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THE ROLE OF S100B IN ACQUIRED EPILEPSY INDUCED BY INFLAMMATION IN A NEONATAL HYPOXIA-INDUCED ANIMAL MODEL

A Thesis

Submitted to the School of Graduate Studies and Research

in Partial Fulfillment of the

Requirements for the Degree

Master of Science

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Indiana University of Pennsylvania

August 2016

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Title: The Role of S100B in Acquired Epilepsy Induced by Inflammation in a Neonatal Hypoxia-Induced Animal Model

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Neonatal hypoxic-ischemic encephalopathy (HIE) is a major cause of mortality and morbidity in infants and children. One of the most important consequences of neonatal HIE is epilepsy. Epilepsy is a common neurodegenerative disorder characterized by recurrent unprovoked seizures due to hyperexcitability and hypersynchrony of neuronal activity. Recent studies uncovered important molecular and cellular aspects of hypoxic-ischemic brain injury that may provide a therapeutic target for intervening in the epileptogenesis in the developing brain. We developed and characterized a model of neonatal hypoxic brain injury to test hypotheses about the interaction of astrocytes and microglia in hypoxia-induced brain injury and early steps in epileptogenesis. Specifically, we hypothesize that hypoxia-induced neuronal injury activates microglia which then starts a reciprocal activation cycle of astrocytes and microglia. A critical step in the cycle is activation of the receptor for advanced glycation end products (RAGE) on microglia by S100-beta (S100B) secreted by astrocytes. In our experiments, we administered a RAGE antagonist (FPS-ZM1) to protect brain from the effect of HI induced damage and inhibit the apoptotic pathway and downstream products, including IL-6. Most importantly, the specific interaction between S100B and RAGE receptors further implicate a role for this astrocytic protein in the pathogenesis of epilepsy. Our findings may create a potential for therapeutic intervention for hypoxia-induced epilepsy.

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It is the time to end the story, but not before thinking all the people who have been part of my thesis. This project is a result of joint efforts of people from different departments and labs and I thankful to all. First I would like to thank my advisor **Dr. Widzowski** who believe in me and gave me the opportunity to work on my project and improve my scientific skills.

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CHAPTER ONE

INTRODUCTION

1.1. General Introduction

The neonatal period is a critical stage of life, in which the risk of seizure is the highest. Recent population-based studies report the incidence of seizure is approximately 1.8-5/1000 live births (Saliba 2001, Jensen 2009, Shetty 2015). Hypoxia or ischemic encephalopathy (HIE), a complex condition characterized by inadequacy in blood flow and shortage in oxygen supply, is a common cause for neonatal seizure and responsible for two-thirds of seizure cases (Jensen 2009). Commonly, these seizures occur within the first or second day of birth. Unfortunately, neonates who have early-onset of seizures are often refractory to available FDA-approved antiepileptic medications and develop long-time consequence, including later-life epilepsy (Shetty 2015, Rudzinski and Meador 2011).

Epilepsy is one of the most common neurodegenerative disorders and accounts for approximately 1% of the global burden of disease (WHO 2016). Each year, 1 in 26 individuals will develop at least one form of epilepsy in the United States (Shafer and Sirven 2013). Epilepsy is defined as a recurrence of spontaneous seizures due to hyperexcitability and hypersynchrony of the brain neuronal activity (Devinsky, et al. 2013). Nonetheless, one seizure does not signify epilepsy, but two and more in frame time of two years do (WHO 2016). Epileptic seizures may range from momentary lapses, uncontrolled muscle movements, loss of consciousness to severe, prolong convulsions; as well as sensational and emotional disturbances. Seizure presentation varies based on the location of onset in the brain; each area of the brain serves specific

body parts and possess specialized functions (NIH 2016). Seizure types can be divided into two categories based on the site of origin: generalized seizures (*Tonic-clonic*) and focal seizures (*Partial*). Generalized seizures are characterized by excessive electrical discharges in both hemispheres, while focal seizures exhibit massive neuronal discharges in a localized part of the brain (e.g., temporal lobe). However, seizures manifest with signs and symptoms that overlap with other diseases, which makes it difficult to define a specific seizure to particular types of epilepsy (NIH 2016, Shneker and Fountain 2003).

Epilepsy is classified into four main categories (Shorvon 2011), including idiopathic, symptomatic, provoked and cryptogenic (Table 1). Idiopathic epilepsy is defined as an epilepsy of predominately genetic or presumed genetic origin and in which there are no gross neuroanatomical or neuropathological abnormalities. Symptomatic epilepsy is an epilepsy of genetic or acquired causes that are associated with structural abnormalities. Provoked epilepsy can be originate from systemic or environmental factors with no structural deformities. Cryptogenic epilepsy is derived from unknown cause of origin. According to William Lennox, about 20% of reported epilepsy cases are purely genetic, 20% are purely acquired, 50% are a mixture of both and about 10% assigned under unknown category (Shorvon 2011).

Table 1 The etiological classification of Epilepsy [a]

Main Category	Subcategory
Idiopathic epilepsy	Pure epilepsies due to single gene disorders Pure epilepsies with complex inheritance
Symptomatic epilepsy 1. Genetic or developmental causation	Childhood epilepsy syndromes Progressive myoclonic epilepsies Neurocutaneous syndrome Disorders of chromosome function Developmental anomalies of cerebral structure
2. Acquired causation	Hippocampal sclerosis Perinatal and infantile causes Cerebral trauma Cerebral Tumor Cerebral infection Cerebrovascular disorders Cerebral immunologic disorders Degenerative and other neurologic conditions
Provoked epilepsy	Provoking factors Reflex epilepsies
Cryptogenic epilepsy	

[a] Shorvon, S D. 2011. "The etiological classification of epilepsy." Epilepsia 52 (6): 1052-1057.

Acquired epilepsy refers to systemic epilepsy that is due to external and environmental causes as well as internal pathogenic processes, including neurodegenerative disorders and auto-immune disorders. Acquired epilepsy results from a CNS injury or metabolic disturbance such as hypoxia that progresses to alteration of neuronal plasticity and which results in spontaneous recurrent seizures (Shorvon 2011).

Although a great number of clinical and experimental studies have revealed some of the evidence with respect to both pathogenesis and consequences of epilepsy and seizures in adults, the molecular basis for developing acquired epilepsy during

childhood is still not completely understood (Hossain 2005). However, an increasing body of evidence has provided strong support for the hypothesis that inflammatory processes might establish a common mechanism in the pathogenesis of various types of epilepsy (Vezzani, et al. 2011). Recent study (Devinsky, et al. 2013) have suggested that glial cells, especially astrocytes and microglia, are significant contributors in the development of epilepsy. Moreover, activated astrocytes and microglia have been found to be responsible for up/down-stream induction of several inflammatory mediators in cell culture models and human tissue from epileptic patients. Furthermore, CNS inflammation is recognized as a common risk factor for epilepsy and associated with seizure development (Devinsky, et al. 2013).

Many studies suggest that most head injuries induce inflammation that causes both malfunction and deformation of cell membrane and blood vessels (Oby and Janigro 2006). In particular, exposure of the brain parenchyma to blood circulation and the release of danger-associated signal molecules from damaged cells can initiate epileptogenic inflammatory cascades. In chronic neuroinflammation, astrocytes and microglial cells act in a destructive manner contributing to sustained release of proinflammatory cytokines, chemokines (such as S100B, interleukins [i.e., IL-1B, IL-6], tumor necrosis factor-alpha (TNF-a), interferon-y-inducible protein-10 (IP-10)), transforming growth factor (TGF) and proteases. The sensitive reactivation process and the inducible overproduction of proinflammatory mediators make astrocytes very important contributors to the neuroinflammation in the early stages of brain injury (Sue and Griffin 2006, Oby and Janigro 2006, Walker and Sills 2012).

