscock et al., 2010; Cheah et al., 2012;

Glasscock et al., 2012; Kalume et al., 2013; Aiba and Noebels, 2015). Central vagal circuit plasticity is prominent in disease states that affect autonomic homeostasis (Mei et al., 2003; Bach et al., 2015; Bhagat et al., 2015; Boychuk et al., 2015a) and seizure-related derangement of central vagal system function has been briefly described, manifesting as increased likelihood of spreading depolarization in the NTS (Aiba and Noebels, 2015). Reactive neuroplasticity in the central vagal complex, however, has not been investigated in animals with acquired TLE.

We used the pilocarpine-induced status epilepticus (pilo-SE) model of TLE in mice (Shibley and Smith, 2002; Borges et al., 2003; Winokur et al., 2004; Groticke et al., 2007; Bhaskaran and Smith, 2010a; Bhaskaran and Smith, 2010b; Hunt et al., 2013) to identify long-term changes in NTS neuron function coinciding with the development of TLE. Because the NTS is the primary integration center for cardiorespiratory reflexes, and GABA neurons in particular are principal participants in vagal reflex activity (Glatzer et al., 2007; Bailey et al., 2008) increased excitability of these neurons would be consistent with an increased propensity for central autonomic failure that could lead to SUDEP in TLE. We tested the hypothesis that reactive plasticity of GABAergic circuitry in the NTS emerges over time in mice that survive pilocarpine-induced SE.

3.2 Methods

3.2.1 ECG Telemetry

Mice were anesthetized with 2.5% isofluorane in 100% O_2 at 1.5 L/min and 2-inch midline incision was made through the skin of the abdomen exposing the

fascia. Hemostats were used to separate the fascia from the skin and create space for the ECG leads underneath the skin. The telemeter body (model ETA-F10; Data Sciences International, St.Paul, MN) was implanted on the right flank with the positive lead near the right pectoral muscle and the negative lead on the left side of the abdomen. The leads were secured by being embedded in the fascia underneath the skin. The incision was closed with staples (autoclips, Kent Scientific Corporation, Torrington, CT) and allowed to heal for two weeks prior to use.

Telemeters were turned on at least 24 hours prior to recordings in order to minimize disturbances and stress to the mice. Data were recorded for 24 hours pre-injection of vehicle or pilocarpine, 24 hours after injection and at 6 and 12 weeks after injection. ECG data were collected with DSI DataQuest A.R.T. 4.31 and Ponemah 6.10 and analyzed with Ponemah 6.10 telemetry software. Data were acquired at a sampling rate of 1000 Hz, which is the standard rate used for mice and results in a smooth physiologic signal when the ECG waveforms are graphed; the telemetry device used a factory preset sampling rate of 200 Hz. No low-pass or high-pass filtering was applied during data acquisition. For ECG waveform analysis, the software was set to use a 40% QRS detection threshold (percentage of the largest derivative peak in QRS segment resulting in an R that satisfies the minimum heart rate criteria), a minimum of R deflection of 0.25 mV, a maximum heart rate of 1000 bpm, and a minimum heart rate of 80 bpm.

Average heart rate was calculated from data from 1 hour recording periods, as described previously (Metcalf et al., 2009a; Ho et al., 2011; Schroder et al.,

2015); all recordings were performed during seizure-free periods. The RR interval was manually examined and filtered for abnormal beats by sorting the RR interval from shortest to longest and deleting cycles that were two standard deviations from the average RR interval, and the ECG channel was subsequently reanalyzed by setting upper and lower limits on RR values (Thireau et al., 2008). Areas of the recording that contained skipped beats or loss of signal were also deleted. The remaining cycles were then averaged to comprise the NN (i.e. RR) interval, which was then used to calculate the standard deviation of the N-to-N interval (SDNN) and the root mean squared of the standard deviation of the RR interval (RMSSD). The SDNN was calculated by taking the square root of the averaged NN interval. The RMSSD was calculated with the following steps: (1) the difference between the NN interval and delayed NN interval was squared; (2) the squared difference was summed; (3) the number of NN intervals was counted; and (4) the sum of the difference squared was divided by the count of NN intervals. RMSSD is reported as the square root of this value.

3.2.2 Electrophysiological Stimulation

Electrical stimulation was performed using a platinum-iridium concentric bipolar electrode (125 μm diameter, FHC, Bowdoinham, ME) that was placed in the solitary tract (ST) (Glatzer and Smith, 2005; Glatzer et al., 2007). Sets of 5 current pulses (30-50 μA; 400 μs) were delivered at interpulse intervals of 30 ms at 50 Hz to the ST and responses in NTS neurons voltage-clamped at -70 mV were recorded and the constant latency and amplitude evoked EPSC (eEPSC) responses were measured. The stimulus intensity was adjusted so that eEPSCs

occurred > 80% of the time in GABAergic NTS neurons when stimulation was applied to the ST. Thirty stimulation sweeps were applied to each cell and averaged for further assessment. The ratio of the second to the first eEPSC (i.e. paired-pulse ratio, PPr) was measured in order to infer changes in the probability of excitatory neurotransmitter release. An increase in the release ratio typically correlates with a decrease in the probability of presynaptic neurotransmitter release, in this case presynaptic input arising from the ST (Schild et al., 1995; Zucker and Regehr, 2002; Pamidimukkala and Hay, 2004; Chen and Bonham, 2005; Glatzer and Smith, 2005; Glatzer et al., 2007; Laaris and Weinreich, 2007). Frequency-dependent depression (FFD) is a common characteristic of second-order NTS neurons that receive input from the ST (Miles, 1986; Doyle and Andresen, 2001; Bailey et al., 2008; Kline, 2008). In order to assess the FDD, the ratio of the amplitudes of the 5th eEPSC to the 1st eEPSC are measured. Stimulus sweeps were only included if an eEPSC was elicited after each stimulation in that sweep and the constant latency was less than 0.2 ms to ensure that responses to stimulation were monosynaptic in nature (Glatzer et al., 2007; Bailey et al., 2008).

Methods for Chapter 3 also included: methylscopolamine and pilocarpine injections, seizure assessment, and on- and whole-cell patch-clamp electrophysiology as described in Chapter 2 of this dissertation.

3.3 Results

3.3.1 Pilocarpine-induced SE as a model of SUDEP

Spontaneous seizure activity was monitored in a cohort of mice (n=6 control mice; n=6 pilo-SE mice) for 1 week between 11 and 12 weeks post-SE. Similar to

previous reports (Shibley and Smith, 2002; Winokur et al., 2004; Hunt et al., 2013) spontaneous seizures were observed during this period in 83% (5 of 6) pilocarpine-treated mice that survived SE. A separate cohort of mice were monitored for long-term survival after pilocarpine-induced SE. Similar to previous reports (Shibley and Smith, 2002; Winokur et al., 2004), all vehicle-injected mice survived the duration of the monitoring period of 150 days (100%; n=10 mice). Between 1 and 7 days after pilocarpine-induced SE, there was a 13.33% mortality rate (2/15 mice). These mice were not considered to have died of SUDEP, as epilepsy (i.e. with spontaneous seizures) likely had not developed by this time. Of the 13 pilocarpine-treated mice that survived for greater than 7 days post-SE, only 3 mice (23%) survived to 150 days post-treatment, with no obvious trauma or other incident; 60% died after greater than 40 days post-SE (Fig. 3.1). Thus, the survival rate of mice that survived pilo-SE was significantly decreased at 150 days compared to with vehicle-treated control mice (log-rank Mantel-Cox; $p = 0.0002$).

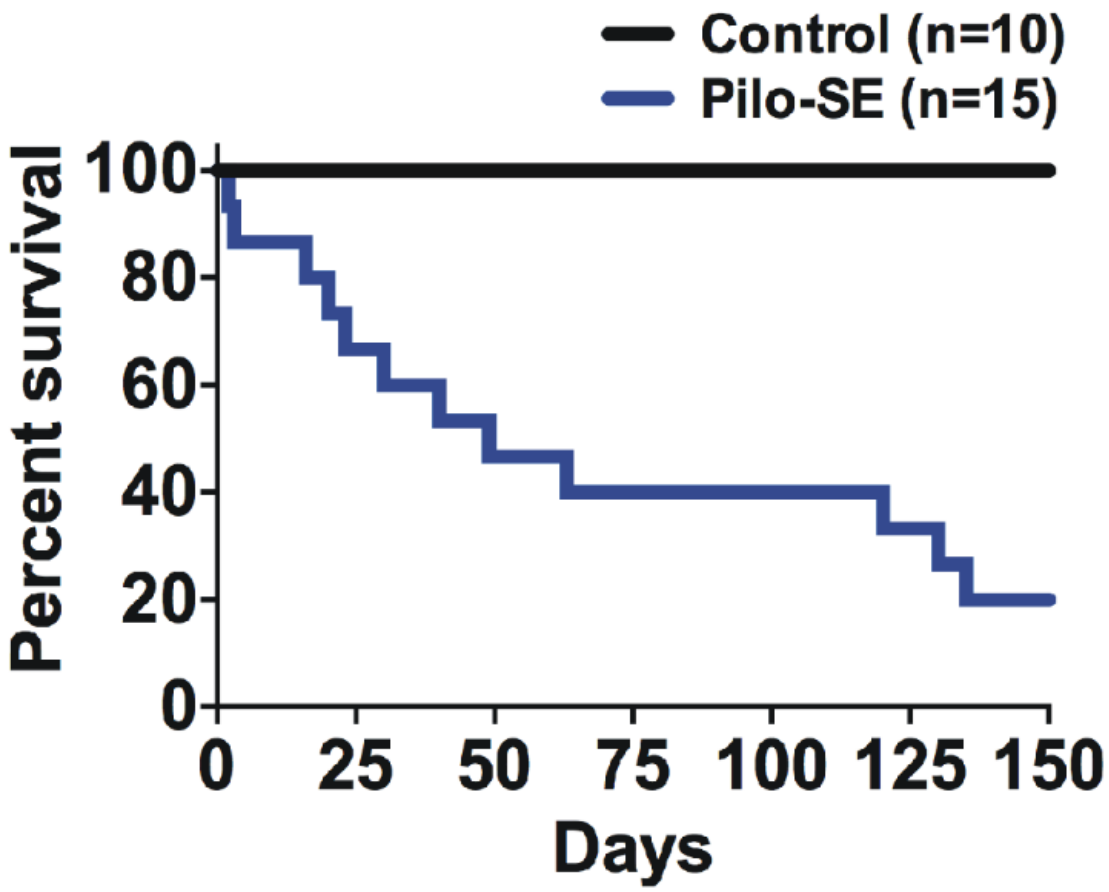


Figure 3.1 Pilocarpine-induced SE (pilo-SE) increases the risk of sudden death. Pilocarpine-treated mice (Pilo-SE; n=15) have a decreased survival rate (23%) compared to control mice (n=10, 100%; Log-rank Mantel-Cox; p = 0.0002). Mice that survived SE died suddenly and unexpectedly at post-SE time points associated with the development of spontaneous seizures.

3.3.2 Increased action potential firing in GABAergic NTS neurons from pilo-SE mice is glutamate receptor dependent

Seizure-induced depolarization in the NTS of mice with genetic epilepsies originates in the lateral NTS (Aiba and Noebels, 2015), and area densely comprised of GABAergic neurons (Blessing, 1990; Fong et al., 2005; Glatzer et al., 2007). We hypothesized that GABAergic NTS neurons, most of which receive primary viscerosensory input from the vagus nerve (Glatzer et al., 2007; Bailey et al., 2008), are altered functionally post-SE. On-cell recordings of GABAergic NTS neurons were performed to determine whether spontaneous action potential firing differed between control and pilo-SE mice (Fig. 3.2A,B,E). One week post-treatment, GABAergic NTS neurons from seven pilo-SE mice displayed significantly higher action potential firing frequency (3.35 ± 0.46 Hz; n=20 cells) compared with NTS GABAergic neurons from seven age-matched control mice (1.32 ± 0.30 Hz; n=15 cells; p = 0.002). Spontaneous action potential firing in GABAergic NTS neurons was also significantly increased 6 weeks after treatment in five pilo-SE mice (3.32 ± 0.65 Hz; n=16 cells) compared with eight age-matched control mice (2.10 ± 0.25 Hz; n=26 cells; p = 0.046). Similarly, action potential firing frequency remained significantly greater in GABAergic NTS neurons from seven age-matched pilo-SE (4.27 ± 0.96 Hz; n=24 cells) than seven age-matched control mice (2.21 ± 0.27 Hz, n=23 cells; p = 0.0048) 12 weeks post-treatment (Fig.3.2E). Therefore, action potential frequency was consistently higher in GABAergic NTS neurons in mice that survived SE than in control mice.

To determine whether the increase in firing frequency was due to increased activation of ionotropic glutamate receptors, spontaneous action potential firing was recorded in the presence of the ionotropic glutamate receptor antagonist, kynurenic acid (KYN; 1mM; Fig.3.2C,D,F). In the presence of KYN, the action potential firing frequency in GABAergic NTS neurons from pilo-SE mice was similar to that of control mice 1, 6, and 12 weeks post-treatment (week 1: $p = 0.47$; week 6: $p = 0.83$; week 12: $p = 0.78$; Fig.3.2F). Therefore, increased action potential firing in GABAergic NTS neurons from mice that survived SE depended on activation of ionotropic glutamate receptors, implicating increased glutamate-mediated, excitatory synaptic drive to these neurons during epileptogenesis.

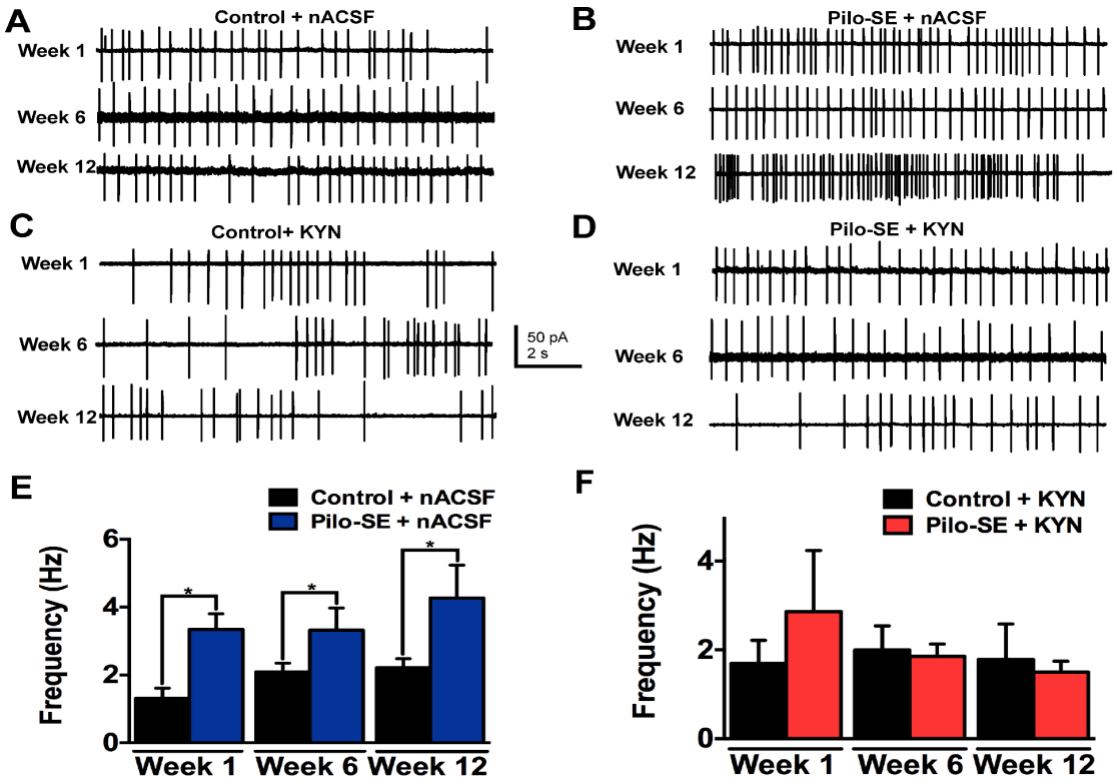


Figure 3.2 Action potential frequency in GABAergic nucleus tractus solitarius (NTS) neurons from pilo-SE mice is dependent on glutamate receptor activation. A) Representative traces showing action potential firing (Na^+ currents) in GABAergic NTS neurons in slices from control mice recorded in normal ACSF (nACSF) at three different time points (i.e., 1, 6, and 12 weeks) after vehicle treatment. (B) Representative traces showing action potential firing in GABAergic NTS neurons from mice that survived pilo-SE under normal recording conditions (nACSF) at three different time points after SE. (C) Representative traces of action potential firing in control mice in the presence of kynurenic acid (KYN; 1mM) at these same time points. (D) Representative traces of action potential firing in the presence of KYN in pilo-SE mice. (E) Action potential firing frequency is significantly greater in pilo-SE mice compared to age-matched control mice at 1, 6, and 12 weeks post-treatment (unpaired *t*-test; *denotes significance at $p<0.05$). (F) In the presence of KYN, action potential firing frequency in NTS GABA neurons from pilo-SE mice was not significantly different than in control mice (unpaired *t*-test; $p>0.05$).

3.3.3 GABAergic NTS neurons display increased excitatory regulation

An increase in spontaneous action potential firing in GABAergic NTS neurons from pilo-SE mice could occur because of altered intrinsic and/or synaptic properties. To determine whether intrinsic properties were altered post-SE, we measured the resting membrane potential and input resistance in GABAergic NTS neurons and found that there were no significant differences between control and pilo-SE mice at any time point (Table 3.1). Because firing rate differences were abrogated by KYN, we hypothesized that excitatory glutamatergic synaptic input was increased after pilo-SE. To test this hypothesis, whole-cell patch-clamp recordings were used to examine the frequency and amplitude of spontaneous and miniature EPSCs in GABAergic NTS neurons from age-matched control and pilo-SE mice (Fig 3.3). One week post-treatment, sEPSC frequency in GABAergic NTS neurons from six pilo-SE mice (4.98 ± 0.98 Hz; 12 cells) was significantly greater than seven control mice (1.61 ± 0.40 Hz; n=10 cells; p = 0.007). The increased sEPSC frequency was also seen at 6 weeks post-SE (five control mice: 2.17 ± 0.46 Hz; n=9 cells; seven pilo-SE mice: 3.40 ± 0.36 Hz; n=13 cells; p = 0.045) and 12 weeks post-treatment in seven pilo-SE mice (2.57 ± 0.46 Hz; n=15 cells) compared with 11 age-matched control mice (1.55 ± 0.27 Hz; n=20 cells; p = 0.03; Fig3.3C). There was no significant difference in sEPSC amplitude at any time point post-treatment (week 1: p = 0.82; week 6: p = 0.89; week 12: p = 0.20; Fig3.3D). Therefore, glutamate release onto NTS GABAergic neurons was increased after pilo-SE, and this increased release period persisted for at least 3 months post-SE.

Table 3.1 Resting Membrane Potential and Input Resistance.
Resting membrane potential (RMP) and input resistance of GABAergic NTS neurons in mice that survived SE is not significantly different from age-matched control mice at any time point (unpaired *t*-test; $p > 0.05$).

