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The effects of hypothermia on status epilepticus-induced acquired epilepsy

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THE EFFECTS OF HYPOTHERMIA ON STATUS EPILEPTICUS-INDUCED ACQUIRED EPILEPSY

A dissertation submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy at Virginia Commonwealth University.

by

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List of Abbreviations

AE	Acquired epilepsy
AEDs	Anti-epileptic drugs
AMPA	±-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid
AM	Acetoxymethylester
ANOVA	Analysis of variance
APV	2-amino-5-phosphonovaleric acid
ATP	Adenosine triphosphate
BAPTA	1,2-bis(2-aminophenoxy)ethane-*N'*-*N'*-*N'*-*N'*-tetraacetate
Ba^{2+}	Barium ion
Ca^{2+}	Calcium ion
$[Ca^{2+}]_i$	Intracellular calcium concentration
Ca^{2+}-ATPase	ATP-driven calcium pump
CA1	Cornu ammonis area 1
CB	Calbindin-D_{28K}
CICR	Calcium induced calcium release
CM	Conditioned media
CNS	Central nervous system
CNQX	6-cyano-7-nitroquinoxaline-2,3-dione
DIV	Days in vitro
DMSO	Dimethyl sulfoxide
ECoG	Electrocorticography
EEG	Electroencephalograph
ER	Endoplasmic reticulum
FBS	Fetal bovine serum
HEPES	N-[2-Hydroethyl]piperazine-N'[2-ethanesulfonic acid]
HNC	Hippocampal neuronal cultures
HS	Horse serum
IP_3R	Inositol (1,4,5)-triphosphate receptor
IP_3	Inositol (1,4,5)-triphosphate
IML	Inner molecular layer
IL	Interleukin
K^+	Potassium ion
KA	Kainic acid
Mg^{2+}	Magnesium ion
MEM	Minimal essential media
MK-801	(+)-5-methyl-10,11-dihydro-5*H*-dibenzo[*a*,*d*]cyclohepten-5,10-imine maleate
mRNA	Messenger RNA
Na^+	Sodium ion

NBQX	2,3-dihydroxy-6-nitro-7-sulfamoylbenzol[*f*]quinoxaline
NMDA	N-methyl-D-aspartate
pBRS	Physiological basal recording solution
PBS	Phosphate buffered saline
PIP_2	Phosphatidyl 4,5-bisphosphate
PLC	Phospholipase C
RyR	Ryanodine receptor
SE	Status epilepticus
SEM	Standard error of the mean
SERCA	Sarco/endoplasmic reticulum Ca^{2+} ATPase
SOC	Store-operated channel
SREDs	Spontaneous recurring epileptiform discharges
TBI	Traumatic brain injury
TLE	Temporal lobe epilepsy
TNF-±	Tumor necrosis factor alpha
VDCC	Voltage-dependent calcium channel

Abstract

THE EFFECTS OF HYPOTHERMIA ON STATUS EPILEPTICUS-INDUCED ACQUIRED EPILEPSY

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A dissertation submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy at Virginia Commonwealth University.

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Status epilepticus (SE) is a type of neurological injury characterized by continuous seizure activity and can lead to molecular and pathophysiological alterations leading to plasticity changes. SE can lead to the development of AE by the process of epileptogenesis, which is a phenomenon that describes the transformation of normal brain tissue into a hyperexcitable neuronal population. It has been demonstrated both in vivo and in vitro that calcium (Ca^{2+}) dynamics are severely altered during and after SE, and these changes play a major role in the progression of epileptogenesis. It has also been reported that preventing the rise in intracellular Ca^{2+} ($[Ca^{2+}]_i$) immediately following injury (the Ca^{2+} plateau) prevents the plasticity changes and ultimate development of epilepsy. Currently, there are no treatments available that can be administered following an injury to prevent the development of AE. Therefore it is clinically important to develop a therapy that can be administered after an injury to block epileptogenesis.

Hypothermia is a potential therapeutic intervention. Hypothermia is used clinically to provide neuroprotection following various neurological insults such as stroke and traumatic brain injury (TBI). However, no studies have been performed to evaluate the therapeutic potential of hypothermia following SE. Hypothermia provides protection via multiple mechanisms, one of which includes modulating excitotoxic neurotransmission. It is believed to reduce Ca^{2+} influx by reducing NMDA receptor activation. It is unclear how hypothermia affects Ca^{2+} through other modes of entry. This dissertation evaluates the effects of hypothermia on the Ca^{2+} plateau and demonstrates the novel finding that hypothermia induced post-SE blocks the development of the Ca^{2+} plateau and reduces the development of AE.

Chapter 1: Introduction

Acquired Epilepsy and Status Epilepticus

Epilepsy is a common neurological condition characterized by recurring spontaneous seizures. It affects 1 to 2 percent of the population worldwide [1-3] and approximately 2.7 million Americans [3]. At least 40 percent of epilepsy cases are the result of a previous neurological insult and are classified as acquired epilepsy (AE) [1, 4-5]. The remaining epilepsy cases are considered idiopathic, meaning there is no known cause for the development of epilepsy [6]. In AE a known cause or injury damages the brain and produces a plasticity change that leads to the development of epilepsy [1-2, 20]. The transformation of normal brain tissue into a hyperexcitable neuronal population manifesting spontaneous recurrent epileptic discharges, or seizures, is called epileptogenesis [1-2, 7]. Epileptogenesis is the process responsible for the development of spontaneous seizures following a neurological insult [8].

The most common brain injuries that lead to AE are stroke, traumatic brain injury (TBI), and status epilepticus (SE) [9]. SE is a common neurological emergency that affects 102,000-152,000 people in the United States each year and is associated with approximately 55,000 deaths annually [10]. Epidemiological studies indicate that up to 43% of all SE patients develop epilepsy [11], and at least 10% of all acquired epilepsies develop after SE [1, 12-13], thus making SE-induced AE important to study. Therefore, the studies performed in this dissertation will utilize SE-induced AE models to study SE-induced epileptogenesis.

Seizures are typically short-lived events lasting 20-120 seconds that terminate on their own and have been shown to not cause brain damage. However, some seizures do not stop on their own. Seizures that continue for a duration of greater than 30 min or multiple seizures that occur without regaining consciousness are classified as SE [9]. SE is defined by the International League against Epilepsy as a seizures that persists "for a sufficient length of time or is repeated frequently enough that recovery between attacks does not occur" [10]. SE can be caused by acute processes such as metabolic disturbances, infection of the central nervous system (CNS), stroke, head trauma, or hypoxia. Chronic conditions that can precipitate SE include preexisting epilepsy, chronic ethanol abuse, and discontinuation of anti-epileptic drugs (AEDs) [14]. In this study the rat pilocarpine and the hippocampal neuronal culture models of SE will be used to study the effects of SE on epileptogenesis. These models have been well characterized and are ideally suited to study how SE-induced neuronal injury causes AE [15, 16].

Considerable research has been done to study the effects of brain injury on producing AE. There are typically three phases underlying injury-induced epileptogenesis [9]. The first phase is the injury phase where some cells die and some cells survive. This is followed by the latent phase, where seizure activity is absent and neuronal plasticity changes are initiated. Epileptogenesis is believed to occur during the latent period. The latency phase varies in duration among various models of epileptogenesis and in humans. The neurons that survive the injury phase are the neurons which can undergo epileptogenesis. Thus, the latent phase serves as a window of opportunity for anti-epileptogenic interventions. The last phase is the chronic epilepsy phase, where recurring spontaneous seizures are present [17-18]. The presence of a latent period

preceding epilepsy is a cardinal feature of injury-induced epilepsy in humans [19]. These phases of AE can be replicated in various models of SE-induced AE.

Models of SE-induced AE

In vivo models: The Pilocarpine Model.

Animal models have greatly improved our understanding of epileptic disorders [21]. Several animal models exist that have provided insight into the various mechanisms of neuronal injury following SE. The most common methods of inducing SE in animals include producing SE by using chemoconvulsants and direct electrical stimulation (Figure 1-1). The pilocarpine model is a well established animal model commonly used to study temporal lobe epilepsy (TLE) because it is highly isomorphic to human TLE [22]. The chemoconvulsant pilocarpine, a muscarinic receptor agonist, induces SE by causing over-activation of muscarinic receptors, leading to the excessive release of neurotransmitters including glutamate. This causes overwhelming neuronal excitation leading to SE. The pilocarpine model is commonly used to study epileptogenesis due to its ability to produce SE in a similar presentation as that seen in human as well as similar pathophysiological consequences. Following SE-induced injury, there is a latent period characterized by the absence of seizures. This is followed by development of spontaneous recurrent seizures, or chronic epilepsy [23-24, 37]. In addition to sharing a similar progression in the development of AE, the third phase in the pilocarpine model closely resembles the seizure phenotype and pathology seen in human TLE. Some of these characteristics include widespread lesions associated with neuronal network reorganization in the hippocampus, such as mossy fiber sprouting and interneuron loss [25], and poor control of seizures with the use of anti-epileptic

drugs (AEDs) [26]. The in vivo studies performed for this dissertation utilized the pilocarpine model of AE.

In vitro model: The Hippocampal Neuronal Culture Model.

While the pilocarpine model has the advantage of presenting a clinical disorder in an intact animal, it is difficult to control many of the variables necessary to study the molecular mechanisms associated with SE and epileptogenesis. Therefore, developing an in vitro correlate of SE has provided us with a useful tool to investigate the molecular mechanisms underlying epileptogenesis in an in vivo preparation that is more suitable to molecular biological and biophysical studies. Our laboratory has developed an in vitro model of SE and SE-induced AE called the "low magnesium" hippocampal neuronal culture model [16]. Hippocampal neurons are cultured in vitro and used to study epileptogenesis. This model uses hippocampal neuronal cultures placed in a buffer solution containing no added magnesium (Mg^{2+}) to trigger epileptiform discharges, the in vitro correlate of SE. The low Mg^{2+}-induced electrographic SE induced in the cultures is essentially identical to the electrographic features of SE in humans [16]. These cultures are treated with low Mg^{2+} for 3 h. During this time, the cultures manifest continuous epileptiform discharges with a frequency greater than 10 Hz consistent with characteristics seen in clinical electrographic seizure activity in humans. After 3 h of low Mg^{2+}, Mg^{2+} is returned to the buffer solution and the neurons no longer exhibit SE-like epileptiform discharges and return to baseline activity. After 12 h (latency phase) following the low Mg^{2+} treatment, the neurons manifest spontaneous recurrent epileptiform discharges (SREDs). The SREDs share the same electrophysiological characteristics seen in human seizure activity. The neurons continue to manifest SREDs for the life of the neurons in culture [27]. In addition to

using an injury similar to SE to cause the development of SREDs, this model also shares other characteristics seen in the in vivo models and the clinical condition such as changes in Ca^{2+} homeostasis, cell death, and resistance to AEDs [16, 28].

Although these models are powerful tools to investigate the molecular mechanisms of AE and screen potential anti-epileptogenic drugs, both models of epileptogenesis have limitations. Differences from the human condition include the latency period following SE, the percentage of cases that develop AE following SE, and co-morbities associated with the conditions [29]. Despites these limitations, both the animal and in vitro models are useful tools in studying SE and AE.

Calcium dynamics of SE-induced AE

The use of in vivo and in vitro models of SE-induced AE has provided tremendous insight into the molecular mechanisms responsible for the development of AE following a brain injury such as SE [9]. Characterizing these mechanisms allows us to develop therapies that target epileptogenesis and prevent the development of AE. Several studies have implicated intracellular Ca^{2+} ($[Ca^{2+}]_i$) as a major player in mediating many of the pathophysiological consequences observed in AE following an injury such as SE, stroke, and TBI [30-32]. These studies provided evidence that following SE, $[Ca^{2+}]_i$ is elevated compared to naïve controls, and this elevation is persistent for the life of the neuron. This persistent elevation in $[Ca^{2+}]_i$ is called the calcium plateau, and this plateau is responsible for many of the plasticity changes observed during epileptogenesis. The neurons that survive an injury such as SE serve as the substrate for epileptogenesis. Targeting the molecular alterations observed in epileptogenesis such as elevated $[Ca^{2+}]_i$ may offer new approaches to developing anti-epileptogenic approaches [9].

Calcium plays an important role in neuronal function under normal physiological conditions [33]. Ca^{2+} is a divalent, cationic charge carrier that acts as a second messenger in many cell signaling pathways [34]. Under normal conditions, $[Ca^{2+}]_i$ is maintained at low concentrations (100 nM), whereas extracellular Ca^{2+} is significantly higher (1-2 mM) [35]. The large concentration gradient between intracellular and extracellular spaces allows Ca^{2+} to act as a second messenger even upon small changes in intracellular concentration. Neuronal depolarization or receptor activation allows extracellular Ca^{2+} to move down its concentration gradient and enter the neuron and produce second messenger effects. Brief elevations in $[Ca^{2+}]_i$ are necessary for basic neuronal processes such as neurotransmitter release as well as higher functions such as long-term potentiation that occurs in learning and memory consolidation [36]. However, prolonged and overwhelming elevations in $[Ca^{2+}]_i$, such as that seen in glutamate excitotoxicity, lead to acute and delayed cell death [9]. Neurons have several homeostatic mechanisms that work to maintain the low $[Ca^{2+}]_i$. However, neurons that survive an injury such as SE exhibit marked alteration in their ability to handle elevations in $[Ca^{2+}]_i$, indicating an alteration in homeostatic mechanisms (Fig 1-2). This led to the development of the calcium hypothesis of epileptogenesis. The calcium hypothesis postulates that: 1) During the injury phase, Ca^{2+} reaches high levels, but not high enough to induce cell death; 2) During the latency phase, Ca^{2+} remains elevated and initiates second messenger effects that lead to long-lasting plasticity changes (epileptogenesis); and 3) During the chronic epilepsy phase, the persistent elevations in $[Ca^{2+}]_i$ that are associated with the epileptic phenotype play a role in initiating and maintaining recurring spontaneous seizures [9]. Several studies have demonstrated the changes in $[Ca^{2+}]_i$ dynamics during all three phases of AE [30-31, 37-40].

One study used the pilocarpine model to demonstrate the changes in $[Ca^{2+}]_i$ in hippocampal neurons at different time points following SE [37]. Rats remained in SE for 1 h before SE was terminated with diazepam. The rats were sacrificed immediately following SE (acute injury phase), and at various time points following SE (latency and chronic phases). Hippocampal tissue was dissected, neurons were acutely dissociated, and $[Ca^{2+}]_i$ of the hippocampal neurons was evaluated using the Ca^{2+} indicator Fura-2. Immediately after SE, during the acute injury phase, hippocampal neurons exhibited significant elevations in $[Ca^{2+}]_i$ not seen in vehicle controls. 100% of the neurons isolated from pilocarpine-induced SE rats had abnormally elevated $[Ca^{2+}]_i$ with mean neuronal $[Ca^{2+}]_i$ values of 850 ± 59 nM compared to vehicle controls who exhibited mean neuronal $[Ca^{2+}]_i$ values of 90 ± 22 nM. This demonstrated a significant increase (9.4-fold increase) in $[Ca^{2+}]_i$ from SE neurons compared to control neurons, suggesting that $[Ca^{2+}]_i$ is significantly elevated during and immediately following SE.

This paper also evaluated $[Ca^{2+}]_i$ at later time points following SE to determine if the changes in Ca^{2+} were transient or more long-lasting. $[Ca^{2+}]_i$ from acutely isolated hippocampal neurons was measured at 1, 2, 6, 10, and 30 days and 1 year post-SE. $[Ca^{2+}]_i$ remained significantly elevated at all time points compared to control. $[Ca^{2+}]_i$ remains elevated to high levels for the first six days after SE. This has been designated the calcium plateau [9]. In addition, even though $[Ca^{2+}]_i$ drops after the seventh day after SE it still remains significantly elevated compared to control neurons (Fig 1-3). This study identified the presence of the calcium plateau that is maintained long after the initial injury.

Studies have also explored the effects of epileptogenesis on the homeostatic mechanisms that regulate $[Ca^{2+}]_i$.[30, 37, 39-40]. In one study, hippocampal neurons isolated from rats in the injury, latency, and chronic phases were challenged with glutamate to investigate whether Ca^{2+} homeostatic mechanisms were intact [37]. Glutamate is an excitatory transmitter that stimulates the NMDA receptor, allowing Ca^{2+} influx into the neuron. Under normal conditions, neurons are able to buffer the increase in $[Ca^{2+}]_i$ back to baseline levels. However, epileptic neurons exhibit deficits in their ability to buffer the excessive influx of Ca^{2+} and return $[Ca^{2+}]_i$ back to baseline concentrations, indicating altered homeostatic mechanisms. In these experiments, control neurons returned to pre-glutamate levels in less than 6 min following 1 min exposure to 10 µM glutamate. However, $[Ca^{2+}]_i$ in neurons from the SE group at all three phases exhibited a significant delay in returning to baseline (Fig 1-4). Neurons from SE animals were unable to restore $[Ca^{2+}]_i$ back to baseline levels, suggesting alterations in homeostatic mechanisms during all three phases of epileptogenesis. These studies demonstrated that elevated $[Ca^{2+}]_i$ and altered Ca^{2+} homeostatic mechanisms remain long after the initial injury from SE, suggesting that these changes play a role in the development and maintenance of AE.

Alterations in $[Ca^{2+}]_i$ have also been demonstrated in the in vitro model of epileptogenesis. This model induces electrographic SE in hippocampal neuronal cultures by treating neurons with 3 h of low Mg^{2+} solution. The low-Mg^{2+} treatment (SE) produces long-term plasticity changes that result in the development of SREDs that persists for the life of the neuron (“epilepsy”). During SE in this model, $[Ca^{2+}]_i$ rises significantly during low-Mg^{2+} treatment compared to controls neurons [41]. Calcium homeostasis has also been shown to be affected in this model. Thus, the in vitro model manifests the same Ca^{2+} plateau as in the pilocarpine model [9].

Evidence that Ca^{2+} in necessary for the development of AE

Glutamate, a major excitatory neurotransmitter, mediates the neuronal injury that occurs during SE. SE triggers the release of large amounts of glutamate which causes excessive stimulation of glutamate receptors including the Ca^{2+}-permeable NMDA receptor. Although glutamate acts at several receptors including NMDA, kainic acid, AMPA, and metabotropic glutamate receptors, it is the activation of the NMDA receptor that most significantly contributes to the pathology that occurs following injury and during epileptogenesis. The hippocampal neuronal culture model of AE has been used in several studies to demonstrate that epileptogenesis is a Ca^{2+}-dependent and NMDA receptor-mediated phenomenon [37, 41-42].

It has been shown that increased $[Ca^{2+}]_i$ during SE was necessary for the development of SREDs. [41]. SE was induced using low Mg^{2+} treatment in the presence of low extracellular Ca^{2+} (0.2 mM) or the Ca^{2+} chelator 1,2-bis(2-aminophenoxy)ethane-*N'*-*N'*-*N'*-*N'*-tetraacetate (BAPTA). The development of SREDs was evaluated 48 h post-SE. The presence of both low extracellular Ca^{2+} and the Ca^{2+} chelator during SE prevented the development of SREDs, indicating that Ca^{2+} is necessary for the development of epilepsy. To further prove that Ca^{2+} was necessary for epileptogenesis, Ca^{2+} was replaced with barium (Ba^{2+}), a divalent cation that has been shown to be unable to substitute for many of the second messenger effects produced by Ca^{2+} [43]. Replacing Ca^{2+} with Ba^{2+} during SE also prevented the development of SREDs. These results demonstrated that elevated $[Ca^{2+}]_i$ during SE is responsible for the plasticity changes the lead to the development of epilepsy [41].

NMDA receptor activation is required for the development of AE

After demonstrating that increased $[Ca^{2+}]_i$ during SE causes SREDs, it was important to determine whether the source of Ca^{2+} was selective. The increase of Ca^{2+} inside the cell during SE could be mediated by several different channels [44-45]. Activation of NMDA receptors by glutamate allows Ca^{2+} to enter the cell. Activation of AMPA, kainic acid, and metabotropic receptors depolarizes the cell and activates voltage-dependent calcium channels (VDCCs) [44]. Ca^{2+} can also enter the cell upon activation of specific AMPA receptor subtypes [44-45].

To investigate the contributions of these receptors and channels in the induction of epileptogenesis, several pharmacological inhibitors were used [41]. The AMPA and kainic acid receptor antagonist 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX) and the more selective AMPA receptor antagonist 2,3-dihydroxy-6-nitro-7-sulfamoylbenzol[*f*]quinoxaline (NBQX) [46] were able to partially block the rise in $[Ca^{2+}]_i$ during SE, reducing $[Ca^{2+}]_i$ from 577nM to 433 nM and 422 nM, respectively. Inhibition of L-type VDCC by the antagonist nifedipine [47] was also effective at partially reducing $[Ca^{2+}]_i$ during SE. However, none of these pharmacological inhibitors were effective at preventing the development of SREDs. The NMDA receptor antagonists 2-amino-5-phosphonovaleric acid (APV) and MK-801 [48-49] were more effective at blocking the rise in $[Ca^{2+}]_i$ during SE, reducing $[Ca^{2+}]_i$ levels from 577 nM to 293 nM and 287 nM, respectively. The NMDA receptor antagonists were also able to completely block the induction of epilepsy in the hippocampal culture model. These results provide direct evidence that NMDA receptor-mediated Ca^{2+} entry is responsible for the rise in $[Ca^{2+}]_i$ that leads to the development of epilepsy.

Several animal models have shown that NMDA antagonism blocks or delays the development of seizure activity and epileptogenesis [37, 42, 50-52]. One study has also shown that NMDA receptor activation in required for the development of AE [42]. In this study rats were treated with pilocarpine to induce SE. In one group, rats were pre-treated with MK-801, an NMDA receptor antagonist. Rats from both groups exhibited comparably severe electrographic SE following pilocarpine injection, suggesting that blockade of the NMDA receptor does not prevent the development and progression of SE. Following SE in both pilocarpine and MK-801 pre-treated groups, EEG activity was monitored for the presence of spontaneous recurring seizures. EEG recordings from control and MK-801 pre-treated pilocarpine rats displayed normal activity with no electrographic seizure activity present. In contrast, EEG patterns from pilocarpine rats exhibited spontaneous electrographic seizures corresponding to behavioral tonic-clonic seizures. Thus, MK-801 pre-treatment prior to SE blocked the development of spontaneous recurring seizures, or epilepsy. The results indicate that NMDA receptor activation is required for the development of AE.

Calcium Homeostasis

Understanding how Ca^{2+} dynamics are altered following SE may lead to better understanding of the mechanisms that contribute to the Ca^{2+} plateau. Elucidating how the Ca^{2+} plateau contributes to epileptogenesis may lead to different therapeutic approaches to blocking the persistent elevation in $[Ca^{2+}]_i$ and thus preventing the development of epilepsy. Several studies have established that the homeostatic mechanisms regulating $[Ca^{2+}]_i$ are altered following SE [30, 37-40, 53-54]. The following presents an overview of Ca^{2+} homeostatic mechanisms and how they are altered following SE. Figure 1-5 provides an illustration of the homeostatic mechanisms.

Calcium entry across the plasma membrane

Calcium can cross the plasma membrane through three main channels: ligand-gated cation channels, voltage-dependent calcium channels (VDCCs), and store-operated Ca^{2+} channels (SOCs). Ligand-gated cation channels include NMDA, AMPA, and KA channels. Multiple forms of VDCCs have been characterized [55-57] and are categorized based on their voltage sensitivities, voltage-dependent inactivation rates, and selective sensitivity to various inhibitors [58]. SOCs are activated when intracellular Ca^{2+} stores are depleted [59].

The NMDA receptor mediates the majority of Ca^{2+} influx into the cell [60]. NMDA receptors, as well as AMPA receptors and kainic acid (KA) receptors, are classified as ionotropic glutamate receptors. Unlike AMPA and KA receptors, NMDA is highly permeable to Ca^{2+}. In addition to glutamate binding, NMDA receptor activation requires the binding of a co-agonist, glycine. The binding of glycine is necessary for the NMDA receptor to enter the open state [60]. Activation of the NMDA receptor is not only ligand-gated but also voltage-dependent. A magnesium (Mg^{2+}) blockade is present within the pore and prevents conductance even in the presence of both glutamate and glycine. Large depolarizations are necessary to expel the Mg^{2+} from the pore. The depolarization is partially mediated by AMPA and KA receptor conductance [61].

NMDA receptor-mediated Ca^{2+} entry has been shown to play a major role in excitotoxic neuronal injury and death. In stroke models, NMDA receptor antagonists reduce the ischemic infarct volume [62] and protect neurons from excitotoxicity [63-64]. In models of SE, NMDA antagonists provide neuroprotection against neuronal death that occurs with the injury from SE [37, 65]. While it is clear that NMDA receptor activation is linked to epileptogenesis, there is no

clear evidence of altered NMDA receptor function in AE from animal models or humans [66]. Thus, NMDA receptor activation accounts for Ca^{2+} entry during the SE injury phase, and this Ca^{2+} has been shown to play an important role in the induction of epileptogenesis in the neurons that survive the injury.

Calcium extrusion

Two major transport systems are responsible for pumping free intracellular Ca^{2+} out of the neuron. These transport systems act against a large concentration gradient and therefore are energy dependent. The dependence on energy makes these pumps vulnerable to ischemic injury [67]. The ATP-driven Ca^{2+} pump (Ca^{2+}-ATPase) uses 1 ATP for every Ca^{2+} ion extruded across the membrane. The Na^{+}- Ca^{2+} exchanger relies on the concentration gradient maintained by the ATP-dependent Na^{+}-K^{+} exchanger. This exchanger extrudes 1 Ca^{2+} ion for every 2 or 3 Na^{+} ions that enter the cell [67]. So far, there is no evidence implicating altered Ca^{2+} extrusion mechanisms in SE or epilepsy [9].

