What doesn't kill you makes you stronger: the paradoxical effect of antibodies in epilepsy

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ABTS	2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid)
ALS	Amyotrophic lateral sclerosis
AMPA	α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid
ANA	Antinuclear antibody
ANNA-1	Anti-neuronal autoantibody 1, anti-Hu
ANRE	Anti-NMDA receptor encephalitis
ATP	Adenosine triphosphate
AVM	Arteriovenous malformation
BBB	Blood-brain barrier
BBBD	Blood-brain barrier disruption
Black 6	C57B6/J mice
Ca^{++}	Calcium
CA1	Cornu ammonis 1
CA2	Cornu ammonis 2
CA3	Cornu ammonis 3
CASPR2	Contactin-associated protein-like 2
CB	Chromatin bound
CD4	Cluster of differentiation 4

CNS	Central nervous system
CPS	Complex partial seizures
CSF	Cerebrospinal fluid
CTE	Chronic traumatic encephalopathy
DAB	3,3'-Diaminobenzidine
DAPI	4',6-diamidino-2-phenylindole
DNA	Deoxyribonucleic acid
EEG	Electroencephalogram
ELISA	Enzyme-linked immunosorbent assay
EMG	Electromyogram
F(ab')2	Fragment antigen-binding
FcR	Fragment crystallizable receptor
FIRES	Febrile infection related epilepsy syndrome
FITC	Fluorescein isothiocyanate
GABA	Gamma-aminobutyric acid
$GABA_B$	Gamma-aminobutyric acid receptor beta
GAD	Glutamic acid decarboxylase
GFAP	Glial fibrillary acidic protein
GluR5	Glutamate receptor 5
HCL	Hydrocholoric acid
HEp-2	Human epithelial type 2 cells
HPLC	High- performance liquid chromatography

HRP	Horseradish peroxidase
IgG	Immunoglobulin gamma
IHHS	Idiopathic hemiconvulsion-hemiplegia syndrome
IL-1ra	Interleukin 1 receptor antagonist
IP	Intraperitoneal
IRS	Infection-related seizures
IV	Intravenous
IVIg	Intravenous immunoglobulin
K^+	Potassium
KA	Kainic acid
Kir 4.1	Inward rectifying potassium channel 4.1
LE	Limbic encephalitis
LGI 1	Leucine-rich glioma inactivated 1
Log (P)	Octanol/water partition coefficient
Ma 1&2	Novel neuron and testis specific proteins 1 and 2
MAP2	Microtubule-associated protein 2
Mg^{++}	Magnesium
MHC-1	Major histocompatability complex 1
MMP-9	Matrix metallopeptidase 9

MRI	Magnetic resonance imaging
MS	Multiple sclerosis
NCBI	National Center for Biotechnology Information
NMDA	N-methyl-D-aspartate
NR1	Subunit of NMDA receptor
NZB	New Zealand black
NZBWF1	New Zealand black/white F1 cross
NZW	New Zealand white
PBS	Phosphate-buffered saline
PVDF	Polyvinylidene fluoride
RE	Rasmussen's encephalitis
RIPA	Radioimmunoprecipitation assay
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
SE	Status epilepticus
SEM	Standard error of the mean
SLE	Systemic lupus erythematosus
SN	Soluble nuclear
SPS	Simple partial seizures
SQIg	Subcutaneous immunoglobulins
SUDEP	Sudden unexpected death in epilepsy
TBI	Traumatic brain injury
TCS	Tonic-clonic seizures

TGF-β	Transforming growth factor beta
TJ	Tight junction
UV/Vis	Ultraviolet/visable
VGKC	Voltage-gated potassium channel
ZO	Zonula occludens

For Jillian

Without your unwavering support and strength
I would never have started or finished
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Abstract

Many types of epilepsy, autoimmune or otherwise, are associated with the presence of autoantibodies against neuronal proteins. Paradoxically, antibodies (IVIg) have also been used to treat epilepsy. The goals of this research were twofold: 1) Determine the CNS location of antibodies in patients with non-autoimmune epilepsies and the targets of these antibodies; and 2) Examine the effects of endogenous and exogenous specific and non-specific antibodies in two status epilepticus (SE) models