	Input Resistance (MΩ)		Resting Membrane Potential (mV)	
	Control	Pilo-SE	Control	Pilo-SE
Week 1	1160 ± 600	1590 ± 490	-47.65 ± 5.35	-51.69 ± 4.25
Week 6	1369 ± 346	1180 ± 293	-52.23 ± 3.69	-53.32 ± 3.63
Week 12	2477 ± 611	1905 ± 429	-55.93 ± 3.31	-57.57 ± 3.33

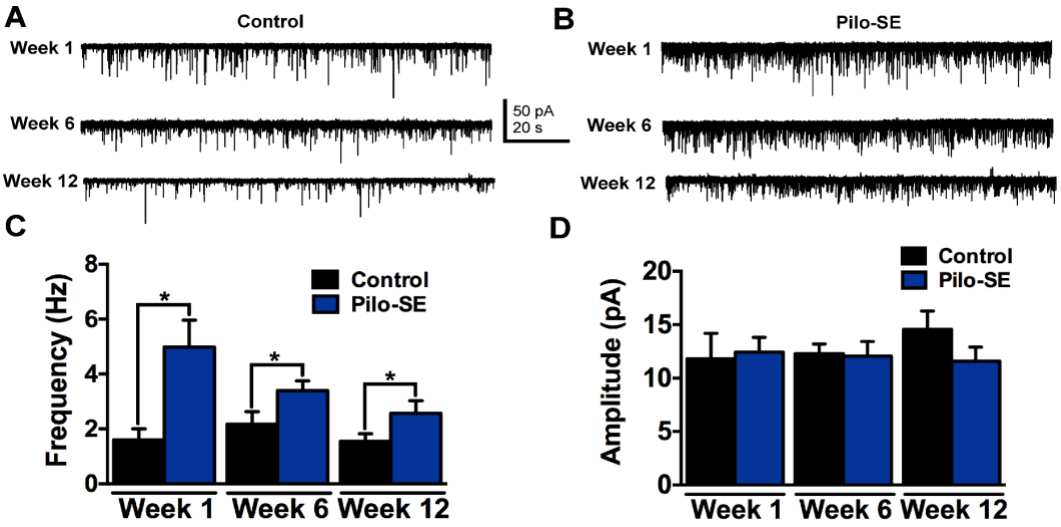


Figure 3.3 Significantly increased sEPSC frequency in GABAergic NTS neurons from pilo-SE mice.
(A) Representative traces showing sEPSCs in a GABAergic NTS neuron from control mice 1, 6, and 12 weeks post-treatment. (B) Representative traces showing sEPSCs in a GABAergic NTS neuron from pilo-SE mice 1, 6, and 12 weeks post-treatment. (C) sEPSC frequency is significantly higher in GABAergic NTS neurons from pilo-SE mice compared to control mice 1, 6, and 12 weeks post-treatment (unpaired *t*-test; *denotes significance). (D) sEPSC amplitude is not significantly different (unpaired *t*-test; $p>0.05$) between control and pilo-SE mice at any time point.

The hypothesis that the increased release of glutamate depended on action potentials in afferent neurons within the slice was tested by measuring the frequency and amplitude of mEPSCs in the presence of tetrodotoxin (TTX, 1 µM), which was added to ACSF to block Na^+ channels and prevent action potential firing (Fig.3.4). Unlike for sEPSCs at 1 week post-treatment, mEPSC frequency was not significantly increased in five pilo-SE mice (2.37 ± 0.35 Hz; n=9 cells) compared with three control mice (1.88 ± 0.34 Hz; n=8 cells; p = 0.33). Miniature EPSC frequency was significantly increased, however in GABAergic NTS neurons at 6 weeks (five control mice: 1.36 ± 0.24 Hz; n=15 cells; six pilo-SE mice: 3.10 ± 0.47 Hz; n=13 cells; p = 0.003) and 12 weeks (five control mice: 1.46 ± 0.13 Hz; n=15 cells; seven pilo-SE mice: 2.52 ± 0.38 Hz; n=12 cells; p = 0.007; Fig 3.4C). Miniature EPSC amplitude in GABAergic NTS neurons from control and pilo-SE mice was not significantly different at any time point post-treatment (week 1, p = 0.85; week 6, p = 0.18; week 12, p =.10; Fig 3.4D). Therefore, glutamate release was increased in GABAergic NTS neurons after pilo-SE, and the increase detected after 6 weeks did not depend on action potential firing in glutamatergic neurons contained within the slice preparation, suggesting that changes at the level of the synaptic terminals contributed to the development of altered glutamate release in the NTS during epileptogenesis.

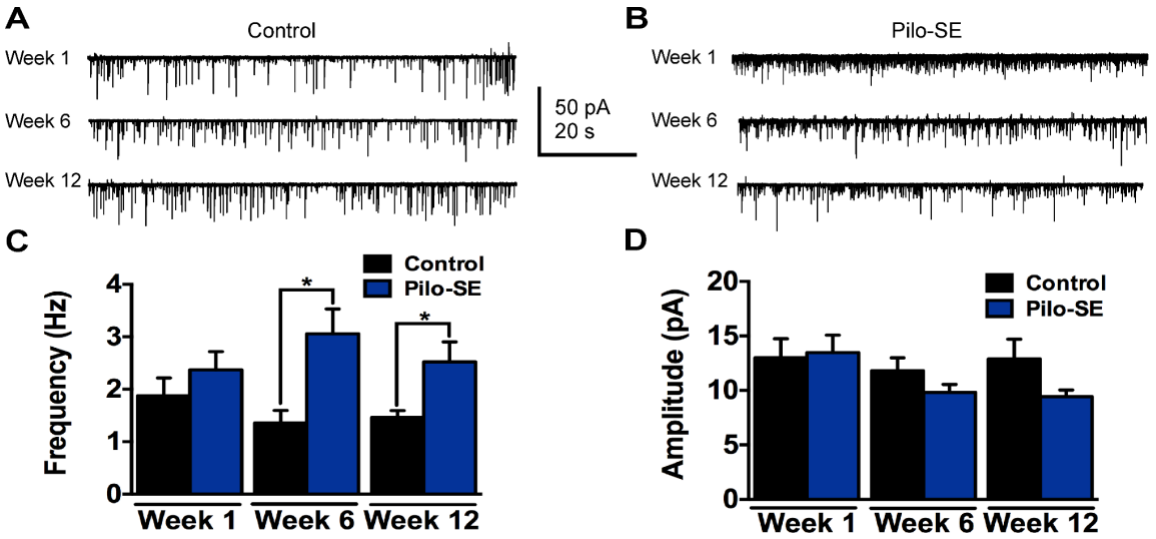


Figure 3.4 Significantly increased mEPSC frequency in GABAergic NTS neurons from pilo-SE mice.
(A) Representative traces showing mEPSCs in a GABAergic NTS neuron from a control mouse 1, 6, and 12 weeks post-treatment. (B) Representative traces showing mEPSCs in a GABAergic NTS neuron from a pilo-SE mouse 1, 6, and 12 weeks post-treatment. (C) mEPSC frequency is significantly higher in GABAergic NTS neurons from pilo-SE mice compared to control mice at 6 and 12 weeks post-treatment (unpaired *t*-test; *denotes significance at $p<0.05$). (D) mEPSC amplitude is not significantly different between control and pilo-SE mice at any time point (unpaired *t*-test).

3.3.4 Primary vagal afferent input to GABAergic NTS neurons was not altered in pilo-SE mice

The increase in mEPSC frequency in GABAergic NTS neurons from pilo-SE mice suggests an increase in the probability of presynaptic glutamate release, possibly including from vagal afferent terminals. Nerve terminals of viscerosensory primary vagal afferents synapse directly onto second order sensory NTS neurons, including GABAergic neurons, the majority of which receive primary vagal input (Glatzer et al., 2007; Bailey et al., 2008). Synaptic responses evoked after stimulating vagal afferents exhibit paired-pulse inhibition and frequency-dependent depression (Miles, 1986; Doyle and Andresen, 2001; Glatzer and Smith, 2005; Bailey et al., 2008). We therefore tested the hypothesis that glutamate release from primary vagal afferents was enhanced in pilo-SE mice by measuring synaptic responses to stimulation of the ST in GABAergic NTS neurons. Examples of responses in NTS GABA neurons to repetitive stimulation of the ST in each group are shown in Fig 3.5. One week post-treatment, the PPr was not significantly different between three control mice (0.71 ± 0.07; n=8 cells) and three pilo-SE mice (0.87 ± 0.10; n=8 cells; p = 0.27). At 6 weeks post-treatment, the PPr was not significantly altered in four pilo-SE mice (1.02 ± 0.13; n=8 cells) compared with five control mice (0.75 ± 0.14; n=5 cells; p = 0.16). There was also no significant difference in the PPr at 12 weeks post-treatment between five control mice (0.71 ± 0.06; n=11 cells) and six pilo-SE mice (0.82 ± 0.06; n=14 cells; p = 0.12; Fig 3.5D). Thus, the increased glutamate release in GABAergic NTS neurons that

developed after pilo-SE was likely not due to modification of synaptic release probability in vagal afferents.

Although changes in the PPr after SE survival are an indicator of alterations in the releasable vesicle pool (Schild et al., 1995; Zucker and Regehr, 2002; Pamidimukkala and Hay, 2004), frequency-dependent depression provides insight into synaptic communication between the vagal afferent fibers and GABAergic NTS neurons that may rely on additional mechanisms (Chen et al., 1999; Atwood and Karunanithi, 2002; Kline, 2008; Zhao et al., 2015). Frequency-dependent depression is a common characteristic of second-order NTS neurons receiving viscerosensory afferent input and has been hypothesized to contribute to central adaptation during cardiovascular and respiratory reflexes (Miles, 1986; Doyle and Andresen, 2001; Kline et al., 2005; Glatzer et al., 2007; Bailey et al., 2008; Kline, 2008).

We also tested the hypothesis that frequency-dependent depression was altered in mice that survived SE by analyzing the amplitude ratios of the 5th to the 1st eEPSC in a train (Fig 3.5F). Similar to the PPr analysis, there was no significant difference between three control mice 1 week (0.55 ± 0.09; n=8 cells) and three pilo-SE mice (0.56 ± 0.08; n=8 cells; p = 0.89), 6 weeks (control: 0.52 ± 0.08; n-=5 cells; pilo-SE: 0.78 ± 0.12; n=8 cells; p = 0.14), or 12 weeks post-treatment (control: 0.56 ± 0.07; n=11 cells; pilo-SE: 0.71 ± 0.07; n=14 cells; p = 0.31; Fig3.5F). These data are consistent with the hypothesis that release properties at vagal afferent synapses with GABAergic NTS neurons are not altered after pilo-SE.

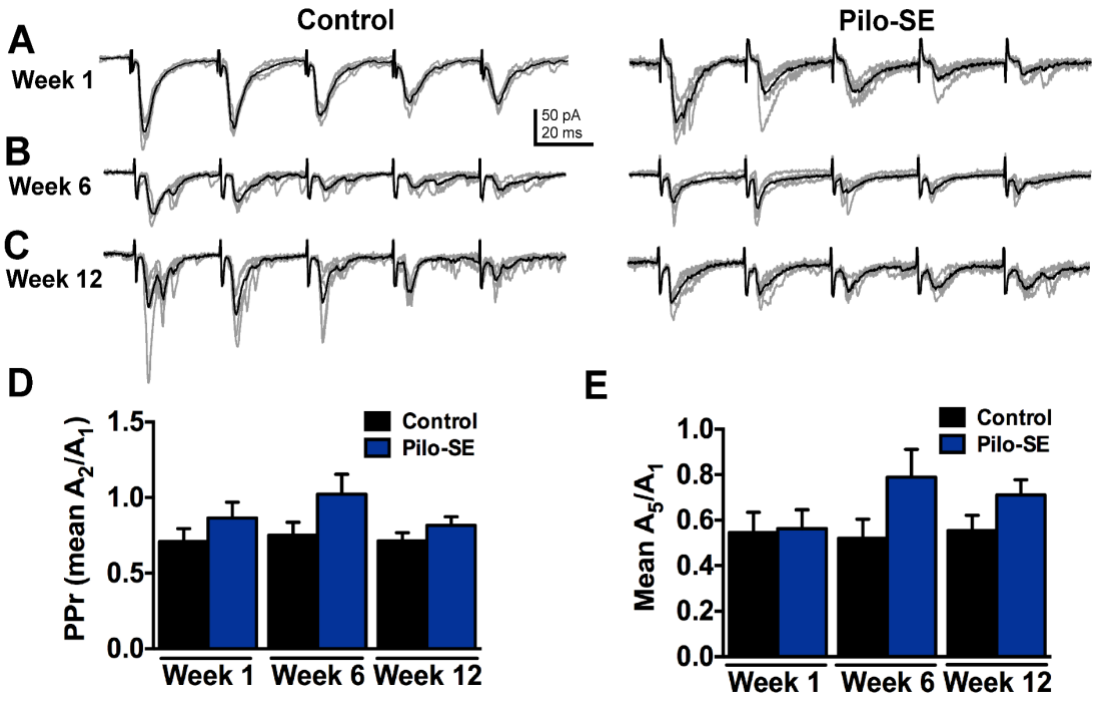


Figure 3.5 Paired pulse ratio (PPr) and frequency dependent depression are transiently altered in pilo-SE mice.
(A) Representative traces of eEPSC responses in GABAergic NTS neurons 1 week post-treatment from control and pilo-SE mice. (B) Representative traces of eEPSC responses in GABAergic NTS neurons 6 weeks post-treatment from control and pilo-SE mice. (C) Representative traces of eEPSC responses in GABAergic NTS neurons 12 weeks post-treatment from control and pilo-SE mice. (D) The PPr was not significantly different between control and pilo-SE mice at any time point post-treatment (unpaired *t*-test; $p>0.05$). (E) The ratio of the 5th response amplitude to that of the 1st response was also not significantly altered in pilo-SE mice at any time point (unpaired *t*-test; $p>0.05$).

3.3.5 Heart rate and heart rate variability in mice surviving SE is not altered long-term

In chemoconvulsant-induced SE models of acquired TLE in rats, changes in cardiac rhythmicity that may reflect plasticity of either central or peripheral vagal regulatory function or cardiac remodeling are detected coincident with epileptogenesis (Metcalf et al., 2009b; Bealer et al., 2010; Biet et al., 2015). We examined mouse ECG activity for changes in heart rate and HRV over time to assess whether ongoing cardiac rhythms were altered following SE. Table 3.2 describes heart rate and two measures of HRV in six control mice and eight pilocarpine-treated mice that survived SE. Heart rate was significantly increased in mice that survived SE compared with their heart rate 24 hours before treatment (baseline, 525.8 ± 22.02 bpm; post-SE, 636.1 ± 39.12 bpm; n=8 mice; $p = 0.018$). This was also true for the SDNN, a measure of HRV. The SDNN was significantly decreased 24 hours post-SE (baseline, 10.77 ± 0.21 ms; post-treatment, 9.85 ± 0.34 ms; $p= 0.038$). No significant differences were detected at any other time points after SE induction in these same mice, nor were any differences detected over the 12-week recording period in the RMSSD (two-way ANOVA; $F(3,46)=0.135$; $p = 0.939$, Table 3.2).

Table 3.2 Heat rate and heart rate variability (HRV) in mice that survived SE.
Heart rate and the standard deviation of the N-to-N interval (SDNN) were increased 24 hours after SE, but no differences were detected at other time points (Heart Rate: Two-Way ANOVA, $F(3,46) = 2.52$, $p = .069$; SDNN: Two-Way ANOVA, $F(3,46) = 2.25$, $p = 0.094$). The root mean squared of the standard deviation (RMSSD) was not significantly different at any time point (Two-Way ANOVA, $F(3,46) = 0.135$, $p = 0.939$).

	Control (n=6)			Pilo-SE (n=8)		
	Heart Rate (bpm)	SDNN (ms)	RMSSD (ms)	Heart Rate (bpm)	SDNN (ms)	RMSSD (ms)
Baseline	539.42 ± 14.23	10.60 ± 0.13	2.96 ± 0.65	525.8 ± 22.02	10.77 ± 0.21	3.22 ± 0.38
24 h	534.66 ± 8.92	10.68 ± 0.08	3.37 ± 0.51	636.10 ± 39.12	9.85 ± 0.34	2.86 ± 0.59
Week 6	549.96 ± 12.93	10.48 ± 0.13	3.21 ± 1.25	556.90 ± 26.26	10.47 ± 0.23	3.32 ± 0.45
Week 12	530.54 ± 9.28	10.68 ± 0.09	3.12 ± 0.66	524.10 ± 18.19	10.75 ± 0.19	3.27 ± 0.62

3.4 Discussion

The present study investigated survival rates and changes in GABAergic NTS neuron function in mice that survived pilo-SE. Mice that died within 1 week of SE were considered to have failed to recover from SE and therefore not to have died of SUDEP, since they likely did not have epilepsy. Of the mice that survived the first week after pilocarpine-induced SE, just 23% survived to 150 days post-SE, whereas 100% of control mice survived the duration of the study. Patients with longstanding epilepsy characterized by frequent generalized tonic-clonic seizures that are relatively poorly controlled are at highest risk for SUDEP (Surges and Sander, 2012; Tolstykh and Cavazos, 2013; Massey et al., 2014; Thurman et al., 2014; Dlouhy et al., 2016). SUDEP risk in patient populations with relatively rare genetic epilepsies such as Dravet's syndrome, which accounts for about 3% of patients with epilepsy, is high (Wu et al., 2015), and many studies have been aimed at elucidating the causes of SUDEP in genetic epilepsies. Patients with TLE represent ~60% of all epilepsies, however, and seizures are medically intractable in about one-third of TLE patients, making this the largest epilepsy population at risk of SUDEP. The pilocarpine-induced SE model in mice represents a consistent and highly replicable TLE model in which mice develop spontaneous seizures within a few weeks after recovery from SE (Shibley and Smith, 2002; Winokur et al., 2004; Scorza et al., 2009; Bhaskaran and Smith, 2010b), promoting this mouse as a reasonable model of SUDEP in TLE. Pilocarpine plasma and brain levels peak in the minutes after injection and fall to almost zero by 2 hours post-injection; it is therefore doubtful that the single exposure to pilocarpine itself is responsible for

our findings, which were measured days to months after injection (Romermann et al., 2015). Additionally, microinjection of muscarinic receptor agonists in the NTS alters function for 4-6 minutes after application without sustained changes (Sundaram et al., 1988). Thus, decreased survivability and increased NTS circuit excitability likely develop coincident with epileptogenesis in this model, rather than as a result of a brief exposure to the muscarinic agonist.

Sudden unexpected death has been documented in mouse models of genetic epilepsy (Goldman et al., 2009; Glasscock et al., 2010; Cheah et al., 2012; Aiba and Noebels, 2015) and unexpected death has been noted anecdotally in models of acquired TLE. In murine Kv1.1-null and Dravet's syndrome genetic epilepsy models, mice begin having seizures by approximately the third week of life, and most animals do not survive past day 90 (Cheah et al., 2012). These models use genetically mediated ion channel derangement to induce epilepsy, and the channelopathies themselves could increase the likelihood of sudden death. They can also result in altered electrical properties of cardiomyocytes, complicating interpretations of the contribution of the effects of seizures-versus the channelopathy itself to death (Auerbach et al., 2013). Conversely, seizures in the pilocarpine-SE model induce reactive neuroplasticity, including ion channel and synaptic reorganization in cortical structures (Shibley and Smith, 2002; Su et al., 2008; Metcalf et al., 2009b; Bealer et al., 2010; Guo et al., 2013), and the present results indicate they also induce remodeling in brainstem neurons or circuits, which could contribute to central autonomic dysregulation. After the initial post-SE period, heart rate and HRV were not affected in this mouse model, but cardiac arrhythmias

have been detected in rats with acquired epilepsy (Powell et al., 2014). An increase in baseline heart rate that coincided with sympathovagal imbalance has been described in rats 2 weeks after pilocarpine injection, before the development of spontaneous seizures (Metcalf et al., 2009a; Bealer et al., 2010). Although similar changes were not detected in the mouse model of TLE used here, further work is necessary to determine whether seizure-related peripheral changes in cardiorespiratory function accompany epileptogenesis in mice, perhaps using isolated hearts to limit the influence of central autonomic regulatory mechanisms (Powell et al., 2014). Our results are consistent with the hypothesis that central autonomic plasticity develops during epileptogenesis in mice, regardless of any potential for cardiac remodeling. Given the critical importance of the vagal complex in regulating cardiac and respiratory reflex function, the development of increased excitability in the NTS during epileptogenesis could reasonably be predicted to increase the propensity for SUDEP in pilocarpine-treated mice.