Ca^{2+} binding proteins

Ca^{2+} binding proteins play an important role in maintaining low basal $[Ca^{2+}]_i$. These binding proteins, such as calbindin and calmodulin, buffer the vast majority of intracellular Ca^{2+} following Ca^{2+} influx [68]. Calbindin-D_{28K} (CB), an important calcium binding protein, is normally expressed in high levels in the dentate granule cell layer of the hippocampus. Several studies have shown that CB expression is altered in animal models of epilepsy [69-72] and in human temporal lobe epilepsy [73]. These studies show that in epilepsy, CB expression is decreased in the dentate granule cells, and this loss of CB is associated with the loss of calcium

binding capacity [69]. In the pilocarpine model of SE, this decreased expression of CB persists for as long as two years, suggesting that the alteration is permanent [72]. These data suggest that the elevated $[Ca^{2+}]_i$ observed during epileptogenesis may contribute to the down-regulation of CB protein and mRNA expression. The decrease in the presence of Ca^{2+} binding proteins may mediate the maintenance of the Ca^{2+} plateau.

Intracellular Ca^{2+} storage and release

Particularly important for maintaining low $[Ca^{2+}]_i$ is the ability of the endoplasmic reticulum (ER) to operate as a dynamic Ca^{2+} store, which is able to actively sequester cytosolic Ca^{2+} ions and store them in the ER lumen [74]. Under normal conditions, the ER is able to sequester large amounts of Ca^{2+}. The ER accumulates Ca^{2+} via uptake by the sarco/endoplasmic reticulum Ca^{2+} ATPase (SERCA). One study using the hippocampal neuronal culture model of epilepsy demonstrated that SERCA activity is decreased in epileptic neurons [40]. Impaired SERCA activity would lead to a decreased capacity to remove elevated intracellular Ca^{2+}, thus contributing to the Ca^{2+} plateau.

Not only is the ER responsible for storing Ca^{2+}, it also releases Ca^{2+} from its internal stores into the cytosol. Release of Ca^{2+} from ER stores is evoked by stimulation of two receptors on the ER. One is the ryanodine receptor (RyR), named for its affinity for the plant alkaloid ryanodine [75]. The RyR is activated by Ca^{2+}, resulting in Ca^{2+}-induced Ca^{2+} release (CICR) [33] that amplifies the Ca^{2+} signal in a positive feedback loop [76]. Pharmacological stimulation of RyR can be achieved using ryanodine and caffeine. At low micromolar concentrations (1 uM), ryanodine stabilizes channels in an open sub-conductive state [77]. Caffeine has proven to be a powerful

pharmacological tool in studying CICR [78-79] due to its ability to sensitize RyR to low cytosolic Ca^{2+} [77]. The other class of receptor that releases Ca^{2+} from ER store is the inositol (1,4,5)-triphosphate receptor (IP_3R). The IP_3R is activated by the second messenger IP_3, which is produced by the cleavage of phosphatidyl 4,5-bisphosphate (PIP_2) by phospholipase C (PLC).

Studies have provided evidence that IP_3 and RyR-mediated CICR play a role in the changes in $[Ca^{2+}]_i$ seen in epilepsy [40, 80]. In the HNC model of epilepsy, IP_3-mediated Ca^{2+} release is increased [40]. In addition, RyR mRNA is up-regulated in the KA model of epilepsy [80]. Furthermore, studies have shown that Dantrolene, a RyR antagonist, is neuroprotective in both in vivo and in vitro models [81-82]. These studies demonstrate that epileptic neurons show an up-regulation in CICR mechanisms, which contribute to the Ca^{2+} plateau, and that blocking CICR with the RyR antagonist Dantrolene provides protection against neuronal death, presumably by inhibiting Ca^{2+}-mediated pathways.

It has also been shown that Ca^{2+} entry following SE is mediated by RyR activation and not NMDA receptor activation [83]. This study utilized the low-Mg^{2+} HNC model of AE to investigate the effects of Dantrolene on $[Ca^{2+}]_i$ when administered after 3 h of in vitro SE. The results demonstrated that following SE, Dantrolene was able to lower $[Ca^{2+}]_i$ to baseline levels whereas MK-801, an NMDA receptor antagonist, did not significantly lower $[Ca^{2+}]_i$ following SE (Fig 1-6). This study also showed that Dantrolene was able to maintain $[Ca^{2+}]_i$ at baseline concentrations for at least 48 h in vitro, suggesting that the effects were long-lasting. Thus, although Ca^{2+} entry during SE is mediated by NMDA receptor activation [37,41-42], NMDA receptors are not the source of the prolonged elevated Ca^{2+} following SE. Intervention with

Dantrolene following SE lowered $[Ca^{2+}]_i$ and blocked the Ca^{2+} plateau, suggesting that RyR play a major role in maintaining the Ca^{2+} plateau [83].

Not only did Dantrolene reduce elevated $[Ca^{2+}]_i$ when administered following SE, it also significantly reduced the development of SREDs in vitro. In neurons treated with 3 h low Mg^{2+}, 84% and 100% exhibited SREDs at 24 and 48 h after SE, respectively. In contrast, only 10% and 20% of neurons treated with Dantrolene following SE showed SREDs at 24 and 48 h following SE, respectively. In addition, Dantrolene reduced cell death when administered after SE. Neuronal death was assessed using propidium iodide (PI) and annexin V conjugated to fluorescein isothiocyanate (FITC). The results suggest that inhibiting the RyR following SE is neuroprotective [83]. Thus, blocking CICR from RyR following SE reduces $[Ca^{2+}]_i$ and blocks the development of the Ca^{2+} plateau, when may prevent the Ca^{2+}-mediated second messenger effects that lead to the development of epileptogenesis. Therefore, blocking CICR is a promising anti-epileptogenic target.

Mossy Fiber Sprouting

The hippocampus is highly vulnerable to a variety of neurological insults including SE. Plasticity changes observed in the dentate gyrus are considered to be important in the pathogenesis of temporal lobe epilepsy (TLE) [84]. A particular form of synaptic plasticity observed in both animal models and human cases of TLE is mossy fiber sprouting [85-90]. Mossy fiber sprouting describes a phenomenon that occurs when mossy fibers, which are axons of dentate granule cells, aberrantly grow and form synapses with dentate granule cells in the inner molecular layer (IML). Normally, mossy fibers arise from dentate granule cells and project across the dentate hilus to the

dendrites of pyramidal neurons in the CA3 region. Very few mossy fibers are normally present in the IML. However, following SE, these mossy fibers grow and sprout across the granule cell layer and form synapses with dendritic spines of dentate granule cells found in the IML [91-92] that normally lack recurrent excitatory synapses [93]. This creates a recurrent excitatory circuit, which may be a mechanism of hyperexcitability observed in epilepsy [84, 94-95]. Epileptic seizures are presumably caused by a disturbance in the balance between excitation and inhibition. The formation of recurrent excitatory synapses may enhance neuronal excitability and lead to epilepsy [84]. The abnormal sprouting of mossy fibers into the IML may serve as a source of a positive feedback loop among granule cells in the dentate gyrus [96-98].

Mossy fiber sprouting can be visualized with a Timm stain, which stains the zinc-containing axon terminals of mossy fibers. Mossy fiber boutons contain a high concentration of zinc, which is sequestered in synaptic vesicles and is co-released with glutamate. The precise effects of zinc release from the mossy fibers are unclear, but it appears to enhance excitability. One possible mechanism by which zinc enhances excitability is by acting as an antagonist to the $GABA_A$ receptor, which mediates the majority of inhibitory transmission. $GABA_A$ receptors appear to be unusually sensitive to the presence of zinc in the dentate gyrus [84]. The aberrant sprouting of mossy fibers can be detected one to two weeks following SE and appear to increase in intensity over several months [93, 99]. Although mossy fiber sprouting is a hallmark characteristic of SE-induced epilepsy, it is not required for the development of epilepsy. However, mossy fiber sprouting has been associated with an increase in both seizure frequency and duration [100-101]. Thus, treatments that block the development of mossy fiber sprouting may prevent the formation of this recurring excitatory synaptic circuit, thus reducing neuronal excitability. In addition,

preventing mossy fiber sprouting may reduce the severity of seizures that develop, thus improving the quality of life for the patient.

Over the last several decades, considerable progress has been made in developing anti-epileptic drugs (AEDs) which treat the symptomology of epilepsy [102]. However, there are no treatments available that prevent epileptogenesis following an injury [103-104]. Thus, one of the major goals in studying the mechanisms of epileptogenesis is to investigate different therapeutic approaches to prevent the development of epilepsy. One possible alternative to pharmacological agents is the use of therapeutic hypothermia.

Clinical Uses of Hypothermia

Hypothermia is defined as cooling the core body temperature below 35°C. Hypothermia can be classified as mild (33-34.9°C), moderate (30-32.9°C), and deep (below 30°C). The neuroprotective benefits of hypothermia have long been recognized. Hypothermia was used clinically 1940's through 1960's [105-112] to improve neurological outcomes after cardiac arrest and TBI. At the time, it was believed that the protective benefits of hypothermia were solely caused by decreasing brain metabolism and oxygen demand, and deep levels of hypothermia were necessary to achieve sufficient neuroprotection [113-114]. However, deep hypothermia was associated with severe side effects such as life-threatening arrhythmias and ventricular fibrillation. The high risk of side effects combined with a lack of controlled cooling methods and an intensive care unit (ICU) to monitor patients led clinicians to abandon further studies of hypothermia. Interest in hypothermia was rekindled in the 1980's when several animal experiments reported improved neurological outcomes with mild to moderate hypothermia that is

associated with far fewer side effects [113-114]. Since then, a number of animal studies and clinical trials have been initiated to determine the effects of hypothermia in various neurological disorders. Currently, therapeutic hypothermia is used clinically to prevent neurological complications associated with out-of-hospital cardiac arrests [115, 116], neonatal hypoxic-ischemia encephalopathy [117], TBI [118-119], and stroke [120-121].

Mechanisms of Hypothermia

The neuroprotective effects of hypothermia are not completely understood. It is likely that hypothermia offers neuroprotective through multiple mechanisms. The mechanisms of hypothermia are complex, but they principally act to attenuate the cascade of events that occur during secondary injury [122]. Secondary injury is injury that occurs following a primary insult such as TBI or stroke. The initial injury initiates a cascade of events that can lead to further neuronal injury and death [123-124]. Most studies investigating hypothermia have been performed in animal models of ischemic injury following stroke or TBI. Some of the various mechanisms will be briefly described below.

Reduction in metabolic rate

It was originally believed that hypothermia's sole protective benefit was due to its ability to reduce cerebral metabolism. For every 1°C reduction in body temperature, there is a 6 to 10% decrease in cerebral metabolism [125-131]. This temperature dependent decrease in metabolism results in a decreased demand for oxygen and glucose in the brain [114]. The decrease glucose and oxygen consumption helps prevent injury caused by a limited supply of oxygen (i.e. stroke).

While there is a decrease in metabolism with hypothermia, we now know that this is only one of the many mechanisms of hypothermia.

Apoptosis and mitochondrial dysfunction

Following neuronal injury such as ischemia, cells may recover, become necrotic, or enter a pathway leading to programmed cell death, or apoptosis. Several studies have shown that hypothermia can interrupt the early stages of the apoptotic pathway, thereby preventing cellular injury from progressing to cell death. Hypothermia appears to reduce apoptosis by inhibiting caspase enzyme activation [132-136] and preventing mitochondrial dysfunction [137].

Inflammation

Many types of brain injury lead to a significant inflammatory response. Following ischemia-reperfusion, large amounts of pro-inflammatory mediators such as tumor necrosis factor-± (TNF-±) and interleukin-1 (IL-1) are released by astroglia and microglia [125, 138]. These inflammatory responses are accompanied by the production of free radicals. A persistent production of cytokines, particularly IL-1, increases the risk and extent of brain injury. Several studies using animal models as well as some clinical evidence have shown that hypothermia reduces ischemia-induced inflammatory reaction [139-142]. Interrupting the inflammatory cascade can significantly attenuate the extent of brain injury and infarct size [143].

Excitotoxicity

The neuroprotective effect of hypothermia has partially been attributed to its capacity to modulate excitotoxic transmission. Traumatic or ischemic insults, as well as SE, induce a

massive release of glutamate which leads to the activation of NMDA receptors and concomitant influx of Ca^{2+} into the cell. This initiates the excitotoxic cascade. Several studies have provided evidence that hypothermia reduces extracellular concentrations of both glutamate and glycine [144-145]. Therefore, hypothermia may be exerting its neuroprotective properties by preventing increases in extracellular concentrations of excitatory transmitters that activate the NMDA receptor, thereby reducing excitatory transmission associated with traumatic injury. Reducing NMDA receptor activation prevents the excessive influx of Ca^{2+} into the cell, thereby preventing Ca^{2+}-mediated neuronal damage. However, no studies have been conducted to investigate the effects of hypothermia on other routes of Ca^{2+} entry or release such as CICR. It is possible that hypothermia attenuates the disruption in Ca^{2+} homeostasis via mechanisms other than reducing NMDA receptor activation. It will be interesting to evaluate the effects of hypothermia on Ca^{2+} homeostasis.

Hypothermia attenuates seizure susceptibility in TBI

Few studies have been performed to evaluate the effects of hypothermia on development of AE. One study investigated the effects of hypothermia on seizure susceptibility after TBI [146]. TBI and SE share a similar pathology that includes the development of aberrant mossy fiber sprouting [86-87, 90, 147-148] and the development of epilepsy following injury [149-150]. In this experiment, TBI was induced in rats using moderate fluid percussion pulse, and mild hypothermia (33.0-33.6°C) was initiated 30 min post-injury and maintained for 4 h. At 12 weeks post-injury, the rats were monitored for seizure susceptibility using electrocorticography (ECoG) recordings. The results of the study indicated that mild hypothermia following fluid percussion injury decreased seizure susceptibility. The study also investigated the effects of hypothermia on

mossy fiber sprouting. The results showed mossy fiber sprouting was present at 12 weeks post-injury, and this pathological abnormality was attenuated in rats that received hypothermia. Therefore, hypothermia treatment may be reducing seizure susceptibility following TBI by reducing the formation a recurrent excitatory circuit associated with mossy fiber sprouting [146]. The results of this study provided promising results that hypothermia may be a potential alternative therapy for preventing AE.

Summary and Rationale

Epilepsy is a common neurological condition affecting 1 to 2% of the population worldwide [1-3]. Approximately 40% of all epilepsy cases are the result of a neurological insult and is termed acquired epilepsy (AE) [1, 4-5]. SE is a common type of injury that can lead to the development of AE by the process of epileptogenesis [9]. The transformation of normal brain tissue into a hyperexcitable neuronal population is called epileptogenesis [2, 7, 9]. Epileptogenesis is divided into three phases: the injury phase (such as SE), followed by a latency period characterized by an absence of seizure activity, and finally the chronic epileptic phase. The latency phase is believed to be the time where pathophysiological and neuroplasticity changes occur in the neuron that leads to the epileptic phenotype [17-18]. The latency phase represents the window of opportunity to introduce therapeutic interventions with the hopes of preventing epileptogenesis and the ultimate development of AE [17-18]. While advances have been made in the development of AEDs to treat seizures in epileptic patients, there are currently no treatments available that can be administered following an injury to prevent the development of AE [103]. Furthermore, approximately half of all cases of AE are refractory to the current anti-epileptic drugs available [1] underscoring the importance of preventing the development of AE after SE. Therefore,

developing a therapy that can be administered after an injury to block epileptogenesis is of paramount importance.

Several studies have provided evidence that implicates Ca^{2+} as being a major player in epileptogenesis. These studies have demonstrated in models of SE, TBI, and stroke-induced AE that surviving neurons exhibit a significant elevation in $[Ca^{2+}]_i$ during the injury phase and this elevation persists for the life of the neurons in both animal and in vitro models. Moreover, these neurons exhibit alterations in Ca^{2+} homeostatic mechanisms [37-38, 40]. The neurons that survive an injury and exhibit this persistent elevation in $[Ca^{2+}]_{i,}$ or the Ca^{2+} plateau, serve as the substrate for epileptogenesis. The persistent elevation in $[Ca^{2+}]_i$ initiates several Ca^{2+}-mediated second messenger effects that lead to the plasticity changes associated with epileptogenesis. This Ca^{2+} plateau serves as a target for further therapies to prevent AE. Interventions that prevent the Ca^{2+} plateau may prevent the progression of epileptogenesis and the eventual development of AE.

One potential therapeutic intervention that may block the Ca^{2+} plateau is therapeutic hypothermia. Hypothermia is defined as lowering core body temperature below 35°C. It is used clinically to improve neurological outcome following cardiac arrest [115-116], neonatal hypoxic-ischemic encephalopathy [117], ischemic stroke [120-121], and TBI [118-119]. The neuroprotective benefits have long been recognized, but the mechanisms of hypothermia have only been evaluated in the last few decades. Hypothermia appears to exert its beneficial effects through several effects which include a reduction in cerebral metabolism, prevention of cell death pathways and mitochondrial dysfunction, reduction in inflammation, and attenuation of

excitotoxic transmission [113-114]. Several studies performed in models of ischemia demonstrated that hypothermia reduces extracellular concentrations of glutamate [144-145], thereby reducing NMDA receptor activation and subsequent Ca^{2+} influx. The ability of hypothermia to decrease excitotoxic transmission and therefore reduce Ca^{2+} entry makes it a promising therapy for preventing epileptogenesis. One study demonstrated that inducing mild hypothermia after TBI attenuated seizure susceptibility and reduced mossy fiber sprouting, which is one type of plasticity change associated with epileptogenesis [146]. Hypothermia has not been evaluated in models of SE, so it will be interesting to investigate the therapeutic potential of hypothermia in models of SE-induced AE.

Experimental models of SE-induced AE have provided valuable insight into some of the mechanisms of neuronal plasticity and epileptogenesis. The pilocarpine model of SE-induced AE is a powerful tool for investigating pathologies associated with SE and AE and for evaluating the therapeutic potential of various interventions. Pilocarpine-induced SE produces a condition that is similar in presentation to that in humans [22] which includes the presence of a latency period after injury followed by chronic spontaneous seizures [23-24]. However, it is challenging to discern molecular mechanisms in an intact animal model. Therefore, the well characterized in vitro low Mg^{2+} model of SE-induced AE will be utilized to elucidate the effects of hypothermia on Ca^{2+} dynamics.

Central Hypothesis

Several studies have demonstrated that injury such as SE leads to a persistent elevation in $[Ca^{2+}]_i$ and blocking this Ca^{2+} plateau can prevent the development of epileptogenesis. Studies have also

showed that hypothermia reduces excitotoxic neurotransmission and thus reduces $[Ca^{2+}]_i$. Based on this evidence, we developed the CENTRAL HYPOTHESIS that hypothermia administered following SE can block the SE-induced calcium plateau, the subsequent plasticity changes such as mossy fiber sprouting, and the ultimate development of AE. To test this hypothesis we have utilized both the in vivo pilocarpine and the in vitro low Mg^{2+} models of SE-induced AE to investigate how hypothermia affects the Ca^{2+} plateau and the eventual development of AE. In order to address this hypothesis, we focused on the following aims:

1) Evaluate the effects of hypothermia on the development of the Ca^{2+} plateau
2) Determine the effects of hypothermia on the development of mossy fiber sprouting
3) Investigate the effects of hypothermia on Ca^{2+}-induced Ca^{2+} release
4) Determine if hypothermia reduces the development of AE

The experiments performed in this dissertation utilized both the in vitro and in vivo models of SE to demonstrate that hypothermia blocks the development of the Ca^{2+} plateau. Based on these results, we hypothesized that hypothermia would be effective at preventing the progression of epileptogenesis to the development of epilepsy. The in vivo rat pilocarpine model was used to demonstrate that hypothermia induced post-SE reduced the development of risk of epilepsy. In addition, hypothermia attenuated the formation of mossy fiber sprouting. The effects of hypothermia on different modes of Ca^{2+} entry were investigated using various pharmacological agents to stimulate Ca^{2+} entry in the in vitro neuronal culture model. The conclusions reached from this dissertation demonstrate that hypothermia effectively reduces the progression of epileptogenesis by blocking the Ca^{2+} plateau and associated plasticity changes.

	Inciting Agent	Model Name
IN VITRO MODEL		
Hippocampal Neuronal Culture	3 hr low Mg^{2+} treatment to mimic SE	Low Mg^{2+} model
IN VIVO MODELS		
Chemical	SE induced by pilocarpine injection	Pilocarpine model
	SE induced by kainic acid injection	Kainic acid model
Electrical	Repeated application of short electrical stimulation	Kindling model

Figure 1-1. Models of SE-induced AE. SE is commonly induced in the whole animal using chemoconvulsants such as pilocarpine and kainic acid or through electrical stimulation in the kindling model. Epileptogenesis can also be studied in vitro by inducing SE in hippocampal neuronal cultures by placing the hippocampal neurons in a solution containing low Mg^{2+} for 3 hours.

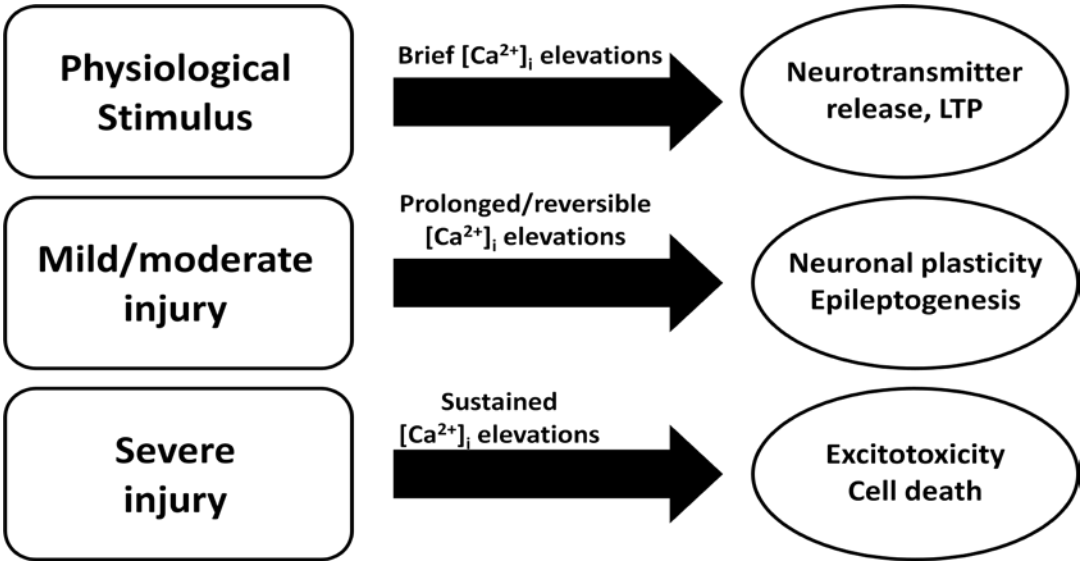


Figure 1-2. Effects of $[Ca^{2+}]_i$ on neurons. Brief elevations in $[Ca^{2+}]_i$ are necessary for normal physiological processes such as LTP. Mild to moderate injuries causes prolonged elevations in $[Ca^{2+}]_i$ that cause plasticity changes in neurons, leading to epileptogenesis. Severe and sustained elevations in $[Ca^{2+}]_i$ initiate cell death pathways, leading to neuronal death.

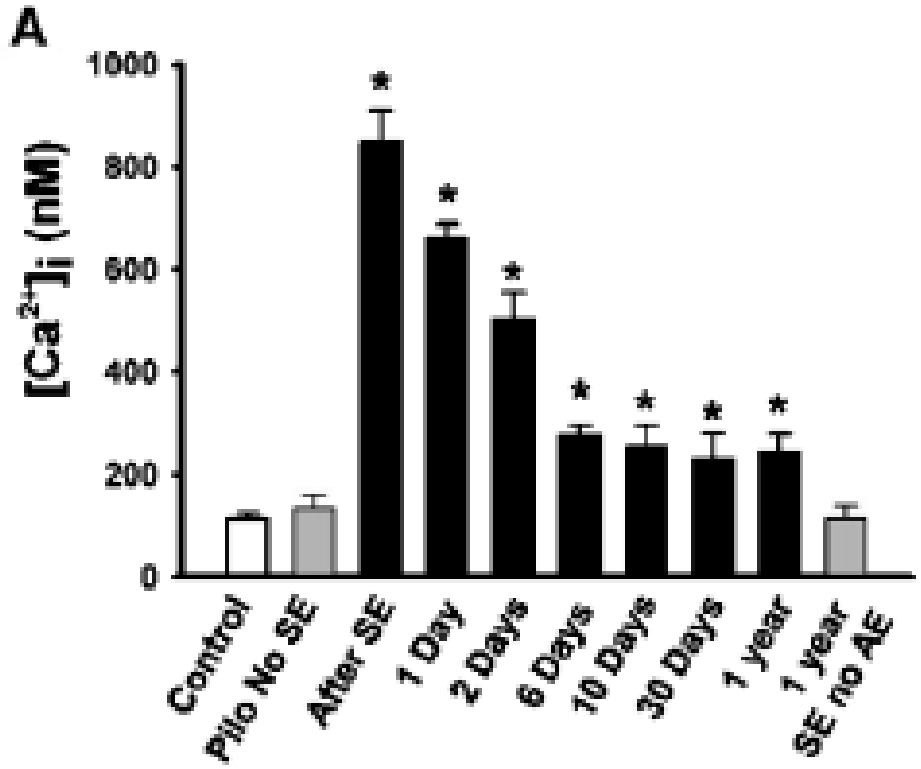


Figure 1-3. $[Ca^{2+}]_i$ in CA1 hippocampal neurons acutely isolated during acute, latent, and chronic phases of epileptogenesis following pilocarpine-induced SE. CA1 hippocampal neurons were acutely isolated to measure $[Ca^{2+}]_i$ following SE during the acute phase (immediately after SE), latent phase (1, 2, 6, and 10 days post-SE), and chronic phase (30 days and 1 year post-SE). The mean $[Ca^{2+}]_i$ during the acute phase was 850 ± 59 nM, which was significantly elevated compared to sham controls (90 ± 22 nM). $[Ca^{2+}]_i$ remained elevated compared to control during the latency phase. During the chronic phase, $[Ca^{2+}]_i$ was still elevated, with mean $[Ca^{2+}]_i$ values of 305 ± 27 nM for 30 days post-SE and 325 ± 35 nM for 1 year post-SE. *$p<0.01$ compared to controls. Data presented as mean ± SEM. From Raza M, et al. PNAS, 2004 [37].