In hypoxia models, microglia are activated and release inflammatory cytokines that in turn activate astrocytes, which release S100B, a danger-associated chemokine. It is a well-known biomarker of brain injury associated with Alzheimer's disease, concussion and epilepsy. Based on literature review, an overproduction of soluble S100B from the astrocytes binds to the receptor for advanced glycation end products (RAGE) leading to various responses including increased reactive oxygen species (ROS) formation, release of pro-inflammatory markers, and activation of stress response kinases resulting in neuronal cell death (Piras, et al. 2016). The ongoing neuronal injury and inflammation further promotes cellular apoptosis and impairs neurogenesis in epileptic brain. Identification of this mechanistic relationship between acquired epilepsy and inflammation may provide an important therapeutic target pathway for prevention of epilepsy (Lu, et al. 2011, Sorci, et al. 2013, E. Leclerc, et al. 2009).

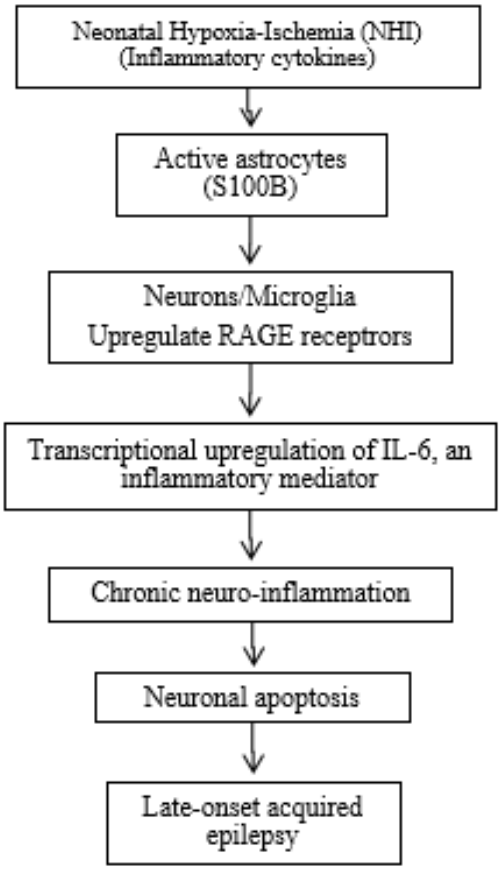

Figure 1.1 Pathogenesis cascade of events leading from inflammation to epilepsy

1.2. The Aims of the Study

The general aim of this project was to investigate the molecular consequences of hypoxia in neonates, also addressing concurrent micro-environmental alteration (Figure 1.1).

The specific aims of the Master thesis were:

1. To study the behavioral aspects, such as seizure-frequency and seizure intensity in neonatal mice during the hypoxia induction
2. To describe the events following hypoxia, including apoptosis, and changes in S100B concentrations.
3. To study pharmacotherapy aspect, that is characterize the effect of administrating RAGE antagonist as a protective factor against hypoxia and assess the afterward consequences, including apoptosis rate, IL-6 levels and S100B concentrations.

CHAPTER TWO

LITERATURE REVIEW

2.1. Astrocytes contribution in inflammation and epileptogenesis

Astrocytes can be classified into two major subtypes, protoplasmic or fibrous which are found in gray matter and white matter respectively (Sofroniew and Vinters 2010). Both subtypes make extensive connections with blood vessels and can act as intermediaries between the vasculature and brain parenchyma (Sofroniew and Vinters 2010). Astrocytes provide a contiguous, non-overlapping substrate (similar to floor tiles) throughout the brain (Sofroniew and Vinters 2010). Under normoxic conditions, astrocytes can sense changes in neuronal activity (e.g., active neurotransmission) and extracellular fluid composition (e.g., changes in extracellular glutamate, GABA). Like neurons, astrocytes are electrically active, expressing potassium and sodium channels and exhibiting oscillations in intracellular calcium, but unlike neurons they do not fire action potentials diseases (Sofroniew and Vinters 2010). Astrocytes can be coupled to each other by gap junctions which facilitates the function of astrocytic networks.

Various stressors and diseases processes (e.g., Alzheimer's disease, Parkinson's disease, inflammation, hypoxia-ischemia), can alter the function of astrocytes and act as regulators of astrocyte reactivity (Sofroniew and Vinters 2010). Indeed, an abnormal increase in astrocytes number (astrogliosis) has been observed in response to the destruction of nearby neurons from CNS trauma, infection, ischemia, stroke and other CNS injuries and diseases (Sofroniew and Vinters 2010). Over the past few decades, there has been accumulating evidence that support the significant role of astrocytes in the CNS immune responses. Astrocytes have been shown to contribute to the initiation,

regulation and amplification of the immune-mediated mechanisms involved in different human CNS diseases, including epilepsy (Farina, et al. 2007, Vezzani, et al. 2013). Immunochemical analyses of different inflammatory cytokines showed that the expression of cytokines in microglia is time-locked to the occurrence of seizures and extension is dependent on the recurrence of seizure. Additionally, astrocytes have shown persistent cytokine expression even after the onset of injury. For instance, interleukin-6 (IL-6) and other inflammatory cytokines are expressed at very low levels in normal brain. However, after the induction of seizure, their protein levels are rapidly increasing, declining to basal levels within 48-72 h of the onset of seizures (Vazzani and Granata 2005, Ravizza, et al. 2008). Moreover, astrocytes from human epileptic tissue can express both inflammatory mediators and receptors, implying that astrocytes can serve as both sources and targets of inflammation molecules (Vezzani, et al. 2011).

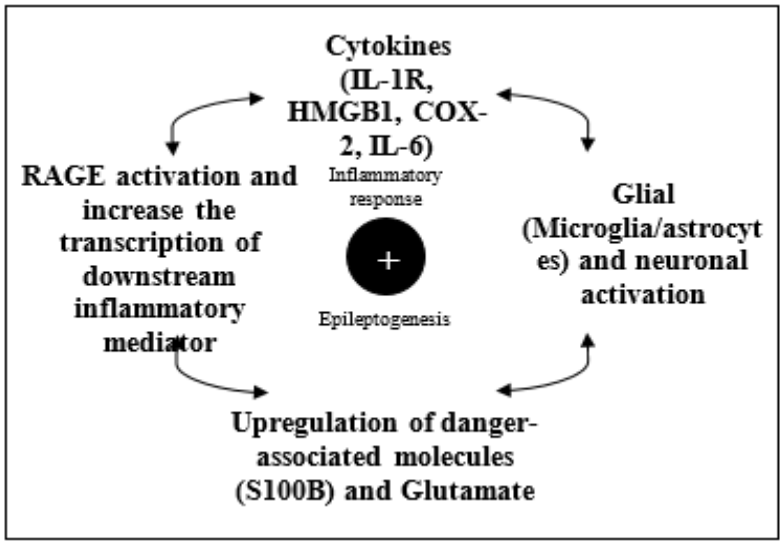

Figure 2.1 Proposed immune mechanism involved in epileptogenesis. The relationship between immune responses and epileptogenesis is presented as a positive feedback loop in which cytokines amplify downstream inflammatory mediators, which lead to epileptogenesis

2.2. Astrocytes and inflammation in experimental models of seizures and epilepsy

Experimental studies have shown the significant contribution of reactive astrocytes in the development of inflammatory responses after a seizure or an epileptogenic brain injury. In rodents, acute seizures were shown to upregulate the prototypical cytokines in microglia and astrocytes in the brain area where seizures originate (Vazzani and Granata 2005). Consequently, a downstream cascade of inflammatory mediators is transcriptionally upregulated that promotes the initiation and propagation of epileptic activity in the brain similar to what have been shown in human epilepsy (Vazzani and Granata 2005, Minagar, et al. 2002). It is proposed that the relationship between immune responses and epileptogenesis is represented as an amplifying feedback loop that enhances the changes in which cytokines perpetuates the development of epilepsy through activation of glial cells and upregulation of downstream inflammatory mediators, which leads to pathophysiological outcomes, including seizure recurrence and epileptogenesis (Figure 2.1, (Xu, et al. 2013)).