The vagal complex in the caudal brainstem controls autonomic output to the thoracic and most abdominal viscera. Within the vagal complex, GABAergic neurons in the NTS receive, filter, and integrate viscerosensory information regarding cardiorespiratory function and modulate both vagal and sympathetic tone. Neuroplasticity in the vagal complex occurs in a variety of diseases (Mei et al., 2003; Zsombok and Smith, 2009; Bach et al., 2015) and these neurons also displayed functional changes weeks to months after SE. GABAergic neurons displayed significantly and chronically increased spontaneous action potential firing after SE. Significant differences in the passive membrane properties of

GABAergic NTs neurons in pilo-SE mice were not detected, but the increase in excitability was accompanied by increased glutamate release, evidence by significantly higher sEPSC and mEPSC frequency versus age-matched controls. Notably, age-related increases in NTS neuron excitability have been documented (Johnson and Felder, 1993), so all comparisons here were made between age-matched groups. The increased activity was eliminated when ionotropic glutamate receptors were blocked, providing further evidence that long-term changes in synaptic function are associated with epileptogenesis in this model.

The increased glutamate release shortly after pilo-SE was action potential dependent, suggesting an initial increase in excitability of local interneurons. Action potential-independent release, however, was significantly increased by 6 weeks, suggesting the development of altered presynaptic release properties or formation of new synapses in the NTS during epileptogenesis. Most GABAergic NTS neurons receive direct vagal input (Glatzer et al., 2007; Bailey et al., 2008) evidenced by eEPSCs with constant response latency (i.e. synaptic jitter <0.2 ms) after ST stimulation. Reduced expression of K^+ channels in the vagus nerve of Kv1.1-null mice with epilepsy has been reported (Goldman et al., 2009; Glasscock et al., 2010; Glasscock et al., 2012), and seizure-induced K^+ channel remodeling in vagal or other afferents could contribute to the increase in glutamate release onto the GABAergic NTS neurons in pilocarpine-treated mice. However, changes in synaptic release properties of vagal afferent terminals were not detected. In addition to synaptic vagal afferent input, these receive glutamatergic synapses originating from local NTS neurons and from other brain areas (Nishimura and

Oomura, 1987; Zhang et al., 1999; Glatzer et al., 2007), consistent with the hypothesis that synaptic reorganization of central neurons contributes to the increased glutamate release in the NTS during epileptogenesis.

The cellular mechanisms underlying the increased glutamate release and enhanced excitability of NTS GABA neurons have yet to be elucidated, but the increase in synaptic excitation is reminiscent of the synaptic rearrangement that occurs in cortical inhibitory interneurons during epileptogenesis (Hunt et al., 2011; Zhang et al., 2011) and is consistent with dysregulation of autonomic control of the thoracic and abdominal viscera. Increased synaptic excitation of GABAergic NTS neurons would be expected to inhibit parasympathetic motor output and suppress autonomic reflex responses in pilo-SE mice. Because NTS neurons also project to neurons that inhibit medullary synaptic circuits (Card et al., 2006), increased activity might also chronically disinhibit sympathetic motor output. Respiratory centers receiving input from NTS neurons with altered excitability may also be affected (Stornetta and Guyenet, 1999).

The chronic increase in glutamate-mediated cellular excitability after SE may also make GABAergic NTS neurons more susceptible to sodium channel inactivation in the event of excessive depolarization, as can occur if seizures spread to this brainstem area (Aiba and Noebels, 2015). Spreading depression and depolarization block have been well studied in cortical neurons and implicated in the pathophysiology of migraine and stroke (Dreier and Reiffurth, 2015; Dreier et al., 2015). NTS neurons are normally resistant to spreading depression (Somjen, 2001; Dreier and Reiffurth, 2015), but focal cortical seizures in mice with

epilepsy induced spreading depolarization in the NTS under conditions of metabolic deprivation, which was followed by cardiorespiratory collapse and sudden death in genetic epilepsy models (Aiba and Noebels, 2015). Spreading depression is typically initiated in the lateral NTS, an area of the nucleus that receives inspiratory vagal afferent input from the lung (Donnelly et al., 1989) and is enriched in GABAergic neurons (Blessing, 1990; Fong et al., 2005; Glatzer et al., 2007). The elevated synaptic excitability in GABAergic NTS neurons in mice that survived pilocarpine-induced SE is consistent with an increased propensity for depolarization block and action potential inactivation in these neurons, which could increase the likelihood that depolarization block and spreading depression could evolve in the NTS (Haller et al., 2001; Larrosa et al., 2006; Sawant-Pokam et al., 2017). Whereas cortical seizures that spread to the NTS can evoke spreading depolarization associated with SUDEP, other coordinated input to the nucleus, such as that which occurs during vagal reflex initiation, might also render the region susceptible to spreading depolarization in mice with TLE. GABAergic NTS neurons play a critical role as mediators of cardiac, respiratory, and baroreceptor reflexes (Andresen and Kunze, 1994; Kanter et al., 1995; Wang et al., 2001a; Wang et al., 2001b; Zoccal et al., 2014). Because we did not see any cardiac-specific phenotypes in the mice with pilocarpine-induced TLE, we hypothesize that the increase in NTS neuron excitability leads to an increased propensity for depolarization block and spreading depression centrally, resulting in sudden death under specific conditions (Aiba and Noebels, 2015). Notably, this is not necessarily superimposed on chronic changes in cardiac function in this model. These central

mechanisms may lead to aberrant baroreceptor or cardiorespiratory reflexes in the pilo-SE mice, but intrinsic changes in cardiac function may not be expressed under nominal conditions.

The present results show that mice that survive SE are susceptible to SUDEP after several weeks. Our findings are consistent with the hypothesis that glutamate release is persistently elevated in the NTS after SE, evidenced by an increase in glutamatergic synaptic input to GABAergic NTS neurons and a corresponding increase in neuronal activity. Chronically increased activity in GABAergic NTS neurons would be expected to impact parasympathetic or sympathetic tone, autonomic reflexes, including cardiorespiratory reflexes, and may underlie seizure-induced depolarization block and spreading depression in the nucleus, leading to cardiorespiratory collapse and SUDEP. Our results also suggest multiple components contribute to the altered excitation of NTS GABA neurons, including an initial increase in glutamate release driven by action potentials in local neurons and a delayed, persistent increase in presynaptic glutamate release from synaptic terminals of central neurons. These changes likely involve seizure-induced synaptic or channel reorganization within the central vagal system. Although the mechanistic cause of SUDEP per se has been debated (Surges and Sander, 2012; Ryvlin et al., 2013; Aiba and Noebels, 2015), it is most likely not due to a single etiology such as cardiac changes, at least in mice with TLE. We posit that in TLE, SUDEP may result from multiple factors (e.g. cardiac or respiratory failure), and the triggers for these are superimposed on dysregulated NTS circuits. Understanding the cellular changes in the NTS that are associated

with seizures may prompt the development of predictive biomarkers for SUDEP in those populations most at risk, and eventually therapies to prevent SUDEP.

Chapter 4 Alterations in A-type current in the nucleus tractus solitarius in acquired temporal lobe epilepsy

4.1 Introduction

Approximately 60% of all individuals with epilepsy have temporal lobe epilepsy (TLE), which is characterized by recurrent, unprovoked electrical activity originating in the medial portions of the temporal lobe. In about 30% of these individuals, seizures are medically intractable. Sudden Unexpected Death in Epilepsy (SUDEP) is defined as when an individual who is otherwise healthy, dies suddenly and unexpectedly for unknown reasons and is one of the leading causes of death in individuals with epilepsy and risk is increased in patients with uncontrolled seizures (Annegers, 1997; Nashef, 1997; Nashef et al., 2012; Tolstykh and Cavazos, 2013; Ellis and Szabo, 2018). Previous studies in mouse models of genetic epilepsy have suggested that functional seizure-related that alterations in brainstem centers that modulate peripheral autonomic and cardiorespiratory reflexes are associated with an increased risk for SUDEP (Goldman et al., 2009; Glasscock et al., 2010; Cheah et al., 2012; Kalume et al., 2013; Simeone et al., 2018). It has also been shown in mouse models of genetic epilepsy that seizures can spread from the cortex to the brainstem, leading to cardiorespiratory collapse and sudden death (Aiba and Noebels, 2015). Importantly, we have recently shown in a model of acquired TLE that γ-aminobutyric acid (GABA) neurons in the nucleus tractus solitarius (NTS), the primary central autonomic integration area in the caudal brainstem, are

hyperexcitable in a mouse model of TLE, which may contribute to increased SUDEP risk in this model (Derera et al., 2017).

The NTS is located in the dorsal vagal complex of the caudal brainstem and is the principal central locus of visceral autonomic integration. NTS neurons receive primary glutamatergic inputs from vagal afferent fibers and project their axons to preganglionic parasympathetic motor neurons in the dorsal motor nucleus of the vagus (DMV) and the nucleus ambiguus (NA) (Andresen and Kunze, 1994; Doyle and Andresen, 2001; Wang et al., 2001b; Davis et al., 2004; Travagli et al., 2006; Glatzer et al., 2007; Browning and Travagli, 2010). Neurons of the NTS project to the hypothalamus and pre-sympathetic areas of the ventral brainstem as well; therefore the nucleus is well-situated to regulate premotor sympathetic and parasympathetic output (Takenaka et al., 1995; Fontes et al., 2001; Irnaten et al., 2001; Affleck et al., 2012; Zoccal et al., 2014). NTS neurons have been shown to alter their function in response to many disease states, including hypertension, hypoxia, and diabetes, and a reduction in voltage-gated K^+ current as been shown to be associated with these pathologies (Mei et al., 2003; Kline et al., 2005; Bach et al., 2015; Boychuk et al., 2015a). While voltage-gated K^+ currents have been investigated in hypertension and hypoxia (Belugin and Mifflin, 2005; Accorsi-Mendonca et al., 2015), epilepsy-related changes in voltage-gated K^+ currents of NTS neurons have not been examined in a mouse model of acquired TLE. We postulate that a portion of the hyperexcitability previously seen in GABAergeric NTS neurons in TLE may be due to a similar reduction in voltage-gated K^+ current.

Several voltage-gated K^+ channels are known to be associated with epilepsy and are expressed in NTS neurons (Moak and Kunze, 1993; Kline et al., 2005; Ramirez-Navarro et al., 2011; Villa and Combi, 2016). Of particular interest is the Kv4 family of voltage-gated K^+ channels, which underlie the A-type K^+ current in NTS neurons (Belugin and Mifflin, 2005; Bailey et al., 2007; Strube et al., 2015). Voltage-gated K^+ channels play a major role in neuronal excitability and K^+ channelopathies can contribute to seizures (Brenner and Wilcox, 2012; D'Adamo et al., 2013; Villa and Combi, 2016). The Kv4 family of voltage-gated K^+ channels prevent action potential back-propagation and regulate neuronal excitability (Birnbaum et al., 2004). There are three subunits that comprise the Kv4 subfamily: Kv4.1, Kv4.2, and Kv4.3. There are also four K^+ channel interacting proteins (KChIPs) that can modulate Kv4 activity and surface expression (Birnbaum et al., 2004; Covarrubias et al., 2008). The Kv4.2 subunit has specifically been associated with TLE and mutations in *KCND2*, which encodes for the Kv4.2 subunit, have been shown to occur in individuals diagnosed with TLE (Singh et al., 2006; Lee et al., 2014). Additionally, when Kv4.2 is deleted in mice, susceptibility to kainite-induced seizures is increased (Barnwell et al., 2009). Several studies have shown a downregulation in Kv4.2, KChIPs, and A-type K^+ currents following pilocarpine-induced status epilepticus (SE). Importantly, these changes occurred not only in the days following SE, but in the chronic seizure phase, months post-SE (Lugo et al., 2008; Monaghan et al., 2008; Su et al., 2008). However, A-type K^+ current has not been examined in NTS neurons in a mouse model of TLE.

We hypothesize that there is an alteration in A-type K^+ current and Kv4 expression is associated with increased excitability of GABAergic NTS neurons that develops during temporal lobe epileptogenesis. Increased excitability in this neuronal subtype may decrease the threshold for spreading depolarization, leading to an increased risk for cardiorespiratory collapse and SUDEP. We used the pilocarpine-induced SE model of TLE to test this hypothesis (Shibley and Smith, 2002; Winokur et al., 2004; Derera et al., 2017).

4.2 Methods

4.2.1 A-Type Potassium Current Recordings

A-type K^+ current (I_A) activation and inactivation were measured in GABAergic NTS neurons using a series of voltage-step protocols. Total K^+ currents (I_K) were determined using the following protocol: from holding potential (-70 mV), a 500 ms hyperpolarizing pre-pulse (-110 mV) was delivered followed by a series of depolarizing voltage steps (-60 mV to +30 mV; 500 ms; 10 mV increments), evoking transient and sustained outward currents. The delayed rectifier K^+ current (I_{KDR}) was determined by delivering a 500 ms depolarizing pre-pulse at -30 mV from holding potential (-70 mV) followed by 500 ms depolarizing voltage steps from -60 mV to +30 mV in 10 mV increments. I_A was calculated by offline subtraction of I_{KDR} from I_K in Clampfit 10.3 (Molecular Devices). I_A inactivation was revealed with the following protocol: from holding potential (-70 mV), conditioning steps from -120 mV to +60 mV (10 mV increments, 500 ms duration), were followed by a test pulse at -30 mV (500 ms duration). The ACSF for all I_A recordings contained tetrodotoxin (TTX, 1 µM), bicuculline (BIC, 30 µM), kynurenic acid (KYN, 1mM),

and tetraethylammonium chloride (TEA-Cl, 10 mM). In order to confirm the presence of I_A, 4-AP (5mM) was added to the ACSF, and the activation and inactivation protocols were repeated. Current is presented as pA/pF in order to adjust for differences in cell size. Series resistance was less than 15 MΩ and compensated between 30% and 70% for all recordings. Series resistance was evaluated between each voltage-step protocol to ensure cell health.

4.2.2 qRT-PCR

Three to four brainstem slices per animal that contained the vagal complex (300 µM) were isolated as previously described for electrophysiological recordings. The vagal complex, including the NTS, was visualized under a dissecting microscope and excised from the rest of the brainstem using a 1 mm diameter punch (Miltex Inc., York, PA). This approach allowed for a more homogenous tissue sample with minimal sampling from other brainstem areas (Boychuk et al., 2015a; Halmos et al., 2015; Boychuk et al., 2017). Tissue samples from one animal were pooled into one sample. RNA was isolated using the RNeasy miniPrep kit (Qiagen, Hilden Germany). Spectrophotometry (ThermoScientific, NanoDrop 2000 Spectrophotometer) was used to determine cDNA quality and quantity. All qRT-PCR reactions were run in triplicate in 96-well optical grade plates using a 7500 Fast Real-Time PCR System (Applied Biosystems, Foster City, CA). Total volume for each run was 20 µL containing 50-100 ng of cDNA. The reaction times and temperatures were 50˚C for 2 minutes, 95 ˚C for 10 minutes, followed by 50 cycles of 95 ˚C for 15s, and 60 ˚C for 1 minute. Primers and probe sets for β-actin (Mm.PT.39a.22214843), Kv4.1(mostly microglia; Mm.PT.58.1430675), Kv4.2

(oligodendrocytes and neurons; Mm.PT.58.29624161), Kv4.3 (neurons); Mm.PT.58.7819122, KChIP1 (neurons; Mm.PT.58.41994313), KChIP3 (astrocytes; Mm.PT.58.43828062) and KChIP4 (neurons; Mm.PT.58.9880505) were purchased from Integrated DNA Technologies (IDT, Skokie, IL). Fold change in each gene of interest was calculated using the formula $2^{-\Delta\Delta CT}$; β-actin was used as a reference gene. Water-only (i.e. no primer/probe) and no-RT controls were also run in each reaction. The positive control contained the whole brainstem instead of the vagal complex tissue sample (Livak and Schmittgen, 2001). The ΔCT was used to determine statistical significance using an unpaired *t*-test (Wood and Giroux, 2003).

Methods for Chapter 4 also included: methylscopolamine and pilocarpine injections, brainstem slice preparation, and whole-cell patch-clamp electrophysiology as described in Chapter 2 of this dissertation.

4.3 Results

4.3.1 Action potential frequency and half-width are altered in GABAergic NTS neurons from Pilo-SE mice

In order to investigate the hyperexcitability seen in GABAergic NTS neurons during epileptogenesis, we assessed action potential firing frequency and half-width in control and pilo-SE mice 9-12 weeks post-treatment. Action potential frequency was significantly greater in GABAergic NTS neurons from pilo-SE mice (2.54 ± 0.37 Hz, n = 10 cells) compared to control mice (1.46 ± 0.24 Hz, n = 11 cells, $p = 0.03$, Fig. 4.1A,C). When measured at a membrane potential of -70 mV, action potential half-width was significantly greater in GABAergic NTS neurons

from pilo-SE mice (2.14 ± 0.16 ms, n = 10 cells) compared to control mice (1.69 ± 0.13 ms, n = 11 cells, *p* = 0.04, Fig. 4.1B,D). The increase in action potential half-width in neurons from pilo-SE mice is suggestive of a lengthening in the repolarization phase of the action potential.

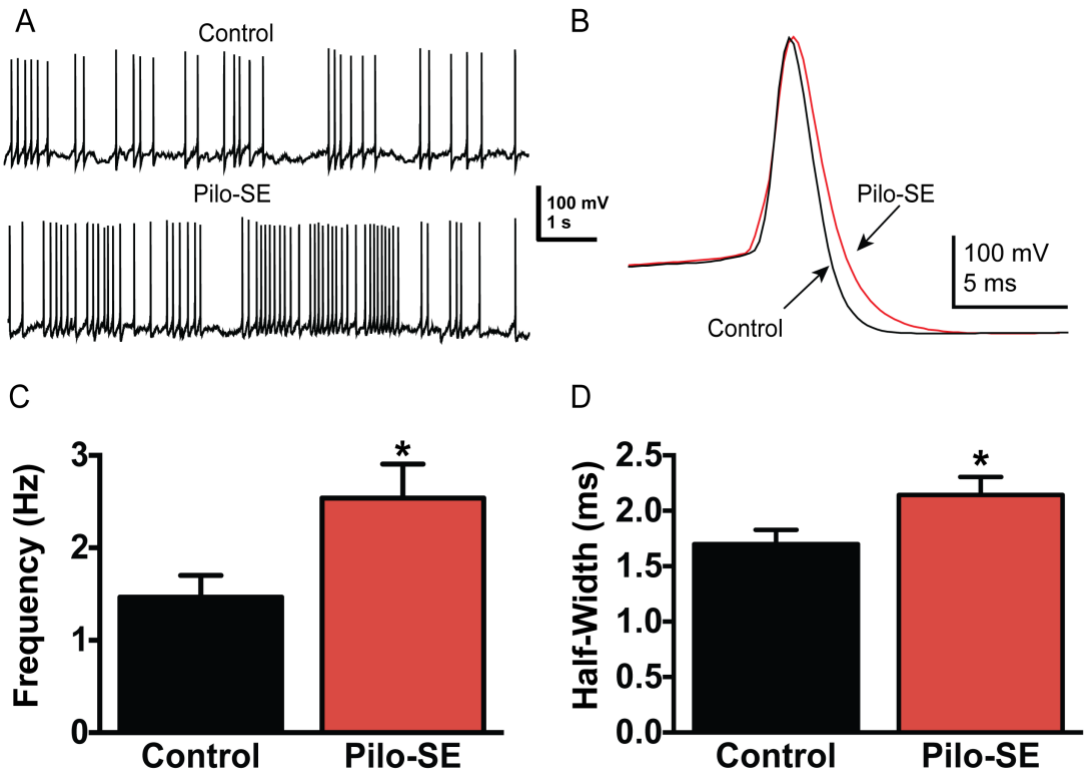


Figure 4.1 GABA NTS neurons from pilo-SE mice have increased action potential firing frequency and half-width**.**
(A) Representative traces showing action potential firing in GABAergic NTS neurons from a control and pilo-SE animal. (B) Representative traces showing action potential half-width in GABAergic NTS neurons from a control and pilo-SE animal. (C) Action potential frequency is significantly increased in GABAergic NTS neurons from pilo-SE mice (unpaired *t*-test; *$p < 0.05$). (D) Action potential half-width is significantly increased in GABAergic NTS neurons from pilo-SE mice (unpaired *t*-test; *$p < 0.05$).