Immunohistochemistry and Western blotting were used to localize antibodies in patients with epilepsy, multiple sclerosis (MS) and arteriovenous malformation. Further analysis by ELISA, HEp-2 assay and immunoprecipitation revealed antibody targets. In mouse model experiments, lupus-prone or C57B6/J mice were injected with pilocarpine or kainic acid and monitored by EEG. Mice were treated with IV or IP injection of native or denatured IgGs, at time of or 12 hours before chemoconvulsant. Tissues were processed for immunohistochemistry and ELISA.

Brain regions from patients with epilepsy contained extravasated IgGs. Intracellular antibodies were found in epilepsy but not in MS brain. In brain from patients with epilepsy, only neurons displayed nuclear IgGs. All subcellular fractions from brain resections of patients with epilepsy contained extravasated IgGs. In the nuclear IgG pool, anti-histone autoantibodies were identified by two independent methods. Serum analysis revealed anti-histone and anti-chromatin antibodies only in patients with epilepsy.

In lupus-prone mice elevated serum IgGs favored post-SE survival. C57B6/J mice injected with native rat IgGs displayed a 40% reduction in pilocarpine-SE compared to control. IgGs extravasated in brains of untreated SE mice, but IgG-treated mice, with no pilocarpine-SE, experienced no parenchymal accumulation of IgGs. IgG leakage was observed in brain samples from KA treated mice and IgG treatment was largely ineffective.

These results show intracellular IgGs in brain of patients with epilepsy are targeted to histones and chromatin. Further, injected non-specific IgGs have a seizure mitigating effect prophylactically or acutely. Intact IgGs prevent blood-brain barrier leakage and SE and may exert their effect on peripheral inflammation. As rat IgGs were used in these experiments, IVIg may exert its effect through a non-Fc receptor mechanism.

CHAPTER 1:

Background and Aims

1.1 Epilepsy: an ancient disease

The oldest known medical records, dating back 4,015 years, detail the first written accounts of epilepsy. These documents written by the Assaryains and Babylonians describe a disease of divine origin caused by the moon god and called "the hands of sin."[1] Treatment of this disease required an equally divine exorcism. Despite the archaic notions of etiology, the Babylonians were able to describe in great detail febrile seizure, tonic-clonic seizures, focal seizures, simple and complex partial seizures and status epilepticus, among others.[2] Further evidence can be found in the Code of Hamurabbi (also Babylonian) describing how to receive a refund for a slave with epilepsy.[3] Outside of Mesopotamia and around the same time period, an Indian writer and

physician described four different types of epilepsy and attributed their origin to loss of consciousness due to memory disturbances (a rather astute hypothesis given the knowledge available).[4]

The ancient Greeks took the divine characterization of epilepsy to an entirely different level of expertise. The Greeks, similar to the Babylonians, believed epilepsy was a disease caused by a "curse" cast upon the soul by the gods. So fascinated were they by this disease that this "sacred disease" (as they called it) wove its way into their mythology, poems[5] and rituals - Spartan babies were washed in wine to "test" them for epilepsy.[6] Paradoxically, many Greeks thought of epilepsy as a "disease of genius," as both the emperor Caesar and Hercules were thought to have had epilepsy.[7]

It was two famous Greeks, Hippocrates and Aristotle, who officially moved epilepsy out of the realm of the supernatural and into the world of medicine. In his work titled *On the Sacred Disease*, Hippocrates writes scathing criticism of physicians who believed that epilepsy was caused by divine intervention, referring to them as "magicians and charlatans".[8] Aristotle discussed epilepsy in his work *Problems.* He determined, as Hippocrates had, that epilepsy was caused by "black bile" and that it induced a state similar to drunkenness and lethargy.[7]