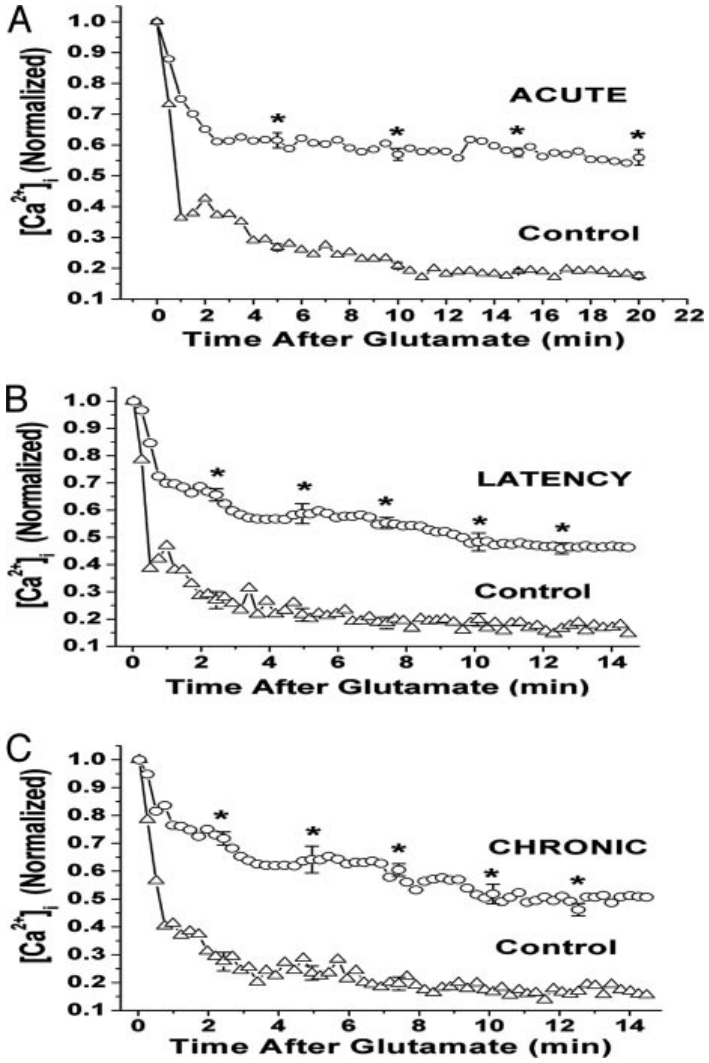


Figure 1-4. Epileptogenesis is associated with alterations in Ca^{2+} homeostatic mechanisms. $[Ca^{2+}]_i$ decay curves for hippocampal neurons isolated during acute *(A)*, latent *(B)*, and chronic *(C)* phases of AE. Values were normalized to the peak value after glutamate exposure (time=0). The epileptic neurons showed a statistically significant delay in returning $[Ca^{2+}]_i$ back to baseline levels following 10 μM glutamate exposure compared to control neurons. *$p<0.05$, student's t-test. From Raza M, et al. PNAS, 2004 [37].

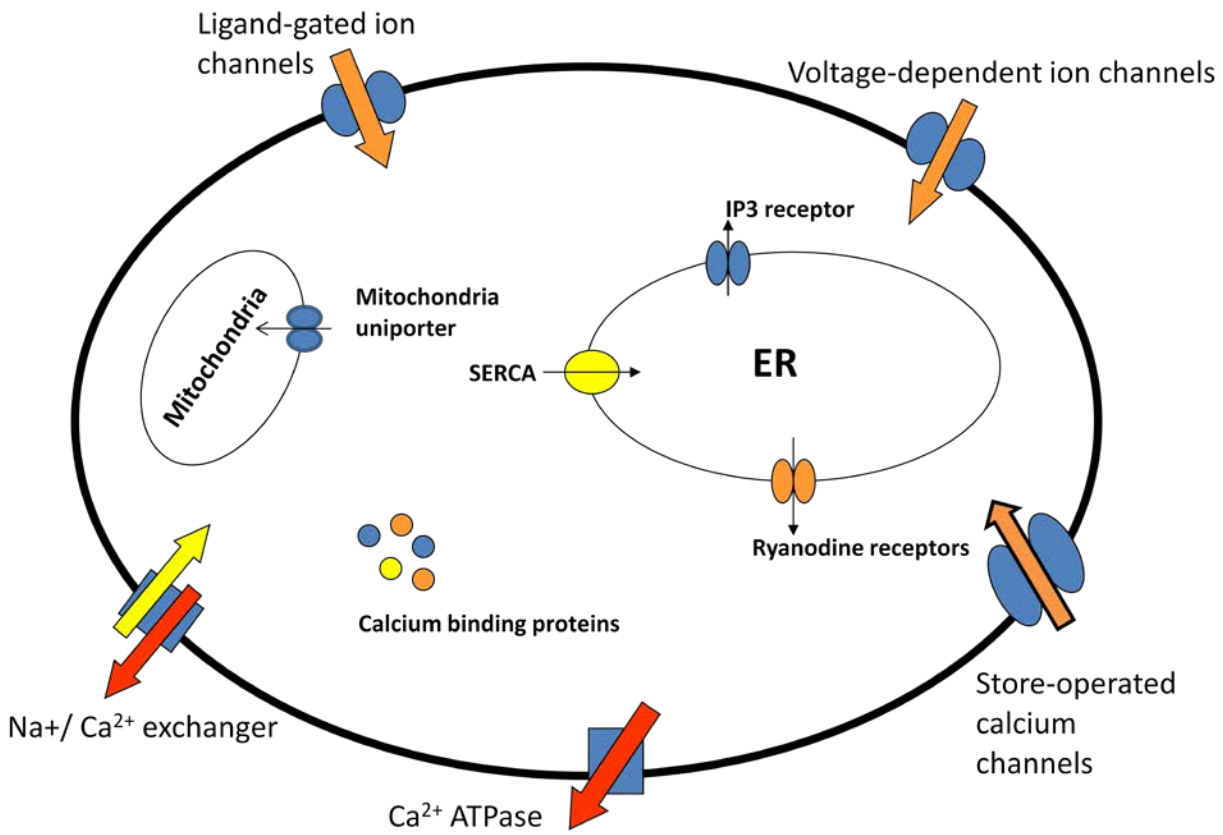


Figure 1-5. Calcium Homeostasis. The majority of calcium enters the neuron across the plasma membrane via voltage-dependent calcium channels and ligand-gated ion channels. Inside the neurons, calcium is buffered by calcium binding proteins. The mitochondria also takes up excess cytosolic calcium via the mitochondria uniporter. The ER serves as a dynamic calcium store. Calcium enters the ER through the sarco/endoplasmic reticulum calcium ATPase (SERCA). Calcium ions can be released from the ER into the cytosol by activation of the IP_3 and ryanodine receptors present on the ER membrane. When ER intralumenal calcium concentrations are depleted, store-operated calcium channels are activated, allowing calcium to enter the cell and be taken up into the ER to replenish ER stores. Calcium extrusion is accomplished via Na^+/Ca^{2+} exchangers and Ca^{2+} ATPases.

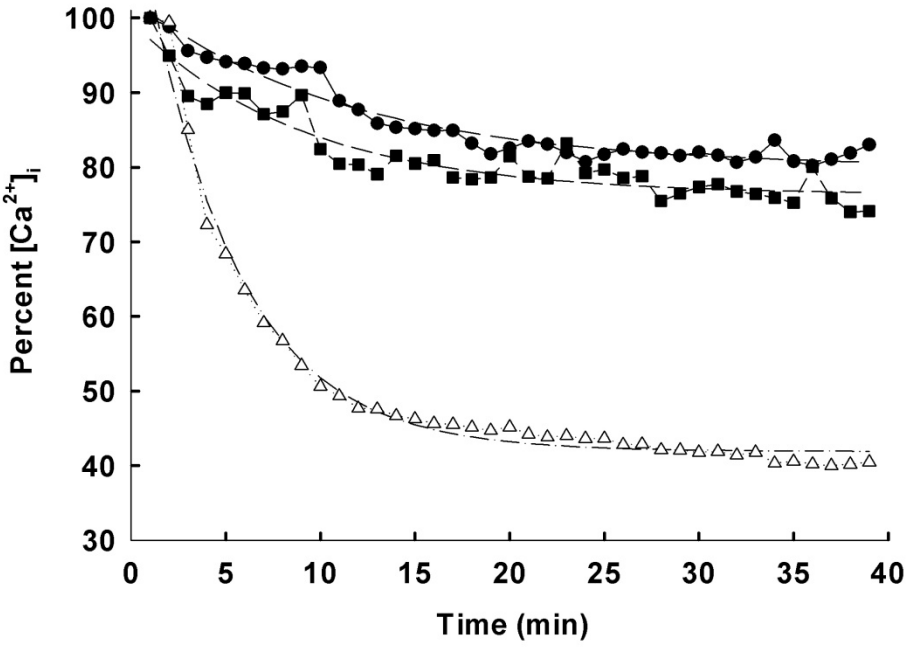


Figure 1-6. Dantrolene lowers $[Ca^{2+}]_i$ to baseline following in vitro SE. Following 3 h SE (time=0), cells were washed with Dantrolene (50 μM, clear triangles), MK-801 (black squares), or pBRS (black circles). 340/380 ratios were recorded every 30 seconds for 40 minutes and normalized to percentage of the peak ratio observed at time=0. n=6 plates per treatment group with ~60 neurons imaged per condition. From Nagarkatti N, et al. EJN, 2010 [83].

Chapter 2: The effects of hypothermia in the in vitro hippocampal neuronal cultures

Introduction

Epilepsy is a common neurological condition affecting 1-2% of the population worldwide [1-3]. Approximately 40% of all epilepsy cases are the result of a previous neurological injury such as stroke, status epilepticus (SE), or traumatic brain injury (TBI). Epilepsy that results from an injury is called acquired epilepsy (AE) [1, 4-5]. Epileptogenesis is defined as the transformation of healthy brain tissue into a hyperexcitable neuronal population that manifests epileptiform discharges [2, 7, 9]. Currently there are no anti-epileptogenic agents that can be administered following a neurological insult to prevent the process of epileptogenesis. Only anti-epileptic drugs (AEDs) exist, which treat the occurrence of seizures in epileptic patients [103]. Therefore developing an anti-epileptogenic therapy that can be administered following an injury is an important therapeutic goal.

It has been demonstrated in both in vivo and in vitro models of stroke, TBI, and SE that Ca^{2+} dynamics are severely altered following injury [30-31, 37-38, 40]. Persistent elevations in intracellular calcium concentrations ($[Ca^{2+}]_i$), termed the Ca^{2+} plateau, as well as changes in Ca^{2+} homeostatic mechanisms have been implicated in playing a major role in the development of AE. As a ubiquitous second messenger responsible for an array of cellular effects including gene transcription and neurotransmitter release, alterations in a neuron's ability to regulate Ca^{2+} could

have huge implications in terms of plasticity changes that lead to epileptogenesis. Therefore preventing the development of the Ca^{2+} plateau may prevent the progression of epileptogenesis and the development of AE.

One study provided evidence that Ca^{2+} entry during SE is mediated via NMDA receptor activation and is responsible for initiating the Ca^{2+} plateau [37]. Excessive stimulation of NMDA receptors by glutamate leads to excessive influx of Ca^{2+} into the neuron. Blocking NMDA receptor activation through the use of the NMDA receptor antagonist MK-801 during SE prevented the development of the Ca^{2+} plateau and the ultimate development of epilepsy. However, administration of MK-801 following SE did not lower $[Ca^{2+}]_i$ or prevent the development of AE [37, 83]. Therefore, other modes of Ca^{2+} entry may be contributing to the maintenance of the Ca^{2+}.

Another study demonstrated that ryanodine receptor (RyR) activation plays a role in mediating the post-SE Ca^{2+} plateau [83]. RyR are part of the Ca^{2+}-induced Ca^{2+} release (CICR) system which is activated by the influx of Ca^{2+} into the neuron through the NMDA receptors. Excessive intracellular Ca^{2+} activates the RyR present on the surface of the endoplasmic reticulum (ER). Ca^{2+} is sequestered in the ER and is released upon activation of either RyR or inositol triphosphate receptors (IP_3R). The in vitro low magnesium (low Mg^{2+}) model of SE was utilized to demonstrate that administration of the RyR antagonist Dantrolene following 3 h of low Mg^{2+}-induced SE rapidly reduced $[Ca^{2+}]_i$ back to baseline levels and maintained this reduction for up to 48 h after SE, suggesting that RyR inhibition blocks the formation of the Ca^{2+} plateau. Not only did Dantrolene reduce elevated $[Ca^{2+}]_i$, it also prevented the development of spontaneous

recurring epileptiform discharges (SREDs) following SE. These results suggest that RyR activation contributes to the maintenance of the Ca^{2+} plateau following SE and inhibiting Ca^{2+} release via the RyR system blocks the Ca^{2+} plateau and the ultimate development of AE. Therefore finding an intervention that reduces $[Ca^{2+}]_i$ following SE is important clinically.

One possible treatment is therapeutic hypothermia. Hypothermia is currently used to prevent neurological injury following a variety of insults including TBI [118-119] and stroke [120-121]. Hypothermia exerts its neuroprotective benefits through a variety of mechanisms. One of these mechanisms includes reduction of glutamatergic stimulation of NMDA receptors. Inhibiting NMDA receptor activation reduces the influx of Ca^{2+}, thereby reducing Ca^{2+}-mediated damage [144-145]. Although it has been demonstrated that hypothermia reduces Ca^{2+} influx via NMDA receptors, it is unknown how hypothermia affects other modes of Ca^{2+} entry and the Ca^{2+} plateau. The purpose of these studies is to investigate how hypothermia affects other modes of Ca^{2+} entry including CICR and whether hypothermia blocks the Ca^{2+} plateau.

Although changes in Ca^{2+} dynamics are observed following different neurological insults, models of SE-induced AE are the most widely used and best characterized models of epileptogenesis. These models produce epilepsy that closely resembles the pathology observed in human cases of AE. The in vivo models have provided important information regarding the pathophysiological changes associated with epileptogenesis. However, the whole animal model is too complex for studying the molecular mechanisms that lead to the Ca^{2+} plateau. The in vitro model is well suited for studying the effects of various treatments at a molecular level. Therefore, these studies will utilize an in vitro hippocampal neuronal culture (HNC) model of

SE-induced AE. The HNC model is a well characterized model that stimulates SE by placing cells in a low Mg^{2+} solution for 3 h. After 3 h, SE is terminated by placing the cells in Mg^{2+}-containing physiological basal recording solution (pBRS). The SE-like activity leads to the development of SREDs, the in vitro correlate of epilepsy. The SREDs manifested in the HNC model are similar to the electrographic features of human epilepsy. Thus, the in vitro model of SE-induced AE is a powerful tool for evaluating the effects of various treatments on Ca^{2+} dynamics.

In these studies, hippocampal neurons will be exposed to 3 h of SE by placing them in low Mg^{2+} solution. The Ca^{2+} indictor dye Fura-2AM will be used to evaluate $[Ca^{2+}]_i$ at the end of SE. At this point, SE will be terminated by washing cells with pBRS at either physiological temperature (37°C) or moderate hypothermic temperature (31°C) and $[Ca^{2+}]_i$ will be measured. In addition, the effect of hypothermia on Ca^{2+} entry through NMDA receptors, voltage-dependent Ca^{2+} channels (VGCC), IP_3R, and RyR will be investigated by stimulating each receptor at either 31°C or 37°C. The results will provide a better understanding of how hypothermia affects Ca^{2+} dynamics.

Materials and Methods

All reagents were purchase from Sigma Chemical Co. (St. Louis, MO) unless otherwise specified. Cell culture media was purchased from Invitrogen (Carlsbad, CA). All animal use procedures were in strict accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals and approved by Virginia Commonwealth University's Institutional Animal Care and Use Committee.

Hippocampal Neuronal Culture Preparation

Hippocampal neurons were harvested from 2-day postnatal Sprague–Dawley rats (Harlan, Indianapolis, IN, USA) in phosphate buffered saline (PBS) with sucrose, N-[2-Hydroethyl]piperazine-N'[2-ethanesulfonic acid] (HEPES) and penicillin (10,000 units/ml). Hippocampal tissue was dissected using a dissection microscope and subjected to 0.25% trypsin at 37°C for 30 min, followed by trituration using fire polished Pasteur pipettes. Cells were counted on a hemocytometer (Scientific Apparatus, Philadelphia, PA). The concentration of cells in suspension was estimated using the trypan blue exclusion method. The single cell suspension was diluted to a concentration of $1x10^5$ cells/mL in either glial feed (minimal essential media (MEM) with Earle's salts (Invitrogen, Carlsbad, CA), 25 mM HEPES, 2 mM L-glutamine, 3 mM glucose, and 10% fetal bovine serum (FBS)) or neuronal feed (MEM with Earle's salts, 25 mM HEPES, 2 mM L-glutamine, 3 mM glucose, 100 µg/mL transferrin, 5 µg/mL insulin, 100 µM putrescine, 3 nM sodium selenite, 200 nM progesterone, 1 mM sodium pyruvate, 0.1% ovalbumin, 0.2 ng/mL triiodothyroxine, and 0.4 ng/mL corticosterone) supplemented with 5% horse serum (HS).

Hippocampal neurons are grown on a glial support layer. To grow glial cultures, $5x10^4$ cells in glial feed were plated onto poly-L-lysine (0.05 mg/mL) coated Lab-Tek 2-well glass chambers (Nunc, Naperville, IL). The cultures were maintained at 37°C in a 5% CO_2/95% air atmosphere. Culture media was replaced with fresh glial feed three times per week. The glia were grown until confluent and then treated with 5 µM cytosine arabinoside for two days to halt cell division. The culture media was replaced with glial feed supplemented with 5% HS instead of FBS on the 10^{th}

day in vitro (DIV). On the 13th DIV, the glial feed was replaced with neuronal feed supplemented with 5% HS.

Cells suspended in neuronal feed plus 5% HS ($8.75x10^4$) were plated onto confluent glial beds on the 14th DIV. The cultures were maintained at 37°C in a 5% CO_2/95% air atmosphere and were fed twice a week with neuronal feed containing 20% conditioned media (CM). Neurons were allowed to mature for 14 days to allow for adequate neuronal maturation, formation of networks, and NDMA receptor development. Experiments were performed on days 14-18.

In vitro SE in hippocampal neuronal cultures

SE was generated in the in vitro cultures using the low Mg^{2+} model developed in the DeLorenzo lab [16]. Neuronal feed plus conditioned media was replaced with a low Mg^{2+} solution containing (in mM) 145 NaCl, 2.5 KCl, 10 HEPES, 10 glucose, 2 $CaCl_2$, and 0.002 glycine, adjusted to pH 7.3, and osmolarity adjusted to 325 mOsm with sucrose, or physiological basal recording solution (pBRS) without Mg^{2+}. The cells were maintained at 37°C under 5% CO_2/95% air for 3 hours (h). After 3 h of SE, the low Mg^{2+} solution was removed and replaced with a Mg^{2+}-containing pBRS to terminate SE and restore Mg^{2+}.

The duration of 3 h of SE was selected because it was previously determined that this treatment duration produced an epileptic state in 95% of neurons in culture. Exposure to low Mg^{2+} for 1 or 2 h did not produce consistent degrees of epileptogenicity and did not produce permanent changes in more than 90% of the neurons. Exposure for 4 h or longer produced a large degree of

neuronal death. Therefore, 3 h exposure to low Mg^{2+} was the optimal duration for producing spontaneous recurring epileptiform discharges (SREDs) [16].

Calcium microfluorometry

Changes in neuronal calcium were measured using the high-affinity (K_d=145 nM), ratiometric fluorescent Ca^{2+} indicator Fura-2-acetoxymethyl ester (AM) (Molecular Probes, Invitrogen, Eugene, OR, USA). The membrane permeable Fura-2AM passes through the cell membrane. Once inside the cell, the AM moiety is cleaved by intracellular esterases, leaving the cell impermeable Fura-2 inside the cell. Once the dye binds to Ca^{2+}, it can be excited by the excitation wavelength 340 nm. Unbound Fura-2 is excited at 380 nm. The emission generated by these wavelengths can be measured at 510 nm. Therefore, alternating excitation wavelengths of 340 nm and 380 nm can be used to determine the relative concentration of Ca^{2+}-bound and Ca^{2+}-free Fura-2. Thus, the resulting ratio of 340/380 corresponds directly to the total $[Ca^{2+}]_i$. Utilizing the ratio is important because it accounts for confounding variables such as unequal Fura-2 loading and variable cell thickness.

Hippocampal neurons were loaded with 1 μM fura-2-AM dissolved 0.1% DMSO and incubated for 20 minutes (min) at 37˚C in 5% CO2/95% air. The dye loading was terminated with three washes with pBRS. The neurons were incubated for an additional 15 minutes to allow for the complete cleavage of the AM moiety from Fura-2. The neurons were then transferred to a heated stage (Harvard Apparatus, Holliston, MA) which maintained the temperature of the cultures at either 31°C or 37°C. Neurons were visualized on an inverted microscope (Olympus IX 70, Olympus America, Melville, NY, USA) using a 20x, 0.7 numerical aperture, fluorite water-

immersion objective (Olympus America). Fura-2 was excited with a 75-W xenon arc lamp (Olympus America) with alternating wavelengths of 340 and 380 nm filtered through a Sutter Filter Wheel (Sutter Instruments Co., Novato, CA USA). Fluorescent emission at 510 nm was captured through a Fura filter cube (Olympus America) with a dichroic mirror at 400 nm using a Hamamatsu ORCA-ER camera (Hamamatsu Photonics, Japan).

MetaFluor (Molecular Devices, Downington, PA, USA) was used to control image acquisition and processing. Specific regions of interest were designated for each neuron in the microscopic field. Image pairs were captured and corrected for non-specific background fluorescence by subtracting images acquired from non-indicator-loaded plates. Ratio measurements for individual neurons were taken at 5 s intervals for 30 min. Ratio values correlate directly with $[Ca^{2+}]_i$.

Data analyses

For Ca^{2+} imagine experiments, a sample size (n) of at least 6 plates per treatment group were used. Experiments were performed over several weeks so that results were representative of multiple cultures. Individual neurons from multiple experiments were pooled to calculate average and standard error of the mean (SEM). Data is presented as mean ±SEM. To determine statistical significance between treatment groups, student's t-test or one-way analysis of variance (ANOVA) were used followed by Tukey post-hoc analysis when appropriate. A p-value of less than 0.05 ($p<0.05$) was considered statistically significant. Statistical analysis was performed using SigmaStat and graphs were drawn using SigmaPlot (Systat Software, San Jose, CA).

Results

Hypothermia rapidly reduced $[Ca^{2+}]_i$ and blocked the development of the Ca^{2+} plateau in the in vitro hippocampal neuronal culture model of SE

In order to determine if hypothermia blocked the development of the Ca^{2+} plateau in an in vitro model of SE-induced AE, neurons were exposed to 3 h of low Mg^{2+} treatment. $[Ca^{2+}]_i$ was measured at the end of the 3 h treatment to get a measurement of $[Ca^{2+}]_i$ during SE. SE was terminated by replacing the low Mg^{2+} solution with pBRS. The low Mg^{2+} solution was washed off with either 31°C (hypothermia) or 37°C (physiological temperature) pBRS, and $[Ca^{2+}]_i$ was measured every 30 sec for 20 min. Data is presented as a percent decay from peak values obtained during SE (in low Mg^{2+} solution).

As illustrated in Fig 2-1, following 3 h of low Mg^{2+} treatment (time 0 on the x-axis), hippocampal neurons exhibited elevated 340/380 ratios, indicating that $[Ca^{2+}]_i$ was elevated after in vitro SE. These ratios were normalized to the peak ratio. At the end of 3 h of low Mg^{2+} treatment, neurons were washed with either 31°C or 37°C pBRS. After 5 min post-treatment, neurons washed with 37°C pBRS showed a slight decrease in 340/380 ratios. However, cells washed with the hypothermia treatment exhibited a larger decrease in 340/380 ratios that were 62% of the peak observed at the end of in vitro SE. At 10 min post-treatment, the 340/380 ratio values were 59% and 74% of the post-SE peak in cells washed with 31°C and 37°C pBRS, respectively. At 15 min post-treatment, the ratio values were 56% of the post-SE peak for cells washed with 31°C pBRS and 70% of the peak for cells washed with 37°C solution. After 20 min, the cells treated with hypothermia were 58% of the peak. This is significantly lower than the cells washed with 37°C solution whose 340/380 ratio values were 71% of the peak. Within 20

min of hypothermia treatment, $[Ca^{2+}]_i$ returned to baseline ratio values observed in naïve control neurons (data not shown), whereas $[Ca^{2+}]_i$ remained significantly elevated in cells washed with 37°C pBRS. The ratio values of neurons not treated with hypothermia fell by only 29% after 20 min compared to hypothermia treated cells whose ratio values fell by 42%. There was a significant difference in the 340/380 ratio values between the two groups at each time point after 5 min of treatment. This experiment demonstrated that hypothermia markedly enhances the cell's ability to reduce elevations in $[Ca^{2+}]_i$. In addition, hypothermia lowered $[Ca^{2+}]_i$ back to control levels, indicating that hypothermia treatment prevents the development of the Ca^{2+} plateau in an in vitro model.