Various families of voltage-gated K^+ channels modulate the repolarization phase of the action potential. We hypothesized that in pilo-SE mice there may be a decrease in voltage-gated K^+ channel function that contributes to both the increase in the action potential half-width and the hyperexcitability seen in GABAergic NTS neurons. In order to test this hypothesis, we measured action potential frequency and half-width before and after bath application of 4-AP (5 mM), a voltage-gated K^+ channel blocker, in GABAergic NTS neurons from control and pilo-SE mice 9-12 weeks post-treatment. Action potential frequency was significantly greater after application of 4-AP in GABAergic NTS neurons from control mice (no 4-AP: 1.56 $\pm$ 0.31 Hz, n=13 cells; after 4-AP: 3.1 $\pm$ 0.65 Hz, n= 6 cells, p = 0.02). However, there was no significant difference in action potential frequency in GABAergic NTS neurons in normal ACSF (3.20 $\pm$ 0.32, n = 13 cells) and after application of 4-AP (4.39 $\pm$ 0.82, n= 8 cells, p = 0.13 Fig. 4.2). When examining action potential half-width, we found that it was significantly longer after application of 4-AP in GABAergic NTS neurons control mice (before 4-AP: 1.63 $\pm$ 0.15 ms, n=8 cells; after 4-AP: 3.52 $\pm$ 0.42ms, n= 5 cells, p = 0.0005). We did not detect a significant difference in action potential half-width in GABAergic NTS neurons from pilo-SE mice in the absence (2.63 $\pm$ 0.44 ms, n= 10 cells) or presence (2.90 $\pm$ 0.22 ms, n= 6 cells, p = 0.66, Fig. 4.3) of 4-AP. The decrease in sensitivity to 4-AP seen in GABAergic NTS neurons from pilo-SE mice suggests that there may be a decrease in the function of voltage-gated K^+ channels, potentially those contributing to the A-type current.

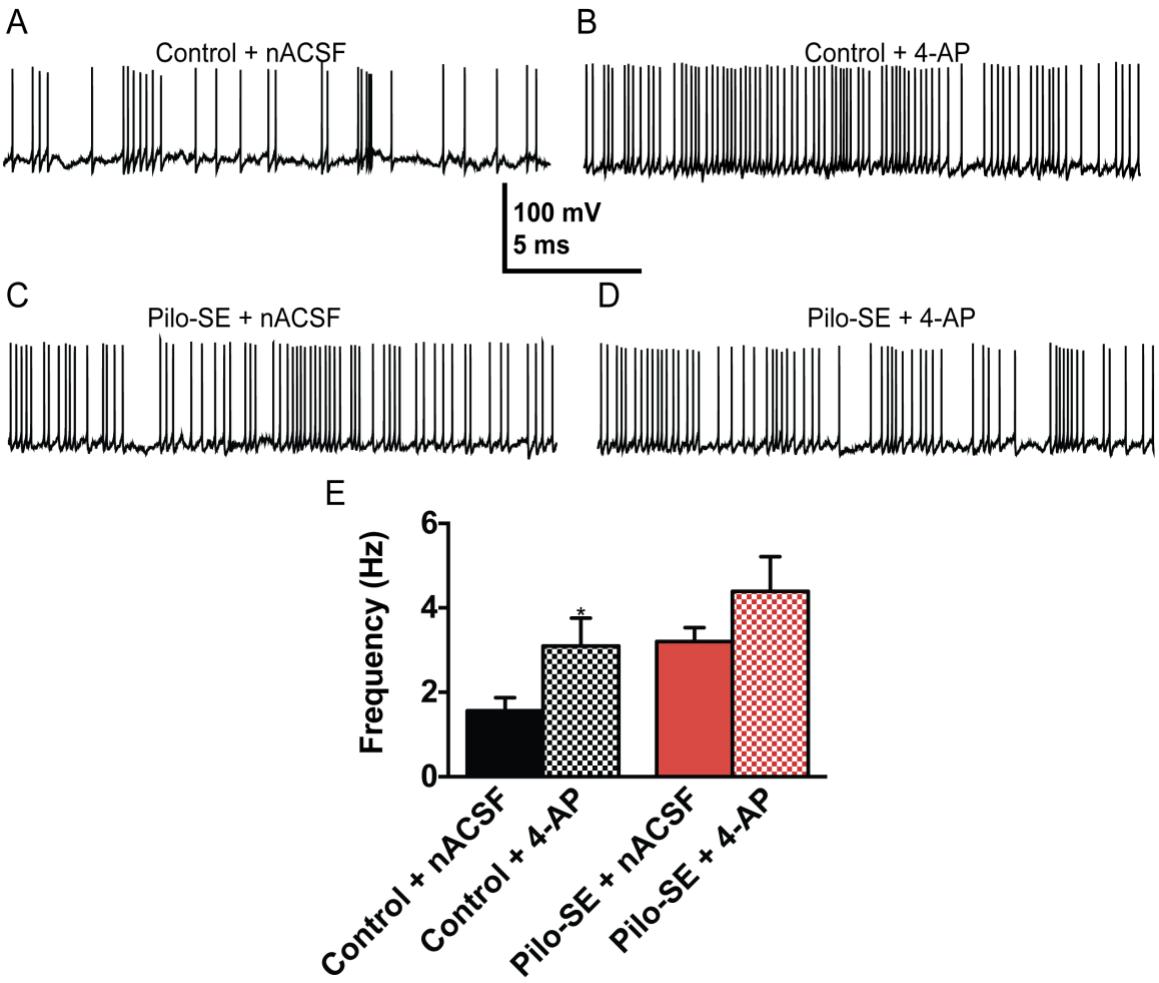


Figure 4.2 The effect of 4-AP on action potential firing frequency in GABA NTS neurons.
(A) Representative trace showing action potential firing in a GABA NTS neuron from a control animal in normal ACSF (nACSF). (B) Action potential firing in the same GABA NTS neuron in the presence of 4-AP (5mM). (C) Representative trace showing action potential firing in a GABA NTS neuron from a pilo-SE mouse in nACSF. (D) Representative trace showing action potential firing in the same GABA NTS neuron in the presence of 4-AP. (E) Action potential frequency is significantly higher after application of 4-AP in GABA NTS neurons form control mice. There is no significant difference in action potential frequency before and after 4-AP application in GABA NTS neurons from pilo-SE mice (unpaired *t*-test; $*p < 0.05$).

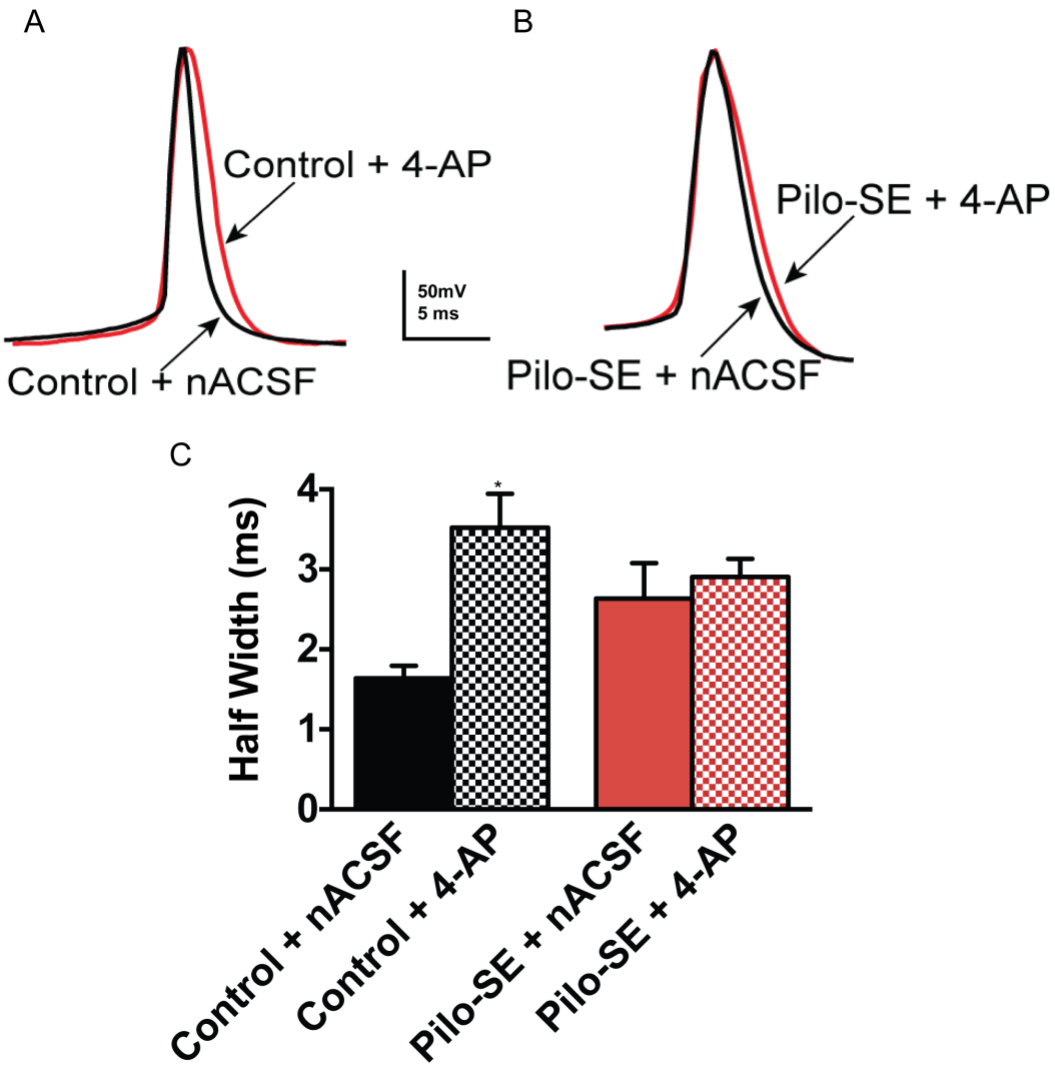


Figure 4.3 GABA NTS neurons from pilo-SE mice are less sensitive to 4-AP.
(A) Representative traces showing action potential half-width in a GABAergic NTS neuron from a control animal in normal ACSF (nACSF) and in the presence of 4-AP (5mM). (B) Representative traces showing action potential half-width in a GABAergic NTS neuron from a pilo-SE mouse in nACSF and in the presence of 4-AP. (C) Action potential half-width is significantly increased in GABAergic NTS neurons from control mice after 4-AP application. There is no significant difference in action potential half-width before and after 4-AP application in GABA NTS neurons from pilo-SE mice (unpaired *t*-test; *$p < 0.05$).

4.3.2 The effect of 4-AP on sEPSC frequency and amplitude in GABAergic NTS neurons

We also assessed the effect of 4-AP on frequency and amplitude of sEPSCs in GABAergic NTS neurons from control and pilo-SE mice 9-12 weeks post-treatment. There was a significant increase in sEPSC frequency in GABAergic NTs neurons after application of 4-AP (before 4-AP: 1.17 $\pm$ 0.23 Hz, n= 7 cells; after 4-AP: 5.34 $\pm$ 0.78 Hz, n= 7, p = 0.003). sEPSC frequency was not significantly different following 4-AP application (before 4-AP: 3.77 $\pm$ 0.87 Hz, n= 6 cells; after 4-AP: 4.54 $\pm$ 0.74 Hz, n= 14 cells, p = 0.55). Application of 4-AP had no significant effect on sEPSC amplitude in either control or pilo-SE mice (control: p = 0.59; pilo-SE: p = 0.67; Fig. 4.4). Changes in synaptic frequency with addition of 4-AP are consistent with previous studies in both the hippocampus (Buckle and Haas, 1982) and NTS (Haji and Ohi, 2010), and the absence of detectable change in pilo-SE mice suggests a functional reduction in 4-AP sensitive channels in GABA NTS neurons.

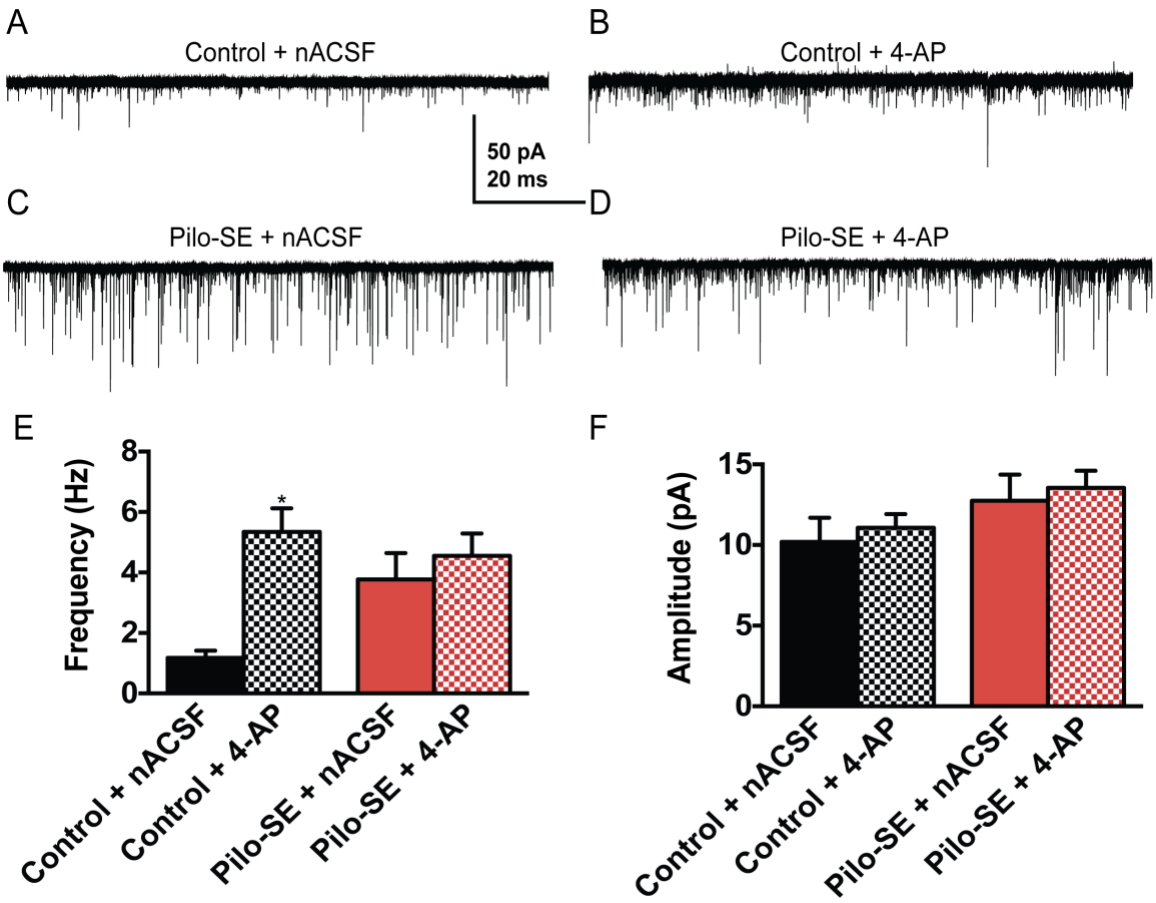


Figure 4.4 The effect of 4-AP on sEPSC frequency and amplitude in GABA NTS neurons.
(A, B) Representative traces showing sEPSCs in a GABA NTS neuron from a control animal in normal ACSF (nACSF) and in the presence of 4-AP (5mM). (C,D) Representative traces showing sEPSCs in a GABA NTS neuron from a pilo-SE animal in nACSF and in the presence of 4-AP. (E) There is significantly higher sEPSC frequency in GABA NTS neurons from control mice after 4-AP and no significant difference in pilo-SE mice after 4-AP application (unpaired *t*-test; $*p < 0.05$). (F) There is no significant difference in sEPSC amplitude before and after 4-AP application in GABA NTS neurons from control or pilo-SE mice (unpaired *t*-test; $p > 0.05$).

4.3.3 A-type potassium current is reduced in GABA NTS neurons from pilo-SE mice

Since 4-AP is a specific blocker of A-type K^+ currents, we hypothesized that the decrease in sensitivity to 4-AP in pilo-SE mice may be due a decrease in A-type K^+ current. First, we assessed the amplitude of the transient outward currents (TOCs) in GABAergic NTS neurons from control and pilo-SE mice. The membrane capacitance was not different between GABAergic NTS neurons from control and pilo SE mice (control: 6.57 $\pm$ 0.20 pF; pilo-SE: 7.39 $\pm$ 0.79 pF; $p = 0.14$). The series resistance also did not differ between groups (control: 8.69 $\pm$ 0.76 MΩ; pilo-SE: 7.86 $\pm$ 1.29 MΩ; p = 0.59). The amplitude of the TOCs (measured 20-30 ms after the voltage step onset) were significantly different between GABAergic NTS neurons from control and pilo-SE mice (F (9,160) = 1.96, $P = 0.04$, control n= 10 cells, pilo-SE n=8 cells, Fig 4.5C). At activation voltages from +10 to +30 mV, the TOCs were significantly reduced in GABAergic NTS neurons from pilo-SE mice compared to control mice (+10 mV: $p = 0.04$; +20 mV: $p = 0.01$; +30 mV: $p = 0.003$). We did not see any significant differences in longer-lasting steady state amplitude in GABAergic NTS neurons between control and pilo-SE mice (F 9,160) = 0.18, $P = 0.99$, Fig 4.5D), measured in the last 50 ms of the voltage step. In a separate cohort of GABAergic NTS neurons, 4-AP was added to the bath solution in order to validate that the A-type voltage-gated K^+ current was being measured (Fig 4.6). We also examined the voltage dependence of activation and inactivation in GABAergic NTS neurons from control and pilo-SE mice. There was no significant difference in the voltage dependence of activation (2-way ANOVA, F (9,330) =

0.21, p = 0.99, Fig 4.7A) or inactivation (2-way ANOVA, F (18, 475) = 0.18, p = 0.99, Fig 4.7B) in GABAergic NTS neurons from control mice and pilo-SE mice.