After Hippocrates and Aristotle, little progress was made toward the understanding of epilepsy. Through the Middle Ages there remained a split

among early physicians between those who classified epilepsy as a divine disease and those who classified it as biological.[9] It should be noted that many physicians were able to better classify and define various components of seizures - most famously Galen who first used the term *aura* to describe pre-ictal sensations.[10]

It was not until the Enlightment began in 1701 that the study of epilepsy, and for that matter the brain, began in earnest. During this time period and continuing throughout the 19th century, scores of French, German and British physicians, surgeons and scientists began studying epilepsy as a treatable disease of biological origin, though the prevailing theory during the 1700s was that epilepsy was linked to sexual deviancy. The primary advances in the understanding of epilepsy during the 18th and 19th centuries were in the establishment of standard lexicon still in use by epileptologists and epilepsy scientists (e.g., 'epileptiform' coined by John Russell Reynolds), the recognition that pre-ictal and ictal behaviors often correlated with anatomical brain regions and the introduction of epilepsy surgery.[11]

1.2 Current epidemiological perspective of epilepsy

At present there are approximately 2 million people living in the United States with epilepsy and an estimated 65 million worldwide.[12] It is the fourth most common neurological disease in the United States after migraine, stroke and

Alzheimer's disease. Epilepsy can effect anyone at any age, though it is most prevalent in the very young and the elderly.[12] The most common causes of epilepsy include traumatic brain injury, central nervous system (CNS) infections, brain tumors and stroke, but the etiology of most remain elusive[13] **(Figure 1.1)**.

Strikingly, nearly 50,000 deaths are associated with epilepsy in the United States each year.[14] While better pharmaceuticals, technological advancement in epilepsy surgery and a greater understanding of the neurobiology of epilepsy has allowed more patients to lead relatively seizure-free lives, 30% of all patients with epilepsy have uncontrolled seizures.[15] Further, the direct cost of epilepsy in the United States is approximately $12.5 billion annually, with the majority of that cost directed towards patients with intractable epilepsy.[16] Both the number of people in the United States with epilepsy and the direct costs of the disease are expected to rise over the next decade as nearly 440,000 soldiers return from

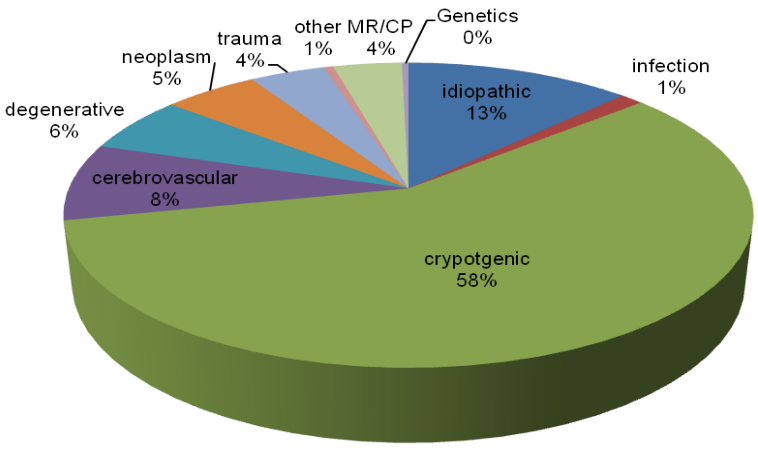

Figure 1.1: Classical etiologies of epilepsy as percent of total cases.

Adapted from Hauser, WA. in Epilepsy: A comprehensive Textbook (2008)[13]

Iraq and Afghanistan with head injuries. It is estimated that a significant number of these soldiers will develop epilepsy.[17]

1.3 Clinical definitions of epilepsy and classifications of seizures

In 2014, [18] the International League Against Epilepsy published an updated definition of epilepsy. The new operational clinical definition of epilepsy is as follows: "A patient is said to have epilepsy if 1) They have at least two unprovoked (or reflex) seizures occurring less than 24 hours apart; 2) One unprovoked (or reflex) seizure and a probability of further seizures similar to the general recurrence risk (at least 60%) after two unprovoked seizures, occurring over the next ten years; 3) Diagnosis of an epilepsy syndrome." In addition, the updated definition also states that epilepsy is resolved in an individual with an age-dependent epilepsy syndrome when the patient is past the age limit and if the patient has remained seizure-free for the last ten years with no anti-seizure medication for the last five years.