2.3. IL-6 chronic production, leading to epileptogenesis

Accumulating evidence has suggested that the pathogenesis of human epilepsy involves activation of inflammatory cells and increased secretion of inflammatory cytokines in sensitive brain regions. Among different inflammatory cytokines, IL-6 has attracted most attention in clinical studies related to epilepsy. IL-6 is a multifunctional cytokine that regulates inflammatory responses and mediates other immune reactions (Nowak, et al. 2011, Marchi, Granata and Janigro 2014). Glial cells and neurons are the main source of IL-6 in the brain (Juttler, Tarabin and Schwaninger 2002). Functionally,

IL-6 can activate both proinflammatory and immunosuppressive pathways in response to brain injury. Proinflammatory actions of IL-6 include increased permeability of the blood brain barrier, activation of microglial cells and astrocytes and promotion of cell differentiation. In other situations, IL-6 can act as a neuroprotective mediator demonstrating its immunosuppressive activity. The balance between pro and anti-inflammatory pathways is fundamental for maintaining CNS integrity and dysregulation may lead to pathogenic chronic neuro-inflammation and neurodegeneration (Farina, et al. 2007).

A previous study reported the clinical characteristics of refractory epilepsy in association with IL-6 activation (Liimatainen, et al. 2009). IL-6 was found to be higher in patients with refractory temporal lobe epilepsy, which may reflect the chronic cytokine overproduction in the brain. Nonetheless, a recent study showed (Lehtimaki, et al. 2011) that the serum IL-6 of patient with refractory epilepsy was significantly higher than the controls, which implies to inflammation. Moreover, higher seizure frequency was associated with the higher IL-6 levels, which support that suggested pathological action of excessive expression of IL-6 (Lehtimaki, et al. 2011). Altogether, these studies imply that Il-6 overproduction is associated with epileptogenesis and higher seizure frequency in individuals with refractory epilepsies.

2.4. S100B overproduction and activation of RAGE receptors

S100B is the first member of the S100 protein family to be identified. S100B is a calcium binding protein that is highly abundant and exclusively produced by the astrocytes in the brain. S100B is constitutively secreted by astrocytes and its secretion can be regulated by external factors, such as the release of inflammatory cytokines and

chemokines. In physiological conditions, S100B exerts its regulatory activities within the astrocytes, including regulation of protein phosphorylation, energy metabolism, Ca+2 homeostasis and cell proliferation and differentiation. Once S100B is released/secreted, it acts as an extracellular pathogenic signal, in which enhances the secretion of inflammatory cytokines and chemokines (i.e. IL-1β and IL-6), glutamate, and acts as a ligand of the receptor for advanced glycation end product (RAGE). Compelling evidence suggests that extracellular S100B can be considered as a damage-associated molecule pattern (DAMP) that might contribute to the inflammatory responses, as well as the upregulation of RAGE expression that significantly enhance the propagation of inflammation (Sorci, et al. 2010).

The Receptor for advanced glycation end product (RAGE) is a multi-ligand receptor of the immunoglobulin superfamily of cell-surface molecules. The cell-surface ligands of RAGE interact and result in sustained cellular activation via multiple signaling pathways that lead to the propagation of inflammatory responses (Kierdorf and Fritz 2013). Among RAGE ligands, S100B has been suggested to mediate the significant role of RAGE in the pathogenesis of certain human diseases, including Alzheimer's disease, diabetes, multiple sclerosis, and cancer. However, RAGE has been poorly investigated in the epileptogenesis (E. Leclerc, et al. 2009, Guo, et al. 2016). A recent study (Iori, et al. 2013) reported that RAGE was upregulated in temporal lobe epilepsy (TLE) and contributed to experimental seizures, which revealed a new molecular mechanism involving inflammatory pathways. Thus, activation or upregulation of RAGE may have an important role in human epilepsy.

2.5. Apoptosis mechanisms in epilepsy

Cell death can be characterized as necrotic or apoptotic based on the biochemical and morphological criteria (MacManus and Linnik 1997). Necrosis often results from serious injury and is indicated by swelling and disruption of cellular membranes, expelled cytoplasmic contents into the external space and development of a secondary inflammatory response. In contrast, apoptosis is a highly regulated and energy consuming process of self-destruction in which a cell undergoes atrophy, cytoskeletal collapse and DNA fragmentation. The cell undergoes different stages, begin with nuclear condensation and contraction and end with the disruption of membrane and organelles (Vannucci and Hagberg 2004).

Most data suggested that apoptosis as a result, in hypoxia has slightly different morphology than the regular apoptotic cells. Several studies found that cells exhibit a hybrid necrotic-apoptotic phenotypes in case of hypoxia were induced (Rocha-Ferreira and Hristova 2016). However, biochemical analysis found that caspase-3, APAF-1, Bcl-2 and Bax, are essential elements associated with apoptotic processes in hypoxia. An upregulation of these apoptotic proteins was observed in immature brains compared to adult brains, which could be expected to have a significant role in the pathogenesis of epilepsy (Leist and Jaattela 2001, Martin, et al. 1998).

2.6. Overall consequences of astrocyte-mediated brain inflammation on seizures and epileptogenesis in neonatal hypoxia

The neonatal brain is more prone to hyperexcitability than the adult brain because many electrical circuits are enhanced to promote development and maintain plasticity (Hossain 2005). We hypothesized that hypoxia disrupts brain circuits and causes an

overproduction of inflammatory cytokines that target the activation of astrocytes, which, in turn, upregulates the production of S100B proteins. S100B activates microglial and neuronal RAGE receptors that, in turn, activate the downstream inflammatory and apoptotic pathways. As a result, hypoxic insult in neonatal brain ultimately causes neuronal injury and degeneration which eventually can lead to late-onset of acquired, refractory epilepsy (Figure 2.2).

Despite the accumulated body of knowledge, the gaps in our understanding of the mechanisms leading to epileptogenesis in neonates after hypoxic injury hinder the discovery of therapeutic drugs for refractory epilepsy. In this thesis, we used an animal model that mimics the hypoxia-induced epileptogenic environment. This methodology consists of exposing 7-day old pups to a transient period of global hypoxia induced by inhalation of 8% oxygen balanced with nitrogen. The damage can be observed in both hemispheres of the cerebral cortex and the hippocampal formation. Neuronal damage is less commonly seen in the thalamus and striatum. Using this model allows us and others investigate the effects of neonatal hypoxia on inflammatory mediators and markers, S100B regulation and hypoxia-induced apoptotic cell death.

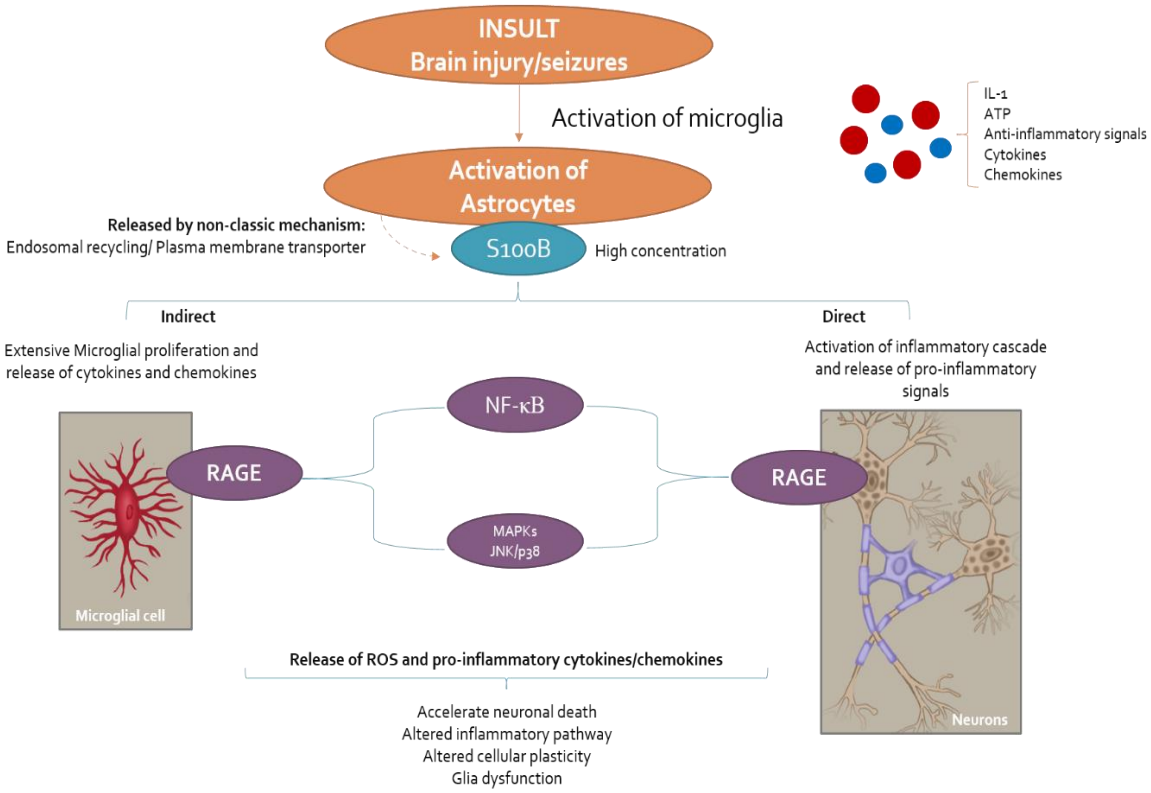

Figure 2.2 The proposed neuropathology cascade events of acquired, refractory epilepsy, begins with hypoxia insult, which lead to neuronal degeneration and apoptosis cell death