Hypothermia reduced NMDA receptor-dependent Ca^{2+} entry

Previous studies have demonstrated that hypothermia reduces the concentration of extracellular glutamate, thereby reducing NMDA receptor activation and Ca^{2+} entry through the NMDA channel [144-145]. The goal of this experiment was to determine what effect hypothermia had on NMDA-mediated Ca^{2+} entry in an in vitro model. Hippocampal neurons were loaded with Fura-2 and incubated for 20 min at 37°C. Fura-2 was washed off, and neurons were incubated for an additional 10 min at 37°C to allow for sufficient cleavage of the AM moiety by cellular esterases. At this point, neurons were incubated at either 31°C or 37°C for 5 min to allow for the cells to acclimate. They were then placed on a temperature controlled stage at either 31°C or 37°C prior to Ca^{2+} imaging.

To determine if hypothermia affected NMDA receptor-mediated Ca^{2+} entry, hippocampal neurons were stimulated with either 31°C or 37°C 50 µM glutamate. Baseline 340/380 ratio

values before stimulation were similar between the two treatment groups with values of 0.30±0.01 for neurons in the 31°C group and 0.31±0.02 for neurons in the 37°C group. Upon stimulation with 37°C glutamate, the ratio values peaked to 0.636±0.04. However, stimulation with 31°C glutamate resulted in a diminished response, with ratio values only peaking to 0.38±0.01, as shown in Fig 2-2A. Pseudocolor images further illustrate that 37°C glutamate causes a dramatic increase in $[Ca^{2+}]_i$ whereas 31°C glutamate causes a lesser response (Fig 2-2B). Hypothermia reduced the height of the calcium transient by 74.6% (Fig 2-6). These results indicate that hypothermia significantly reduced Ca^{2+} entry through the NMDA receptor.

Hypothermia did not affect Ca^{2+} entry through voltage-dependent Ca^{2+} channels

Ca^{2+} entry through voltage-dependent Ca^{2+} channels (VDCC) was stimulated using a high potassium solution composed of 105 mM NaCl, 40 mM KCl, 10 mM HEPES, 10 mM glucose, 2 mM $CaCl_2$, 1 mM $MgCl_2$, and 0.002 mM glycine. The high potassium solution depolarizes the neuronal membrane, thus allowing an influx of Ca^{2+} through VDCCs. As demonstrated in Fig 2-3A, the baseline ratio values for neurons in the 31°C group and 37°C group were not significantly different from each other at 0.27±0.01 and 0.27±0.02, respectively. When neurons were stimulated with 37°C high potassium, 340/380 ratio values peaked to 0.54±0.02. Neurons stimulated with high potassium at 31°C exhibited a similar peak of 0.51±0.01. Fig 2-3B further illustrates the similar rises in $[Ca^{2+}]_i$ upon stimulation with 31°C and 37°C high potassium solution with the use of pseudocolor images. Hypothermia reduces Ca^{2+} entry through VDCCs by only 13.4%, as illustrated in Fig 2-6. However, the peak values were not statistically different from each other. In contrast, the peaks of both groups were significantly higher when compared

to 37°C baseline values (One way ANOVA, $p<0.001$). These results show that high potassium causes a rise in $[Ca^{2+}]_i$ and that hypothermia does not affect Ca^{2+} entry through VDCCs.

Hypothermia did not affect IP_3 receptor-mediated Ca^{2+} entry

Bradykinin–induced Ca^{2+} release is mediated by stimulation of IP_3 receptor [151]. Binding of bradykinin to its receptor activates phospholipase C (PLC), which cleaves PIP_2, producing diacylglycerol (DAG) and IP_3. In order to investigate how IP_3 receptor-mediated Ca^{2+} release is affected by hypothermia, hippocampal neurons were stimulated with either 31°C or 37°C bradykinin (1 μM). Before stimulation with bradykinin, similar baseline ratio values of 0.27±0.01 in 31°C group and 0.27±0.01 in the 37°C group were observed, as shown in Fig 2-4. Upon stimulation with 37°C bradykinin, neurons exhibited a significant rise in $[Ca^{2+}]_i$ with ratio values increasing to 0.65±0.03. When stimulated with 31°C bradykinin, neurons also exhibited a significant increase in $[Ca^{2+}]_i$ with ratio values increasing to 0.60±0.01 (Fig 2-4A). Pseudocolor images illustrate the similar rises in $[Ca^{2+}]_i$ following stimulation with 31°C and 37°C bradykinin (Fig 2-4B). Both peak values were significantly elevated compared to baseline ratio values. Hypothermia resulted in a 15.8% reduction in the height of the Ca^{2+} transient (Fig 2-6). However, there was no significant difference between the peak values of the two treatment groups. These results indicate that hypothermia does not affect IP_3 receptor-mediated Ca^{2+} release.

Hypothermia reduced ryanodine receptor-mediated Ca^{2+} release

Ca^{2+} release through ryanodine receptors (RyR) can be evaluated pharmacologically with the use of caffeine [152-153]. Thus, caffeine (10 mM) was used to stimulate RyR-mediated Ca^{2+} release.

Prior to caffeine stimulation, both 31°C and 37°C groups exhibited similar 340/380 ratio values of 0.28±0.01 and 0.34±0.01, respectively. Upon stimulation with a 37°C caffeine solution, neurons exhibited a marked increased in $[Ca^{2+}]_i$ with peak ratio values of 0.72±0.05 (Fig 2-5A). When stimulated with 31°C caffeine solution, neurons exhibited a diminished response with a peak ratio value of 0.44±0.04. Fig 2-5B illustrates the differences in peak $[Ca^{2+}]_i$ through pseudocolor images. Stimulation with 31°C caffeine solution results in a diminished response compared to the peak in $[Ca^{2+}]_i$ observed following 37°C caffeine stimulation. The peak value in the hypothermia condition is significantly lower than the peak response in the 37°C group ($p<0.001$). There was also a significant difference between the peak values of both treatment groups compared to 37°C baseline. However, hypothermia inhibited the height of the caffeine-induced Ca^{2+} transient by 57.1% (Fig 2-6). These results suggest that hypothermia reduces the release of Ca^{2+} from intracellular stores by inhibiting the RyR.

Caffeine stimulates Ca^{2+} release through ryanodine receptors

Several studies have demonstrated that non-physiological concentrations of caffeine (in the millimolar range) function similarly on the RyR as nanomolar concentrations of ryanodine [152-155]. In order to prove that caffeine stimulates Ca^{2+} release from the RyR, neurons were stimulated with 10 mM caffeine in the presence of Dantrolene (1 µM), a known RyR antagonist. Neurons were loaded with Fura-2AM and allowed to incubate at 37°C for 20 min. Baseline 340/380 ratios were obtained in the presence of and the absence of 1 µM Dantrolene. There was no significant difference between these values (data not known), which demonstrated that 1 µM Dantrolene did not affect baseline $[Ca^{2+}]_i$. When neurons were stimulated with caffeine in the presence of Dantrolene, there was not a significant spike in $[Ca^{2+}]_i$ (Fig 2-7). This provided

evidence that caffeine was stimulating Ca^{2+} release from intracellular stores by acting on the RyR. Furthermore, the effects of Dantrolene were reversible. Hippocampal neurons were subjected to 3 h low Mg^{2+} treatment and then washed with 1 µM Dantrolene. Elevated $[Ca^{2+}]_i$ rapidly declined in the presence of Dantrolene. When Dantrolene was replaced with 37°C pBRS, $[Ca^{2+}]_i$ immediately spiked, suggesting that the antagonistic properties of Dantrolene were reversible (Fig 2-8).

Discussion

This study demonstrated that hypothermia can reduce the development of the Ca^{2+} plateau following in vitro SE. In both in vivo and in vitro models of SE, $[Ca^{2+}]_i$ remains elevated following injury, and this persistent elevation in $[Ca^{2+}]_i$ is believed to contribute to the pathological consequences associated with epileptogenesis. Blocking the development of the plateau has been shown to prevent the development of epilepsy in both in vivo and in vitro models of SE-induced AE. The novel finding that hypothermia reduces elevated $[Ca^{2+}]_i$ and can prevent the formation of the Ca^{2+} plateau in an in vitro model offers an alternative intervention to pharmacological agents in preventing epileptogenesis.

Furthermore, this study also demonstrated that hypothermia reduces Ca^{2+} entry into the neuron by inhibiting both NMDA and ryanodine receptors. Evidence has shown that the NMDA receptor mediates the majority of Ca^{2+} influx during SE. Inhibition of the NMDA receptors during SE blocks the formation of the Ca^{2+} plateau and prevents the development of AE [37]. The excessive entry of Ca^{2+} into the cell during SE stimulates Ca^{2+}-induced Ca^{2+} release (CICR) via activation of RyR. Evidence has suggested RyR may be responsible for maintaining the Ca^{2+}

plateau following SE [83]. Inhibition of NMDA receptors after the injury does not provide neuroprotection against the Ca^{2+} plateau or development of AE. However, the administration of Dantrolene, a RyR antagonist, following in vitro SE blocks the Ca^{2+} plateau and prevents the development of SREDs.

Although previous studies have demonstrated that hypothermia modulates Ca^{2+} transmission, it is not know how hypothermia exerts its effects on Ca^{2+} transmission. One study showed that hypothermia reduces NMDA receptor activation by reducing glutamate concentrations, thereby reducing Ca^{2+} entry [144-145]. However, no studies have provided evidence that hypothermia reduces Ca^{2+} entry through the NMDA receptor independent of glutamate concentrations, nor have any studies investigated the effects of hypothermia on other modes of Ca^{2+} entry. This study utilized pharmacological agents to stimulate various modes of Ca^{2+} entry. Ca^{2+} influx following stimulation of VDCC with the use of a high potassium solution was unaffected by hypothermia. In addition, hypothermia did not significantly reduce Ca^{2+} entry through bradykinin-mediated IP_3R stimulation. However, hypothermia did have a significant effect on NMDA receptor-mediated Ca^{2+} entry. Hippocampal neurons stimulated under hypothermic conditions exhibited a markedly diminished response to glutamate. This provides evidence that hypothermia reduces Ca^{2+} entry through NMDA receptors despite the presence of extracellular glutamate.

Interestingly, hypothermia also significantly reduced the height of the Ca^{2+} transient upon caffeine induced-RyR stimulation, thereby reducing CICR. Caffeine is an established pharmacological tool for activating RyR-mediated Ca^{2+} release [78-79, 152-155]. Non-physiological concentrations of caffeine have been shown to function similarly to nanomolar

concentrations of ryanodine with the advantage of having faster kinetics and rapid reversibility upon washout [153]. Caffeine at concentrations greater than 5 mM leads to activation of the RyR that is independent of cytosolic Ca^{2+} [156]. Thus, this study employed 10 mM caffeine to study the effects of hypothermia on CICR. To further prove that caffeine was acting on the RyR, neurons were stimulated with caffeine in the presence of the RyR blocker Dantrolene. Caffeine did not elicit a significant peak in $[Ca^{2+}]_i$ in the presence of Dantrolene, which further demonstrated that caffeine stimulates Ca^{2+} release through RyR activation.

The results of this study suggest that hypothermia is a viable therapeutic intervention that can be administered following SE to prevent the development of the Ca^{2+} plateau and possibly the subsequent development of epilepsy. The ability of hypothermia to modulate two important modes of Ca^{2+} entry provides novel information regarding one of hypothermia's mechanisms of action. Hypothermia is presumed to exert its neuroprotective benefits via a variety of actions, and its ability to modulate excitatory transmission, specifically Ca^{2+}, is thought to be one its main mechanisms of action. These experiments were performed in the highly relevant hippocampal neuronal culture model of SE-induced AE. However, in vitro cultures do not represent true anatomical connections. Moreover, there are distinct differences between the in vitro model and human epilepsy including the duration of time between the injury and the development of AE as well as co-morbidities associated with epileptogenesis [29]. The cultures provide a powerful tool for studying molecular mechanisms of SE and hypothermia in a controlled environment. However, it will be essential to study the effects of hypothermia in a whole animal model in order to determine if hypothermia truly is neuroprotective following SE.

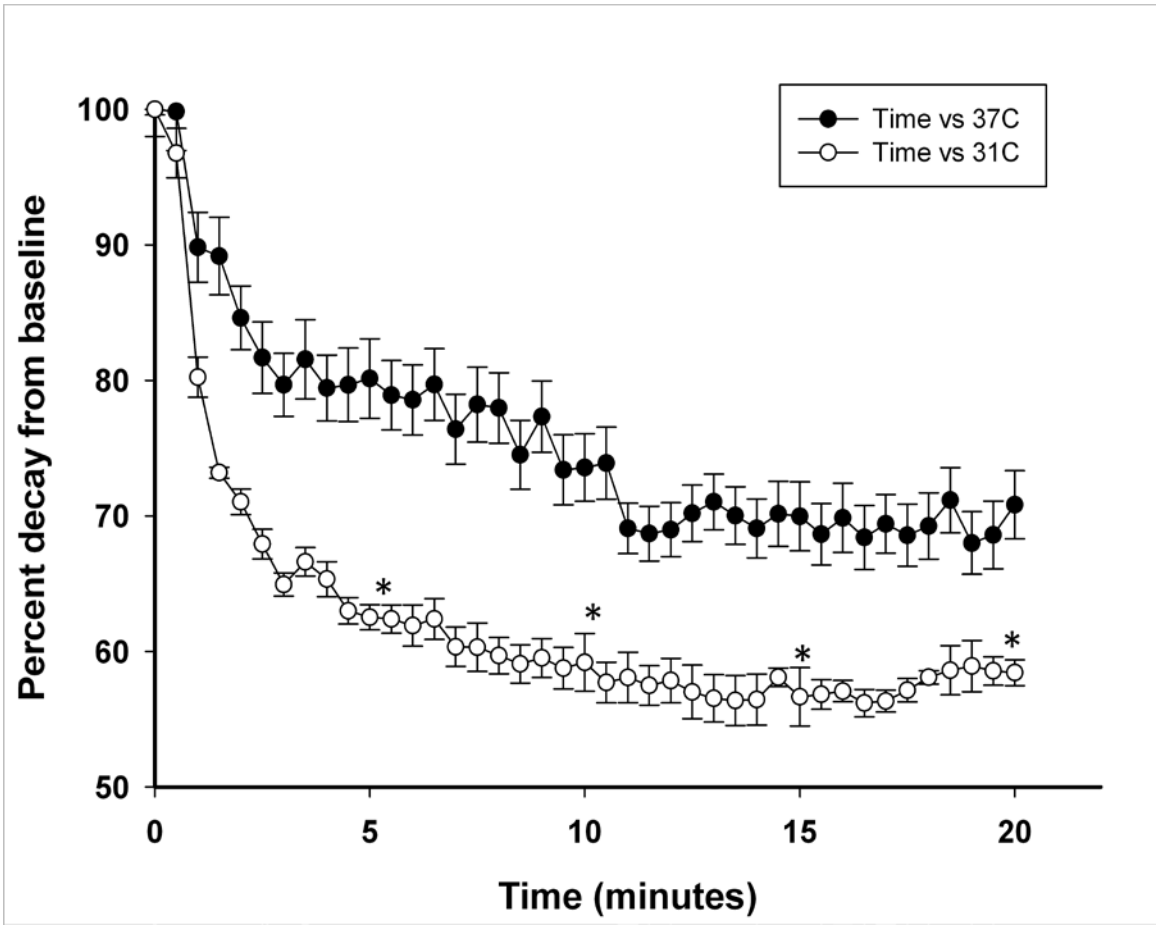
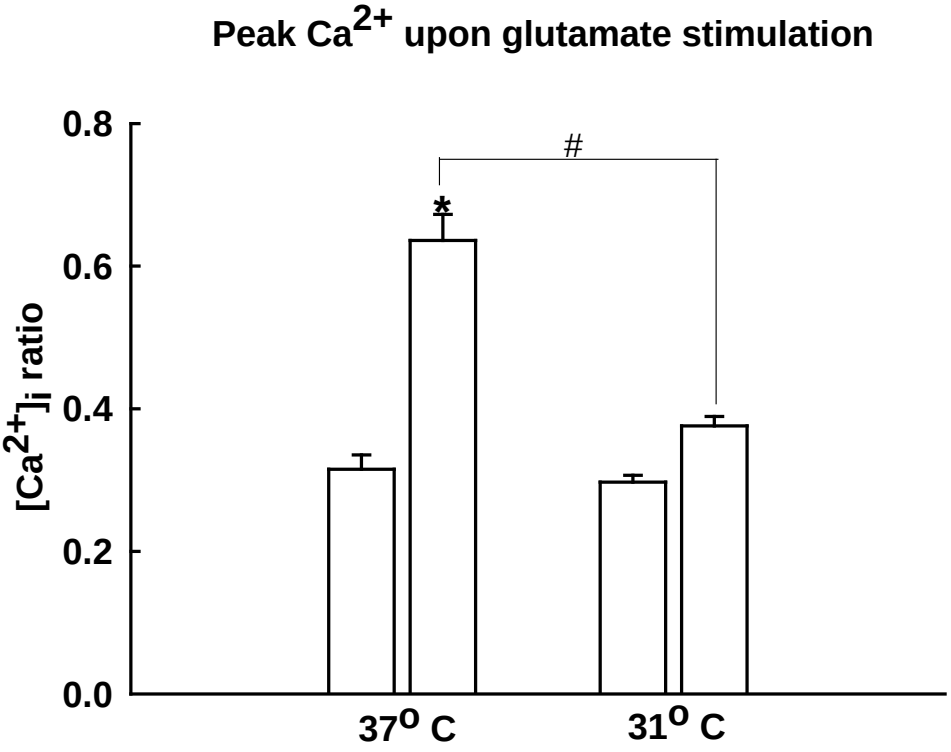


Figure 2-1. Hypothermia blocked the Ca^{2+} plateau after in vitro SE. Following 3 h of in vitro SE (time=0), cells were washed with either 31˚C or 37˚C pBRS. 340/380 ratios were recorded every 30 seconds for 20 minutes and normalized to percent of the peak ratio observed at time=0. n=6 plates per treatment group with 40-60 neurons imaged per group. *$p<0.05$, Student's t-test for all time points after 5 min.

A


Peak Ca2+ upon glutamate stimulation
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B

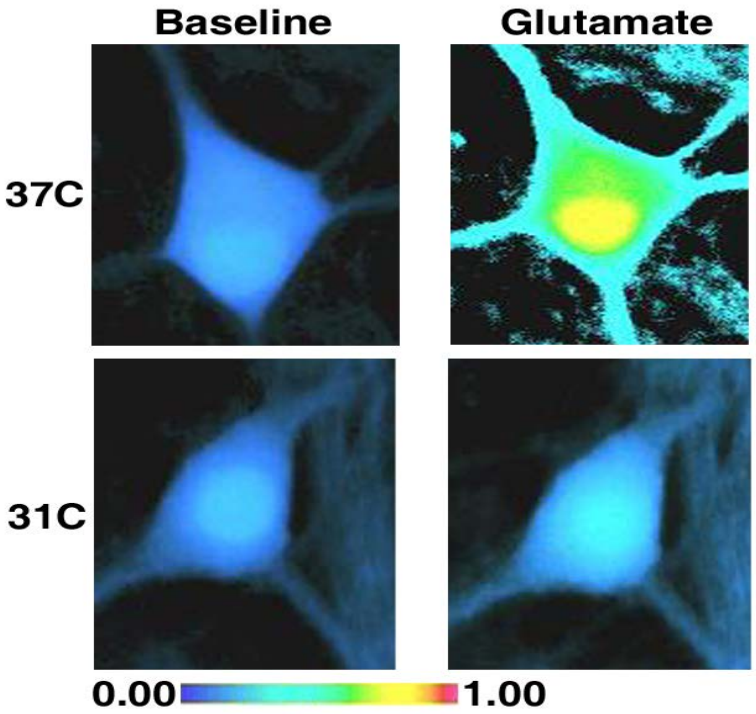
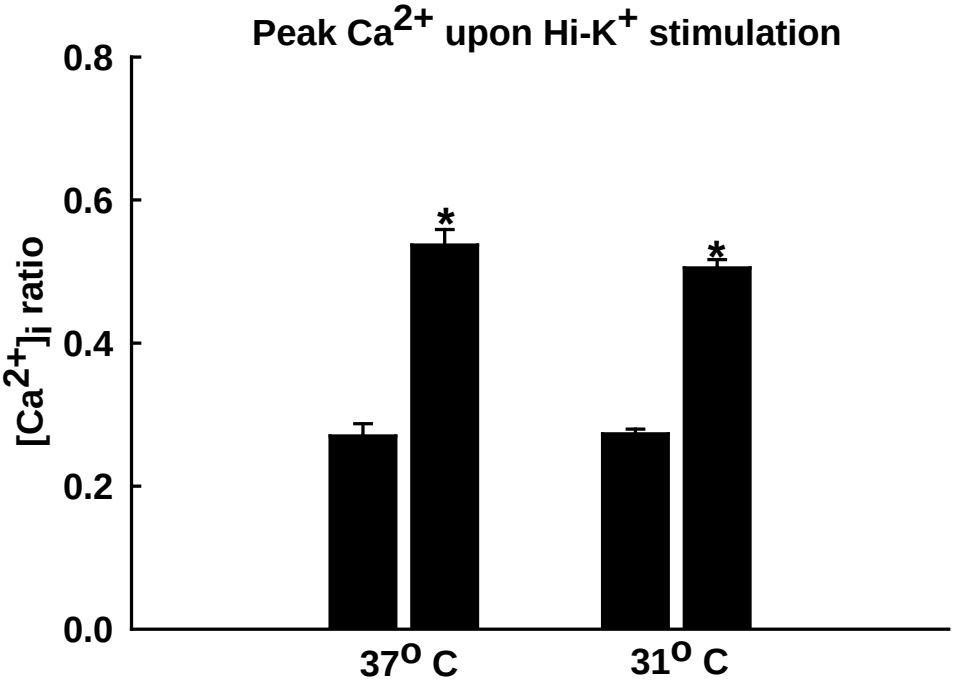


Figure 2-2. Hypothermia reduced NMDA receptor-dependent Ca^{2+} entry. (A) Prior to stimulation with 50 µM glutamate, hippocampal neurons exhibited similar 340/380 baseline ratios of 0.30±0.01 for neurons in the 31°C group and 0.31±0.02 for neurons in the 37°C group. Upon stimulation with 37°C glutamate, 340/380 ratios peaked to 0.636±0.04, which is significantly elevated compared to baseline. When neurons were stimulated with 31°C glutamate, 340/380 ratios peaked to 0.38±0.01. *$p<0.001$ compared to baseline; #$p<0.001$ between 31°C and 37°C peaks, one way ANOVA, n=7 plates per condition. **(B)** Representative pseudocolor images obtained from baseline neuron (left panel), neuron stimulated with 37°C glutamate (top right panel), and neuron stimulated with 31°C glutamate (bottom right panel). Neurons exhibited elevated $[Ca^{2+}]_i$ upon 37°C stimulation and a diminished response to 31°C stimulation.

A


Peak Ca2+ upon Hi-K+ stimulation
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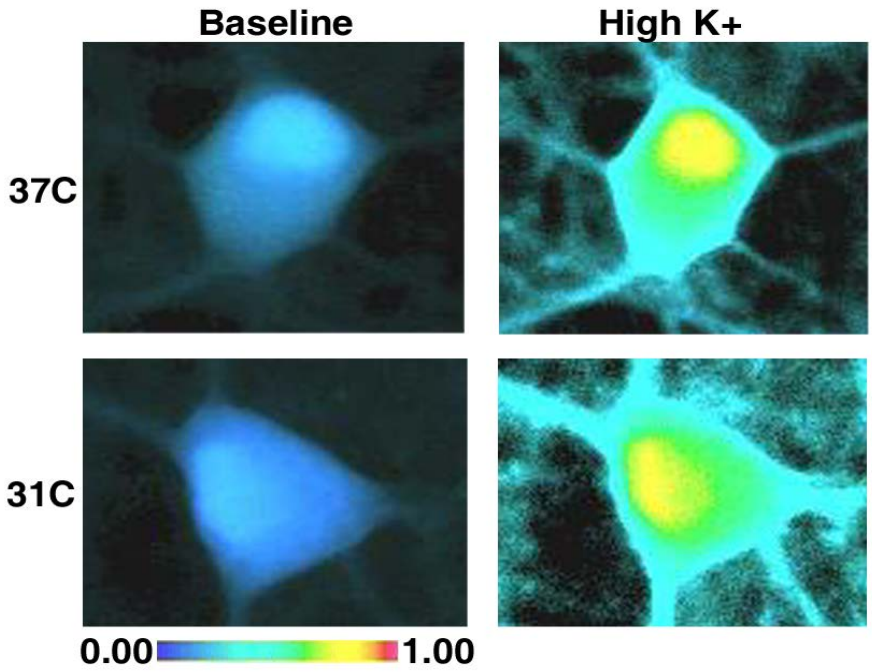
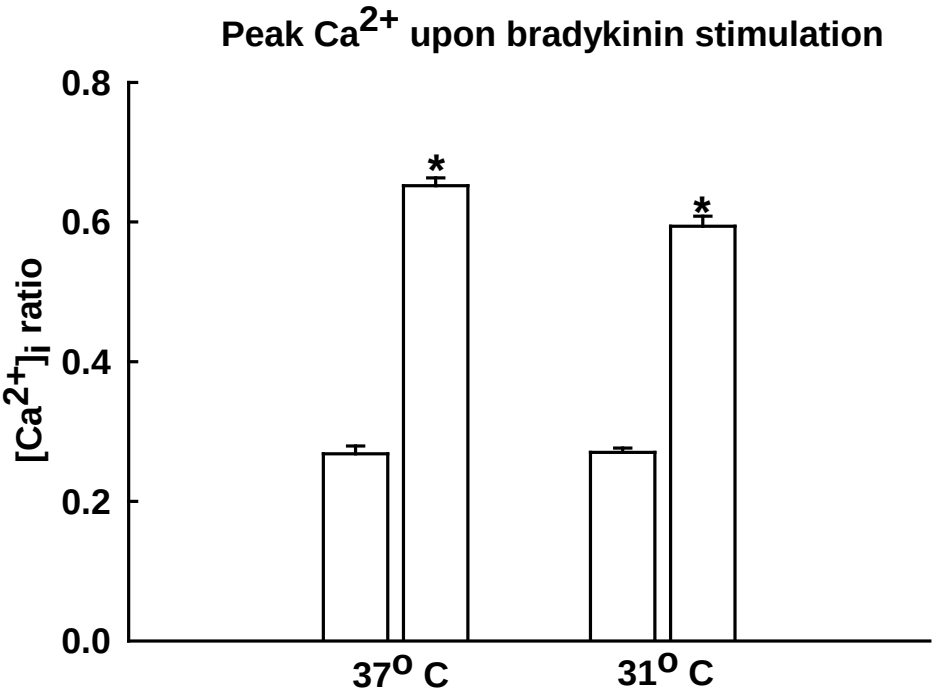


Figure 2-3. Hypothermia did not affect Ca^{2+} entry through VDCCs. (A) Prior to stimulation of VDCCs with high potassium solution, hippocampal neurons from both treatment groups displayed similar 340/380 baseline ratios of 0.27±0.01 in the 31°C group and 0.27±0.02 in the 37°C group. When stimulated with 37°C high potassium solution 340/380 ratios peaked to 0.54±0.02. Stimulation with 31°C high potassium resulted in a similar 340/380 peak ratio of 0.51±0.01. *$p<0.001$ compared to 37°C baseline, one way ANOVA, n=6 plates per condition. **(B)** Representative pseudocolor images obtained from baseline neuron (left panel), neuron stimulated with 37°C high potassium (top right panel), and neuron stimulated with 31°C high potassium (bottom right panel). Neurons from both groups exhibited elevated $[Ca^{2+}]_i$ upon 37°C and 31°C stimulation.