As is clear from the definition above, seizures are the most common clinical manifestation of what is often an extraordinarily complicated disease. As such, seizures have been well classified both behaviorally and electrophysiologically but often lack classification based on underlying

pathophysiology, though certain types of EEG phenomena are associated with specific epilepsy syndromes (e.g., infantile spasms and hypsarrhythmia).[19] The international seizure classifications are described at length below.[20] While this classification describes the most common types of seizures it should be acknowledged that many of them can be "mixed" with other types of seizures that are outside of the international classification (e.g., tonic-absence seizures). In addition, only the major seizure types will be discussed and rare seizure types only discussed as necessary (e.g., gelastic seizures and negative myoclonus).

Partial seizures:

Partial seizures, also called ‘focal’ or ‘localized seizures’, are further divided into complex partial seizures (CPS) and simple partial seizures (SPS). These two subcategories refer to their ability to impair consciousness or not, respectively. It is also worth noting that both types of seizures can evolve into generalized seizures which are termed 'secondary generalizing' seizures.

Simple partial seizures

Simple partial seizures are the most common type of seizure within the first year of life and are estimated to be the sole manifestation of epilepsy in up to 12% of patients.[21] Symptoms of SPS are typically associated with the brain regions affected. For instance, if the epileptogenic focus is located in the temporal lobe, patients experience hallucinations or inappropriately timed

emotions (e.g., fear, anger, etc). Often, SPS associated with hallucinations are misdiagnosed as psychosis or schizophrenia.[22]

A fascinating and well studied phenomenon present in many patients with SPS with motor involvement is the 'Jacksonian march'. This phenomenon occurs when the sensations triggered by the seizure start in the fingertips (or toes) and spread up the arm (or leg) towards the torso. These seizures may also have facial involvement and/or generalize.[23]

Rolandic seizures are yet another type of simple partial seizure. These occur during childhood and are linked to an autosomal dominant mutation on chromosome 15, possibly linked to the acetylcholine receptor.[24] Rolandic seizures are associated with hemifacial paresthesia and hemifacial contractions, among other common sensory symptoms.[25] Most SPS are not associated with a genetic mutation but rather developmental abnormalities in the CNS, trauma, encephalitis, vascular malformations and tumors.[26] Irrespective of the origin, consciousness is maintained for the duration of the seizure which may last from several seconds to minutes.[27]

The wide variety of symptoms that are often seen in patients with SPS can make for a challenging diagnosis. For example, SPS with autonomic involvement may produce only gastrointestinal symptoms that may be misdiagnosed,[28] tingling or numbness sensations that may be confused with migraine and paralysis (such as Todd's paralysis) that may be confused with stroke or tumor.[29]

Further complicating diagnosis is the lack of ictal electroencephalogram (EEG) changes in some patients with SPS. Numerous studies have demonstrated that only a small percentage of patients (21% in one study[30]) will show changes during a simple partial seizure at the scalp EEG level. Additionally, while MRI may reveal a brain lesion associated with these seizures, often patients have negative MRI findings. For patients with intractable SPS, stereo-EEG (depth electrodes) or dural grids may be used to pinpoint and remove the epileptogenic zone.[31]

The prognosis for patients with SPS is typically favorable. Patients with SPS are less likely to have seizure-related accidents (e.g., falls or traffic accidents),[32] but the underlying etiology of the seizures may lead to significant morbidity and mortality and uncontrolled SPS may worsen or develop into generalized seizures. First-line treatment options for patients with SPS that do not generalize are the anti-seizure drugs carbamazepine and lamotrigine.[33,34] For refractory SPS, alternative treatments may be attempted (e.g., steroids and vagal nerve stimulation) and, as a last resort, surgical removal of the lesion.[35,36]