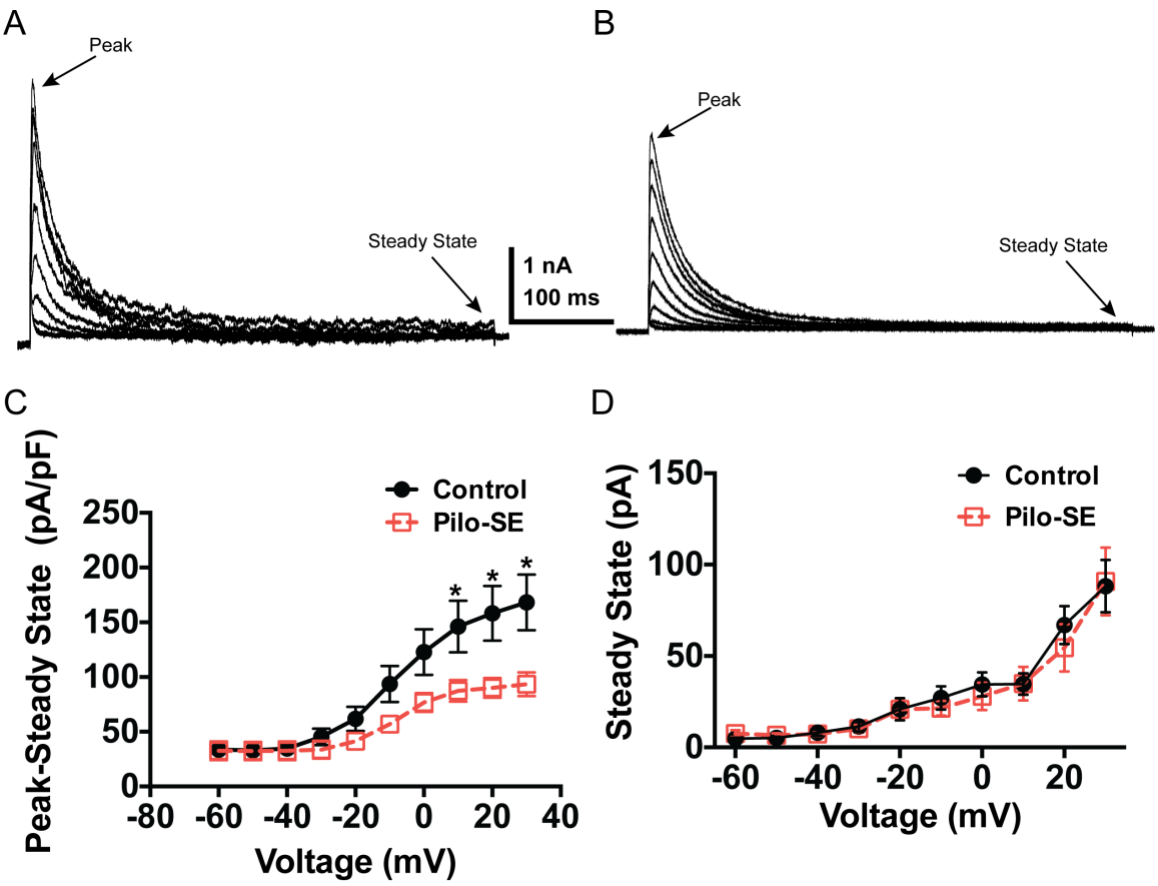


Figure 4.5 Transient outward current amplitude is reduced in pilo-SE mice.
(A) Representative trace of I_A activation in a GABAergic NTS neuron from a control animal. (B) Representative trace of I_A activation in a GABAergic NTS neuron from a pilo-SE animal. (C) Mean amplitude of TOCs (measured as peak-steady state values). GABA NTS neurons from pilo-SE mice had significantly lower TOCs compared to those from control mice (2-way ANOVA, $*P < 0.05$). (D) There was no significant difference in steady-state outward currents in GABA NTS neurons from control and pilo-SE mice (2-way ANOVA, $p > 0.05$).

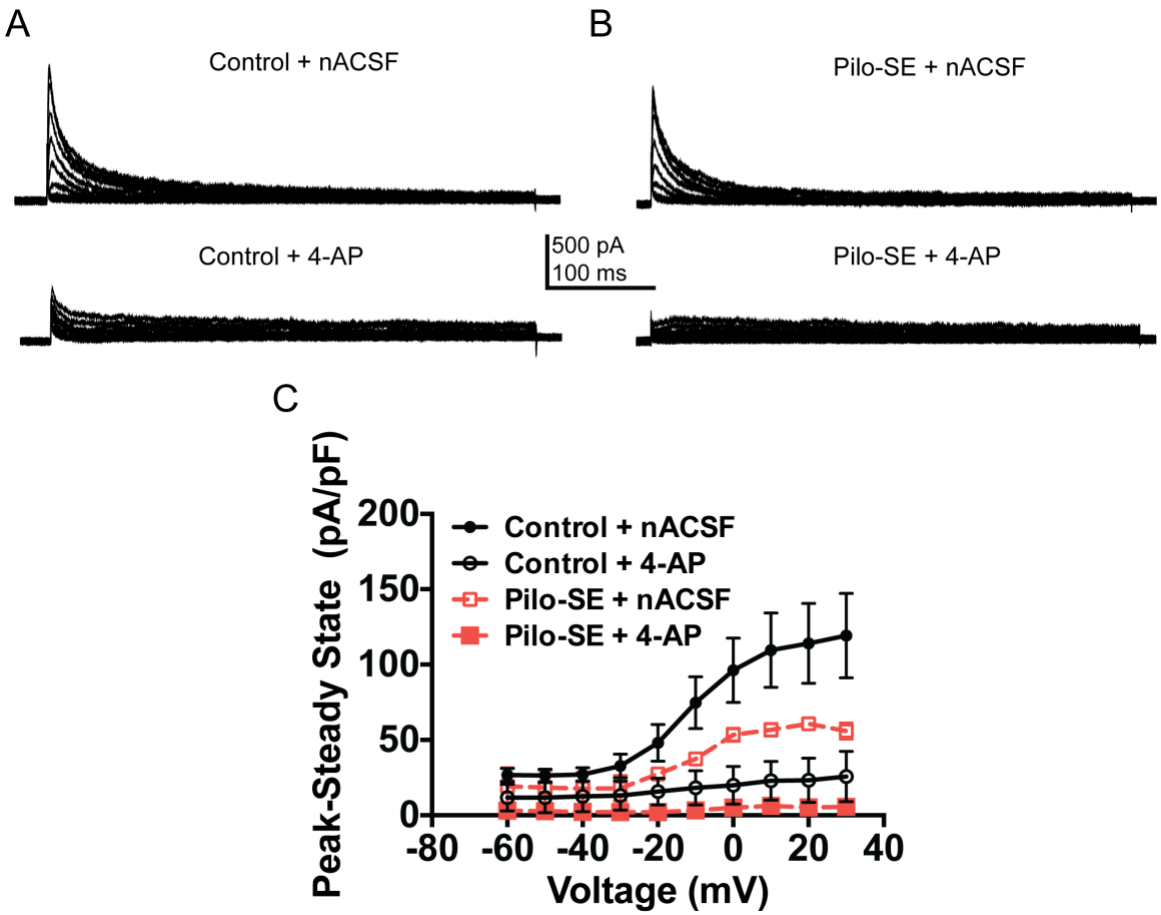


Figure 4.6 The effect of 4-AP on transient outward current amplitudes in GABAergic NTS neurons.
(A) Representative trace of I_A activation in a GABAergic NTS neuron from a control animal in normal ACSF (nACSF) and in the presence of 4-AP (5 mM). (B) Representative trace of I_A activation in a GABAergic NTS neuron from a pilo-SE animal in nACSF and in the presence of 4-AP. (C) 4-AP significantly reduced TOCs in GABAergic NTS neurons from both control and pilo-SE mice (2-way ANOVA, $*p < 0.05$).

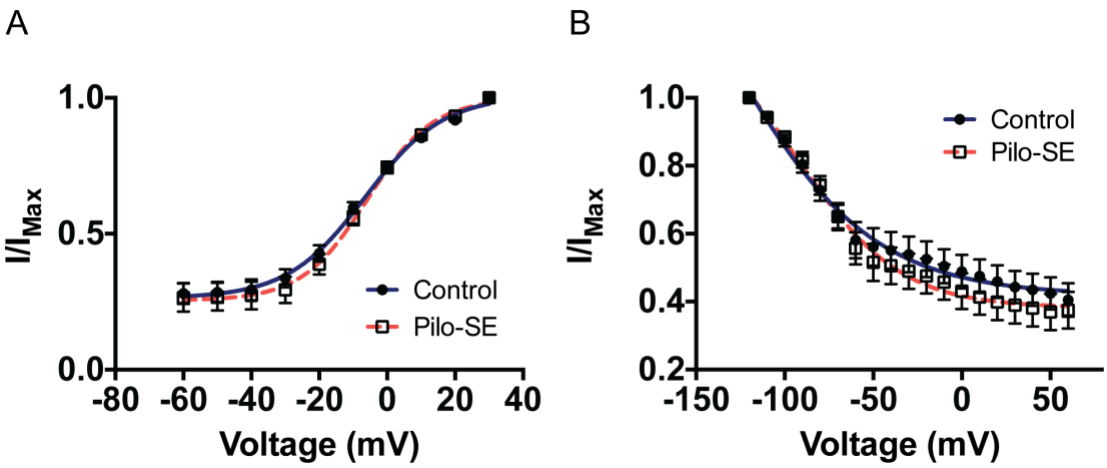


Figure 4.7 Activation and Inactivation of TOCs in GABAergic NTS neurons from control and pilo-SE mice.
(A) Voltage dependence activation of TOCs in GABAergic NTS neurons from control and pilo-SE mice. (B) Voltage dependence inactivation of TOCs in GABAergic NTS neurons from control and pilo-SE mice. (2-way ANOVA, $p > 0.05$).

4.3.4 Quantitative reverse transcriptase PCR

Quantitative RT-PCR was utilized to determine if the reduction in I_A is due to a down regulation in the expression of the genes that encode the Kv4 subfamily of voltage-gated K^+ channels and their associated accessory subunits, KChIP1, KChIP3, and KChIP4. There were no significant differences in the expression of any Kv4 genes or their associated accessory subunits in pilo-SE mice relative to control mice (Kv4.1: $p = 0.81$; Kv4.2: $p = 0.83$; Kv4.3: $p = 0.81$; KChIP1: $p = 0.61$; KChIP3: $p = 0.40$; KChIP4: $p = 0.47$; Fig. 4.8).

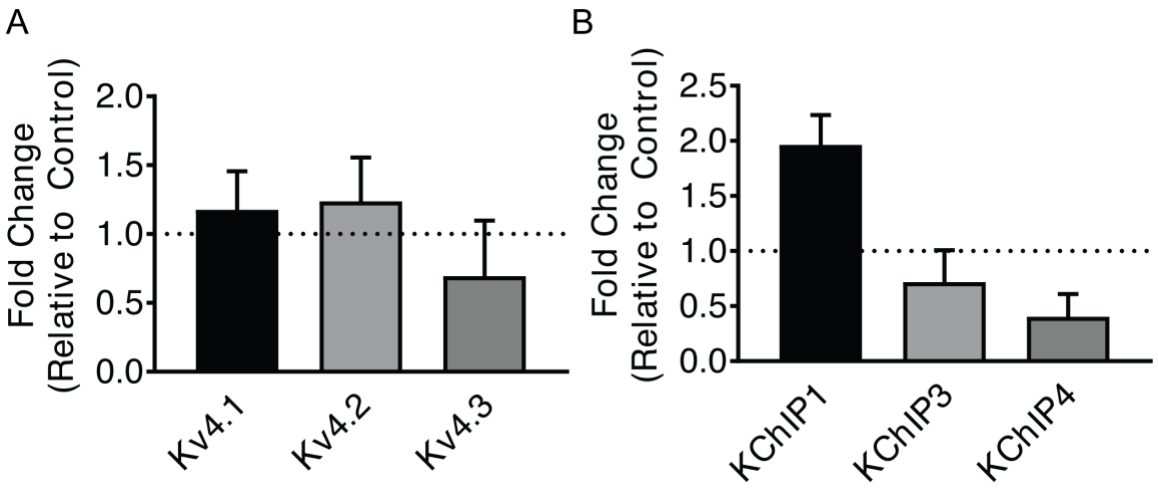


Figure 4.8 Kv4 and KChIP expression levels in the vagal complex from control and pilo-SE mice**.**
There was no significant difference in expression of Kv4.1, Kv4.2, Kv4.3, KChIP1, KChIP2, KChIP3, or KChIP4 (Unpaired *t*-test, $p > 0.05$). These analyses were performed by Dr. Katalin Smith.

4.4 Discussion

The present study investigated the 4-AP sensitive A-type voltage-gated K^+ current in a mouse model of pilocarpine-induced TLE. Previous studies have shown that there is a downregulation in the A-type current as well as voltage-gated K^+ channel protein in the hippocampus of pilocarpine-treated mice. This change was seen both in the hours and months following SE induction (Lugo et al., 2008; Monaghan et al., 2008; Su et al., 2008). SUDEP in genetic epilepsy models established by Kv1.1 channelopathy have been described in the NTS (Glasscock et al., 2010; Vanhoof-Villalba et al., 2018). Since the channelopathies induced the epilepsy phenotype, however, the relevance of the seizure disorder to autonomic dysfunction and SUDEP cannot be easily separated from the effect of the channel mutation itself in these models. Conversely, the functional modification of voltage-gated channels described here develops concurrently with epileptogenesis, implying that epilepsy-related changes in channel function can contribute to central vagal dysfunction in acquired epilepsy.

There have been several previous studies examining functional plasticity of NTS neurons in other disease states (Mei et al., 2003; Belugin and Mifflin, 2005; Kline et al., 2005; Dergacheva et al., 2013; Bach et al., 2015; Boychuk et al., 2015a; Boychuk and Smith, 2016). We have also recently shown that in the pilocarpine-induced SE model of TLE, GABAergic NTS neurons display hyperexcitability 6 and 12 weeks post-SE (Derera et al., 2017). The A-type current has been described in NTS neurons (Moak and Kunze, 1993; Bailey et al., 2002; Bailey et al., 2007) and prominently regulates neuronal excitability and action

potential morphology and repolarization. We first examined action potential frequency and half-width and found that they were significantly increased in GABAergic NTS neurons from pilo-SE mice compared to control mice suggesting a decrease in the function of the voltage-gated K^+ currents that underlie action potential repolarization develops during epileptogenesis. More specifically, the decrease in sensitivity of GABAergic NTS neurons from pilo-SE mice to 4-AP suggests a reduction in A-type voltage-gated K^+ current and Kv4 channel function.

The effect of 4-AP on synaptic activity in GABAergic NTS neurons between control and pilo-SE mice was also assessed. We found that 4-AP elicited a significant increase in sEPSC frequency, consistent with an increase in presynaptic excitatory neurotransmission. This effect of 4-AP has previously been shown in CA1 pyramidal neurons, as well as in NTS neurons involved in the cough reflex (Buckle and Haas, 1982; Gu et al., 2004; Haji and Ohi, 2010). Interestingly, we did not observe a similar increase in sEPSC frequency in GABAergic NTS neurons from pilo-SE mice. These neurons already receive an increase in excitatory neurotransmission (Derera et al., 2017), and combined with the alterations in voltage-gated K^+ current, may contribute to the lack of effect of 4-AP in neurons from pilo-SE mice. Similar to what was reported by others (Haji and Ohi, 2010), there was no significant effect of 4-AP on sEPSC amplitude in either group. Overall, the exact mechanism as to why there is not a significant increase in sEPSC frequency with 4-AP in pilo-SE mice, needs to be fully elucidated and the increase in excitatory neurotransmission may be one factor of many that is

contributing to the hyperexcitability of GABAergic NTS neurons in this model of TLE.

The Kv4 subfamily of voltage-gated K^+ channels is the primary regulator of the A-type current and includes the Kv4.1, Kv4.2, and Kv4.3 subunits, which are widely expressed in brain tissue (Rudy, 1988; Salkoff et al., 1992; Birnbaum et al., 2004; Covarrubias et al., 2008). More specifically, immunolabelling has revealed the presence of Kv4.2 and Kv4.3 subunits in neurons of the NTS (Strube et al., 2015). Our data on average TOC amplitude and voltage dependence of activation and inactivation in control animals are consistent with results from previous studies in NTS neurons (Moak and Kunze, 1993; Bailey et al., 2002). Interestingly, we found a reduction in average TOC amplitude in GABAergic NTS neurons from pilo-SE mice, but no changes in voltage-dependent activation or inactivation were observed. This suggests that there may be a decrease in the number of voltage-gated K^+ channels at the membrane, rather than an alteration in the channel or voltage-sensing pore contributing to a reduction in the A-type current amplitude.

The cellular mechanisms involved in Kv4 membrane expression and function in TLE in the NTS remains to be elucidated. We did not detect a significant decrease in the molecular expression of Kv4.1, Kv4.2, or Kv4.3 in pilocarpine-treated mice relative to control mice. I_A can also be modulated via KChIPs, which associate with Kv4 subunits and increase their surface expression and conductance (Hoffman et al., 1997; Beck et al., 2002; Lien et al., 2002; Birnbaum et al., 2004; Covarrubias et al., 2008). Previous studies suggest that expression of these accessory subunits is reduced in pilocarpine-treated mice (Monaghan et al.,

2008; Su et al., 2008). Upon measuring mRNA levels of KChIP1, KChIP3, and KChIP4 there was no significant difference in expression in pilocarpine-treated mice relative to controls. Kv4 function and expression can be altered in several different ways other than through accessory subunit modulation. A decrease in the export of Kv4 subunits from the endoplasmic reticulum, potentially via phosphorylation of the PDZ domain of the channel can also decrease surface expression of the channel (Birnbaum et al., 2004). Once the Kv4 channel is shuttled from the endoplasmic reticulum to the membrane, surface expression and channel opening can also be modified through phosphorylation by several kinases, including protein kinase C (PKC), protein kinase A (PKA), calmodulin-dependent protein kinase II (CAMKII), extracellular signal-related kinases/mitogen-activated protein kinases (ERK/MAPK), and protein tyrosine kinase (PTK) (Birnbaum et al., 2004). It has been shown in kainate-induced SE, that a decrease in the surface expression Kv4.2 correlates with ERK phosphorylation of the channel suggesting additional mechanisms for a decrease in the A-type current associated with epilepsy (Lugo et al., 2008). An increase in PKC-mediated phosphorylation in concert with a decrease in channel expression has been shown to be associated with a reduction of I_A in a rat model of TLE (Bernard et al., 2004). Other post-translational modifications, such as glycosylation and palmitoylation are known to increase channel stability and enhance surface localization of the channel (Birnbaum et al., 2004), but if and how this may occur during epileptogenesis is unclear.

This region of the brain has played a significant role in the research for potential SUDEP mechanisms. A previous study using genetic epilepsy models has showed that after a chemically-induced cortical seizure spreads to the brainstem, NTS neurons undergo spreading depolarization and the animal suffers from cardiorespiratory collapse and death (Aiba and Noebels, 2015). In other brain regions, increases in neuronal excitability have been associated with a decreased threshold for spreading depression (Somjen, 2001; Dreier and Reiffurth, 2015). Additionally, previous studies in individuals with TLE have shown evidence of Kv4 modification (Villa and Combi, 2016). We propose that reduced A-type current contributes to the increase in GABA NTS neuron hyperexcitability seen during TLE and that this may increase the risk for cardiorespiratory collapse. Understanding both the cellular mechanisms and functional consequences of alterations in the A-type current in the context of TLE and SUDEP may allow for the development of novel biomarkers and/or treatment options for individuals at risk of sudden death.

Chapter 5 Chemogenetic manipulation of GABA NTS neurons in TLE

5.1 Introduction

Sudden unexpected death in epilepsy (SUDEP) is one of the leading causes of death in individuals with temporal lobe epilepsy (TLE), which comprises 60% of the total epilepsy population (Nashef, 1997; Tolstykh and Cavazos, 2013). Patients with TLE display autonomic dysfunction such has depressed heart rate variability and abnormal cardiac rhythms (Tomson et al., 1998; Mayer et al., 2004; Ronkainen et al., 2005; Suorsa et al., 2011; Romigi et al., 2016). Additionally, studies utilizing genetic epilepsy models have suggested that dysfunction in central autonomic control centers in the caudal brainstem are involved in SUDEP (Glasscock et al., 2010; Aiba and Noebels, 2015).