A


Peak Ca2+ upon bradykinin stimulation
0.8
0.6
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[Ca2+]i ratio
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37O C
31O C

B

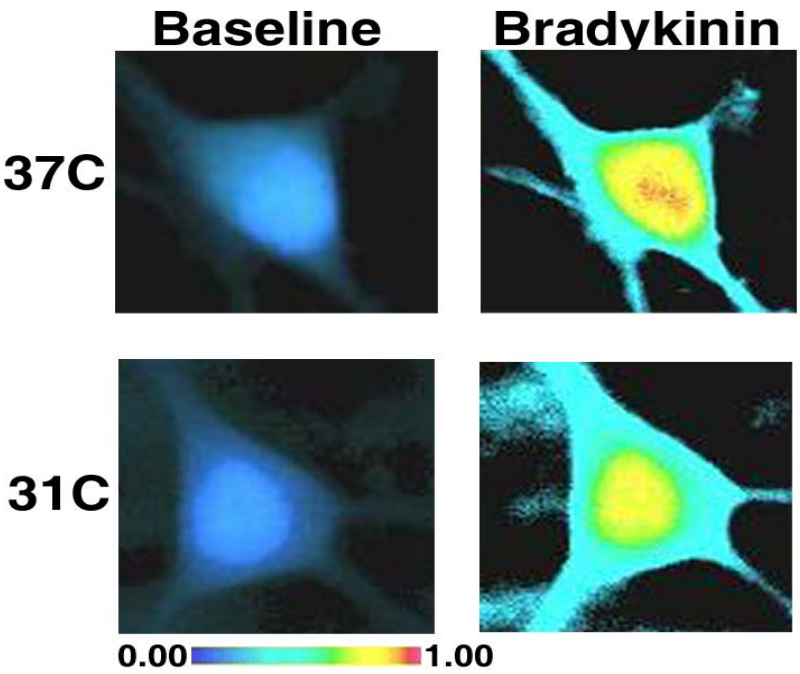
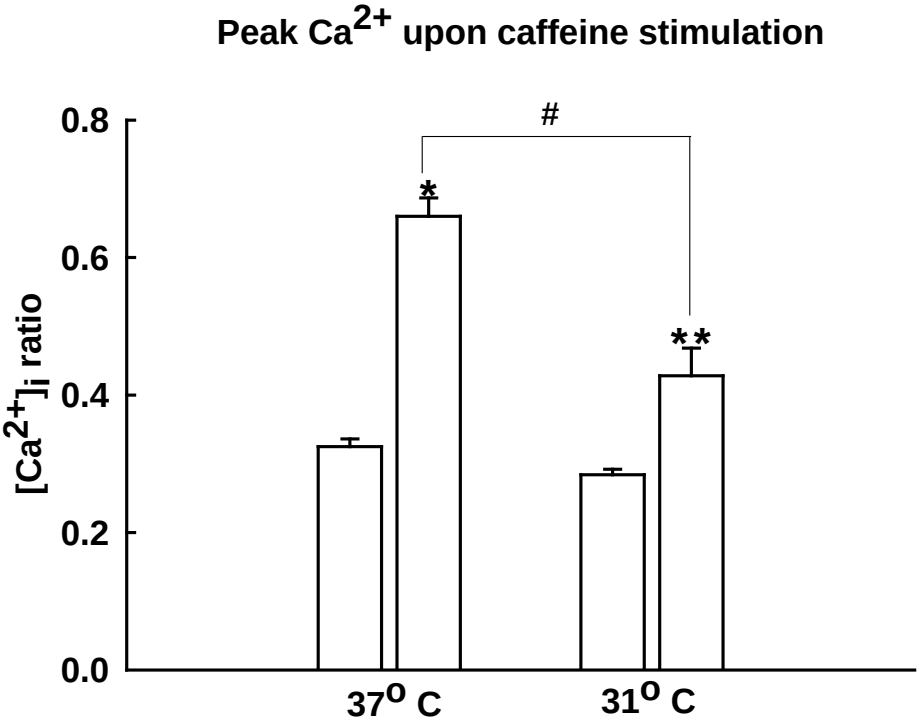


Figure 2-4. Hypothermia did not affect IP_3 receptor-mediated Ca^{2+} entry. (A) Prior to bradykinin-mediated stimulation of IP_3 receptors, neurons from 31°C and 37°C treatment groups displayed similar 340/380 baseline ratios of 0.27±0.01 and 0.27±0.01, respectively. Stimulation with 37°C bradykinin resulted in a peak 340/380 ratio of 0.65±0.03. Similarly, when stimulated with 31°C bradykinin, 340/380 ratios peaked to 0.60±0.01. *$p<0.001$ compared to 37°C baseline, one way ANOVA, n=5 plates per condition. **(B)** Representative pseudocolor images obtained from baseline neuron (left panel), neuron stimulated with 37°C bradykinin (top right panel), and neuron stimulated with 31°C bradykinin (bottom right panel). Neurons in both groups exhibited elevated $[Ca^{2+}]_i$ upon stimulation with 37°C and 31°C bradykinin.

A


Peak Ca2+ upon caffeine stimulation
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0.8
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[Ca2+]i ratio
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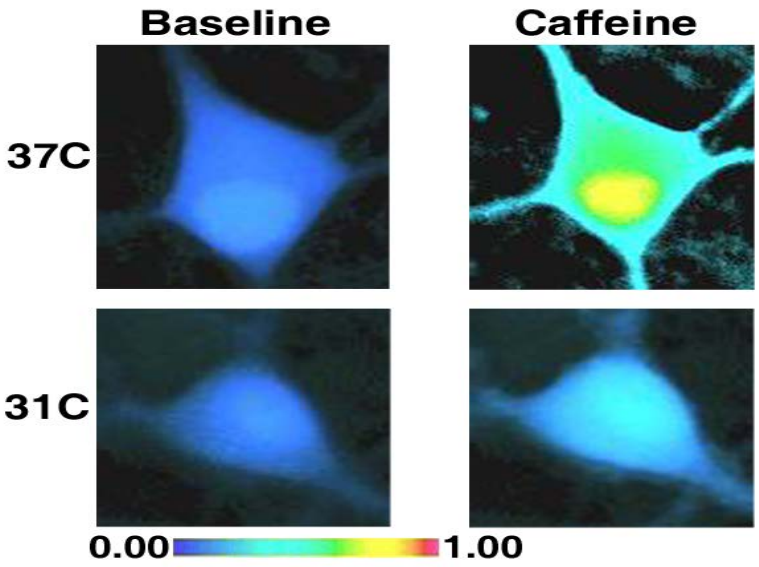

Figure 2-5. Hypothermia reduced ryanodine receptor-mediated Ca^{2+} induced Ca^{2+} release. **(A)** Prior to caffeine-mediated RyR stimulation, baseline 340/380 ratio values from both treatment groups were not significantly different with values of 0.28±0.01 in the 31°C group and 0.34±0.01 in the 37°C group. Upon stimulation with 37°C caffeine, 340/380 ratios peaked to 0.72±0.05. When stimulated with 31°C caffeine, neurons exhibited a diminished response with a peak ratio of 0.44±0.04. *p<0.001 compared to 37°C baseline, **p<0.05 compared to 37°C baseline, #p<0.001 between 31°C and 37°C peaks, one way ANOVA, n=10 plates per group. **(B)** Representative pseudocolor images obtained from baseline neuron (left panel), neuron stimulated with 37°C caffeine (top right panel), and neuron stimulated with 31°C caffeine (bottom right panel). Neurons exhibited elevated $[Ca^{2+}]_i$ upon 37°C stimulation and a diminished response to 31°C stimulation.

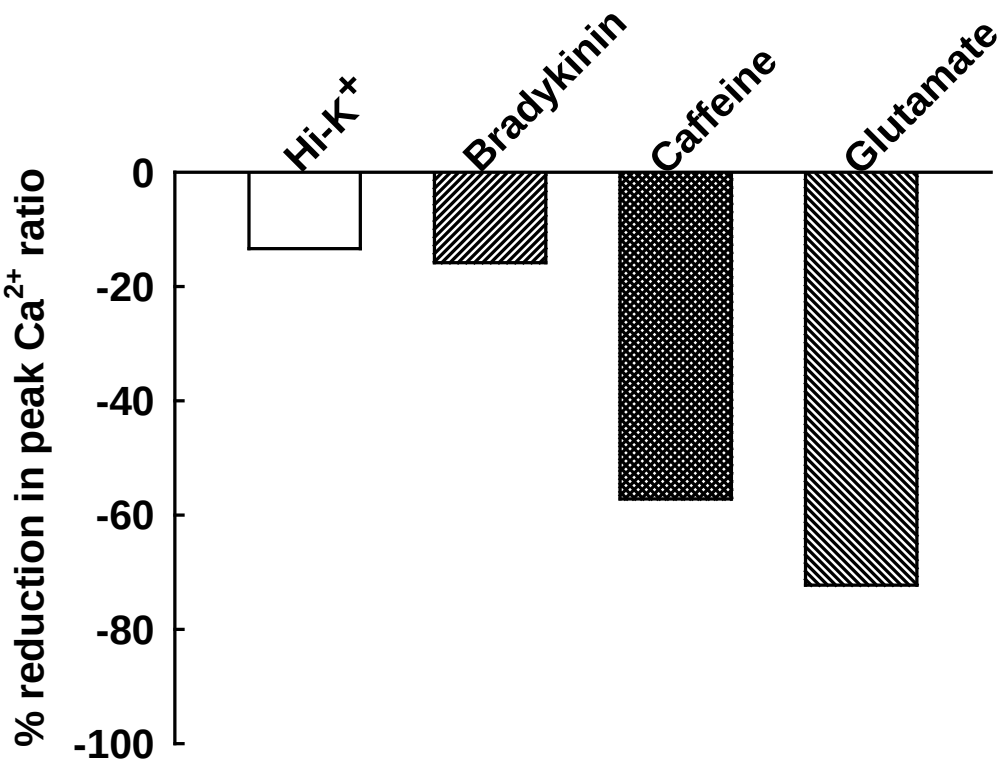

Figure 2-6. Percent reduction in Ca^{2+} transient caused by hypothermia. Hypothermia causes a 13.4% reduction in high potassium-induced Ca^{2+} transient, a 15.8% reduction in bradykinin-induced Ca^{2+} transient, a 57.1% reduction in caffeine-induced Ca^{2+} transient, and a 74.6% reduction in glutamate-induced Ca^{2+} transient.

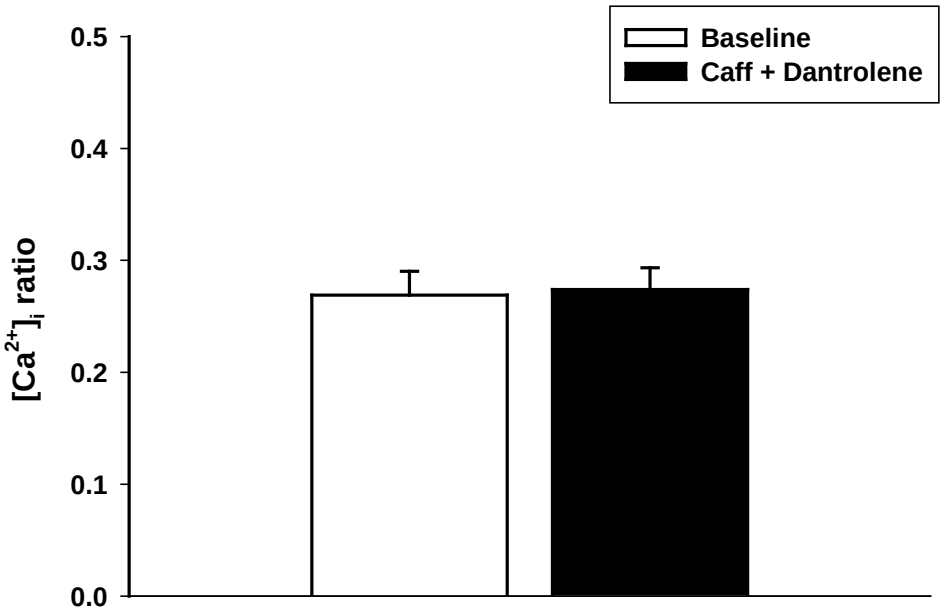

Figure 2-7. Dantrolene inhibits caffeine-induced Ca^{2+} release. Prior to caffeine stimulation, baseline 340/380 ratio values were 0.27±0.02. When stimulated with caffeine in the presence of 1 µM Dantrolene, ratio values remained at 0.27±0.02. Data presented as mean ratio ± SEM, n=5 plates per group.

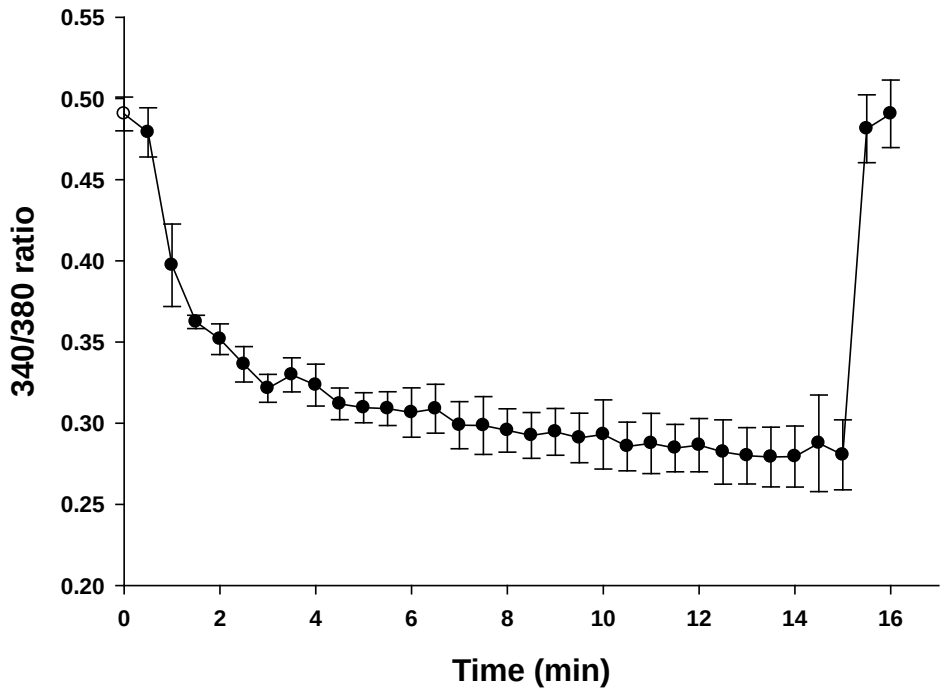

Figure 2-8. Antagonistic properties of Dantrolene are reversible. Following 3 h low Mg^{2+} treatment, cells were washed with 1 µM Dantrolene (time=0). 340/380 ratios were recorded every 30 seconds for 15 minutes. At 15 min post-wash, Dantrolene was removed and replaced with 37˚C pBRS.

Chapter 3: Effects of hypothermia in the in vivo rat pilocarpine model of SE

Introduction

Epilepsy is a common neurological condition affecting approximately 1 to 2% of the population worldwide [1-3]. Acquired epilepsy (AE) results from a previous neurological insult such as stroke, TBI, or SE, and accounts for at least 40% of all epilepsy cases [1, 4-5]. In AE, a known injury leads to plasticity changes that transform normal brain tissue into a hyperexcitable brain tissue, giving rise to epilepsy. This phenomenon is called epileptogenesis [2, 7, 9].

Several studies have implicated Ca^{2+} as a major player in mediating many of the pathophysiological consequences associated with AE. Ca^{2+} is a ubiquitous second messenger that is highly regulated to maintain low intracellular concentrations. One study demonstrated using the in vivo rat pilocarpine model of SE-induced AE that Ca^{2+} dynamics are significantly altered during SE and following injury [37]. This study showed that hippocampal neurons acutely isolated from rats immediately following SE exhibited significantly elevated $[Ca^{2+}]_i$. Over time, the $[Ca^{2+}]_i$ gradually fell but reached a plateau that was significantly elevated 1 year after SE, demonstrating that this Ca^{2+} plateau is a long-lasting consequence of SE. As an important second messenger, Ca^{2+} plays a role in synaptic plasticity and gene expression. Thus, altered Ca^{2+} dynamics could have significant implications in terms of plasticity, leading to the development of AE. This study suggests that the Ca^{2+} plateau plays a role in the pathophysiology of

epileptogenesis. Therefore, modulating the rise in $[Ca^{2+}]_i$ immediately after SE may prevent the Ca^{2+}-mediated effects that lead to the development of AE.

Besides elevated $[Ca^{2+}]_i$, epilepsy resulting from neurological insult is also associated with the plasticity change called mossy fiber sprouting. Mossy fiber sprouting is a hallmark characteristic of temporal lobe epilepsy (TLE) and has been observed in both animal models and human cases of TLE [85-90]. It is a process where mossy fibers abnormally grow and form synapses with dentate granule cells in the inner molecular layer (IML) of the hippocampus [91-92], forming a recurrent excitatory circuit in the dentate gyrus [84, 94-95]. Although mossy fiber sprouting is not required for the development of AE, it is associated with an increase in both seizure frequency and duration [100-101]. Therefore, treatments following injury that block the development of mossy fiber sprouting may prevent the formation of this recurring excitatory synaptic circuit, thus reducing neuronal excitability.

One potential intervention is hypothermia. Hypothermia is used clinically to prevent neurological complications associated cardiac arrests [115-116], neonatal hypoxic-ischemia encephalopathy [117], TBI [118-119], and stroke [120-121]. The neuroprotective effects of hypothermia are not completely understood, but it is likely that protection is conferred through multiple mechanisms. One of these mechanisms is attenuating excitotoxic transmission and modulating intracellular Ca^{2+}. Studies have demonstrated that hypothermia reduces extracellular glutamate concentrations, thereby reducing NMDA receptor activation and thus, preventing the excessive influx of Ca^{2+} into the cell [144-145].

Few studies have been performed to evaluate the effects of hypothermia on epileptogenesis. One study demonstrated that mild hypothermia induced following moderate TBI decreased seizure susceptibility and reduced mossy fiber sprouting [146]. No studies have been performed to examine the potential of hypothermia in a model of SE-induced AE. Therefore, it will be clinically relevant to determine if hypothermia has any promise in preventing epileptogenesis and the development of AE.

The purpose of these studies was to determine the effects of hypothermia in the *in vivo* model of pilocarpine-induced SE. The studies evaluated the ability of hypothermia to block to development of the Ca^{2+} plateau and reduce the occurrence of mossy fiber sprouting. These studies also sought to determine if hypothermia induced following SE reduced or prevented the development of AE. There are currently no treatments available that can be administered following an injury to prevent the development of AE [103]. Therefore, developing a therapy that can be administered after an injury to prevent the development of AE is clinically important. Hypothermia may be a promising alternative to pharmacological agents.

Materials and Methods

Sprague–Dawley male rats (Harlan) weighing 200–250 g were used in strict accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals and approved by Virginia Commonwealth University's Institutional Animal Care and Use Committee. Animals were housed in single cages on a 12-h/12-h light/dark cycle and were provided food and water ad libitum.

Reagents

All the reagents were purchased from Sigma Chemical Co. (St. Louis, MO) unless otherwise noted.

Pilocarpine preparation

Prior to pilocarpine injections, rats were administered methyl scopolamine nitrate (1 mg/kg) intra-peritoneally (i.p.) to minimize peripheral, parasympathetic effects of pilocarpine treatment. The muscarinic agonist pilocarpine nitrate (375 mg/kg, i.p.) was administered 30 min later. Both scopolamine and pilocarpine were dissolved in 0.9% saline and filter-sterilized. Onset of SE typically occurred within 20–30 min after pilocarpine injection and was determined when the animal displayed continuous moderate-to-severe behavioral seizures characterized by forelimb clonus, rearing, and falling. Sixty minutes after the onset of SE, rats were administered diazepam (5 mg/kg, i.p.) followed by additional diazepam injections at 3 and 5 h after the onset of SE to control further seizure activity. Control groups were composed of naïve animals and sham control animals that received methyl scopolamine nitrate and diazepam injections only.

Induction of moderate hypothermia

Moderate hypothermia was rapidly induced by gently spraying rats with chilled ethanol (17°C) to speed the process of cooling. They were then placed in a cold room (5-8°C) for 8-10 minutes. Surface cooling methods were used because they are non-invasive and cost-effective. Core body temperature was determined every 2-3 min using a rectal probe (2100 Tele-thermometer; YSI, Inc., Yellow Springs, OH USA). Rectal temperature regarded clinically as a valid estimate of core temperature and is most often used in inducing hypothermia [157]. Once core temperatures

reached a 32-33°C, the rats were returned to their home cages in a room temperature environment. Core temperatures were continuously monitored and maintained between 30-33°C (moderate hypothermia range) with the intermittent use of ice packs and heating pads.

Acute isolation of hippocampal neurons

Hippocampal CA1 neurons were acutely isolated using methods established in our laboratory. Rats with injected with MK-801 (1 mg/kg i.p.) 15 min prior to anesthesia to inhibit NMDA receptor activation and increase neuronal call viability. Following anesthesia via isoflurane inhalation, the rats were decapitated and the brains rapidly removed, dissected, and placed in a 4°C oxygenated (95% O_2/5% CO_2) artificial cerebrospinal fluid (aCSF) [composed of (in mM) 201 sucrose, 10 glucose, 1.25 NaH_2PO_4, 26 $NaHCO_3$, 3 KCl, 7 $MgCl_2$, and 2 $CaCl_2$]. MK-801 (1 µm) was added to all solutions to increase neuronal viability and was removed 15 minutes prior to recording. Hippocampal slices of 450 µm were cut with a vibratome sectioning system (Series 2000, Technical Products International, St. Louis, MO) and placed in an oxygenated dissociation medium [composed of (in mM) 13 NaCl, 10 glucose, 1.25 NaH_2PO_4, 26 $NaHCO_3$, 3 KCl, 7 $MgCl_2$, and 0.2 $CaCl_2$] for 30 min. The CA1 region was then visualized with a dissecting microscope and 1 mm^2 thick slices were excised in the presence of an oxygenated PIPES buffer medium [containing (in mM) 120 NaCl, 25 glucose, 20 piperazine-*N, N'*-bis[2-ethanesulfonic acid] (PIPES), pH adjusted to 7.2 with NaOH]. Slices were then treated with 8 mg/ml Protease XIV (Sigma Chemical Co.) in PIPES-containing medium for 6-8 minutes and then thoroughly rinsed. The tissue preparations were then triturated in the PIPES-containing medium containing the calcium indicator Fura-2-AM at 4°C. During the trituration process, the hippocampal slices were passed through a series of Pasteur pipettes of decreasing diameter, resulting in an even

suspension of acutely isolated CA1 hippocampal cells plated on poly-l-lysine and Cell-Tak (BD Biosciences, Franklin Lakes, NJ) coated 2-well chambers allowing appropriate adherence for Ca^{2+} imaging. The culture dishes were immediately placed in a humidified oxygenated dark chamber at 37°C (5% CO_2/95% air atmosphere) and allowed to incubate for 30 minutes. The Fura-2 was then washed off using the PIPES buffer without the presence of MK-801 and allowed to incubate for an additional 15 minutes to allow the cellular esterases to cleave the dye from its AM moiety.

Calcium microfluorometry

The calcium indicator Fura-2-AM (1μM) was used to measure $[Ca^{2+}]_i$ in acutely isolated neurons. Cells were loaded with Fura-2-AM in a PIPES buffer and allowed to incubate for 30 min at 37°C in 5% CO_2/95% air atmosphere. The cells were then washed to terminate the dye loading and allowed to incubate for an additional 15 min to maximize esterase cleavage of AM moiety.