Complex partial seizures

Complex partial seizures are the primary seizure type in 35% of patients with epilepsy. They are also the most common type of seizure in adults, particularly those older than 60 years of age. CPS are also common in areas

where cysticercosis (a disease caused by a parasite in pork) is endemic, as it can lead to neurocysticercosis that may evolve into epilepsy.[37]

Simple partial seizures often precede CPS (referred to as an *aura*). CPS involve impairment to consciousness, though the impairment may not be readily apparent to a casual observer. CPS are also associated with automatisms[38], that is, movement - often of the extremities or face - that is involuntary, stereotyped and repetitive (e.g., lip smacking and patting) usually appearing after consciousness is lost. As with SPS, CPS symptoms are associated with the region of the brain from which they arise.[39] Eighty percent of patients with CPS have an epileptogenic focus in the mesial temporal lobe and most patients have some degree of hippocampal sclerosis (based on MRI findings).[40] As such, typical auras include déjà vu, euphoria and hallucinations.

Scalp EEG is the most commonly used method to detect CPS. Unfortunately, numerous subcortical structures may be involved in CPS and therefore these seizures many not be readily apparent on scalp EEGs; this has often lead to misdiagnosis of CPS as psychiatric conditions.[41] MRIs are particularly useful in diagnosing CPS as hippocampal sclerosis is frequently observed by this method.[42]

Patients with CPS are at a greater risk of seizure-related accidents than patients with SPS due to loss of consciousness.[43] In addition, patients with CPS are also at greater risk of sudden unexpected death in epilepsy (SUDEP) than

patients with SPS.[44] Standard anti-seizure medications are used to treat CPS (carbamazepine, lamotrigine, levetiracetam, etc),[45] but as a last pharmacological resort vigabatrine may be used.[46] Further treatment options include vagal nerve stimulators and mesial temporal lobectomy.[47]

Generalized seizures:

These seizures involve synchronous electrical activity across both sides of the brain. As with partial seizures, this general electrical phenomenon can have any number of etiologies. The major types of generalized seizures are tonic-clonic seizures (formerly *grand mal*), atonic seizures, absence seizures (formerly *petit mal*), myoclonic seizures, tonic seizures and clonic seizures.

Tonic-clonic seizures

Tonic-clonic seizures (TCS) are the "prototypical" epileptic seizure, perhaps due to their dramatic presentation. They comprise 25% of seizures[12], but are the least common seizure type in infants and elderly individuals. In addition to the typical cause of seizures (TBI, stroke, genetic diseases, etc.), TCS can be caused by low electrolyte levels (e.g., magnesium), alcohol or drug withdrawal.[48] Diabetic shock is also a trigger for TCS which has been used to the advantage of clinicians in the form of electroconvulsive therapy.[49] TCS may also have no known origin.

Presentation of tonic-clonic seizures can be divided into four distinct phases: pre-ictal, tonic, clonic and post-ictal. The most common presentation of

the pre-ictal state in TCS is a pre-ictal cry caused by rapid contraction of the chest and abdominal muscles. The pre-ictal state may also include prodromes (e.g., insomnia and mood disturbances) that occur hours to days before a seizure.[50] This is distinct from secondary generalizing seizures where patients experience an aura.[51] The tonic phase involves loss of consciousness and intense muscle contraction throughout the body. The patient can be seen with arms and legs pulled in towards the body or with extremities extended away from the body; in either instance the patient will fall if standing or sitting unsupported. This phase of the seizure is the shortest, lasting less than a minute.[52]