The autonomic nuclei that receive information regarding cardiorespiratory function and subsequently modulate output to the periphery are located in the dorsal vagal complex (DVC) of the causal brainstem. The DVC receives viscerosensory information via the afferent fibers of the vagus nerve, which synapse onto neurons in the nucleus tractus solitarius (NTS). NTS neurons project to preganglionic motor neurons within the nucleus ambiguus and dorsal motor nucleus of the vagus (DMV) to regulate parasympathetic output (Andresen and Kunze, 1994; Doyle and Andresen, 2001; Wang et al., 2001b; Davis et al., 2004; Glatzer et al., 2007). We have recently shown that GABAergic neurons in the NTS are hyperexcitable in a mouse model of TLE (Derera et al., 2017). However, it is still unknown if direct GABAergic NTS neuron activation is involved in seizures or SUDEP.

The aim of this study is to determine if selective activation of GABAergic NTS neurons can modulate in vivo seizure threshold and *in vitro* electrophysiological properties of neurons within the DVC. An excitatory designer receptor exclusively activated by designer drugs (DREADDs) was used to selectively modulate GABAergic NTS neuron function in a mouse model of pilocarpine-induced TLE. Excitatory DREADDS have been used to modulate animal behavior in other brain regions and pathologies but have yet to been utilized in a mouse model of TLE and SUDEP (Armbruster et al., 2007; Urban and Roth, 2015; Roman et al., 2017). We hypothesized that if GABAergic NTS neurons are directly involved in TLE, activation of these neurons via an excitatory DREADD may either induce a seizure or decrease seizure threshold in TLE mice.

5.2 Methods

5.2.1 Stereotaxic Injection of DREADD Virus

Four weeks after pilocarpine or vehicle treatment, age-matched Vgat-ires-Cre mice were injected with the excitatory DREAAD, pAAV8-hSyn-DIO-hM3D(Gq)-mCherry into the NTS (pAAV8-hSyn-DIO-hM3D(Gq)-mCherry was a gift from Bryan Roth, Addgene, Cambridge, MA; plasmid #44361) (Krashes et al., 2011). Mice were weighed and placed under 5% isofluorane and 1.5% 100% O_2 for initial anesthesia. Once the animal was fully anesthetized, isofluorane was reduced to 2.5 % with 1.5% 100% O_2 and the animals head was shaved. Animals were then placed in a stereotaxic device and given a subcutaneous injection of the analgesic buprenorphine (0.1 mg/kg in 0.9% saline) to mitigate post-operative pain. A midline incision was made across the scalp and bregma, lambda, and the

occipital bone were exposed. Stereotaxic coordinates were measured from bregma: AP: 7.0, ML: 0.1, DV: 0.36. A drill was used to make small hole (<1 mm in diameter) at the injection site and 250-500 nL of pAAV8-hSyn-DIO-hM3D(Gq)-mCherry was slowly injected into the NTS. Mice were given 1 mg/kg carprofen post-operatively. After 10-14 days sutures were removed. Mice were used for in vivo and in vitro studies 3-4 weeks after the injection of the DREADD.

5.2.2 Flurothyl-Induced Seizures

Flurothyl (Bis(2,2,2-trifluroethyl)ether (10% flurothyl in 95% ethanol; Sigma) was used to induced a single acute seizure in Vgat-ires-Cre control and pilo-SE mice 3 weeks after intracranial injection of pAAV8-hSyn-DIO-hM3D(Gq)-mCherry. All experiments using flurothyl were performed within a certified fume hood. Mice were given an i.p. injection of either saline mixed with DMSO or CNO (1mg/kg) 15-20 minutes prior to being exposed to flurothyl. Each animal received the opposite treatment 3-4 days later to have a counter-balanced design. The 10% flurothyl was dripped at a rate of 6 mL/hour with a syringe pump (KD Scientific, Holliston, MA) onto a gauze pad within the plexiglass container. One animal at a time was placed in the container for each trial. Once the animal developed a seizure (i.e. loss of posture), the flurothyl was stopped and the animal was exposed to open air, after which the seizure ended (Kadiyala et al., 2016; Ferland, 2017; Kadiyala and Ferland, 2017). Once the animal resumed normal behaviors (i.e. after five to 10 minutes), it was returned to its home cage and the latency to seize was recorded.

5.2.3 Electrophysiological Recordings

Brainstem slices were transferred to a chamber mounted on a fixed stage under an upright microscope (BX51WI; Olympus) where they were superfused with continuously warmed (30-32°C) oxygenated ACSF. VGAT-containing neurons in the NTS that were transfected with pAAV8-hSyn-DIO-hM3D(Gq)-mCherry, were mCherry-expressing and were targeted under epifluorescence (Krashes et al., 2011). DMV neurons were targeted based on their morphological characteristics (Bach and Smith, 2012; Bach et al., 2015). Patch pipettes for recordings were pulled from borosilicate glass (open tip resistance 3-5 MΩ; King Precision Glass Co, Claremont, CA). The pipette solution contained (in mM): 130 K^+ gluconate, 1 NaCl, 5EGTA, 10 HEPES, 1 $MgCl_2$, 1 $CaCl_2$, 3 KOH, 2 ATP. Recordings were obtained using an Axon 700B amplifier (Molecular Devices; San Jose, CA), low pass filtered at 2-3 kHz, digitized at 20 kHz, and recorded onto a computer (Digidata 1440A, Molecular Devices).

Prior to recording, cells were allowed to equilibrate with the pipette recording solution for approximately five minutes. Whole-cell patch clamp recordings of spontaneous action potentials in NTS neurons were recorded in current-clamp at resting membrane potential. Resting membrane potential was recorded in I=0 in NTS neurons. Spontaneous inhibitory postsynaptic currents (sIPSCs) were recorded in voltage-clamp in DMV neurons. DMV neurons were clamped at a holding potential of 0 mV to isolate inhibitory currents. The effect of clozapine-N-oxide (CNO, 30 μM) was assessed on resting membrane potential and action potential frequency in NTS neurons and on sIPSC frequency and

amplitude in DMV neurons from control and pilo-SE mice. Series resistance was less than 25 MΩ and monitored periodically throughout the recordings. If the series resistance changed by more than 20% over the course of a recording, it was discarded.

5.2.4 Statistical Analyses

A repeated measures two-way ANOVA (Tukey's *post hoc*) was used to determine the effect of CNO treatment on flurothyl induced seizure latency between control and pilo-SE mice (GraphPad Prism; La Jolla, CA). Action potential frequency (2-minute recording segment) and resting membrane potential were analyzed using Clampfit 10.2 (Molecular Devices). Two-minute segments of continuous recordings of sIPSCs from DMV neurons was assessed with MiniAnalysis (Synaptosoft, Decatur, GA). For electrophysiology data, a paired *t*-test was used to determine the effect of CNO on NTS and DMV neurons from control and pilo-SE mice. Statistical significance for all measurements was set at $p < 0.05$.

Methods for Chapter 5 also included: mice used, methylscopolamine and pilocarpine injections, and brainstem slice preparation as described in Chapter 2 of this dissertation.

5.3 Results

5.3.1 Spontaneous Seizures

It has been previously established in various mouse strains that pilocarpine-induced SE leads to the development of chronic spontaneous seizures four to six weeks after treatment (Shibley and Smith, 2002; Groticke et al., 2007). However,

spontaneous seizure development has not yet been established in the Vgat-ires-Cre mouse strain. Mice were passively monitored for seizures as described in Chapter 2 beginning at four weeks post-pilocarpine treatment. None of the mice that received vehicle treatment or did not have SE developed spontaneous seizures, whereas 100% of mice that survived pilocarpine-induced SE developed spontaneous seizures (Table 5.1).

Table 5.1 Development of spontaneous seizures.

	Spontaneous Seizures		
	Occurrence in SE survivors	**Days after SE to 1st Spontaneous Seizure**	**Seizure Rate (seizures/h)**
Control (n=4)	-	-	0
Mice that survived SE (> 3 S3 seizures; n=3)	100%	48.67 ± 16.44 (range: 30-61)	0.13 ± .04
Mice that died during SE (n=8)	-	-	-
Non-SE mice (n=3)	0%	-	0

5.3.2 Effect of GABAergic NTS neuron activation on Flurothyl-Induced Seizure Latency

Control and pilo-SE mice were randomly administered either CNO or saline and the latency to seize upon flurothyl exposure was measured. Three to four days later, animals that were previously given saline, received CNO treatment, and vice-versa. A repeated measures two-way ANOVA showed a significant interaction effect between saline and CNO administration in control and pilo-SE mice ($F(1,13)=5.71$; $p = 0.03$). Pilo-SE mice had a significantly shorter seizure latency (462 $\pm$ 37 sec; n=7 mice) compared to control mice (609 $\pm$ 38 sec; n=8 mice; $p = 0.03$) when administered saline. While CNO did not significantly alter seizure latency in pilo-SE mice (saline: 462 $\pm$ 37 sec; CNO: 482 $\pm$ 37 sec; n=7 mice; $p = 0.99$), it did significantly shorten the seizure latency in control mice (saline: 609 $\pm$ 38 sec; CNO: 458 $\pm$ 22 sec; $p = 0.02$, Fig.5.1).

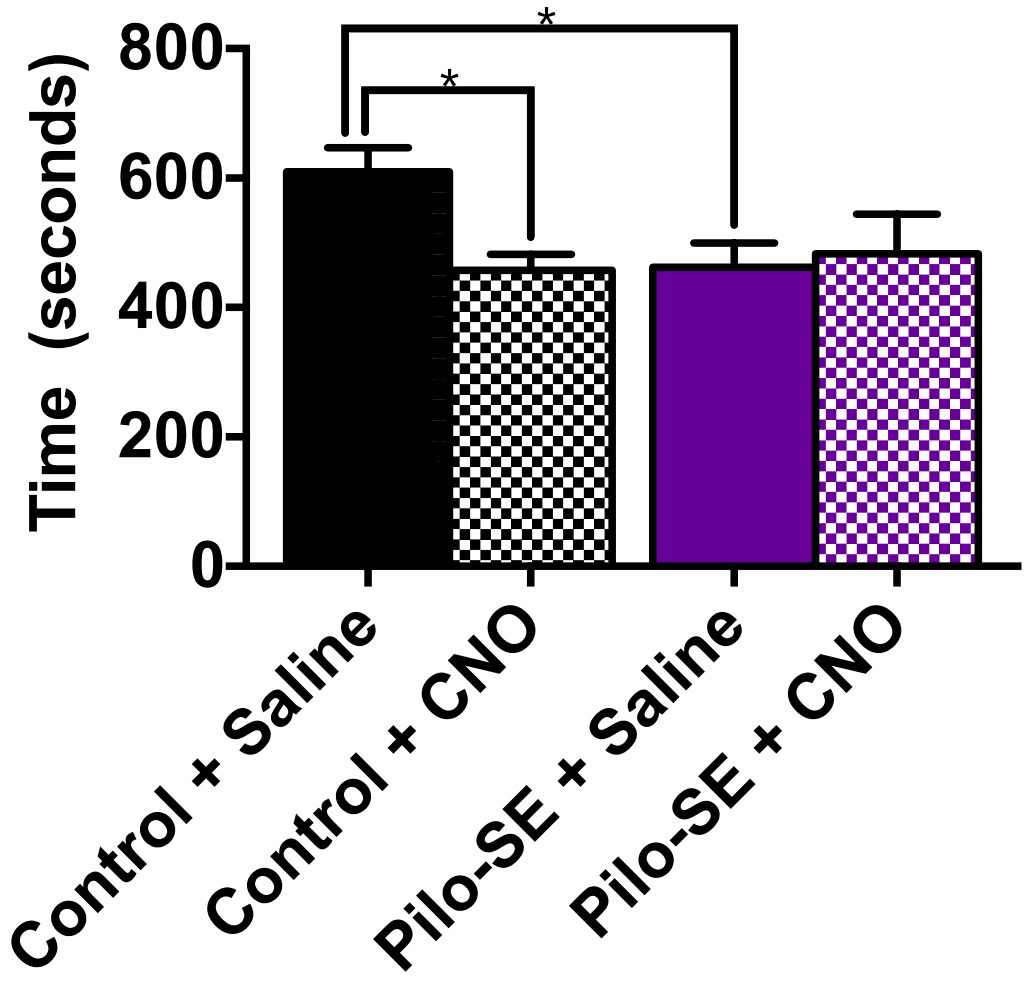


Figure 5.1 Chemogenetic modulation of seizure latency in control and pilo-SE mice.

There was a significantly shorter latency to seize in pilo-SE mice (n=7) compared to control mice (n=8) when treated with saline. CNO alone reduced seizure latency in control mice, but this did not further shortern the latency in pilo-SE mice. (p > 0.05). Two-way repeated measures ANOVA: F (1,13) = 5.71; p = 0.03. *p < 0.05.

5.3.3 GABAergic NTS neuron activation in control and pilo-SE mice

The effect of GABAergic NTS neuron activation on action potential frequency and resting membrane potential was assessed in control and pilo-SE mice. The resting membrane potential was significantly more depolarized after CNO application in control mice (ACSF: -46.06 ± 1.48 mV; CNO: -42.57 ± 0.76 mV; p = 0.02, n= 10 cells, Fig. 5.2D). Activation of GABAergic NTS neurons from pilo-SE mice also had a significantly depolarized resting membrane potential (ACSF: -45.83 ± 0.82 mV; CNO: -41.21 ± 1.06 mV; p = 0.001; n=10 cells Fig. 5.2D). In the same cells, the CNO-induced activation of GABAergic NTS neurons was concomitant with an increase in action potential frequency in both control (ACSF: 1.72 ± 0.39 Hz; CNO: 2.36 ± 0.38 Hz; p = 0.03; n=10 cells) and pilo-SE mice (ACSF: 2.49 ± 0.47 Hz; CNO: 3.90 ± 0.80 Hz; p = 0.03; n=10 cells; Fig. 5.2C).

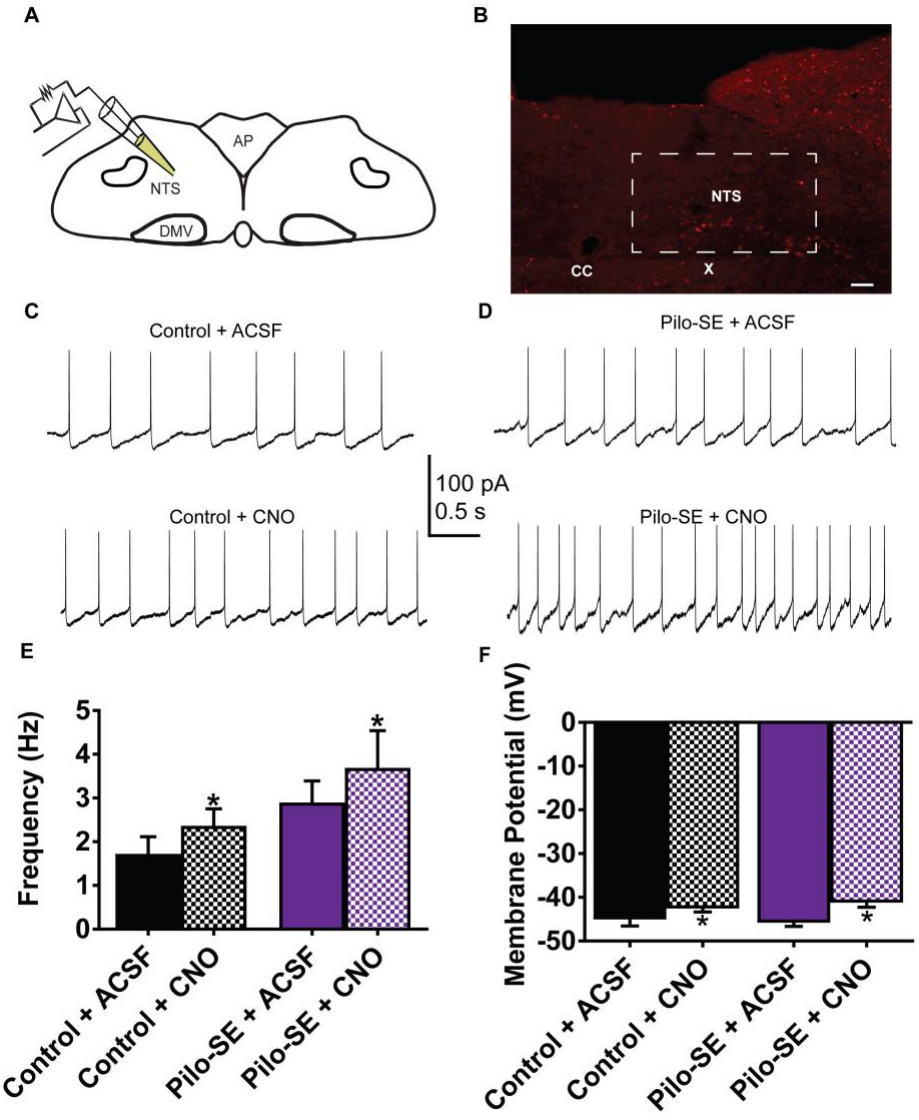


Figure 5.2 Chemogenetic modulation of GABAergic NTS neurons from control and pilo-SE mice.
(A) Illustration of pipette location in the NTS. (B) Representative image of mCherry expression in the NTS four weeks after DREADD injection. (C) Representative traces showing the effect of clozapine-N-oxide (CNO, 30 µM) on action potential firing in a GABAergic NTS neuron from a control animal. (D) Representative traces showing the effect of CNO on action potential firing in GABAergic NTS neuron from a pilo-SE animal. (E) CNO significantly increases the action potential frequency in control mice and pilo-SE mice. (F) CNO significantly depolarizes membrane potential in GABAergic NTS neurons from control and pilo-SE mice. Sample Sizes: control: n=8 mice; pilo-SE: n=8 mice. *$p < 0.05$.

5.3.4 Inhibition of Vagal Motor Neurons via GABAergic NTS Neuron Activation

GABAergic NTS neurons project to preganglionic parasympathetic motor neurons in the DMV (Travagli et al., 1991; Davis et al., 2004; Babic et al., 2011). We tested the hypothesis that activation of mCherry-expressing NTS neurons would increase inhibition of DMV neurons, assessed via sIPSC frequency and amplitude. GABAergic NTS neuron activation in slices from control animals significantly increased sIPSC frequency (ACSF: 0.53 ± 0.1 Hz; CNO: 0.96 ± 0.21 Hz; $p = 0.02$) but not amplitude (ACSF: 36.97 ± 4.45 pA; CNO: 30.73 ± 1.75 pA; $p = 0.22$; n=6 cells) of DMV neurons. There was a similar effect in DMV neurons from pilo-SE mice, where sIPSC frequency increased (ACSF: 0.98 ± 0.35 Hz; CNO: 1.72 ± 0.45 Hz; $p = 0.001$), but there was no significant change in amplitude (ACSF: 43.39 ± 9.20 pA; CNO: 53.58 ± 19.28 pA; $p = 0.48$; n=8 cells; Fig. 5.3).