Neurons were visualized on an inverted microscope (Olympus IX 70, Olympus America, Melville, NY, USA) using a 20x, 0.7 numerical aperture, fluorite water-immersion objective (Olympus America) maintained at 37°C with a temperature-controlled stage (Harvard Apparatus Inc., Holliston, MA, USA) maintained at 37°C. Fura-2 was excited with a 75-W xenon arc lamp (Olympus America) with alternating wavelengths of 340 and 380 nm filtered through a Sutter Filter Wheel (Sutter Instruments Co., Novato, CA USA). Fluorescent emission at 510 nm was captured through a Fura filter cube (Olympus America) with a dichroic mirror at 400 nm using a Hamamatsu ORCA-ER camera (Hamamatsu Photonics, Japan).

MetaFluor (Molecular Devices, Downington, PA, USA) was used to control image acquisition and processing. Specific regions of interest were designated for each neuron in the microscopic field. Ratio measurements for individual neurons were taken at 5 s intervals for 30 s. Image pairs were captured and corrected for non-specific background fluorescence by subtracting images acquired from non-Fura-loaded plates. Individual neurons from multiple experiments were pooled to calculate mean and ±SEM. The resulting 340/380 ratios correspond directly to the total concentration of Ca^{2+} inside the cell.

Video monitoring

Rats were monitored for spontaneous behavioral seizure activity using multi-camera video acquisition system (GeoVision, Inc., Irvine, CA). Rats were monitored at various time points following SE for 5 days at a time for the presence of spontaneous behavioral seizures. Spontaneous seizures are characterized by unprovoked behavior characteristic of seizure activity that includes forelimb clonus, rearing, and falling.

Timm stain

Rats were briefly anesthetized by isoflurane prior to injection of a ketamine/xylazine cocktail (75 mg/kg/7.5 mg/kg; i.p.). Once fully anesthetized, rats were transcardially flushed with saline, then perfused with 1.2% sodium sulfide and 4% paraformaldehyde in a 0.1 M phosphate buffer (pH 7.4). Perfusion was continued until full body fixation was observed. Brains were removed and post-fixed in the same fixative. The brains were then placed in a 30% sucrose solution in sodium phosphate buffer for cryoprotection for 3-4 days until fully saturated. Brains were frozen at -80°C until sliced. 40 µm coronal sections were cut on a Leitz cryostat (Leica Microsystems,

Wetzlar, Germany) maintained at -20°C and mounted onto gelatin-subbed slides and allowed to air dry.

Timm staining was used to detect mossy fiber sprouting due to its ability to label intracellular zinc located in the axon terminals of mossy fibers. The method of Timm staining used was adapted from Mello et al. [89]. Mounted sections were immersed in developing solution consisting of 180 mL 50% gum arabic and 30 mL aqueous solution of 7.65 g citric acid and 7.05 g sodium citrate to which 5 g hydroquinone (dissolved in 90 mL water) and 1.5 mL 15% silver nitrate aqueous solution were added in darkness just prior to use. The slides were developed in the dark for 30 min, and then washed in distilled water for 5 min in the dark room. Slides were removed from the dark room, washed again in distilled water, dehydrated and cover-slipped.

Subjective scoring was adapted from Tauck and Nadler [94]. Sections were assigned a score from 0 to 3. Scoring was performed by a reviewer blind to the treatment groups. A score of 0 indicated no presence of mossy fiber sprouting in the IML of the dentate gyrus; a score of 1 indicated light and scattered staining in the IML; a score of 2 indicated patches of heavy staining or a continuous band of intermediate intensity in staining in the IML; and a score of 3 indicated a dense, continuous band of mossy fiber staining in the IML.

Electrode implantation and EEG monitoring

Under general anesthesia (5% isoflurane), rats were stereotaxically implanted with three skull surface electrode screws with teflon-insulated stainless steel wire (Plastics One, Roanoke, VA). Electrode screws were positioned through burr holes above the right and left frontal cortices

(anteroposterior, 3 mm, and mediolateral, ±3 mm from bregma). A third surface electrode screw was positioned over the cerebellum to serve as reference, and two additional (non-electrode) skull screws were inserted for structural support. The electrode screws were seated to contact but not penetrate the dura mater. Female amphenol terminals connected to the electrode wire were seated into an electrode pedestal (Plastics One), and was secured to the skull with Cerebond adhesive (Plastics One). Rats were allowed 1 week of recovery time before the start of the EEG monitoring. Wire leads were securely connected into the threaded electrode pedestal on the rat and connected to an electrical-swivel commutator (Plastics One) to allow for free movement of the rat while maintaining continuity of EEG signals. EEG signals were amplified using a Grass model 8-10D (Grass Technologies, West Warwick, RI) and digitized with Powerlab 16/30 data acquisition system (AD Instruments, Colorado Springs, CO). Evaluation of digitally acquired EEG was performed with Labchart (AD Instruments). EEG acquisition occurred simultaneously with video surveillance.

Electrographic seizures were identified by having discrete epileptiform events characterized by episodes of high frequency (more than 2 per second) and increased voltage multi-spike complexes and/or synchronized spike or wave activity lasting for 10 seconds or longer. Interictal spikes or epileptiform discharges that lasted less than 10 seconds were not included.

Seizure severity classification

Behavioral seizures were scored using the Racine scale for seizure severity. Seizures were assigned a score between 0 and 5 based on the observed characteristics. A score of 0 indicates no detectable seizure activity; a score of 1 is assigned for behavioral such as immobility and facial

clonus; a score of 2 for head nodding, rigid posture, and tail extension; a score of 3 for repetitive movements and forelimb clonus; a score of 4 for rearing, forelimb clonus with rearing, and rearing and falling; and a score of 5 for continuous rearing and falling and general tonic-clonic seizure activity. Scoring was performed by a reviewer blind to the treatment condition.

Data analyses

Data was statistically analyzed using SigmaStat and graphed using SigmaPlot (Systat Software, San Jose, CA). To determine statistical significance, student's t-test or one-way analysis of variance (ANOVA) were used followed by post-hoc Tukey test when appropriate. A p-value<0.05 was considered statistically significant. Data is presented as mean ±SEM.

Results

In order to determine the effects of hypothermia on the Ca^{2+} plateau, mossy fiber sprouting, and the development of AE, hypothermia was induced 1 hr post-SE and was maintained for 4 h. Animals were classified as either pilocarpine, hypothermia, or control. Pilocarpine and hypothermia rats received pilocarpine, scopolamine, and diazepam injections. Hypothermia rats received hypothermia following SE, whereas rats in the pilocarpine group did not receive hypothermia intervention. Control rats did not receive pilocarpine or hypothermia. There was no difference between naïve controls and sham controls, nor was there a difference in controls compared to naïve rats who received hypothermia (results not shown). Therefore, control group rats are naïve controls.

Pilocarpine-induced SE caused significant elevations in $[Ca^{2+}]_i$

The Ca^{2+} indicator Fura-2AM was used to determine $[Ca^{2+}]_i$ following SE. Alternating excitation wavelengths of 340 nm and 380 nm produced fluorescent ratio values. The 340/380 ratios correspond directly to $[Ca^{2+}]_i$. Therefore, the data is presented as a ratio of 340/380 nm. Similar to previously published results from our laboratory [37], hippocampal neurons isolated 24 h after pilocarpine-induced SE (n=13) exhibit significant elevations in $[Ca^{2+}]_i$ compared to naïve controls (n=13). The average ratio values increased from 0.65±0.04 in controls neurons to 0.86±0.07 in neurons isolated from pilocarpine-treated rats (Fig 3-1). The ratios obtained from pilocarpine-treated rats were significantly higher than those obtained from naïve controls. The results confirm that pilocarpine-induced SE results causes significant elevations in $[Ca^{2+}]_i$ 24 h after SE.

Hypothermia prevented the development of the Ca^{2+} plateau

We tested whether moderate hypothermia (31-33°C) was able to lower hippocampal neuronal $[Ca^{2+}]_i$ when induced 1 h after SE and maintained for 4 h. Hippocampal neurons were acutely isolated 24 h after SE to evaluate $[Ca^{2+}]_i$. As previously stated, pilocarpine-induced SE causes a rise in $[Ca^{2+}]_i$. Hypothermia appears to block this elevation in $[Ca^{2+}]_i$. Neurons isolated from hypothermia-treated rats (n=12) exhibit an average ratio value of 0.65±0.07, which is the similar to control neurons. This value is significantly lower than ratios obtained from pilocarpine-alone treated groups (one-way ANOVA, $p < 0.05$) (Fig 3-2). Therefore, hypothermia induced following SE appears to block the development of the Ca^{2+} plateau.

Hypothermia reduced the occurrence of spontaneous behavioral seizures

Rats from both pilocarpine (n=9) and hypothermia groups (n=16) were monitored using video surveillance over a period of 5 consecutive days for the presence of spontaneous behavioral seizures, or epilepsy. Monitoring took place at 3 months and 1 year post-SE. Rats experiencing spontaneous behavioral seizures exhibit unprovoked behavior characteristic of seizure activity that includes forelimb clonus, rearing, and falling.

At 3 months post- SE, 33.3% (±16.67) of rats from the pilocarpine group exhibited spontaneous behavioral seizures. In comparison, only 18.75% (±10.08) of the rats that received hypothermia experienced this spontaneous seizure activity. Although statistical significance was not found (Student's t-test, p=0.43), hypothermia caused a 43.7% reduction on the occurrence of epilepsy at 3 months after injury (Fig 3-3).

The rats were re-monitored at 1 year. At this time point, 66.9% (±16.6) of the rats in the pilocarpine group were classified as epileptic. This rate of AE is similar to the rate in previous studies using the pilocarpine model of AE. Of the rats who received hypothermia following SE, only 31.25% (±11.9) exhibited spontaneous seizures (Fig 3-4). Although the results were not statistically significant (Student's t-test, p=0.16), hypothermia caused over a 50% reduction in the development of AE following SE.

Hypothermia reduced mossy fiber sprouting

After animals were monitored for seizure activity at the 1 yr post-SE time point, they were sacrificed for the purpose of histological stains. Timm stains were performed on slices from each

group to detect for the presence of mossy fiber sprouting. Slices from each animal were assigned a score ranging from 0 to 3 based on the degree of Timm staining present. The scorer was blind to the treatment group. A full explanation of the scoring guidelines can be found in the Materials and Methods section (page 67).

Of the animals in the pilocarpine group (n=7), 85.7% demonstrated the presence of mossy fiber sprouting (Fig 3-5). The average score of Timm staining intensity was 2.43±0.43, indicating that pilocarpine-induced SE led to robust mossy fiber sprouting in the majority of the animals. In contrast, only 28.6% of rats in that received hypothermia following SE (n=7) exhibited any degree of Timm staining. The average score in intensity of Timm staining from this group was 0.8571 ± 0.5530 (Fig 3-6). The average score for the hypothermia group was significantly lower than the average score from the pilocarpine group (t-test, $p < 0.05$). Figure 3-7 illustrates a representative slice of the mossy fiber sprouting present in a pilocarpine rat compared to the lack of sprouting observed in a control and hypothermia treated rat. A distinct dark line is visible in IML in the slice obtained from a pilocarpine-treated rat, which indicates the presence of mossy fiber sprouting. This dark staining is not present in the slices obtained from control and hypothermia rats. These results demonstrate that hypothermia reduces the occurrence of mossy fiber sprouting following SE.

Inducing hypothermia earlier was more protective

The window of opportunity following an injury is unclear, but it is generally agreed upon that earlier intervention provides more protection. The goal of this study was to determine if inducing hypothermia 30 min earlier provided more protection against the development of AE. In this

study, hypothermia was induced 30 min post-SE instead of 1 h post-SE and was maintained for 4.5 h. The rats were monitored for 5 consecutive days at 3 months and 1 year post-SE for the presence of spontaneous behavioral seizures.

At 3 months post-SE, 30.7% (±13.32) of pilocarpine-treated rats (n=13) exhibited spontaneous seizures, which is similar to the rate seen in our previous group (33.3% ±16.67) at this time point (Fig 3-8). No rats receiving hypothermia (n=10) in this group demonstrated spontaneous behavioral seizures at this time point. The difference in the development of AE at 3 months was statistically significant (Student's t-test, $p<0.05$).

To determine if this hypothermia protocol delayed the development of or abolished AE, rats were re-monitored at 1 yr post-SE. At this time point, 76.9% (±12.16) of the rats in the pilocarpine group were epileptic, which is not significantly different from the rate of epilepsy in the previous group or previously published results. In contrast, only 20.0% (±13.33) of the rats that received hypothermia 30 min post-SE exhibited spontaneous behavioral seizures (Fig 3-9). In this group, hypothermia significantly reduced the occurrence of spontaneous behavioral seizures (t-test, $p <0.05$). These results suggest that earlier induction of hypothermia is more protective against the development of AE. In addition, the results also suggest that earlier intervention with hypothermia delays the development of AE.

Hypothermia reduced the severity of seizures

Video surveillance only allows us to detect behavioral seizures; seizure activity may still be present but not easily visible. However, EEG analysis allows us to detect seizure activity that is

not normally visible during video monitoring. Thus, animals from both pilocarpine and hypothermia groups were implanted with electrodes for EEG analysis. EEG monitoring occurred simultaneously with video surveillance to allow us to analyze the behavioral characteristics of each seizure and grade the seizure intensity. Animals were implanted with electrodes and allowed a minimum of one week to recover before EEG monitoring began. Rats were monitored for a period of five consecutive days. Seizure activity was detected on the EEG and compared with video surveillance to score the behavior. A representative trace of baseline and seizure activity can be found in Fig 3-10.

Seizures were graded using the modified Racine scale, which ranges from a score of 0 for no observed seizure activity to a score of 5 for severe seizure activity. Rats in the pilocarpine group (n=4) had an average Racine score of 3.75 (±0.25), indicating that pilocarpine treatment led to the development of severe seizures. Rats treated with hypothermia 30 min following SE (n=7), however, had an average Racine score of 2.14 (±0.26) which is significantly lower than the pilocarpine group (t-test, $p<0.005$) (Fig 3-11). Figure 3-12 illustrates the distribution of the scores. The rats treated with hypothermia received a score between 1 and 3, with the majority of the animals receiving a score of 2. However, rats in the pilocarpine group exhibited more severe seizures, with the majority of the animals receiving a score of 4. The results demonstrate that hypothermia treatment after SE reduces the severity of seizures in epileptic rats. Reducing the severity of seizures in epileptic patients vastly improves their quality of life. Therefore finding a treatment that improves seizure intensity would be especially useful in epilepsy patients who are refractory to current AEDs.

Hypothermia reduced seizure frequency

High seizure frequency is also associated with reduced qualify of life for epileptic patients. Therefore reducing the frequency of seizures is a necessary goal. Seizure frequency describes the average number of seizures observed per day over a 5 day monitoring period. Seizure frequency is illustrated in Fig 3-13. Animals in the pilocarpine group exhibited an average seizure frequency of 15.75 (±3.68) per day. Rats treated with hypothermia displayed a reduced frequency of seizures, with an average seizure frequency of 9.6 (±2.14) seizures per day. Fig 3-14 illustrates the distribution of the frequency. The majority of the rats treated with hypothermia exhibited between 6 and 10 seizures per day and never more than 16 seizures per day. However, the majority of the rats in the pilocarpine displayed at least 17 seizures per day. Although the results were not statistically significant (Student's t-test, $p=0.21$), these results demonstrate a trend in decreasing seizure frequency with hypothermia treatment after SE.

Discussion

The results of this study demonstrate that hypothermia administered following SE is effective at blocking the development of the Ca^{2+} plateau and reduces the development of AE. Moderate hypothermia induced 1 h after SE was able to block the rise $[Ca^{2+}]_i$ that is typically observed following pilocarpine-induced SE as well as reduce the rate of epilepsy at 1 yr post-SE. These results confirm the similar observation that hypothermia blocks the Ca^{2+} plateau in the in vitro model of SE. Furthermore, these studies demonstrated that hypothermia reduced the formation of mossy fiber sprouting, a plasticity change commonly associated with SE-induced AE. These results provide evidence that hypothermia is a beneficial intervention in preventing epileptogenesis.

It has been demonstrated in the pilocarpine model of SE-induced AE that SE caused a significant rise in $[Ca^{2+}]_i$ and these elevations persist in animals that eventually develop epilepsy [37], which suggests that the Ca^{2+} plateau is involved the progression of epileptogenesis to the epileptic phenotype. Blocking the formation of the Ca^{2+} plateau with the use of NMDA receptor antagonists prior to and during SE prevents the development of AE [37, 42], further suggesting that elevations in $[Ca^{2+}]_i$ are involved in the pathophysiology associated with epileptogenesis. However, NMDA antagonists are ineffective at preventing epilepsy if administered after the injury. Currently, there are no anti-epileptogenic drugs that can be administered following a neurological insult [103]. Therefore, it is clinically important to develop a therapy that can block epileptogenesis when administered post-injury.

Hypothermia has been shown to attenuate Ca^{2+} influx by reducing the activation of NMDA receptors by reducing extracellular glutamate concentrations [144-145]. A previous study also demonstrated using the in vitro model of SE that hypothermia rapidly reduced elevated $[Ca^{2+}]_i$ and prevented the formation of the Ca^{2+} plateau (see Chapter 2 Results). This current study provided novel evidence that hypothermia induced 1 hour after pilocarpine-induced SE blocks the formation of the Ca^{2+} plateau. Ca^{2+} is a common second messenger responsible for initiating an array of effects ranging from neurotransmission to plasticity changes to neuronal death. Blocking the rise in $[Ca^{2+}]_i$ following injury is a major aim for preventing the development of epileptogenesis and AE. Therefore developing new treatment strategies that target the Ca^{2+} plateau and inhibit the Ca^{2+}-mediated second messenger effects associated with epileptogenesis remains an important clinical goal.

Hypothermia is a promising therapeutic alternative. It has been shown to reduce neurological injuries associated with cardiac arrest [115-116], ischemic stroke [120-121], and TBI [118-119]. However, very few studies have evaluated the potential benefits of hypothermia following SE. Although they have different inciting injuries, stroke, TBI, and SE share the common pathology of elevated $[Ca^{2+}]_i$ and the eventual development of epilepsy [9]. Therefore it stands to reason that hypothermia would provide similar neuroprotection after SE as it does with stroke and TBI. The results of these studies indicate that hypothermia induced 1 hour after SE was protective against the development of epilepsy. Animals were monitored up to one year post-injury. The results demonstrated that hypothermia reduced the percentage of animals exhibiting spontaneous behavioral seizures, or epilepsy, thus providing the first line of evidence that hypothermia may prevent SE-induced AE.

In addition to elevated $[Ca^{2+}]_i$, mossy fiber sprouting is commonly seen in animal models of stroke, TBI, and SE, as well as in human cases of TLE [85-90]. This form of synaptic plasticity is believed to produce a recurrent excitatory circuit in the dentate gyrus of the hippocampus, which enhances neuronal excitability and may lead to the development of AE [84, 94-95]. Preventing the abnormal sprouting of mossy fibers may prevent the excitatory circuitry that leads to a hyperexcitable neuronal population, thus preventing epileptogenesis. One study demonstrated that hypothermia reduced mossy fiber sprouting in the animal model of TBI. In addition, seizure susceptibility was attenuated [146]. In these studies, hypothermia induced 1 hour after SE was effective at reducing the development and severity of mossy fiber sprouting. These findings suggest the hypothermia is effective at reducing a major plasticity change associated with epileptogenesis. One possible way that hypothermia reduces the risk of

developing epilepsy may be by preventing the aberrant sprouting of mossy fibers, thereby blocking the formation of recurrent excitatory synapses and this reducing the risk of epilepsy.

The therapeutic window of opportunity is an important consideration when developing new treatment strategies. The period between the initial neurological insult and the development of epilepsy is called the latency period. This time frame represents the potential window of opportunity to inhibit the plasticity changes associated with epileptogenesis. The duration of this window is unclear and differs among various neurological injuries, but it is generally agreed among clinicians and researchers that earlier hypothermia intervention produces a greater degree of neuroprotection. Therefore, we evaluated the effects of earlier hypothermia induction. Hypothermia was induced 30 min after the start of SE instead of 1 hr post-SE. The earlier intervention resulted in a greater degree of protection against the development of epilepsy. There was a significant reduction in the percentage of rats displaying spontaneous behavioral seizures. In addition, earlier hypothermia appeared to delay the appearance of spontaneous seizures based on the results that no rats exhibited spontaneous seizures at 3 months post-SE compared to the group that received hypothermia 1 hr after SE, in which 19% exhibited seizure activity. These results offer promising evidence that hypothermia is effective at reducing epileptogenesis.

The seizure activity of the hypothermic rats that developed epilepsy was analyzed to determine if hypothermia had any effect on the severity and frequency of the seizures. Seizures were detected using EEG analysis and analyzed for behavioral seizure characteristics which were scored using the Racine scale. The results indicate that hypothermia significantly reduced the severity of the seizures. The epileptic rats that did not receive hypothermia following SE exhibited severe

seizures characterized by rearing, falling, and general tonic-clonic activity. In comparison, the rats that received hypothermia did not display these robust seizures. Instead, the majority of their seizures were characterized by mild forelimb clonus and facial contractions that were often difficult to detect visually. In addition, the frequency of seizures per day was reduced in the animals that received hypothermia following SE. The animals in the pilocarpine experienced a seizure on average of once an hour during the wake cycle. The rats that received hypothermia exhibited far less seizures per day. These results demonstrate that not only is hypothermia capable of reducing the development of AE, it is effective at reducing the severity and frequency of seizures in rats that do develop epilepsy. Reducing seizure severity and frequency would provide a greater quality of life for patients that do develop epilepsy.

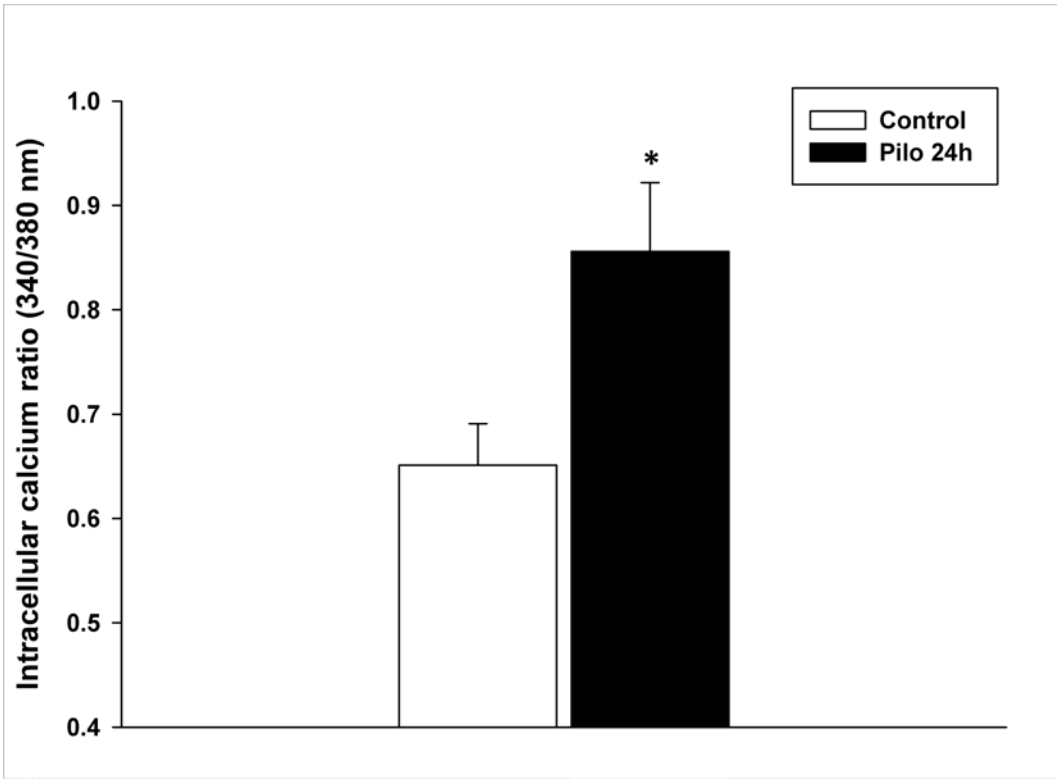

Figure 3-1. Pilocarpine caused significant elevation in $[Ca^{2+}]_i$ 24 h post-SE. (A) Hippocampal neurons isolated from control rats (n=13) exhibited 340/380 ratio values of 0.65±0.04. Neurons acutely isolated from pilocarpine rats 24 h post-SE (n=13) exhibited significantly elevated ratios of 0.86±0.07 (data presented as mean ± SEM). *p<0.05, Student's t-test.