The clonic phase of the seizure is characterized by repeated, rhythmic and rapid contraction and relaxation of the muscles throughout the body (convulsions). In many cases cyanosis and incontinence occurs. In addition, many patients may bite their tongue during the seizure.[53] The final phase of a TCS is the post-ictal phase which lasts approximately 30 minutes, though it can be longer.[54] This state is marked by an altered level of consciousness in which a patient may be unresponsive, confused, nauseous or have a headache, among other symptoms. While the precise mechanisms that lead to the post-ictal state are unknown, cascades of events have been observed that could produce what is experienced by these patients. These changes include depletion of neurotransmitters,[55] acidosis and decreased cerebral blood flow relative to metabolism.[56]

Patients with TCS will undergo a standard EEG and imaging work-up in the clinic. Frequently pseudoseizures present as TCS; to help differentiate a true tonic-clonic seizure from a pseudoseizure a prolactin test may be administered during the post-ictal period[57] if standard EEG assessment fails to delineate between the two types. Presumably, prolactin is increased after seizures due to limbic activation of the hypothalamus which leads to dopamine depletion and the subsequent release of prolactin.[58] Treatment options are numerous for patients with TCS. In addition to anti-seizure medication,[33] vagal nerve stimulation[59] and surgery, a ketogenic diet has shown great promise in decreasing seizure burden in these patients, though the exact mechanism has yet to be determined.[60]

Tonic seizures

While tonic seizures are a generalized seizure type that is fairly uncommon, most patients with Lennox-Gastaut Syndrome, a rare but devastating childhood epilepsy, experience them. Onset of tonic seizures is sudden and seizures last less than a minute. As this is a generalized seizure, the patient will lose consciousness at the onset of the seizure, which may lead to injury if the patient falls. Tonic seizures present as a rapid onset of muscle contraction, but not relaxation, throughout the body. Patients may have difficulty breathing during the seizure when the chest and abdominal muscles contract tightly.[61] Onset of these seizures often occurs during sleep or just after waking. The post-ictal period of these seizures is short with mild confusion.[62]

As tonic seizures are associated with Lennox-Gastaut Syndrome and the seizures are generalized, scalp EEG recordings showing tonic patterns make diagnosis relatively straightforward. Treatment of tonic seizures diagnosed under the umbrella of Lennox-Gastaut syndrome can be particularly difficult. Anti-seizure medications are prescribed but are rarely effective alone and no single drug has shown greater efficacy than any other.[63] In addition to standard treatments, corticosteroids or intravenous immunoglobulins (IVIg) have been shown to reduce seizure burden in these patients.[64]

Myoclonic and clonic seizures

Myoclonic and clonic seizures involve the same basic involuntary movements but differ in duration and associated epilepsy syndromes. Myoclonic seizures involve momentary (less than one second) contraction and relaxation of a particular muscle often described as "jumps" or "jerks." There is no detectable lapse in consciousness by an observer but spikes may be observed on EEG that can then be correlated to spikes on electromyogram (EMG).[65] Most myoclonic events (e.g., hiccups[66]) are not associated with a neurological disease but several epilepsies are specifically linked to myoclonic seizures; these include juvenile myoclonic epilepsy[67] and progressive myoclonic epilepsy.[68] Juvenile myoclonic epilepsy is a relatively common epilepsy comprising up to 10% of epilepsy cases and is associated with a myriad of ion channel mutations.[69] Progressive myoclonic epilepsy is an extremely rare disorder that falls under the

veil of a number of genetic childhood neurological diseases that are almost always fatal.[70]

First-line treatments for myoclonic seizures are clonazepam, valproic acid or lamotrigine.[71,72] Carbamazepine should be avoided as it can exacerbate these seizures.[73] A number of other treatment options remain available including hormone therapy and serotonin.[74]

Clonic seizures are not associated with any particular epilepsy syndrome but have the same origin as many seizure types including stroke, CNS infection, tumor, traumatic brain injury, etc.[13] These seizures are differentiated from myoclonic seizures by their duration (minutes) and clear loss of consciousness.[75] Treatment involves standard anti-seizure medications or surgery when an identifiable lesion is present.