CHAPTER THREE

PROCEDURES

3.1. Animals

Male and female C57BL/6 mice (7-9 – day – old, 3 – 4g; Indiana University of Pennsylvania; Indiana, PA) with their mothers (dams), were housed in a temperature – controlled environment with a 12:12 h light-dark cycle and had free access to Lab Diet 5015 diet (LabDiet, ST. Louis, MO) and water for 7-9 days. Procedures involving animals and their care were conducted in conformity with the Institutional Animal Care and Use Committee (IACUC) (Authorization No. 02-1516-R3).

3.2. Induction of seizures and drug treatment

Mice (PND7-9, n=36) were exposed to nonlethal graded acute hypoxia for 90 minutes in an airtight chamber (volume = 1L, 6”x 3.75” x 2.25”; Scivena scientific, Clackamas, OR) equipped with inlet and outlet valves (Figure 3.1). The chamber was immediately flushed with a gas mixture (79% N2, 21% O2) for two minutes at a flow-rate of 2.5 L/min to ensure a >99% gas exchange in the chamber which was then sealed. The oxygen concentration was maintained at 8% to induce hypoxia. The changes in oxygen levels were monitored by an oxygen meter (Medex supply, Monsey, NY). Each animal was scored for the number of convulsions and seizures during the hypoxia. After termination of hypoxia and maintain recovery, animals were returned to their dams. Littermate controls were separated from their dams and kept at room air (21% O_2) for the equal duration of the experimental group. Body temperature was maintained between 36-38^{o}C.

Thirty minutes prior to hypoxia induction, mice (n=9) were injected with one-time dose of 1.0 mg/kg i.p. of a RAGE antagonist, EPS-ZM1 (EMD Millipore; Billerica, MA, USA) or vehicle (0.9% Saline). Development of seizures and convulsions was recorded and evaluated by behavioral assessment (see section 3.4).

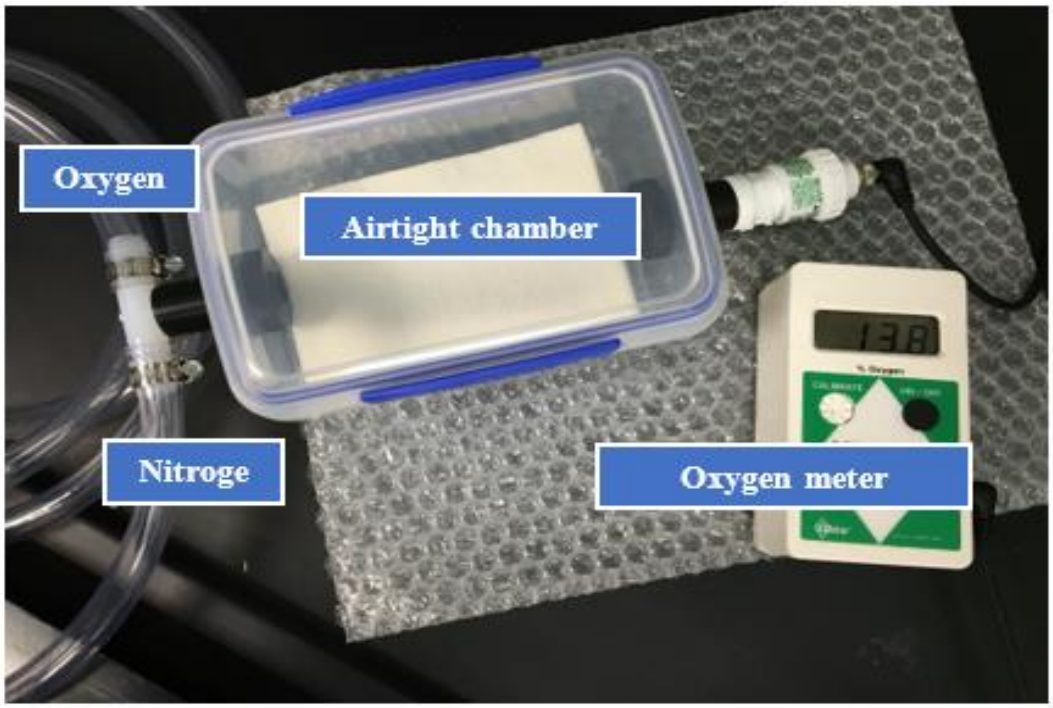

Figure 3.1 Hypoxia induction chamber. The hypoxia induction chamber consists of an airtight chamber with oxygen/ nitrogen intel and oxygen sensor outlet

3.3. Assessment of seizures and convulsions after acute hypoxia

Seizures during hypoxia were videotaped, reviewed and scored for the number and cumulative duration of tonic-clonic seizures. The mice were classified according to the following scale: 0 = normal; 1 = hyperactivity; 2 = immobility and ataxia; 3 = Circling, shaking, and clonic convulsions; and 4 = tonic-clonic convulsions (Table 2.).

Table 2 Seizure scale scores of mice during hypoxia experiment

Stage	Behavior	Description
0	Normal	Normal explorative behavior
1	Hyperactivity	Hyperactive behavior, jumping and rearing
2	Immobility Ataxia	Sudden total immobility (3-10 seconds) Unsteady, jerky gait
3	Circling Shaking Clonic convulsions	Running tight circles (2 circle/ second) Whole-body shaking Contractions of hind-and forelimbs with reduced consciousness
4	Tonic-clonic convulsions	continuous tonic-clonic convulsions with loss of consciousness, loss of righting reflex

3.4. Brain samples preparations and cryosectioning

Mice (PND7-9, n =25) were anesthetized using Isoflurane (0.6 ml/L) in an induction chamber (1 L, 4” x 4” x 4”, Braintree scientific Inc. Braintree, MA) for 2-5 min. Depth of anesthesia was assessed by monitoring the toe pinch withdrawal reflex. Brain samples were harvested and fixed with 3.7% formaldehyde in 0.1 M phosphate buffered saline (PBS) for 24 h at room temperature. The samples were then dehydrated in 70% ethanol. Subsequently, the samples cut into thick blocks and immersed into cryo-protection buffer, consisting of 3 M sucrose in 0.1 M PBS buffer until the samples sink.

Samples were placed in a plastic tray, embedded in OCT cutting matrix (provided by IUP faculty) and stored in -80^{o}C. Ultrathin cryo-sections (20 µm) were cut at -20^{o}C with disposal blades at 17^{o}- 20^{o} angle. The sections were collected on gelatin-subbed slides and stored at 4^{o}C.