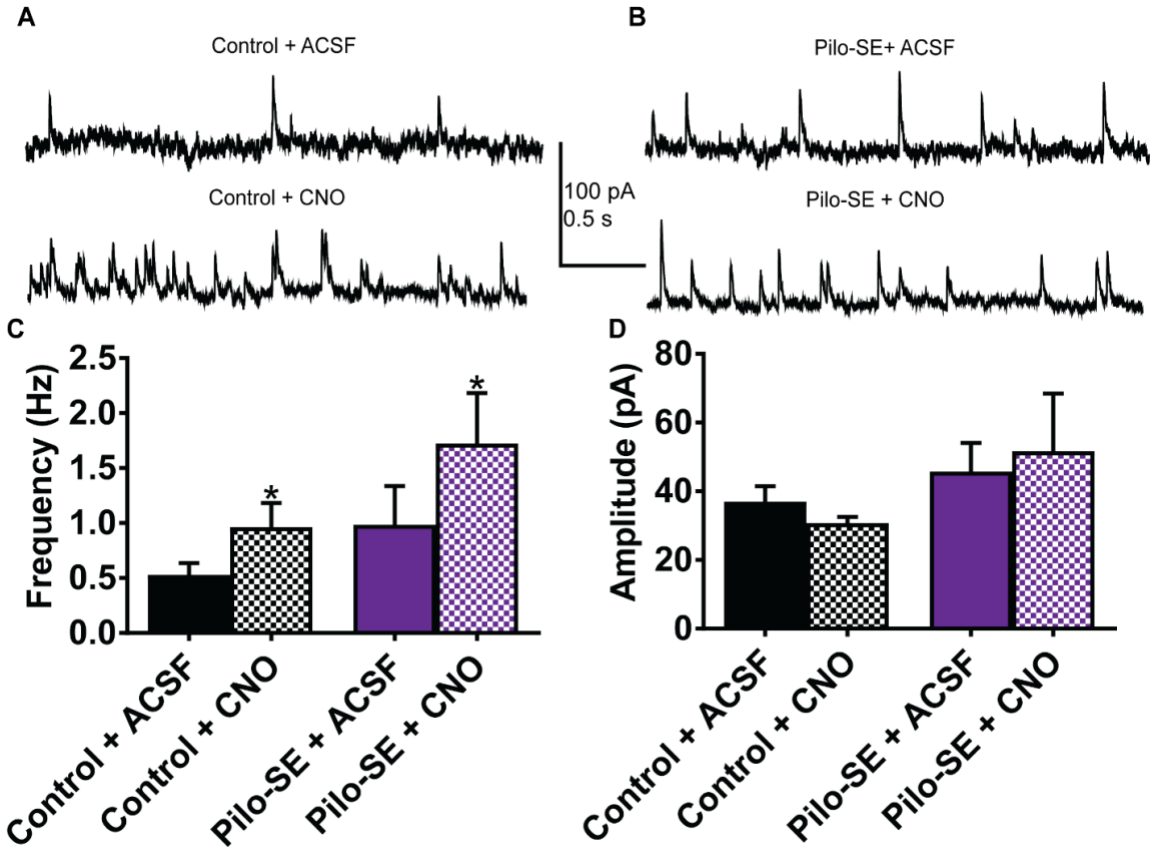


Figure 5.3 Inhibition of preganglionic parasympathetic motor neurons in control and pilo-SE mice.
(A) Representative traces showing the effects of CNO (30 µM) on sIPSCs in a DMV neuron from a control mouse. (B) Representative traces showing the effect of CNO on sIPSCs in a DMV neuron from a pilo-SE mouse. (C) Application of CNO significantly increases sIPSC frequency in DMV neurons from control and and pilo-SE mice. (D) CNO does not significantly alter sIPSC amplitude in DMV neurons from control and pilo-SE mice. Sample sizes: control: n=4 mice; pilo-SE: n=5 mice. *$p < 0.05$.

5.4 Discussion

Previous research has utilized DREADDs to excite or inhibit specific neuronal subtypes in order to modulate whole animal behavior and neuron function (Krashes et al., 2011; MacLaren et al., 2016; Roth, 2016; Whissell et al., 2016). Inhibitory DREADDs have been used to block seizure activity in organotypic slice cultures and kindling models (Avaliani et al., 2016; Wicker and Forcelli, 2016). While blocking seizure activity with inhibitory DREADDs in these studies has been successful, there have been no studies using excitatory DREADDs to induce seizure activity. This study tested the hypothesis that activation of GABAergic NTS neurons via the excitatory DREADD, pAAV8-hSyn-DIO-hM3D(Gq)-mCherry, can modulate whole animal seizure threshold and single vagal complex neuron activity.

We first investigated the effect of DREADD-mediated activation of GABAergic NTS neurons on animal behavior, namely on flurothyl-induced seizure latency. When pilo-SE mice received no treatment, their latency to seize was significantly shorter compared to control animals. While CNO did not appear to have a significant effect on seizure latency in pilo-SE mice, it did significantly reduce seizure latency in control mice. This result was unexpected as I hypothesized that CNO would decrease seizure latency in pilo-SE mice when compared to control mice. Because GABAergic NTS neurons from pilo-SE mice have been shown to become hyperexcitable, it is possible that there is a ceiling effect, which could explain why CNO activation of mCherry-expressing NTS neurons does not further shorten seizure latency. Control animals did not exhibit any prior seizure activity and therefore may be more susceptible to a reduction in

seizure latency when mCherry-expressing neurons are activated via CNO administration. Aiba and Noebels have shown that when a seizure is chemically elicited in the cortex of an anesthetized mouse with Dravet's Syndrome, it spreads to the brainstem initiating cardiorespiratory collapse and sudden death (Aiba and Noebels, 2015). Additionally, they have shown that chemically-induced depolarization of NTS neurons in Kv1.1 knockout mice results in seizure-like waveforms and brief EEG suppression in the cortex. This suggests that spreading depression in the brainstem effects cortical activity, supporting the hypothesis that seizures propagation can affect NTS function and vice-versa, potentially influencing SUDEP risk. Perhaps similar studies would be necessary for a seizure in pilo-SE mice administered CNO to occur.

There has also been concern that CNO may not be as specific as initially shown, as recent studies have suggested that CNO metabolizes into clozapine, which may produce off-target effects (Gomez et al., 2017; Manvich et al., 2018). While there is the possibility that this occurred in the whole animal studies, effects of CNO were seen in less than 5 minutes during in vitro slice electrophysiology. Electrophysiological results suggest that CNO increases excitation of GABAergic NTS neurons, subsequently leading to increased inhibition of downstream motor neurons that go on to regulate parasympathetic function. This would be expected to increase the inhibition of parasympathetic output to the periphery, thus leading to autonomic imbalance, similar to what is seen in patients with TLE (Ronkainen et al., 2005; Romigi et al., 2016). Additionally, GABAergic NTS neurons were slightly more sensitive to CNO-induced excitation (ΔmV: control: 7.58%; pilo-SE:

10.08%). This small differential effect may partially be due to epileptogenesis this model.

These data show that pilo-SE mice have a reduced seizure latency and are more easily excited by CNO compared to control animals, leading to increased inhibition of preganglionic parasympathetic motor neurons. While CNO administration decreased seizure latency in control mice, the lack of effect in pilo-mice suggests that increased excitability in GABAergic NTS neurons is not the sole factor responsible for influencing seizure threshold and contributing to SUDEP. Others have suggested that brainstem autonomic nuclei are the prime mediators of SUDEP in models of genetic epilepsy (Glasscock et al., 2010; Aiba and Noebels, 2015), however, acquired epilepsies may be more complicated and SUDEP could be due to a multitude of factors including altered respiratory function (Ryvlin et al., 2013; Sowers et al., 2013; Zhan et al., 2016). More work needs to be done examining the function of neurons in other brain regions that may contribute to SUDEP in concert with autonomic nuclei. This would provide greater insights to mechanisms of SUDEP and allow for the development of potential biomarkers or preventative treatment options.

Chapter 6 Discussion

6.1 Summary of Findings

This dissertation focused on alterations in GABAergic NTS neuron function in a mouse model of pilocarpine-induced TLE and SUDEP. While several studies have used genetic epilepsy models to study SUDEP (Faingold et al., 2010; Glasscock et al., 2010; Cheah et al., 2012; Auerbach et al., 2013; Kalume et al., 2013; Aiba and Noebels, 2015), this dissertation is the first to examine sudden death and NTS neuron function in a model of acquired TLE. The main findings of this dissertation are as follows: 1) the pilocarpine-induced SE model of TLE is a model of SUDEP, 2) pilo-SE mice did not display long-term, chronic abnormal heart rhythms or heart rate variability, 3) GABAergic NTS neurons display acute (i.e. 1 week post-SE) and long-term (i.e. 6-12 weeks post-SE) hyperexcitability, 4) reduced A-type current contributes to hyperexcitability, 5) chemogenetic activation of GABAergic NTS neurons alone is not sufficient to induce a seizure or SUDEP, but lowers seizure latency in control mice, 6) Activation of an excitatory DREADD in GABAergic NTS neurons increases inhibition of preganglionic parasympathetic motor neurons. This study is the first to examine the pilocarpine-induced SE model of TLE in the context of SUDEP. This study is the first to quantify mortality rates from SUDEP in pilo-SE mice. Additionally, these results indicate that GABAergic NTS neurons, which modulate parasympathetic output to the periphery are functionally altered in TLE mice, potentially contributing to SUDEP.

6.2 The Pilo-SE model of TLE as a model of SUDEP

6.2.1 Survival of Pilo-SE mice

Many studies have examined SUDEP in the context of genetic epilepsies, with a focus on channelopathies that are associated with seizures. In studies using a mouse model of Dravet's Syndrome, sudden death has ranged from postnatal day 16 to postnatal day 150 (Yu et al., 2006; Cheah et al., 2012; Auerbach et al., 2013; Cheah et al., 2013; Kalume et al., 2013). There does appear to be difference in the onset of sudden death occurrences in a global heterozygous Nav1.1 knock-in model (Yu et al., 2006) versus a cre-driven deletion of Nav1.1 (Cheah et al., 2012). However, mice with genetic mutations to recapitulate Dravet's Syndrome only have a small number of seizures (e.g. 1-12 seizures total) before dying suddenly (Ogiwara et al., 2007; Cheah et al., 2012; Auerbach et al., 2013). By comparison, pilo-SE mice have approximately 2 seizures per day beginning at 4-6 weeks post-SE (Shibley and Smith, 2002; Groticke et al., 2007; Hunt et al., 2013). *KCNA1*-null mice also experience behavioral seizures and SUDEP beginning at postnatal day 14-21 (Glasscock et al., 2010), presumably as consequence of the deletion of Kv1.1 potassium channels, but no data has been published regarding seizure frequency prior to death (Gautier and Glasscock, 2015).

Not including mice that died 1-7 days post-SE, there is a 23% survival rate 150 days post-treatment in pilo-SE mice compared to control mice. Mice that died within the week following pilocarpine treatment were not considered to have died of SUDEP because they did not develop spontaneous seizures. Additionally, mice begin to suffer from SUDEP 16 days post-SE, with 60% having died after 6 weeks

(Derera et al., 2017). This study is the first to show the mice that develop and survive status epilepticus go on to die of SUDEP.

6.2.2 Cardiac changes in pilo-SE mice

Individuals with TLE who are at risk for SUDEP or eventually die from SUDEP display cardiac abnormalities such as increased heart rate, depressed HRV, and arrhythmias (Mayer et al., 2004; Ronkainen et al., 2005; Suorsa et al., 2011; Romigi et al., 2016). Mouse models of genetic epilepsy also display cardiac abnormalities, such as in Dravet's syndrome, where mice have increased heart rate directly preceding sudden death (Auerbach et al., 2013). *KCNA1* knockout mice have arrhythmias and autonomic blockade decreases the frequency of atrioventricular block, suggesting that a central autonomic change might be responsible for these abnormal heart rhythms (Glasscock et al., 2010).

This dissertation examined heart rate and heart rate variability at 4 time points: 24 hours pre-treatment, 24 hours post-treatment, 6 weeks, and 12 week post-treatment in control and pilo-SE mice. While there was a significant increase in heart rate and decrease in HRV 24 hours post-treatment in pilo-SE mice, there were no significant differences in the later time points when animals should have developed spontaneous seizure activity. Previous studies using the pilocarpine-induced SE model of TLE had examined heart rhythms and autonomic balance 1-2 weeks post-treatment and used rats instead of mice (Metcalf et al., 2009a; Bealer et al., 2010; Bealer et al., 2011). At rest, mice have a significantly higher heart rate compared to rats, which may contribute to heart rate differences seen in pilocarpine-treated rats compared to mice (Lujan et al., 2012). Within 30 minutes

of SE, there is a significant increase in heart rate in pilocarpine-treated rats (Metcalf et al., 2009b). This increase persists 1 and 2 weeks post-SE (Metcalf et al., 2009a). This study also concluded that a decrease in vagal tone, leading to a dominance in sympathetic drive is responsible for autonomic imbalance and a persistent increase in heart rate, increasing the risk for sudden death (Metcalf et al., 2009a). Overall, alterations heart rate and HRV occur acutely in pilo-SE mice, but more work needs to be done to determine if long-term changes occur as a result of chronic spontaneous seizure activity resulting in increased SUDEP risk.

6.2.3 Future directions

While this study shows that pilo-SE mice die from SUDEP, it was unable to pinpoint seizures in relation to sudden death or the actual death itself. It would have been more impactful to record video-EEG in these animals. This would have allowed for the exact time of death to be determined and record if a seizure event directly preceded SUDEP. The video component would have been useful in determining if there was any altered respiratory function, such as gasping behavior after a seizure and/or prior to death.

In the future, the addition of video-EEG to the cardiac monitoring would be a valuable way to decipher pre-, inter-, and post-ictal periods during a spontaneous seizure occurrence. It would allow for more specific examination of cardiac rhythms. For example, the use of EEG would allow for the comparison of heart rate and HRV between seizure periods instead of during random time points, making this study more similar to those examining individuals with epilepsy. This could allow for more clear interpretation of autonomic alterations during epileptogenesis

as well, because inter-ictal autonomic changes may be more predictive of SUDEP risk than changes that occur surrounding a specific seizure event. The addition of continuous ECG monitoring instead of only recording at 4 time points would also be more informative of cardiac changes. There were 2 animals that were initially part of this study that died suddenly just prior to the 8 weeks-post SE, but because there was not continuous ECG recording, it was impossible to determine if cardiac abnormalities occurred prior to death. Ultimately, these additions could allow for a better interpretation of autonomic function over time in association with seizure development.

6.3 Alterations in GABAergic NTS neuron excitability

6.3.1 Synaptic and intrinsic changes in GABAergic NTS neurons

GABAergic NTS neurons receive glutamatergic, or excitatory, input primarily from the postsynaptic terminals in the afferent fibers of the vagus nerve that terminate at the ST. Previous research shows that GABAergic NTS neurons alter their function as a consequence of various pathological states such as hypoxia and diabetes (Zsombok and Smith, 2009; Kline, 2010; King et al., 2012; Boychuk et al., 2015a). This dissertation examined GABAergic NTS neuron function in a mouse model of TLE and SUDEP. While there were no changes in the resting membrane potential or input resistance of GABAergic NTS neurons following pilocarpine-induced SE, there were significant alterations in the synaptic properties of these neurons. One week post-SE these neurons displayed significant increases in spontaneous action potential and sEPSC frequency. Even more interesting, there appeared to be sustained long-term changes in excitability.

This was evidenced by increases in spontaneous action potential, sEPSC, and mEPSC frequency 6 and 12 weeks post-SE. Spontaneous and mEPSC frequency increases were not accompanied by increases in amplitude, indicative of an increase in excitatory neurotransmitter release from presynaptic terminals, such as the vagal afferent terminals in the ST. Additionally, when the ionotropic glutamate blocker kynurenic acid was applied to slices, there was a significant decrease in excitability as indicated by a decrease in sEPSC frequency in GABAergic neurons from pilo-SE mice.

A significant increase in mEPSC frequency but not amplitude is suggestive of an increase in presynaptic release probability of glutamate (i.e. readily releasable vesicle pool) or an increase in the number of functional synapses (Kline, 2008; Pinheiro and Mulle, 2008; Queenan et al., 2012). To test the hypothesis that there was a change in either of these properties, paired pulse and frequency-dependent depression was examined. It has been theorized that this type of synaptic plasticity in the NTS serves to prevent excessive alterations to blood pressure (Liu et al., 2000; Kline, 2008). While this dissertation is the first to examine paired pulse and frequency-dependent depression in GABAergic NTS neurons in a mouse model of TLE, there were no significant differences in either of these parameters. Therefore, the increase in excitability seen in GABAergic NTS neurons from pilo-SE mice is likely not due to an increase in the vesicle pool or an increase in synapses stemming from vagal afferent terminals. Local glutamatergic neurons within NTS circuitry can also influence GABAergic firing properties (Champagnat et al., 1986; Kawai and Senba, 1996). For example, increases in the

amount of excitatory neurotransmitter or slow turnover within the synaptic cleft can also alter excitability (Chen and Bonham, 2005; Kline, 2008). Additionally, there exists multiple subgroups within the NTS neuron population that are dependent on the location of vagal afferent fiber terminals. Cardiovascular afferents terminate in the lateral and medial NTS, while afferents from the stomach terminate in the commissural NTS (Barraco et al., 1992; Babic et al., 2015). Electrophysiology recordings of evoked EPSCs in paired pulse and frequency-dependent depression studies did not differentiate between these subpopulations. Thus, recordings from various NTS subpopulations may have confounded effects of presynaptic glutamate release from vagal afferents.

6.3.2 Future directions

To examine vagal afferent firing properties further, future studies should focus on GABAergic neurons within the lateral and medial NTS, in order to better isolate alterations in synaptic plasticity from cardiovascular afferents. Additional work could also be done examining local glutamatergic NTS neurons. Immunohistochemical studies labeling vesicular glutamate transporter 2 (VGLUT2), which is present in the NTS (Hermes et al., 2014; Bach et al., 2015), would provide insight into the sites of origin for increased glutamate neurotransmitter release. As labeling for VGLUT2 would also presumably label vagal afferent terminals, this could allow for differentiation between glutamate release in the ST versus local glutamatergic NTS neurons. To more closely examine the structural relationship between presynaptic terminals from local glutamatergic NTS neurons, VGLUT, and postsynaptic 2nd order GABAergic NTS

neurons, electron microscopy could be used. This could provide insight into the differences in the number of glutamate vesicles in the presynaptic terminal between control and pilo-SE solely in local glutamatergic NTS neurons. The relationship between the potential modulation of excitatory neurotransmission onto 2nd order GABAergic neurons by local glutamatergic NTS neurons has not been well-examined, and these future studies would provide insight from both basic science and pathological perspectives.

6.4 Effects of TLE on voltage-gated K^+ current in GABAergic NTS neurons

6.4.1 Reduced I_A contributes to hyperexcitability in GABAergic NTS neurons from pilo-SE mice

I_A plays a prominent role in maintaining neuronal excitability by modulating action potential repolarization (Mitterdorfer and Bean, 2002; Birnbaum et al., 2004). I_A is present in NTS neurons and has been altered in response to various pathological in states (Moak and Kunze, 1993; Bailey et al., 2002; Belugin and Mifflin, 2005; Bailey et al., 2007; Accorsi-Mendonca et al., 2015; Strube et al., 2015). This dissertation examined the effects of 4-AP, an I_A blocker, on action potential frequency and half-width and also isolated I_A in GABAergic NTS neurons. GABAergic NTS neurons from pilo-SE mice displayed a decrease in sensitivity to 4-AP, while in contrast, GABAergic NTS neurons from control animals showed significant increases in both action potential frequency and half-width. There was also a significant increase in sEPSC frequency upon application of 4-AP. This occurs because 4-AP depolarizes pre-synaptic terminals, increasing neurotransmitter release, which is similar to what others have seen in the

hippocampus and NTS (Buckle and Haas, 1982; Gu et al., 2004; Haji and Ohi, 2010). Peak transient outward A-type current in GABAergic NTS neurons was significantly reduced, with no changes in the kinetics of activation or inactivation in pilo-SE mice compared to control mice. The lack of change in activation or inactivation kinetics suggests that there is not a change in the voltage-sensing pore region of the voltage-gated K^+ channels (Birnbaum et al., 2004; Jerng et al., 2004). This dissertation was first to examine I_A in GABAergic NTS neurons in the pilocarpine-induced SE model of TLE. It also shows that TLE can induce long-term alterations in a crucial modulator of neuronal excitability.