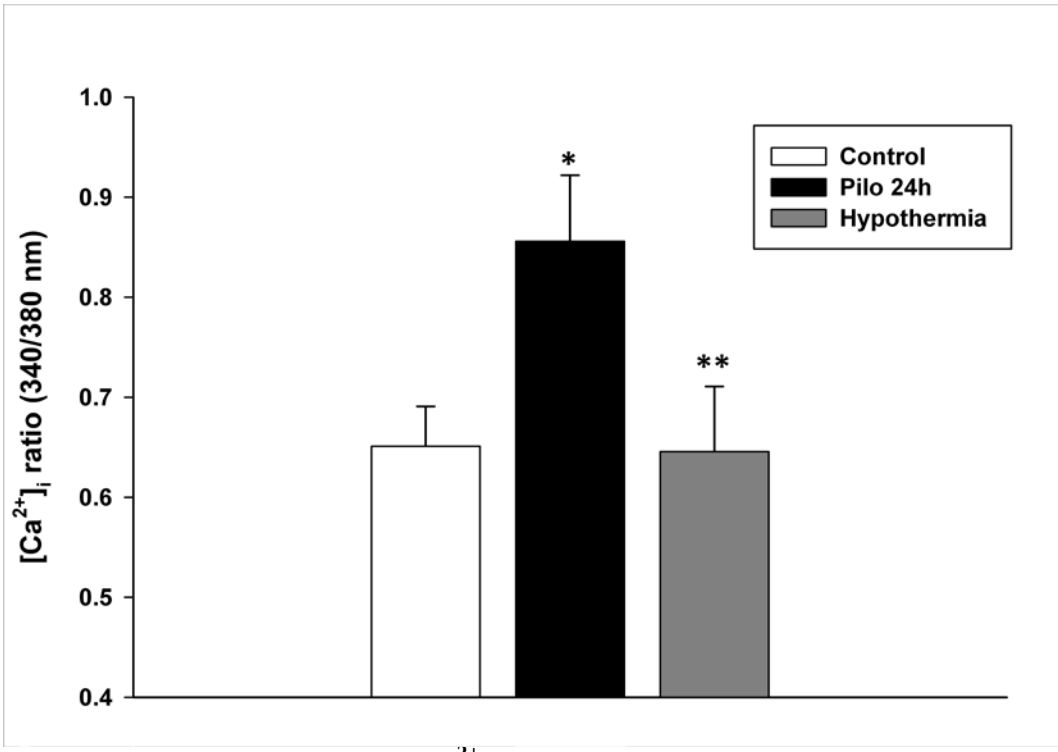

Figure 3-2. Hypothermia blocked the Ca^{2+} plateau following SE in vitro. (A) Hippocampal neurons acutely isolated from rats 24 h after hypothermia (n=12) was induced post-SE displayed 340/380 ratio values of 0.65±0.07, which were similar to control neurons (n=13). These values were significantly lower than the ratio values of neurons isolated from rats in the pilocarpine group (0.86±0.07). Data presented as mean ratio ± SEM. No significant difference observed between neurons acutely isolated from control and hypothermia-treated rats. *$p<0.05$ compared to control, **$p<0.05$ compared to pilocarpine, one-way ANOVA followed by post-hoc Tukey analysis

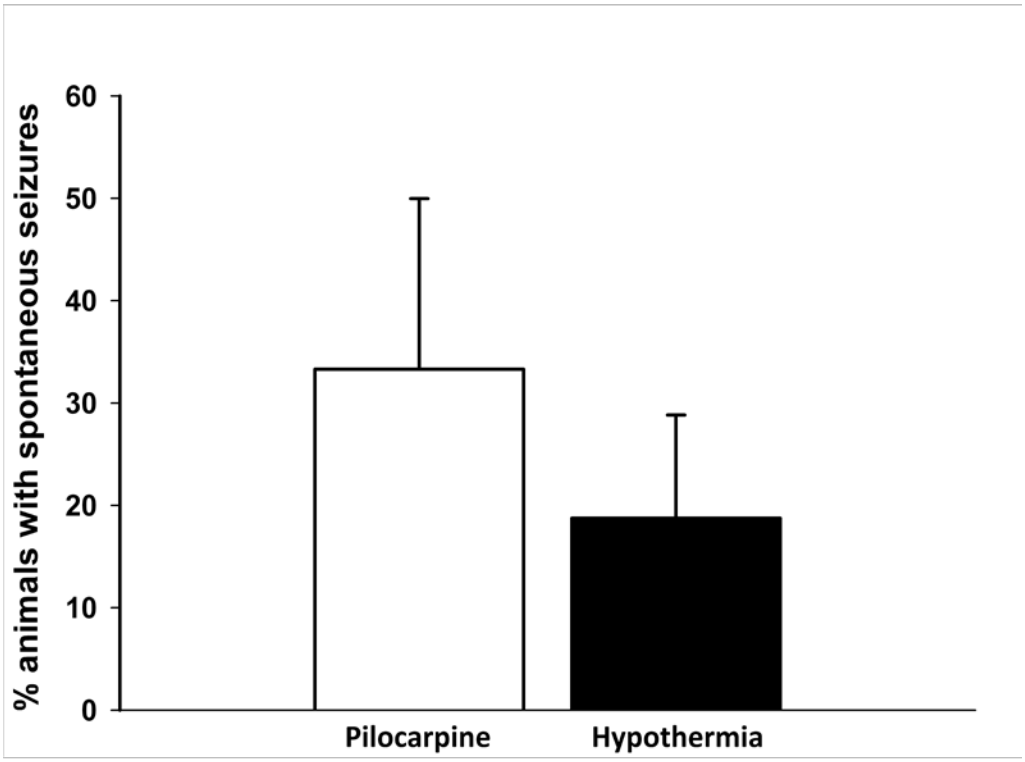

Figure 3-3. 4h of hypothermia reduced development of epilepsy 3 months post-SE.
Behavioral monitoring demonstrated that 33.3% ±16.67 of the rats in the pilocarpine group (n=9) exhibited spontaneous behavioral seizures compared to only 18.75% ±10.08 of the rats who received hypothermia 1 hour post-SE (n=16). Data presented as percent epileptic ± SEM. Results not statistically significant (student's t-test).

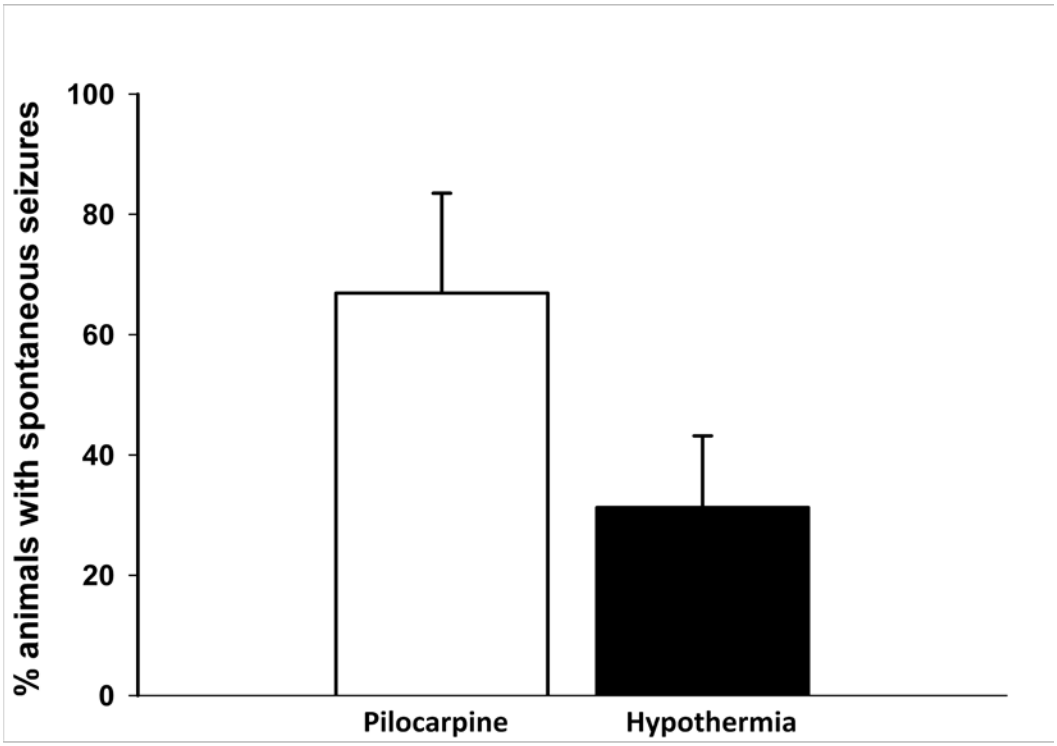

Figure 3-4. 4 h of hypothermia reduced development of epilepsy 1 yr post-SE. At one year post-SE, 66.9% ±16.6 of rats in the pilocarpine group (n=9) exhibited spontaneous behavioral seizures. Rats who received hypothermia 1 hour post-SE (n=16) displayed a lower rate of 31.25% ±11.9. Data presented as percent epileptic ± SEM. Results not statistically significant (student's t-test).

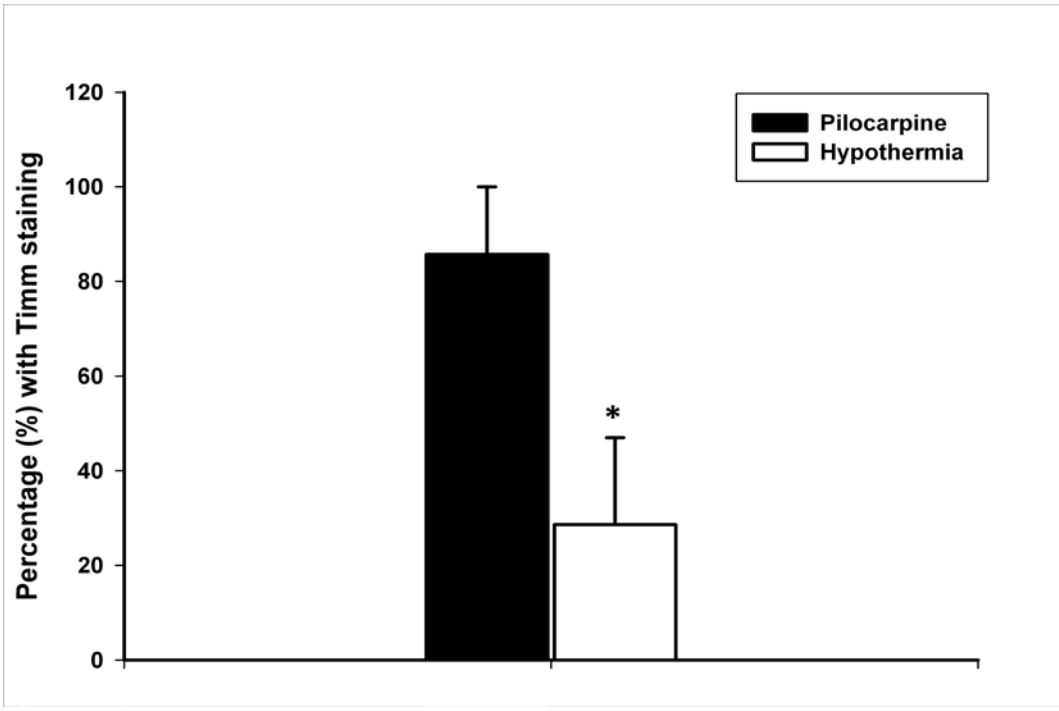

Figure 3-5. Hypothermia reduced the occurrence of mossy fiber sprouting. At one year post-SE, 85.7% ±14.3 of pilocarpine rats (n=7) displayed Timm staining. Rats that received hypothermia 1 hr post-SE (n=7) displayed a significantly lower rate with 28.6% ±18.4 of the rats exhibiting any degree Timm staining. Data presented as percent displaying Timm staining ± SEM. *$p<0.05$, Student's t-test.

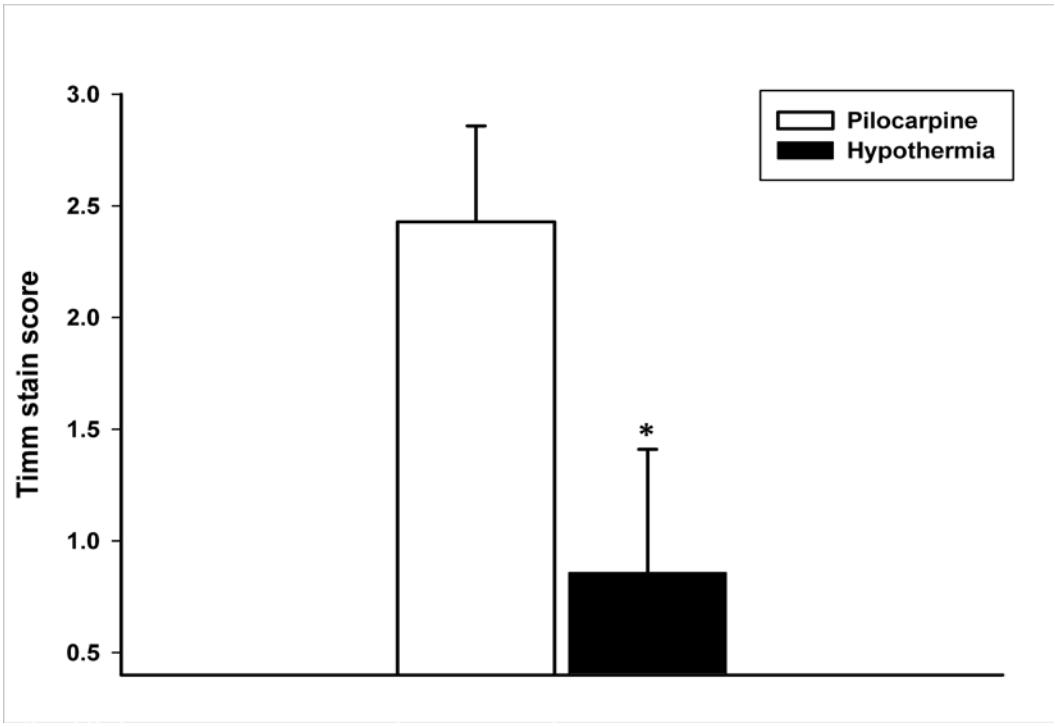

Figure 3-6. Hypothermia reduced the intensity of mossy fiber sprouting. Timm staining intensity was scored on a scale of 0 to 3. A score of 0 was assigned for no staining present; a score of 1 for light and scattered staining in the IML; a score of 2 for darker but patchy staining in the IML; and a score of 3 for continuous and robust staining in the IML. Rats in the pilocarpine group (n=7) had an average score 2.43 ± 0.43, which was significantly higher than the average score of 0.86 ± 0.55 in the hypothermia group (n=7). Data presented as mean score ± SEM. *$p < 0.05$, Student's t-test.

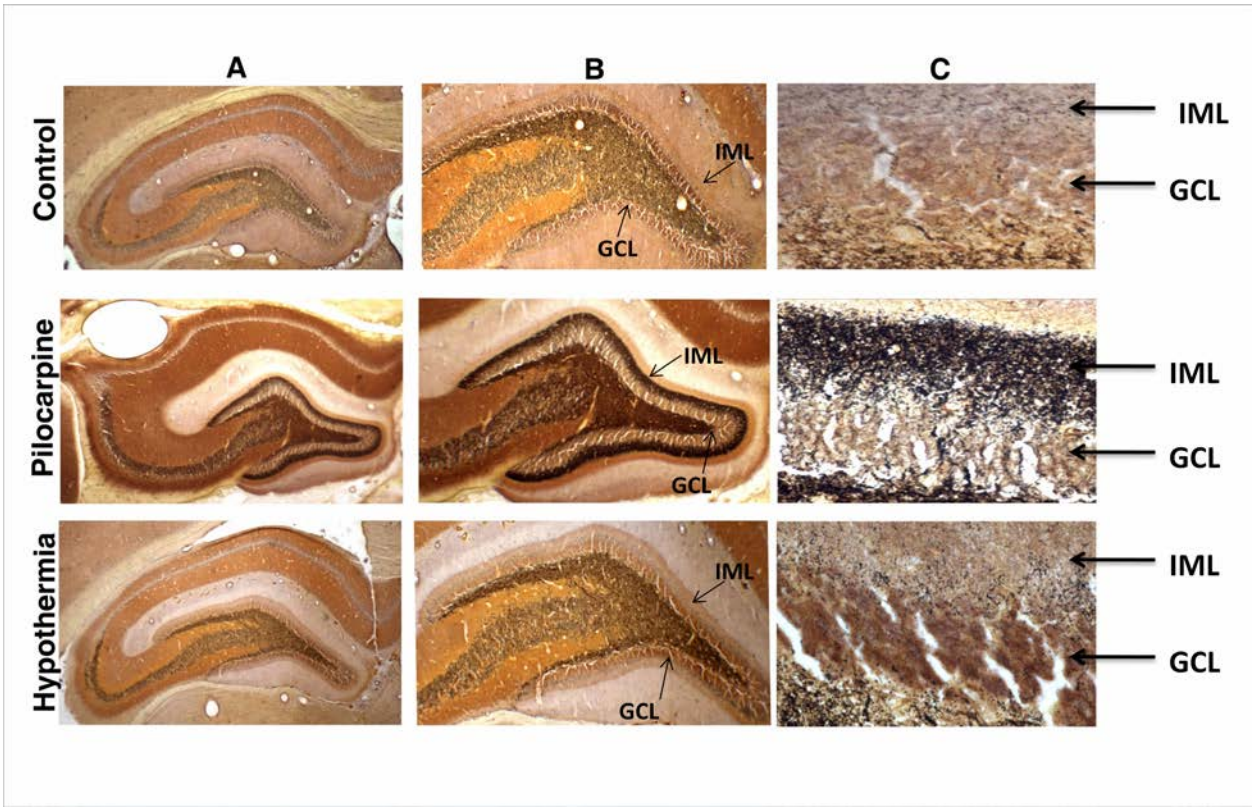

Figure 3-7. Timm stain images. Representative images of Timm staining in slices from control (top row), pilocarpine (middle row), and hypothermia rats (bottom row). Column A depicts the hippocampus (3X magnification). Column B depicts the dentate gyrus (10X magnification). Column C represents a high magnification image of the mossy fiber sprouting (40X magnification). Mossy fiber sprouting is not present in the IML of control or hypothermia slices, but robust sprouting is present in the IML of the pilocarpine slice. *IML*, inner molecular layer, *GCL*, granule cell layer.

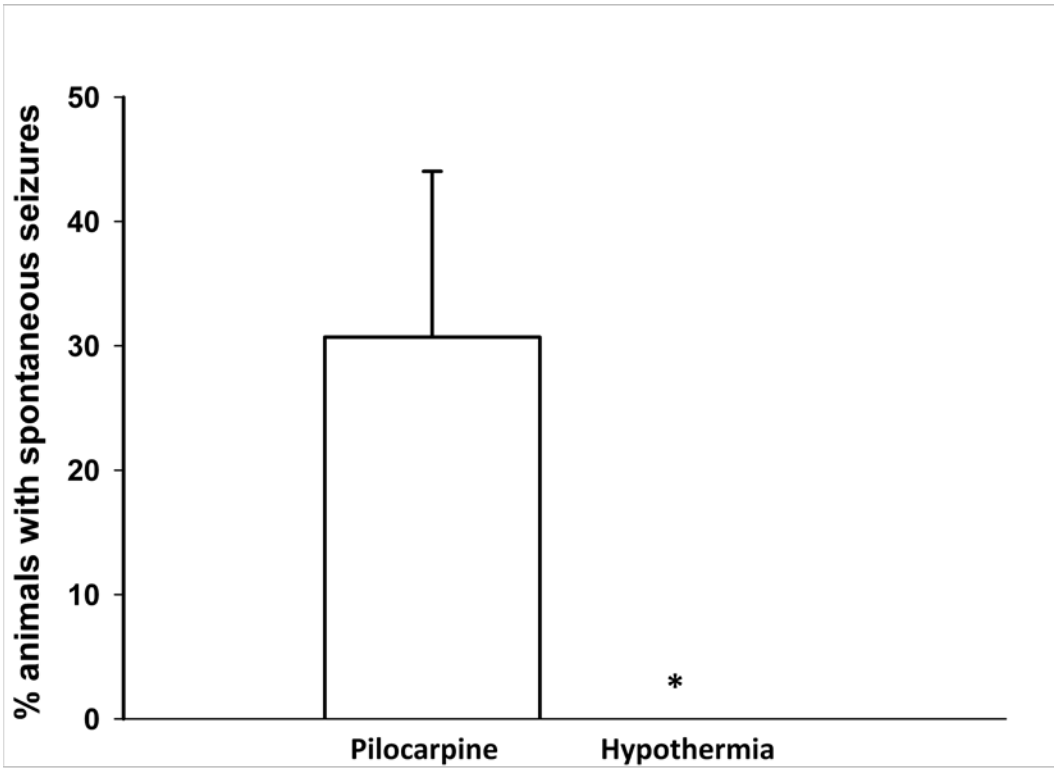

Figure 3-8. Earlier induction of hypothermia significantly reduced development of epilepsy, 3 months post-SE. At 3 months post-SE, 30.7% ±13.32 of the rats in the pilocarpine group (n=13) displayed spontaneous behavioral seizures. This was significantly higher than the rate of 0% in the animals treated with hypothermia 30 min post-SE (n=10). Data presented as percent epileptic ± SEM. *$p<0.05$, Student's t-test.

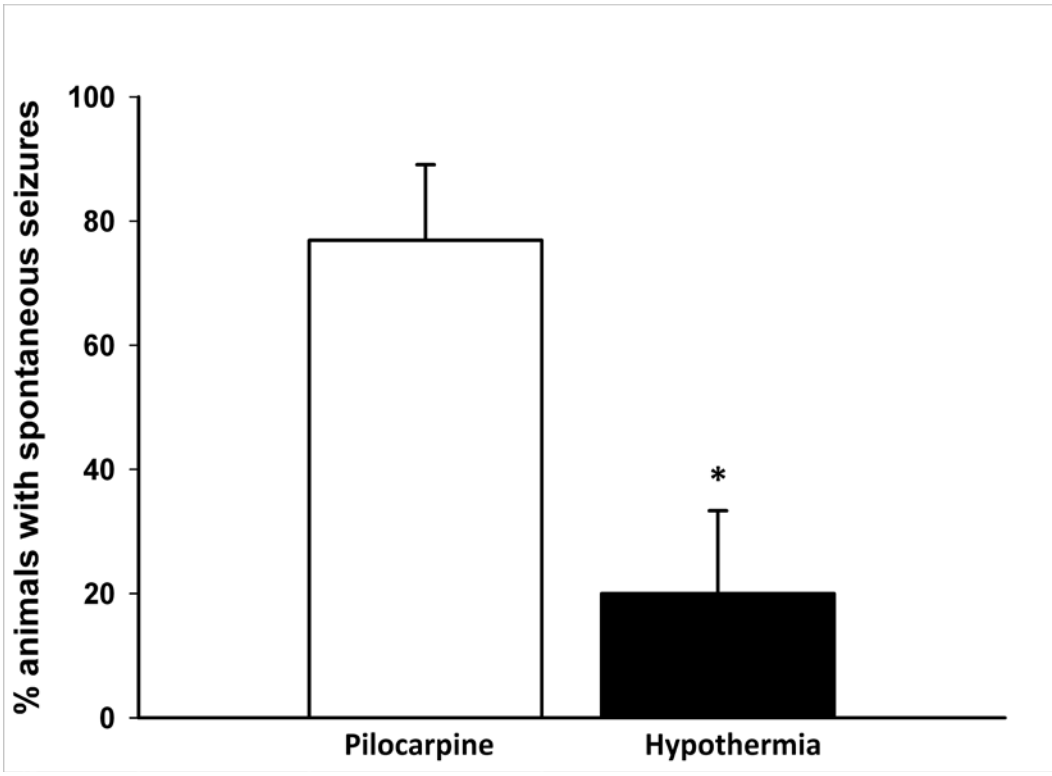

Figure 3-9. Earlier induction of hypothermia significantly reduced development of epilepsy, 1 yr post-SE. At 1 yr post-SE, 76.9% ±12.16 of the rats in the pilocarpine group (n=13) displayed spontaneous behavioral seizures. This was significantly higher than the rate of 20.0% ±13.33 in the animals treated with hypothermia 30 min post-SE (n=10). Data presented as percent epileptic ± SEM. *$p<0.05$, Student's t-test.

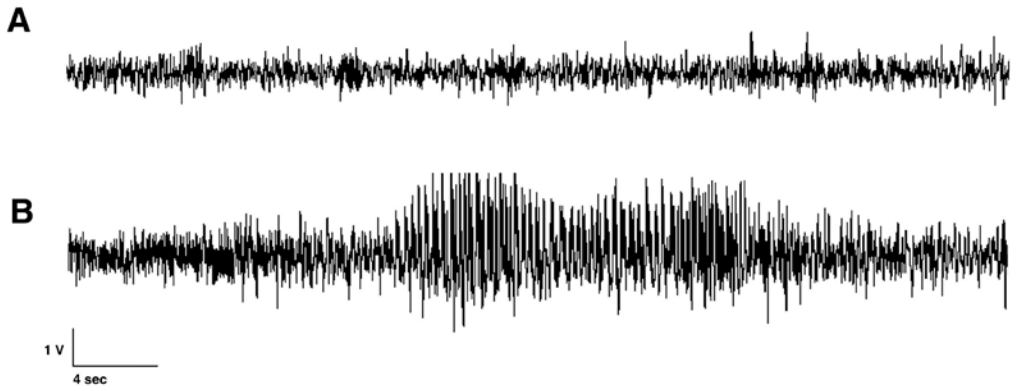

Figure 3-10. Representative EEG trace. (A) EEG trace from baseline does not exhibit seizure activity. **(B)** EEG trace representing seizure activity.

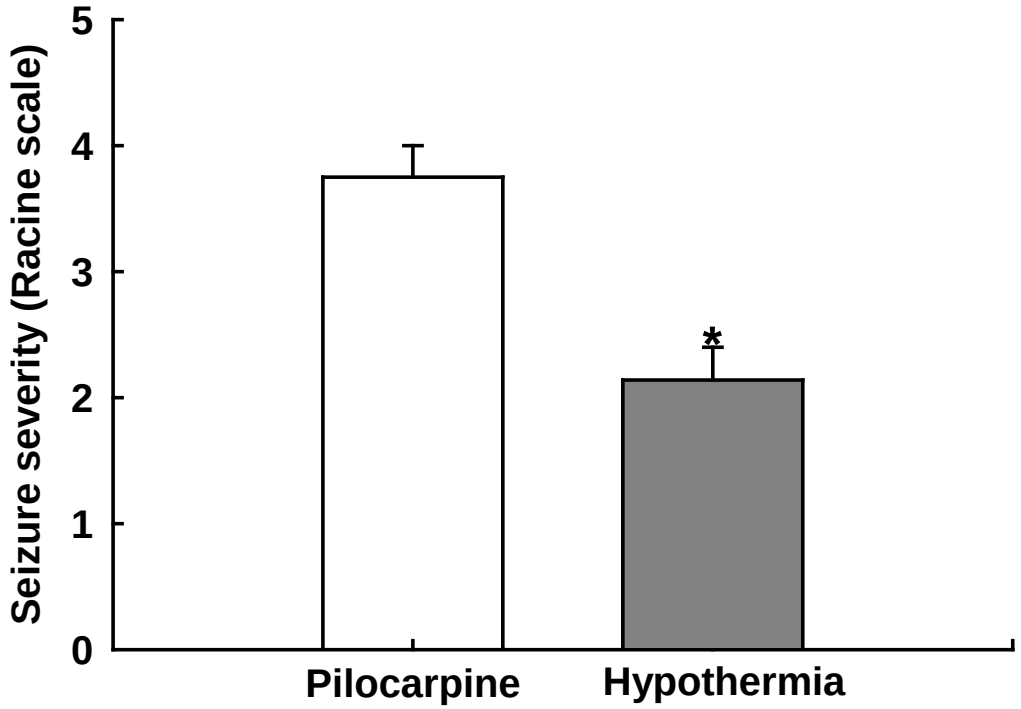

Figure 3-11. Hypothermia reduced the severity of seizures following SE. Seizure severity was scored from 0, for no detectable seizure activity to a 5, for severe tonic-clonic seizure activity based on the Racine scale. Rats in the pilocarpine group (n=4) displayed an average seizure severity score of 3.75±0.25. Rats in the hypothermia group (n=7) exhibited a significantly lower score of 2.14±0.26. Data presented as average score ± SEM. *$p<0.005$, Student's t-test.