3.5. Apoptosis Assay

We tested the effects of hypoxia and of RAGE antagonist administration after acute hypoxia on cell death events. Mice injected with vehicle and placed in the chamber for 90

minutes under 21% oxygen conditions were considered intact (negative, no apoptosis) controls. Mice administered vehicle and placed in 8% oxygen for 90 minutes were considered the positive (with apoptosis) control and mice administered 1 mg/kg of FPS-ZM1 and placed in 8% oxygen for 90 minutes were the experimental group. Intra-neuronal apoptosis was detected with the Trevigen TACS-XL in Situ apoptosis detection kit (TUNEL; Trevigen Inc., Gaithersburg, MD) according to manufacturer's instructions. Briefly, prepared brain slices were treated with Proteinase K (50 µl) followed by quenching for endogenous peroxidase activity. The samples were then incubated with a labeling buffer containing brominated nucleotide and terminal deoxynucleotidyl transferase (TdT) at 37°C. The incorporated 5-bromo-2' deoxyuridine was detected by anti-5-bromo-2' deoxyuridine, followed by streptavidin–horseradish peroxidase and a proprietary TACS Blue label. Staining was documented with an Electronic microscope and scaled as: 0% no staining, 10-30% fine staining, 40-60% mild staining, and 70-100% high staining.

3.6. Brain/Serum proteins extraction

After the recovery period, mice were anesthetized (Isoflurane, 0.6 ml/L) for 2-5 min until they achieve surgical plane of anesthesia which was assessed by monitoring the pinch withdrawal reflex. Brains were collected, halved along the sagittal midline, and then frozen. All brains were inspected visually for any sign of bleeding during collection (Figure 3.2). Also, blood collected by cardiac puncture and clotted to separate out the serum (Figure 3.3).

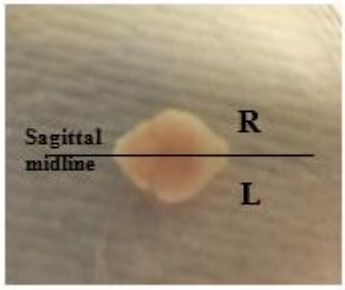

Figure 3.2 Brain collection method. The brain sample was halved along the sagittal midline: right half (R) was assigned for apoptosis assay, while lift side (L) for the ELISA assay

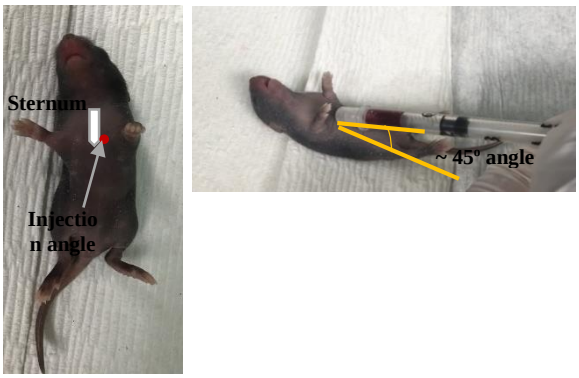

Figure 3.3 Cardiac Puncture method. The mouse was position on the back and the injection was inserted under the sternum at 45° angle. The blood collected using Insulin needles

Brain tissue proteins were obtained using the Thermo Scientific T-PER Tissue Extraction Reagent (Thermo Scientific, Rockford, IL) according to manufacturer's instructions. In brief, a ration of ~1 g of tissue to 20ml of T-PER reagent was used. Samples were homogenized and centrifuged at 10,000 x g for 5 minutes; tissue debris was discarded and sample supernatants were collected for further analysis (see section 3.7).

Blood serum proteins were extracted using the Thermo Scientific Pierce Albumin/IgG Removal Kit (Thermo Scientific, Rockford, IL) as instructed by manufacture. The Albumin Removal column was loaded with 75µl of diluted serum [10-15 µl of serum diluted to final volume of 75µl with 25 mM Trid – NaCl, 0.01% sodium azide; pH 7.5, binding/washing buffer]. Further, 75µl of binding buffer ware applied and the eluent was collected (albumin-free serum). The albumin-free serum was used for further analysis (see section 3.8).

3.7. Astrocytic S100B ELISA

Levels of S100B in the brain were analyzed by the enzyme-linked immunosorbent assay (ELISA, MyBioSource; San Diego, CA) following the manufacturer's instructions. In brief, the experiment uses double-sandwich ELISA technique, the pre-coated antibody is a mouse S100B monoclonal antibody and the detecting antibody is a polyclonal antibody with biotin labeled. Samples and biotin labeling antibody were added into ELISA plate wells and washed out with PBS. Then Avidin-peroxidase conjugates were added in order. TMB substrate was added for coloring after reactant thoroughly washed out by PBS. The data were determined using two wavelength plate and the concentrations of S100B were found by checking the OD 450 reading.

3.8. IL-6 Western blotting

Levels of Interleukin-6 was analyzed by the western blotting, following adapted protocol. In brief, 25 µl of serum protein extract sample was mixed with 25 µl of sample loading buffer to get a total of 50 µl, heated at 90 ^{o}C for 10 minutes. 14 µl of ladder was loaded and 10 µl of the samples separated by 10% Tris-HCL SDS-PAGE at 10 mA for 2 hours. Separated proteins were transferred onto PVDF membrane at ~285-300 mA for 1

hour. The blotted membrane was blocked with 5% nonfat milk in 10X PBS contains 0.1% Tween-20 (PBST) for overnight at 4 ^{o}C. After washing the membrane with PBST, Rat anti-mouse IL-6 antibody (Southern Biotech; Birmingham, AL), diluted in PBST containing 0.02 final sodium azide, was added and incubated for 1 hour. The bound antibodies were detected by anti-rat Ig secondary antibody, diluted in PBST, followed by Pierce West Pico substrates. The blot was scanned using a blot scanner to determine IL-6 levels.

3.9. Statistical analysis

Group measures are expressed as mean ± standard error of the mean (SEM); error bars also indicated SEM. Statistical analysis of differences between groups means were performed using t-test and one-way analysis of variance (ANOVA) for multiple group comparison.

CHAPTER FOUR

DATA AND ANALYSIS

4.1. Frequency of seizures and convulsions after hypoxia

Consistent with previous studies (Vannucci and Hagberg 2004), exposure to global hypoxia induced acute seizures in 95% of the mice (17 of 18 mice ≥ 5 convulsive episodes). Seizures were characterized by hyperactivity, immobility, shaking and tonic-clonic convulsions. During the 90-minute hypoxia episode, vehicle-treated mice averaged 13±1.2 seizures, while RAGE administration turn down the average to 9.3±1.0 seizures (Table 3., Figure 4.1). The hypoxia model works in neonatal mice since there were more convulsions in hypoxic than normoxic control mice. However, there was no statistically significant (P= 0.889) acute effect of RAGE inhibitor FPS-ZM1 on convulsions during hypoxia. We did not test whether or not the RAGE inhibitor would protect against delayed epileptogenic effects of neonatal hypoxia.

Table 3 The number of mice exposed hypoxia-induced convulsions according to the behavioral scale

	Control	Hypoxia-Vehicle	Hypoxia-RAGE
n	7	9	9
Normal (0)	7	0	0
Hyperactivity (1)	0	9	7
immobility and ataxia (2)	0	9	9
Circling, shaking, and clonic seizure (3)	0	8	6
tonic-clonic convulsions (4)	0	6	4

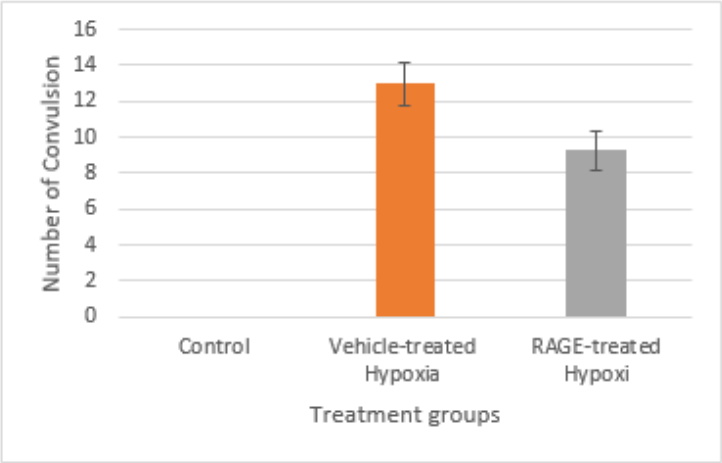

Figure 4.1 Convulsions number in mice treated with RAGE inhibitor (EPS-ZM1). Vehicle-treated mice (*n*=9) averaged 13±1.2 seizures, while RAGE-treated mice (n=9) averaged 9.3±1.0 seizures. Mean ± SEM, Error bars indicate SEM. *P-value* = 0.889

4.2. Effects of RAGE on Cell Death during development

To evaluate the cell death rate in the hippocampus and cortex of 25 mouse pups, a histological apoptosis assay was performed. In vehicle-treated mice (*n*=9) the distribution of positive stained cells was high in different anatomical regions: cortex and hippocampus. Based on our scale of apoptosis, RAGE administration (*n*=9) caused 80% reduction in apoptosis levels seen in vehicle-treated CA1 region of the hippocampus (Figure 4.2A, B, and C, S1). In agreement with previous reports, pyramidal cells of CA1 have a selective sensitivity to hypoxia insult (MacDonald, Xiong and Jackson 2006). In addition, increased distribution of apoptotic cells was detected in the dentate gyrus within the hippocampus cross all the different samples.