6.4.2 Kv4 and KChIP expression in GABAergic NTS Neurons

It was hypothesized that the reduction in I_A was due to a downregulation in the expression of genes that encode the Kv4 family of voltage-gated K^+ channels. Kv4.1, Kv4.2, and Kv4.3 modulate the A-type current and can therefore affect I_A amplitude (Birnbaum et al., 2004; Strube et al., 2015). In one study a viral construct was transfected into hippocampal cell cultures leading to a downregulation in Kv4.2, as evidenced by action potential broadening and frequency increases (Kim et al., 2005), similar to the electrophysiological data described in Chapter 4 of this dissertation. When qRT-PCR was utilized to test for differences in expression in Kv4.1, Kv4.2, and Kv4.3 there were no significant alterations in mRNA expression in tissue samples from pilo-SE mice relative to control mice. This in contrast to other studies examining the Kv4 subfamily in the hippocampus in TLE rodents, which found decreases in Kv4.2 and Kv4.3 protein (Tsaur et al., 1992; Monaghan et al., 2008; Su et al., 2008). However, a separate study found there were no

changes in I_A or Kv4.2 and Kv4.3 in pilocarpine-treated rats that developed spontaneous seizure activity (Ruschenschmidt et al., 2006). KChIPs can also modify the conductance of Kv4 channels (Beck et al., 2002; Birnbaum et al., 2004; Covarrubias et al., 2008), so qRT-PCR was utilized to test for differences in expression in KChIP1, KChIP3, and KChIP4. No significant differences were found in any of the KChIPs in pilo-SE mice relative to controls. Again, this differs from the literature where studies have shown a downregulation in KChIP1 months after pilocarpine-induced SE (Monaghan et al., 2008; Su et al., 2008).

6.4.3 Future directions

There are several reasons why there may be a reduction in I_A, suggestive of increased excitability, without a concomitant decrease in Kv4 or KChIP expression. Studies that examined Kv4 and KChIP expression in the hippocampus of pilocarpine treated mice did not also examine the electrophysiology of the A-type current (Tsaur et al., 1992; Monaghan et al., 2008; Su et al., 2008) and electrophysiological studies did not also examine gene expression (Bernard et al., 2004). The 1mm tissue samples that the RNA was isolated from contained the majority of the vagal complex, including neurons from NTS, DMV, and area postrema (Bach et al., 2015; Boychuk et al., 2015a; Boychuk et al., 2017). Although the majority of neurons within the NTS are GABAergic (Chan and Sawchenko, 1998), the tissue sampling process used in this dissertation inevitably samples from a heterogeneous cell population. An alternative approach to more accurately measure Kv4 and KChIP expression includes single cell qRT-PCR which would allow for differences in expression to be measured from single

GABAergic NTS neurons, identified by GFP. In the hippocampus, it has been shown that increased channel phosphorylation via protein kinase C (PKC) in neurons from TLE mice is associated with altered A-type current excitability (Bernard et al., 2004). Additionally, biochemical studies have shown that site-directed mutagenesis of phosphorylation sites on Kv4 channels alters the A-type current (Nakamura et al., 1997; Schrader et al., 2002). Antibodies have been developed for several phosphorylation sites on Kv4.2 (Birnbaum et al., 2004), and could be used to examine differences in the amount of phosphorylated channel in GABA NTS neurons. Western blotting could also be utilized to determine differences in phosphorylated Kv4 channel protein compared to total Kv4 channel protein in control and pilo-SE mice. Overall, more work is necessary to determine why there is a reduction an I_A but no change in RNA expression.

6.5 Chemogenetic modulation of vagal complex neurons in TLE

6.5.1 Activation of GABAergic NTS neurons in whole animals

The use of DREADDs to modify whole animal behavior has become increasingly popular in studies examining feeding, addiction and anxiety (Whissell et al., 2016; Roman et al., 2017; Grafe et al., 2018; Ray et al., 2018; Runegaard et al., 2018; Zhang et al., 2018). This dissertation used an excitatory DREADD that allowed for the activation of GABAergic NTS neurons via CNO to test the hypothesis that these neurons were directly involved in seizure activity and SUDEP. A study using genetic epilepsy models showed that in anesthetized mice, a pharmacologically induced cortical seizure can spread to the caudal brainstem resulting in spreading depression and SUDEP (Aiba and Noebels, 2015). This is

significant because the caudal brainstem contains the NTS, which is directly implicated in SUDEP because of this study. However, until this dissertation, this has not been similarly tested in TLE. GABAergic NTS neurons were activated via i.p. CNO injection 20 minutes prior to the induction of an acute seizure. None of the animals died from SUDEP. Additionally, CNO did not have an effect on seizure latency in pilo-SE animals. Interestingly, CNO significantly reduced seizure latency in control animals. DREADDs-mediated activation of GABAergic NTS neurons from control mice may have had a greater effect on seizure latency compared to pilo-SE mice because the control animals never exhibited spontaneous seizures prior to flurothyl, so they may be more susceptible to the effect of CNO on seizure latency. Whereas GABAergic NTS neurons from pilo-SE mice are already hyperexcitable, and therefore CNO may have less of an effect on seizure latency. This study also shows that DREADDs are capable of modulating seizure activity in control animals.

This dissertation differed significantly from that of Aiba and Noebels in many ways. Aiba and Noebels used mouse genetic epilepsy models to determine if brainstem structures were involved in SUDEP. Using both *in vivo* and *in vitro* techniques, they showed that NTS is particularly susceptible to spreading depression, ultimately leading to cardiorespiratory collapse and sudden death (Aiba and Noebels, 2015). In their study a chemoconvulsant was applied directly to cortical tissue, cortical EEG was measured during this application, and direct current was recorded in the caudal brainstem. This dissertation used a different seizure induction method and did not record EEG or brainstem current. However,

the study by Aiba and Noebels is confounded by their use of genetic epilepsy models because their results may be due to genetic mutations instead of the seizures (Aiba and Noebels, 2015). Additionally, while these genetic epilepsy models have a reduction in Na^+ current in regions of the cortex (Yu et al., 2006; Cheah et al., 2012; Kalume et al., 2013; Ogiwara et al., 2013), this has not been investigated in NTS neurons.

Interestingly, in the Aiba and Noebels study, when KCl was injected directly into the NTS, seizure-like waveforms occurred in the cortex (Aiba and Noebels, 2015). The NTS does project to and receive projections from higher brainstem centers such as the amygdala and hypothalamus (Ricardo and Koh, 1978; van der Kooy et al., 1984; Takenaka et al., 1995; Fontes et al., 2001; Affleck et al., 2012). Additionally, changes in NTS neuron activation in response to cortical seizure activity can occur independently of blood pressure changes, showing that input from vagal afferents is not necessary for NTS neuron plasticity to occur (Kanter et al., 1995) . One treatment for refractory epilepsy whose mechanism is poorly understood but appears to involve these pathways is vagal nerve stimulation (VNS). VNS consists of a device that is implanted under the chest skin with a wire attached to the vagus nerve delivering electrical pulses to the nerve preventing seizures. While successful, it has not been well-established exactly how VNS is capable of preventing seizures (Uthman, 2000; Howland, 2014; Giordano et al., 2017). One study has suggested that electrical activation of the NTS attenuates amygdala-kindled seizures (Magdaleno-Madrigal et al., 2002), however specific neuronal subtypes were not investigated. This dissertation has shown that NTS

neurons experience functional changes in association with epileptogenesis, but this area could also be targeted for potential epilepsy treatments in individuals with refractory TLE.

In other epilepsy models, inhibitory DREADDs have been utilized to attenuate seizure activity (Avaliani et al., 2016; Wicker and Forcelli, 2016). In one study using kindled mice, the dose of CNO that was most effective in reducing seizure severity was much greater (2.5, 5, and 10 mg/kg) compared to this study (1 mg/kg) (Wicker and Forcelli, 2016). Additionally, Wicker and Forcelli used a 1mg/kg dose, but it did not significantly suppress seizure severity (Wicker and Forcelli, 2016).

There are additional concerns regarding the conversion of CNO to clozapine (MacLaren et al., 2016; Gomez et al., 2017; Raper et al., 2017; Manvich et al., 2018). CNO is the compounds that binds to and activates DREADDs. Studies optimizing DREADDs have shown that it is a pharmacologically inert metabolite of the antipsychotic compound of the drug clozapine (Armbruster et al., 2007; Roth, 2016). Additionally, while clozapine does have high affinity for DREADDs, CNO is supposed to have a more potent and therefore efficacious effect (Armbruster et al., 2007). One caveat to these drug binding studies is that they were done in yeast cultures and not whole animal systems. The studies asserting that CNO is reverse metabolized to clozapine, binds to DREADDs receptors with high affinity, and produces clozapine effects were done using rodents, which are typically utilized in chemogenetics (Gomez et al., 2017; Manvich et al., 2018). Additionally, one of the studies showed that clozapine

crosses the blood brain barrier more effectively then CNO, but the sample sizes were extremely small, with 2 rats per group (Gomez et al., 2017). However, a separate study concluded that while CNO metabolizes to clozapine, it does so in insignificant amounts, and therefore does not have an effect (Guettier et al., 2009). In a small cohort of animals that did not contain mCherry-expressing GABAergic NTS neurons, there were no off-target effects of CNO in vitro. However, this was not tested in the in vivo studies with flurothyl-induced seizures in this dissertation.

Behavioral and motor testing can be done on mice to ensure that CNO does not produce clozapine-like effects. Antipsychotic agents, such as clozapine, can cause catalepsy, which is characterized by behavioral immobility and muscle rigidity. To determine if CNO administration causes a clozapine-like effect on behavior, the open field test can be used to test for behavioral immobility. If clozapine-like effects are evident, there should be a decrease in overall movement in the open field test and periods of immobility where the animal is stationary. This can also be compared to systemic injection of clozapine and vehicle in mice with and without DREADDs.

6.5.2 Activation of GABAergic NTS neurons using in vitro slice electrophysiology

The hypothesis that CNO was able to activate mCherry-expressing GABAergic neurons and also inhibit downstream vagal motor neurons was tested in control and pilo-SE mice. CNO application elicited a depolarization in mCherry expressing neurons from both control and pilo-SE mice. Interestingly, this effect was slightly stronger in pilo-SE mice, where mCherry-expressing neurons were

depolarized 2.5% more than neurons from control mice. There was also a significant increase in action potential frequency in GABAergic NTS neurons from control and pilo-SE mice. CNO-mediated activation of GABAergic NTS neurons led to the inhibition of downstream DMV neurons, a portion of which are preganglionic parasympathetic neurons. DMV neurons from pilo-SE mice were 5.62% more inhibited after mCherry-expressing neuron activation than control mice. This suggests that GABAergic NTS neurons from pilo-SE mice are 1) more susceptible to CNO-induced depolarization and 2) their activation leads to increased downstream inhibition of DMV neurons. These data suggest that pilo-SE mice have an increased propensity for parasympathetic inhibition upon activation of GABAergic NTS neurons, which could lead to autonomic balance, one of the main risk factors for SUDEP.

6.5.3 Future directions

In the future the in vivo studies should be repeated with higher dose of CNO. In a study using kindling to induce epilepsy combined with an inhibitory DREADD to silence seizures, a much higher doses of CNO was needed to reduce seizure severity compared this dissertation (Wicker and Forcelli, 2016). Additionally, CNO was dissolved at a much higher concentration in the aforementioned study compared to the in vivo seizure induction in this dissertation (Wicker and Forcelli, 2016). The dose and concentration of CNO in this dissertation were chosen were based on efficacy in previously published studies and to minimize potential conversion of CNO to clozapine (Guettier et al., 2009). Perhaps modifying the concentration and dosing of CNO would elicit alterations in seizure behavior.

To avoid an effect of the flurothyl treatment, alternative methods similar to that of Aiba and Noebels could be used to elicit a seizure (Aiba and Noebels, 2015). One option would be to apply a chemoconvulsant, such as 4-AP directly to cortical tissue and measure brainstem currents. Another option includes microinjection of KCl into the brainstem to elicit spreading depression in an anesthetized animal after i.p. injection of CNO. In this case it could be determined if CNO causes a pilo-SE animal to develop spreading depression more quickly than without CNO and compared to control animals in the same conditions. This has technique has been used successfully by others to elicit spreading depression in both the cortex and brainstem (Aiba and Noebels, 2015; Nasretdinov et al., 2017).

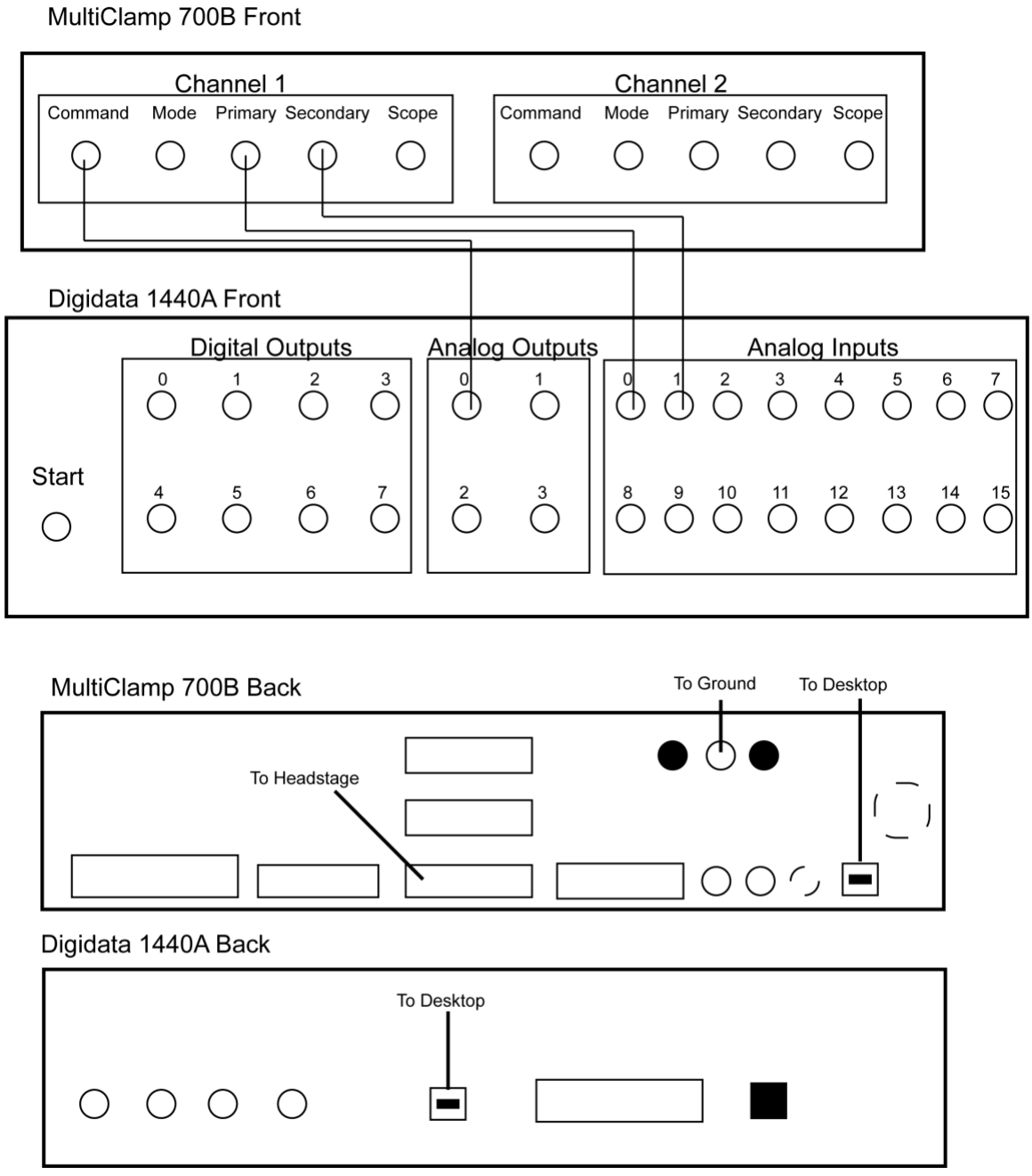
6.6 Summary

While potential mechanisms of SUDEP have been heavily studied in genetic epilepsy models (Glasscock et al., 2010; Cheah et al., 2012; Auerbach et al., 2013; Kalume et al., 2013; Aiba and Noebels, 2015), this dissertation is the first to do so in a mouse model of TLE, which is a model of acquired epilepsy. This is impactful on the field since over 50% of individuals with epilepsy have TLE and over half of those are at high risk for SUDEP, compared to the 1-2% of individuals that have genetic epilepsies. Genetic epilepsies also tend to be more severe and have more comorbidities than those with TLE. This dissertation was the first to show that TLE mice die from SUDEP and that functional changes in GABAergic NTS neurons occur in response to pilocarpine-induced TLE. These neurons are crucial integrators of cardiorespiratory information and modulators of peripheral output of

parasympathetic tone, impacting autonomic balance. This dissertation has shown that these neurons develop an increase in excitability, which in part is due to a reduction in the A-type K^+ current. However, this study did not find that chemogenetic activation of GABAergic NTS neurons could directly lead to a seizure or SUDEP in this mode. The exact role that these changes play in SUDEP in TLE remains to be elucidated and SUDEP in this model is most likely due to a combination of factors including alterations in autonomic, cardiac, and respiratory systems.

Appendix

Appendix 1.1 Electrophysiology Recording Setup



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DERERA, I.D., Delisle, B.P., Smith, B.N. “Functional Neuroplasticity in the Nucleus Tractus Solitarius and Increased Risk of Sudden Death in Mice with Acquired Temporal Lobe Epilepsy”. eNeuro. **4**(5): ENEURO.0319-17.2017.

PRESENTATIONS

Oral Presentations

Physiology Department Retreat. University of Kentucky, Lexington, KY. May 2018. Altered Voltage-Gated K^+ Current in GABA NTS Neurons in a Mouse Model of TLE and SUDEP.

Physiology Departmental Seminar. University of Kentucky, Lexington, KY. April 2015. Reduced Voltage-Gated K^+ Channel Function in GABAergic NTS Neurons in a Murine Model of Acquired TLE and SUDEP.

Poster Presentations

Bluegrass Society for Neuroscience Spring Neuroscience Research Day. Lexington, KY. April 2018. SUDEP and Functional Remodeling of Vagal Complex Activity in a Mouse Model of Temporal Lobe Epilepsy.

Society for Neuroscience Conference. Washington D.C. November 2017. Reduced Voltage-Gated K^+ Channel Function in GABAergic NTS Neurons in a Murine Model of Acquired TLE and SUDEP.

Kentucky Neuroscience Institute Clinical and Translational Research Symposium. Lexington, KY. September 2017. Reduced Voltage-Gated K^+ Channel Function in GABAergic NTS Neurons in a Murine Model of Acquired TLE and SUDEP.

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American Epilepsy Society Annual Meeting. Philadelphia, PA. December 2015. SUDEP and Functional Remodeling of Vagal Complex Activity in a Mouse Model of Temporal Lobe Epilepsy.

Physiology Departmental Retreat. Jabez, KY. August 2014. Temporal Lobe Epilepsy and Its Implications in Heart Rate Variability.

HONORS AND AWARDS

Graduate School Domestic Travel Award – University of Kentucky, 2016

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