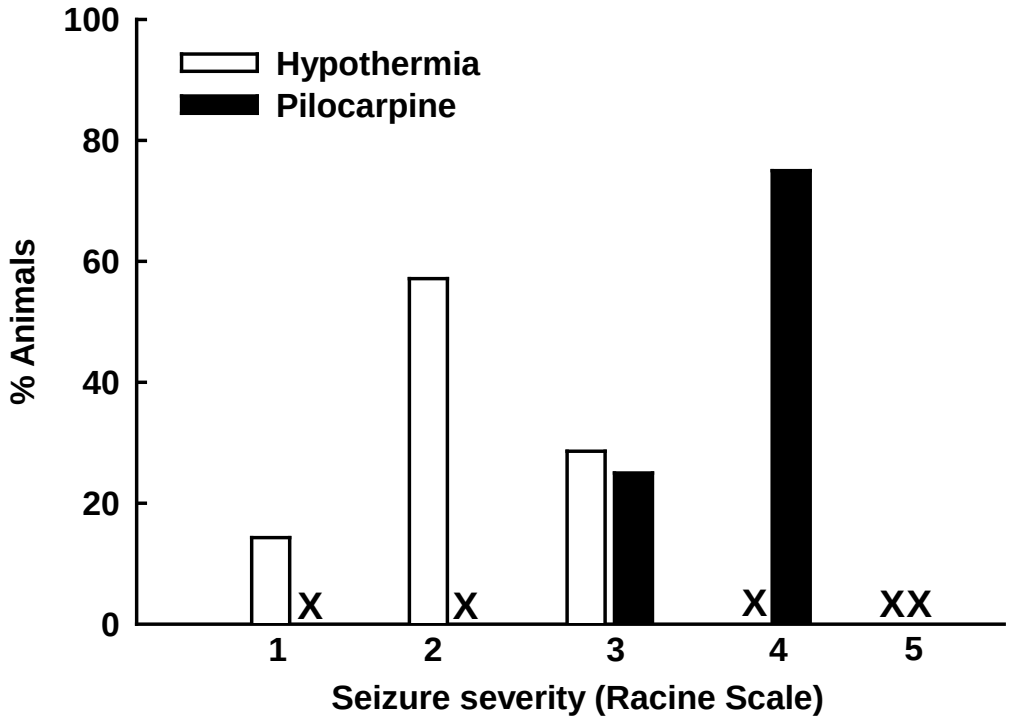

Figure 3-12. Seizure severity distribution. Bar graph depicting the distribution of seizure severity scores across the pilocarpine (n=4) and hypothermia (n=7) groups. 100% of the rats in the pilocarpine group exhibited a minimum average seizure severity score of 3 with 75% exhibiting a score of 4. In contrast, the highest score exhibited by rats in the hypothermia group was a 3, with 71% exhibiting a seizure severity score of 2 or less.

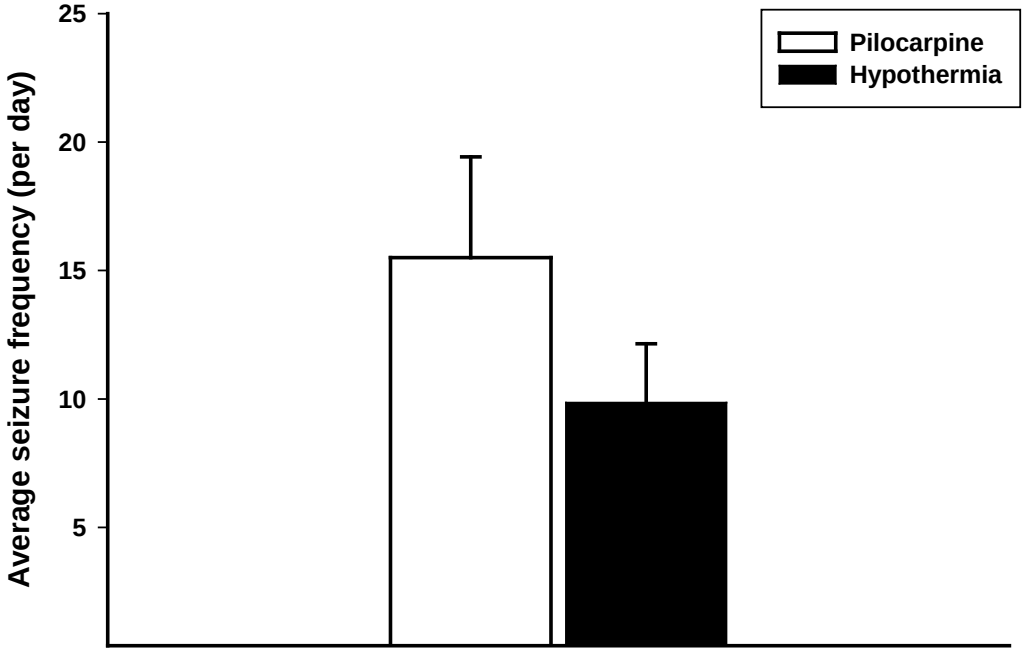

Figure 3-13. Hypothermia reduced seizure frequency. Rats in the pilocarpine group exhibited an average of 15.5±3.9 seizures per day. Hypothermia rats exhibited a reduced number of seizures per day with an average of 9.6±2.1. Data presented as average seizure frequency ± SEM. Results not statistically significant (student's t-test).

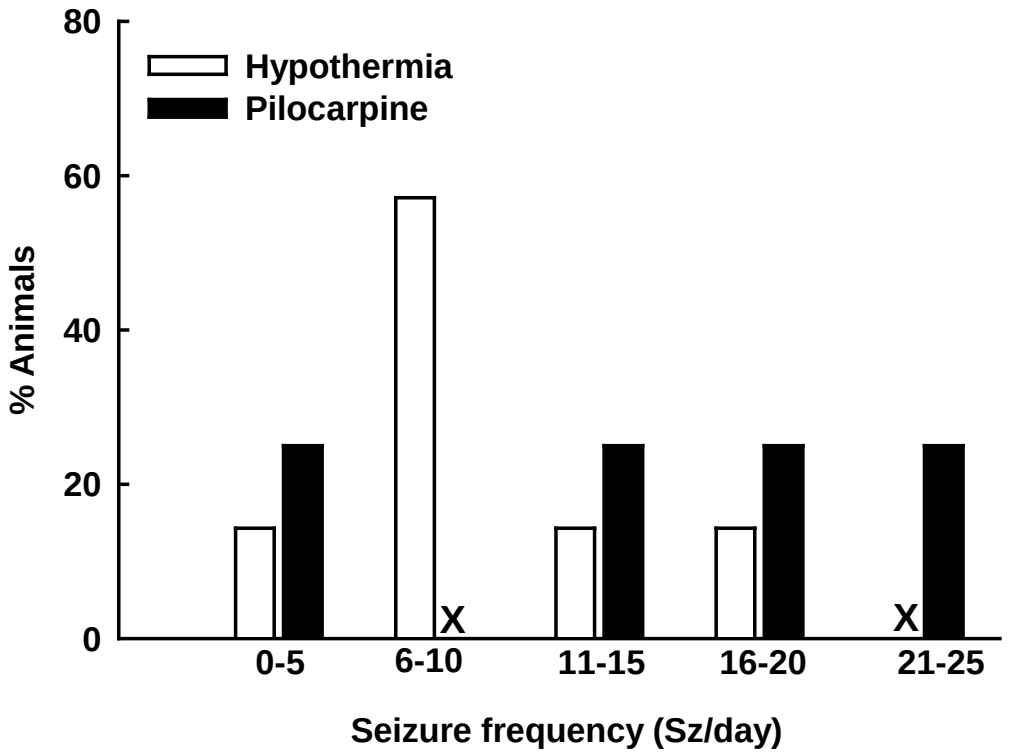

Figure 3-14. Seizure frequency distribution. Bar graph illustrating the distribution of seizure frequency among pilocarpine (n=4) and hypothermia (n=7) rats. 75% of the rats in the pilocarpine group exhibited at least 15 seizures per day. In comparison, 71.4% of the rats receiving hypothermia exhibited 10 or less seizures per day.

Chapter 4: Discussion

The purpose of these studies was to find a suitable treatment to be administered following an injury such as SE that can prevent epileptogenesis. Hypothermia is considered to be a potential intervention based on extensive literature supporting its neuroprotective effects. The use of hypothermia following SE has significant clinical ramifications because it is currently used to improve neurological outcome following stroke and TBI [118-121], two neurological injuries that can lead to the development of AE [9].

The results from these studies suggest that hypothermia is a potentially useful clinical intervention that can be induced following injury such as SE to prevent the progression of epileptogenesis. Both in vivo and in vitro models of SE-induced AE demonstrate that SE causes a persistent elevation in $[Ca^{2+}]_i$, and this plateau may be responsible for the long-term plasticity changes that ultimately lead to the development of AE. Developing new therapies aimed at blocking the Ca^{2+} plateau may provide neuroprotection and prevent epileptogenesis. These studies provided evidence that hypothermia blocks the Ca^{2+} plateau in both in vitro and in vivo models, making hypothermia a possible alternative intervention to pharmacological agents in reducing the development of AE.

The rationale behind studying the effects of hypothermia following SE is based on the fact that the cellular cascade of events following ischemic stroke and TBI are very similar to what occurs

during SE. Despite differing in the type of inciting insult, all these injuries share a common pathology of elevated $[Ca^{2+}]_i$ after injury followed by the development of epilepsy. Hypothermia is currently used clinically to reduce neurological injury associated with both ischemic stroke and TBI. Therefore, it stands that hypothermia may be effective at reducing neuronal injury associated with SE. Furthermore, fever has been shown to further potentiate the events following any brain injury, whereas hypothermia has been shown to prevent or interrupt the excitotoxic cascade [114].

The neuroprotective benefits of hypothermia have long been known, but its mechanisms of actions are still unclear. It is assumed that hypothermia protects against neuronal damage through a myriad of factors including reducing cerebral metabolism, decreasing inflammation, and modulating excitatory transmission and intracellular Ca^{2+}. The studies performed in this dissertation focused on the effects of hypothermia on intracellular Ca^{2+} as the role of hypothermia on Ca^{2+} dynamics in not well understood. These studies specifically focused on the ability of hypothermia to block the Ca^{2+} plateau. Studies have demonstrated that hypothermia prevents Ca^{2+}-mediated neuronal damage by reducing extracellular glutamate accumulation, thereby reducing NDMA receptor activation and concomitant Ca^{2+} influx [144-145]. However, no studies have examined the effects of hypothermia on other regulators of Ca^{2+} dynamics. Intracellular Ca^{2+} is regulated by a variety of other mechanisms including membrane Ca^{2+} channels, Ca^{2+} binding proteins, and regulators of intracellular Ca^{2+} storage and release. Studies have demonstrated that various regulators of intracellular Ca^{2+} are altered, and the alterations in Ca^{2+} dynamics contribute to the development and maintenance of the Ca^{2+} plateau. Ca^{2+} is a ubiquitous second messenger involved in a variety of cellular processes including

neurotransmitter release and transcription, as well as long-term potentiation. Excessive elevations in $[Ca^{2+}]_i$ activate several downstream effectors leading to plasticity changes and eventually activation neuronal death pathways. Therefore modulating the rise in $[Ca^{2+}]_i$ may be effective in blocking the Ca^{2+}-mediated cascade that leads to plasticity changes associated with epileptogenesis, thus preventing epilepsy.

The in vitro hippocampal neuronal culture model is a powerful tool for determining which systems contribute to the Ca^{2+} plateau. It provides a controlled environment that allows for manipulation of the systems without the presence of confounders found in the intact brain. The in vivo model is a more complex model that allows us to further test the effectiveness of various pharmacological and non-pharmacological treatments following injury. The in vivo pilocarpine model produces a pathology similar to that seen in human TLE [22]. It has been instrumental in providing a further understanding of epileptogenesis. The studies performed in this dissertation utilized both models to better understand the mechanisms and applications of hypothermia following SE. The results of these studies demonstrated that hypothermia blocked the development of the Ca^{2+} plateau following SE in both in the vitro and in vivo models. The novel finding that hypothermia blocked the Ca^{2+} plateau led to the hypothesis that hypothermia may block the plasticity changes associated with epileptogenesis and reduce or prevent the development of epilepsy.

Studies have demonstrated that NMDA receptors are responsible for the majority of Ca^{2+} influx during SE, and pre-treatment with NMDA receptor antagonists prior to SE inhibited the formation of the Ca^{2+} plateau and the eventual development of AE. Blocking the NMDA

receptors after injury, however, does not offer neuroprotection against epileptogenesis [37, 42]. Therefore, the Ca^{2+} plateau must be maintained by other mechanisms following SE. One study demonstrated that the RyR system may be involved. The RyR is part of the CICR system, and it releases Ca^{2+} from intracellular stores in ER by gauging cytosolic Ca^{2+} concentrations. This study demonstrated in the in vitro model that administration of the RyR blocker Dantrolene immediately following SE rapidly reduced the elevation in $[Ca^{2+}]_i$, blocked the formation of the Ca^{2+} plateau, and prevented the development of SREDs, the in vitro correlate of epilepsy. These results suggest that the RyR may be involved in the maintenance of the Ca^{2+} plateau following SE and may be partially responsible for the progression of epileptogenesis [83].

The studies performed in this dissertation evaluated the effects of hypothermia on different modes of Ca^{2+} entry. The in vitro hippocampal neuronal culture model was utilized to investigate if hypothermia affected Ca^{2+} entry through various channels. The results demonstrated that hypothermia does not affect Ca^{2+} entry through VDCCs on the cell membrane or IP_3R-mediated Ca^{2+} release from ER. However, hypothermia did reduce Ca^{2+} entry via NMDA receptor activation independent of extracellular glutamate, and it reduced RyR-mediated Ca^{2+} release from intracellular stores. This provided the first piece of evidence that hypothermia directly impacts intracellular Ca^{2+} through multiple means.

The pilocarpine model of SE-induced AE is a well established and commonly used model in studying epileptogenesis based on its ability to produce a condition that is highly similar to the pathology observed in human TLE [22]. The in vivo pilocarpine model has provided powerful evidence supporting our hypothesis that hypothermia may reduce or prevent the development of

epilepsy. Hypothermia was rapidly induced in rats 1 hour after SE began and maintained for 4 hours. Epilepsy typically begins to develop around 2 months post-SE, but can be as early as 1 week post-SE and as late at several months. Therefore, the rats were monitored for the presence of spontaneous behavioral seizures at both 3 months and 1 year post-SE. Our observations showed that the rats treated with hypothermia had a reduced rate of spontaneous behavioral seizures. These promising results demonstrated that hypothermia was effective at inhibiting the progression of epileptogenesis in the pilocarpine animal model.

The therapeutic window of opportunity is unclear, and it differs vastly depending on the type of injury. For example, in cases of stroke, there is evidence that the most effective window of opportunity is before the start of secondary injury [158]. It is generally agreed upon that earlier intervention provides a greater degree of neuroprotection. Therefore, we investigated whether earlier induction of hypothermia was more beneficial in preventing epilepsy. Monitoring at 3 months and 1 year post-SE provided exciting results that earlier hypothermia appears to delay to development of AE and further reduces the rate of developing spontaneous behavioral seizures.

The results from these studies provide important information regarding the mechanisms of epileptogenesis. As previously stated, the NMDA receptors are largely responsible for the influx of Ca^{2+} during SE, but the RyR system is at least partially responsible for the maintenance of Ca^{2+} plateau. The first treatment paradigm induced hypothermia at the end of 1 hour of SE, where the RyR system is assumed to dominate. The results from a previous study that blocking the RyR after SE prevented SREDs, combined with the results from these studies that hypothermia reduces RyR-mediated Ca^{2+} release, allows us to deduce that hypothermia is

blocking the Ca^{2+} plateau and reducing epileptogenesis by inhibiting the RyR. However, when hypothermia was induced during SE, it had an even greater effect at preventing epilepsy. Hypothermia is most likely reducing Ca^{2+} entry through NMDA receptors during SE and is acting on the RyR after SE. This double impact on Ca^{2+} entry appears to provide a greater degree of protection.

Not only did hypothermia block the Ca^{2+} plateau, it also reduced the occurrence of mossy fiber sprouting. Mossy fiber sprouting is a unique form of synaptic plasticity commonly observed in animal models of AE and human cases of TLE [85-90] Mossy fiber sprouting describes the abnormal sprouting of the axons of dentate granule cells (mossy fibers) into the inner molecular layer of the dentate gyrus which form synapses with dentate granule cells [91-92]. These connections are excitatory, thus creating a recurrent excitatory circuit in an area that normally lacks this circuitry. This is believed to contribute to the excitability observed in epilepsy [84, 93-95]. Although the presence of sprouting is not required for the development of AE, studies have correlated mossy fiber sprouting with increased seizure frequency and duration [100-101]. Thus, finding a treatment that blocks the formation of this aberrant sprouting may reduce neuronal excitability, as well as reduce the severity of the seizures, thereby improve the quality of life for the epileptic patient. One study demonstrated that hypothermia induced following TBI attenuated the development of mossy fiber sprouting in the dentate [146]. In our model of pilocarpine-induced SE, hypothermia reduced the development of mossy fiber sprouting as well as diminished the degree of intensity of sprouting. These results demonstrate that hypothermia reduces this form of plasticity change associated with epileptogenesis, which may be contributing to the decreased occurrence of epilepsy.

The molecular determinants for mossy fiber sprouting are not known. Therefore, understanding how hypothermia affects mossy fiber sprouting is still unclear. One possible mechanism is that hypothermia affects the expression of semaphorins, which are secreted proteins that guide neural development by dictating axon growth based on repulsive and attractive signals. Semaphorin are considered a stop signal for growing axons, thereby producing repulsive axonal guidance signals which leads to growth cone collapse [159]. Following SE and in TLE, mRNA levels of various semaphorin molecules are decreased [160-161], which may allow for the growth of aberrant axons of granule cells and facilitate the development of recurrent excitatory networks. Hypothermia has been reported to regulate transcription factors in the hippocampus [162], although it is not known if hypothermia has a direct effect on semaphorins. Thus, hypothermia could possibly be regulating the transcription of semaphorins.

Although hypothermia did not completely abolish epilepsy in our animal model, it did reduce the severity and frequency of seizures present in the small percentage of rats that did developed epilepsy. Rats exhibited a significant reduction in the severity of seizures and showed a trend towards reduced frequency. It is possible that the reduced severity and frequency is associated with the attenuation of mossy fiber sprouting. High seizure severity and frequency negatively affects the quality of life for epileptic patients. Reducing these two characteristics could vastly improve their quality of life, and these aspects should be taken into consideration when developing therapies for AE [163].

Further studies will be directed at determining the best window of opportunity to induce hypothermia after SE and the optimal duration of treatment. In human cases, hypothermia ranges

anywhere from a few hours to several days [121]. Rat models of ischemia have shown that prolonged hypothermia provides persistent neuroprotection [164-165]. Our model utilized a treatment duration of 4 and 4.5 hours. It is highly likely that a longer duration of hypothermia will yield a higher degree of protection against epileptogenesis. It is important to better define the treatment paradigm in terms of window of opportunity and treatment duration before hypothermia can be successfully translated to human application.

It is important to note that the Ca^{2+} plateau is not solely responsible for the development of AE. In addition to Ca^{2+} dysfunction, epilepsy is also associated with inflammation [166]. Most types of brain injury produce a significant inflammatory response [114]. Glial cells contribute to epileptogenesis through a variety of mechanisms including mediating an inflammatory response. Upon insult to the brain, microglia and astrocytes commonly undergo activation which is accompanied by the release of pro-inflammatory cytokines including interleukin (IL)-1², IL-6, and TNF-± [125, 138, 167-170]. This is often followed by a cascade of downstream events including activation of NFº B and chemokines [171-173]. The inflammatory response is accompanied by free radical production, which can cause significant additional damage [114]. Furthermore, the degree of brain inflammation has been shown to be positively correlated with seizure frequency and severity in both patients and animal models [174-176]. Therefore reducing inflammation caused by brain injury is another approach in preventing epileptogenesis. Not only does hypothermia reduce elevations in $[Ca^{2+}]_i$, it also modulates the inflammatory response. Several animal studies and some clinical evidence have demonstrated that hypothermia suppresses the induction of inflammatory reactions and the release of pro-inflammatory

cytokines [139-142]. Attenuating the inflammatory response significantly reduces the degree of brain injury [143].

Metallomatrix proteinases (MMPs) are a family of highly homologous neutral proteases that regulate cell-matrix composition [177-178]. MMPs have been implicated in various neurological diseases including epilepsy. Injuries that lead to the activation of an inflammatory response, such as stroke, TBI, and SE, stimulate the activation of various MMPs, including MMP-9 [173, 179-181]. Animal models of AE including the pilocarpine and kainic acid models of SE-induced AE have demonstrated that MMP-9 mRNA and activity are selectively up-regulated [182-184]. Furthermore, emerging evidence has implicated MMP-9 in contributing to processes involved in epileptogenesis, including neuronal death, abnormal synaptic plasticity, and inflammation [185]. The effects of MMP-9 appear to be temperature-dependent, and thus are inhibited by hypothermia [186]. Therefore, not only does hypothermia block the Ca^{2+} plateau, it is also effective at reducing inflammation associated with brain injury and epileptogenesis. Further exploration of how hypothermia affects the inflammatory process in models of SE-induced AE could provide valuable insight into the mechanisms of epileptogenesis and hypothermia.

Hypothermia's effects are multi-factorial, making it a powerful technique. Because hypothermia does not simply target one specific cascade or mechanism, it has the advantage over pharmacological interventions that only target specific events. It would be interesting to investigate whether the combination of hypothermia and a pharmacological agent would be more effective at preventing epileptogenesis. One possible combination is Dantrolene plus hypothermia. In vitro studies have demonstrated the Dantrolene prevents the development of

SREDs. However, its anti-epileptogenic effects have yet to be demonstrated in vivo. It is possible that hypothermia may work synergistically with Dantrolene in preventing Ca^{2+}-mediated plasticity changes and may be more effective at blocking the development of AE. In addition, Dantrolene and hypothermia are both FDA-approved treatments; Dantrolene is approved for the treatment of malignant hyperthermia, and hypothermia is approved for cardiac arrest, neonatal hypoxic-ischemic encephalopathy, TBI, and stroke. Therefore, it may be easier to translate both interventions to clinical applications.

Several studies have demonstrated that a variety of pharmacological agents can prevent epileptogenesis when administered prior to an injury. However, there are currently no anti-epileptogenic agents that can be administered following a neuronal injury. Clinically, it is not feasible to treat a patient prior to an injury such as SE with the hopes of preventing epileptogenesis. Although advances have been made in developing effective AEDs, approximately 40% of all epileptic patients are refractory to currents AEDs [1], which further underscores the importance of developing new strategies of preventing epileptogenesis. Therefore, developing a novel therapy that can be administered after an injury to block epileptogenesis is clinically important. In addition, it is important to define the window of opportunity in which therapeutic treatment would be most effective. The results the studies performed in this dissertation suggest that hypothermia is a viable intervention for preventing epileptogenesis. It is a clinically relevant therapeutic technique in that it can be effective when induced after the injury. Furthermore, the results provide additional evidence that supports the theory that earlier intervention is more protective.

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Vita

Kristin Fenton Phillips was born in Jacksonville, FL on September 24, 1984. She was raised in Keflavik, Iceland and Virginia Beach, VA, where she graduated from Frank W. Cox High School with an advanced studies diploma in June 2002. She attended Virginia Polytechnic Institute and State University (Virginia Tech) for her undergraduate education, where she became a member of Virginia Tech Autism Clinic. During this time, she worked as a research assistant for the Autism Clinic as well as for the Cognitive and Developmental Sciences Lab in the Department of Psychology. She graduated with honors from Virginia Tech with a Bachelor of Science in Psychology in May 2006. She joined the Department of Pharmacology and Toxicology at Virginia Commonwealth University in August 2006.

Research Experience

- Dissertation research, Dr. Robert DeLorenzo, Dept. of Pharmacology & Toxicology and Neurology, 2007-2011
- Research Assistant, Dr. Imad Damaj, Dept. of Pharmacology and Toxicology, 2006
- Research Assistant, Dr. Angela Scarpa, Virginia Tech Autism Clinic, 2004-2006
- Research Assistant, Dr. Martha Ann Bell, Dept. of Psychology, 2004-2006

Awards and Honors

- Who's Who Among American College Students, 2006
- Dean's List, 2002-2006
- Golden Key National Honor Society, 2005
- Psi Chi National Honor Society, 2004
- NATO Azalea Festival Princess, representing Iceland, 2002

Volunteer Experience

- Personal Aide, Autism Clinic, Blacksburg, VA, 2004-2006