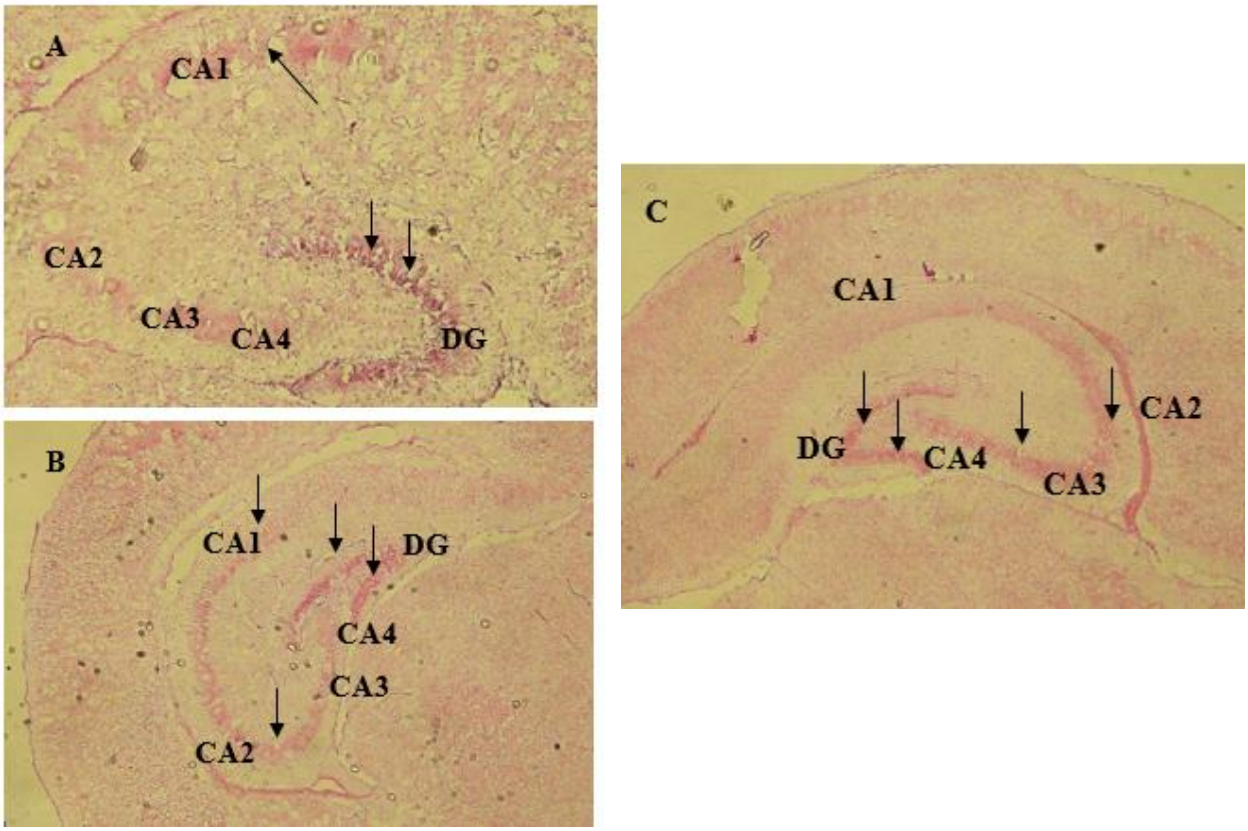

Figure 4.2 RAGE administration decrease cell death in 7-day mouse pups. Illustrated magnified photomicrographs of TUNEL staining on 20 µm coronal sections of brains after hypoxia induction. (A) Positive control (control brain tissue treated with nuclease to express the maximum apoptosis rate), (B) vehicle (0.9% saline) (n=9), (C) RAGE inhibitor (EPS-ZM1) (n=9). Apoptosis cells can be seen as dark pyknotic nuclei (arrows denote examples). RAGE-treated mice showed a mild decrease in apoptosis in CA1 part of hippocampus compared to vehicle. The positive control showed numerous apoptotic cells. Regions shown here: DG: dentate gyrus; CA1, CA2, CA3, CA4

4.3. Brain S100B levels following RAGE inhibitor administration

To evaluate the expression of S100B in the brain – specifically hippocampus and cortex – of mice exposed to hypoxia-induced seizures, immunocytochemical analysis was performed in 25 mouse pups (n=7 control, n=9 vehicle, n=9 RAGE, S2) using an ELISA assay. The expression of S100B was significantly upregulated in vehicle-treated 8% hypoxic mice compared to control (21%) normoxic mice (*p-value* = 0.018). Administration of the RAGE inhibitor FPS-ZM1 resulted in a variable level of S100B in

the brain tissue of hypoxic mouse pups that was not significantly different from that of vehicle treated hypoxic mice (Figure 4.3). Interestingly, RAGE-treated mice – specifically # 3, 4 and 5 – showed the highest levels of S100B (S1, 2), which may indicate a loss of sensitivity to the administrated drug. It is common among individuals to have different response to drugs due to differences in size, genetic mutations, age, and body weight (Rowland and Slutz 2015). In addition, it may be we did not have the optimal time or dose for seeing effects on FPS-ZM1 on S100B. It is also possible that the acute convulsion-induced seizures are more related to the early release of glutamate, and that later effect of hypoxia-induced epileptic seizures may be more driven by RAGE and S100B.

4.4. Serum IL-6 protein expression following RAGE inhibitor administration

Western blot was performed to quantify the amount of Interleukin-6 in 12 mouse pups (n=2 control, n=5 vehicle-treated, n=5 RAGE inhibitor treated, Figure 4.4). The western blot analysis determined a prominent band with molecular weights of approximately 26 kDal in all samples. As can be seen from Figure 4.4., in healthy mouse (negative control), low levels of IL-6 were detected in the serum. It is very likely to observe a similar expression in individuals with normal brain function (Yan, et al. 1992). In contrast, in data obtained from vehicle-treated mice IL-6 expression increased in 80% (4 of 5) mice pups. Despite the significant decrease (*p-value* = 0.012) in expression of RAGE inhibitor treated mice, an interesting increase of expression was also detected (Figure 4.4). IL-6 antibody labeled additional band of higher molecular weight (58 kDal), which may correspond to IL-6 dimer. A visual estimation of blot bands

concentration indicated an increase of IL-6 after exposure to hypoxia in mice-treated with vehicle but not in mice treated with RAGE inhibitor (Figure 4.5).

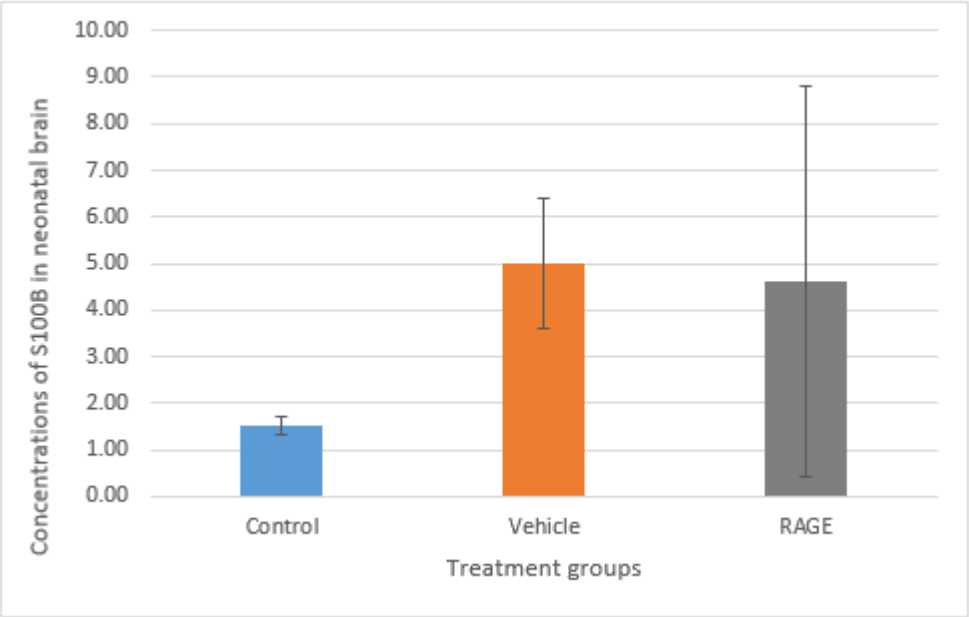

Figure 4.3 Brain S100B levels following RAGE inhibitor treatment. S100B levels were determined by ELISA assay, vehicle-treated mice showed significant increase in levels of S100B (*p-value* = 0.018) with average of 5.00±1.4 µl/. RAGE-treated mice (average 4.61±4.2) did not reduce brain S100B (*p-value* = 0.816). Mean ± SEM, Error bars indicate SEM

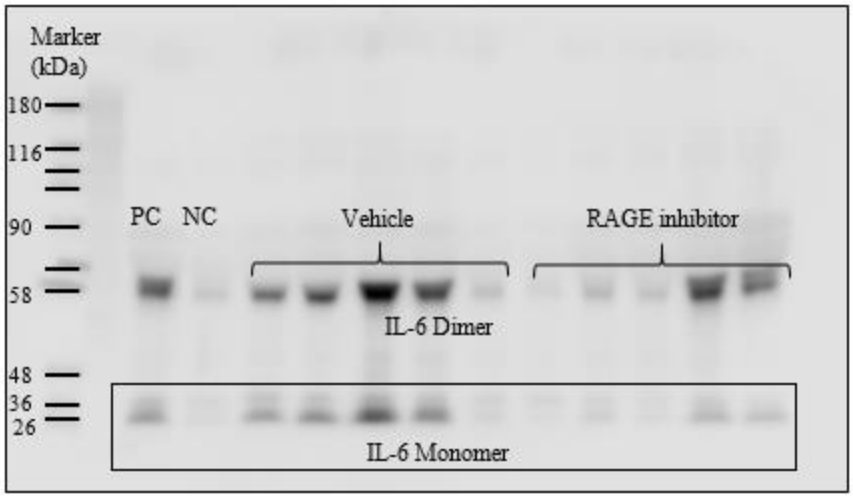

Figure 4.4 Serum IL-6 levels following RAGE inhibitor treatment. Using western blot, IL-6 protein levels were assessed in the blood serum obtained from 7-day mouse pups 24 hours after hypoxia-induced seizures (vehicle or RAGE inhibitor treated), and compared to age match controls. A total of 10 µl of protein extract was loaded in each well and 14 µl of ladder. PC: positive control (Hypoxia w/o vehicle), NC: negative control (no hypoxia, no vehicle/RAGE)

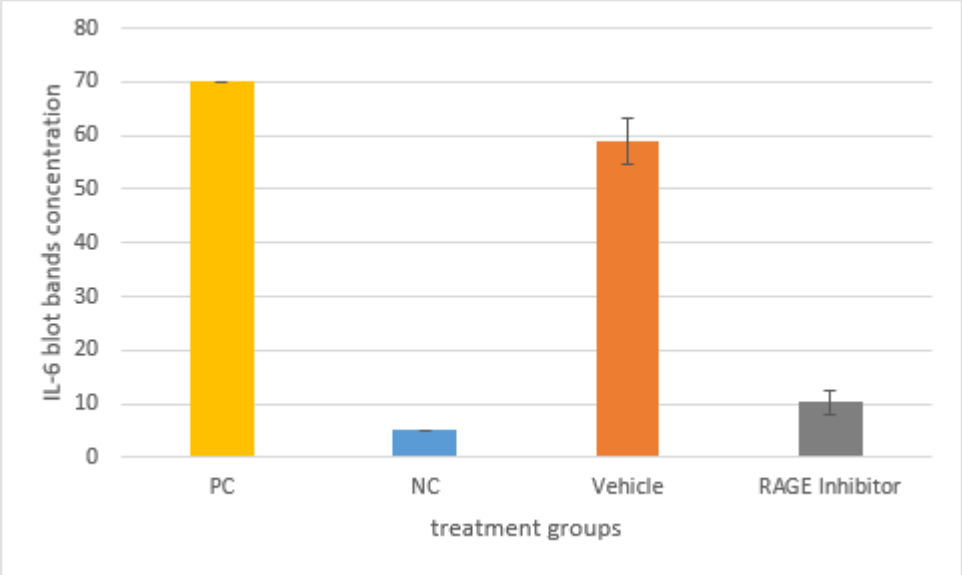

Figure 4.5 Serum IL-6 blot bands concentration. A visual estimation of western blot depicted the concentrations of IL-6 protein in the blood serum obtained from 7-day mouse pups 24 hours after hypoxia-induced seizures (vehicle or RAGE inhibitor treated), and compared to age match controls. Vehicle-treated mice exhibited a higher concentration (%56±4.03) compared to mice treated with RAGE inhibitor has a concentration of %10±2.2 (*p—value* = 0.012). PC: positive control (Hypoxia w/o vehicle), NC: negative control (no hypoxia, no vehicle/RAGE). Mean ± SEM, Error bars indicate SEM

CHAPTER FIVE

SUMMARY, CONCLUSIONS, RECOMMENDATIONS

5.1. Summary

In the last two decades, hypoxia was proposed to be the number one brain injury that accounts for a great amount of mortality and morbidity (Hossain 2005, Vannucci and Hagberg 2004). One of the most common consequences of hypoxia is epilepsy. Epilepsy is one of the most common neurodegenerative disorder that affect 1% of the global population, approximately 30% of which are pharmacoresistant. Understanding the role of S100B in the pathology could provide new targets for the development of alternative treatments for these forms of epilepsy. The general aim of this thesis was to investigate the contribution of S100B in RAGE activation and upregulation of inflammatory mediators following neonatal hypoxia. The specific objectives included: a) investigation the contribution of S100B in inflammatory responses; b) examination of the effect of RAGE antagonist administration on the genesis of epilepsy; and c) evaluate IL-6 levels after induction of acute hypoxia. We used a variety of approaches, including animal model aiming to contribute to the elucidation of mechanisms underlining untreatable epilepsy, in an attempt to find new molecular target for therapy. The results of these pilot studies were encouraging, but not conclusive. The results suggest that RAGE inhibitor FPS-ZM1 have some activity on both S100B and IL-6. As we found that RAGE inhibitor significantly reduced the expression of IL-6 in 60% of the treated mice along with reduction in apoptosis phenotype in brain slices. In contrast, RAGE inhibitor did not significantly reduce convulsions or S100B, although, in some cases, was almost significant. This may relate to dose, time-course and could be explained by that

hypoxia-induced convulsions are mostly driven by glutamate rather than RAGE-S100b interactions. Overall, the RAGE inhibitor showed some efficacy, but not in all areas we expected. However, the results were encouraging, yet we need to take in consideration the pathophysiology and pharmacology related to epilepsy and RAGE inhibitor.

After a general introduction on epilepsy and related key components of the research (section 1.1 -1.6), in section 2.1 we reviewed current evidence regarding the contribution of astrocytes in inflammation. In section 2.2 we reviewed the inflammatory responses in neonatal epilepsy and the pathogenesis consequences. In chapter 3 we provided detailed methods to investigate the cellular concentration of S100B after hypoxia; examine the protective effect of RAGE antagonist drug (FPS-ZM1); evaluate the level of the inflammatory cytokines, IL-6; and measure the apoptosis rate after acute hypoxia. Finally, in chapter 4 we discussed the significance of our results and contextualized our findings.

5.2. Conclusions

Different experimental model data suggest the critical role of S100B, RAGE and inflammatory mediators in the development of spontaneous, refractory seizures leading to the pathogenesis of epilepsy (Sorci, et al. 2013, Iori, et al. 2013, Liimatainen, et al. 2009). Several mechanisms are proposed, including an upregulation of RAGE (Bianchi, et al. 2011). In addition, RAGE may contribute to increase downstream inflammatory mediators, which is a significant player in the process of chronic refractory epileptogenesis (Iori, et al. 2013).

The present study was designed to test the hypothesis that increased expression of S100B can upregulate RAGE receptor and consequently increase the transcription of

downstream inflammatory markers in mice exposed to graded global hypoxia-induced acute seizures. Therefore, brain sections obtained from mouse pups after hypoxia episode was examined with ELISA assay. In addition, blood serum samples obtained from mice exposed to hypoxia were examined with Western blot analysis to determine the level of IL-6. We further assess the distribution of apoptotic cells after RAGE inhibitor administration, to define the potential protective role of EPS-ZM1 and therapeutic target in the pathogenesis of epilepsy.

5.3. Recommendations

To gain a better understanding of the significant role of S100B in the process of epileptogenesis, *in vitro* experiment must be performed to obtain data that complement the *in vivo* results. Also, increasing the sample size to avoid ambiguous variation. In addition, we may test different doses of RAGE inhibitor EPS-ZM1 at different time-course and have a western blot and ELISA analyses for both S100B and IL-6 to determine the real effects of RAGE on their concentrations in the blood serum and the brain.

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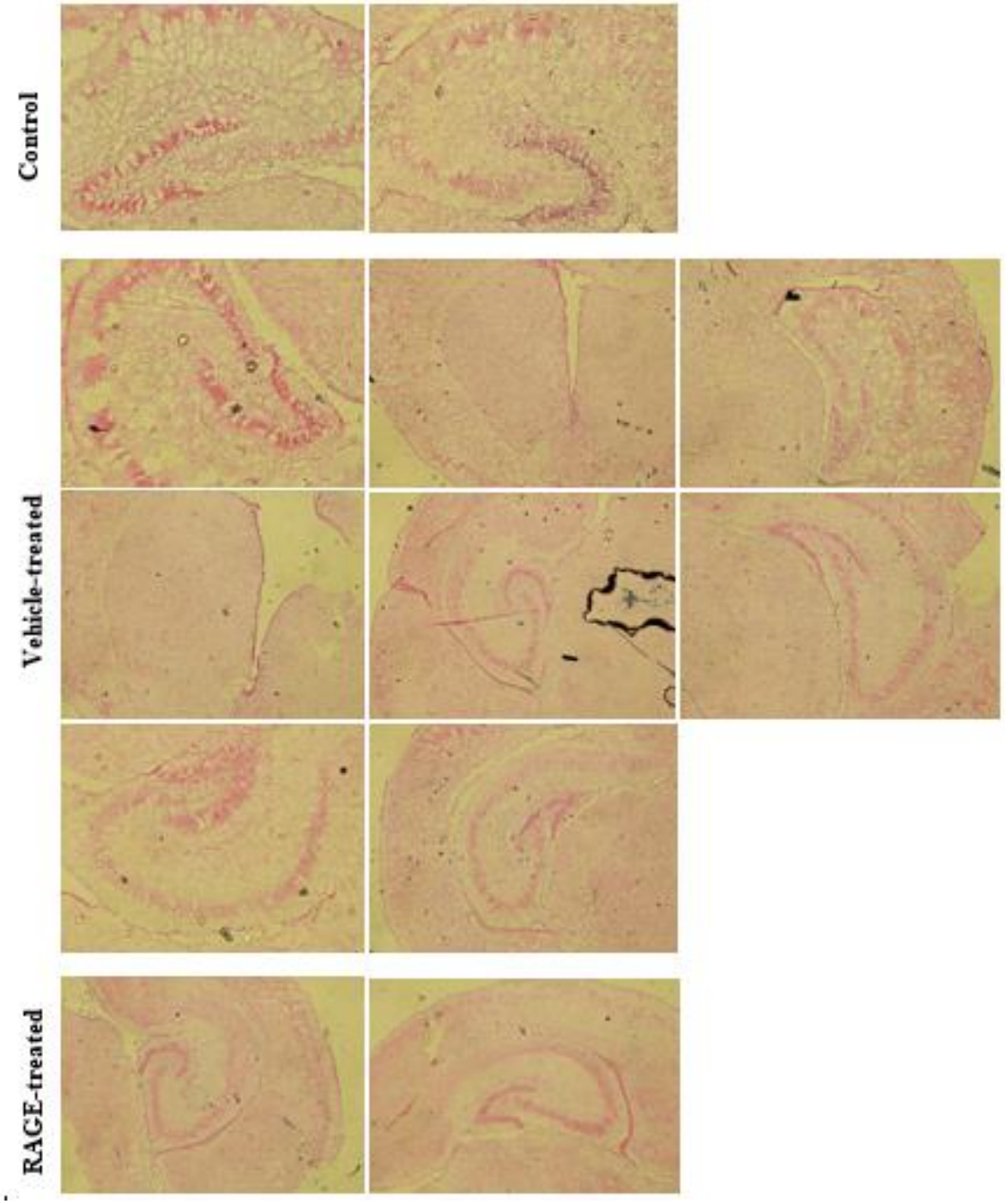
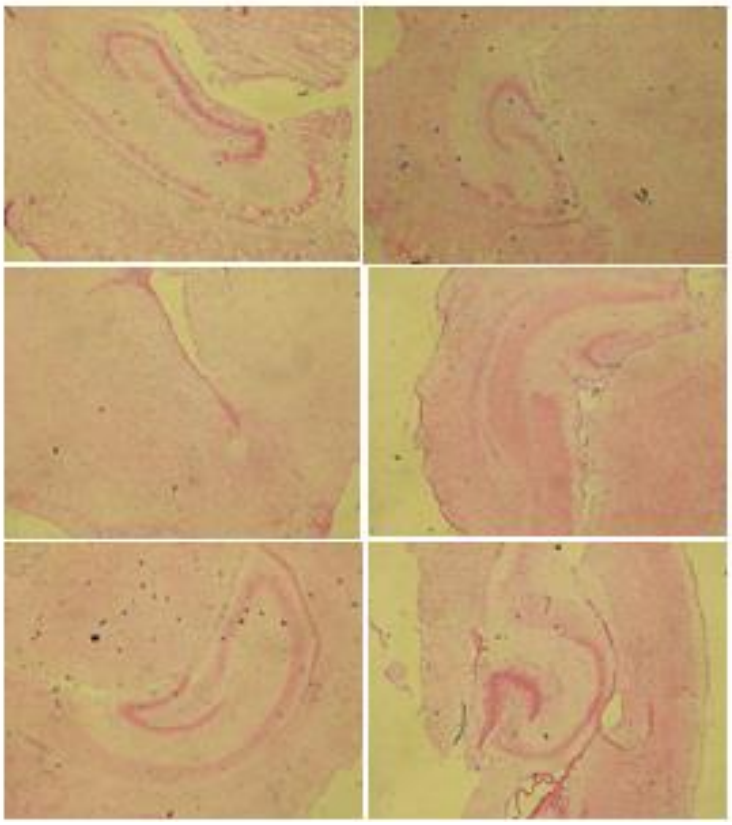
APPENDIX A

Supplements

S1. Histology of the brain coronal sections. Dark red indicate apoptotic cells. Most section depicted the hypothalamus, cortex, and striatum. (A) Control, (B) Vehicle treated and (c) RAGE inhibitor. Each figure represents one mouse.





S2. S100B levels in the brain.

# of mice	Control	Vehicle	RAGE
1	1.80	10.68	1.99
2	1.59	3.95	1.85
3	1.19	1.86	22.14
4	1.24	3.53	24.23
5	1.53	9.09	10.39
6	1.75	6.86	3.85
7		3.30	4.25
8		2.48	5.33
		3.26	