Atonic seizures

These seizures, also called drop seizures, cause a sudden and brief loss of muscle tone. Symptoms may include head nodding, drooping eyelids or, in severe cases, complete loss of muscle tone resulting in the patient falling. While they may occur in the absence of a broader neurological syndrome, they are most frequently associated with Lennox-Gastaut Syndrome.[76]

To confirm diagnosis of atonic seizures, Scalp EEG is coupled with EMG.[77] Atonic seizures are notoriously difficult to treat and refractory seizures

often result in injury, leading to the patient wearing protective equipment when standing. In the context of Lennox-Gastaut Syndrome, rufinamide has shown efficacy in reducing these seizures significantly.[78] In addition, while standard anti-seizure medications are ineffective in reducing the number of atonic seizures a patient has, the ketogenic diet has shown tremendous efficacy in preventing these seizures. Many studies using the ketogenic diet have demonstrated a greater than 50% reduction in seizures without additional anti-seizure therapy.[60]

Absence seizures

Of all the seizure types described herein, absence seizures are the most diverse between patients and the number of variables (timing, duration, automatisms, autonomic involvement and muscle tone) are nearly limitless.[79] For purposes of brevity and clarity, these seizures will be discussed in general by their two major types - typical and atypical.

In general, absence seizures are brief (less than 30 seconds) generalized seizures with little or no post-ictal period. In severe cases seizures may occur up to 100 times per day. Children are most susceptible to these seizures and they rarely occur for the first time in patients older than 13 years.[80]

Absence seizures classified as typical are characterized by specific EEG patterns faster than 2.5 Hz in a '3 per second spike and wave' formation.[81] Typical absence seizures have no known cause and are not associated with a broader epilepsy syndrome or developmental syndrome.[82] Atypical absence

seizures are characterized by 'spike and wave' formations less than 2.5 Hz on scalp EEG in addition to abnormal interictal EEG patterns. These seizures occur concomitantly with other juvenile and developmental neurological syndromes.[83] Frequently, absence seizures are not the only seizure type present in these patients and duration can be variable.[80]

Irrespective of underlying etiology, detection of these seizures occurs through EEG monitoring and seizure induction by hyperventilation.[84] Typical and atypical absence seizure are treatable in the majority of cases with valproic acid or ethosuximide.[85] Drugs that act on gamma-Aminobutyric acid (GABA) receptors (e.g., vigabatrine) should be avoided for the treatment of absence seizures as they can trigger an ictal event.[86]

Status epilepticus

Though not a seizure type in and of itself, status epilepticus (SE) is a seizure lasting greater than five minutes that occurs in patients with generalized or complex partial seizures. Up to 20% of first seizures present as SE.[87] An increased risk of mortality is associated with SE, but rapid treatment and proper precautions can mitigate some of this risk.[88] First-line treatments for SE include rectal diazepam, benzodiazepines or barbiturates.[89] In addition, IVIg has also been effective in treating super-refractory SE.[90]

1.4 Pathophysiology of epilepsy as it relates to seizure threshold

To characterize epilepsy as simply different kinds of seizures betrays an extraordinarily complex and variable set of pathologies that involve multiple systems within and outside of the CNS. Seizures are simply the end product, but not the only end product, of pathologies such as tumors,[91] malformations in cortical development,[92] traumatic brain injury,[93] inflammation/autoimmunity,[94] blood-brain barrier disruption,[95] infection[96] and hypoxic-ischemic injury,[97] among others. Aside from seizures, these pathologies are associated with mental illness (e.g., schizophrenia[98]), intellectual disabilities (e.g., cerebral palsy[99]) and memory loss (via hippocampal sclerosis[100]). Of particular importance to the work contained in the following pages are inflammation, autoimmunity and blood-brain barrier (BBB) disruption.

It is convenient to think of the state induced by the cornucopia of CNS pathologies listed above as contributing to the overall seizure threshold of an individual **(Figure 1.2)**. Everyone has a seizure threshold **(Figure 1.2 A)** and throughout the course of a day an individual's seizure threshold is steady but events may take the CNS closer to that threshold. For example, head injury can promote an excitatory and inflammatory environment in the brain which may

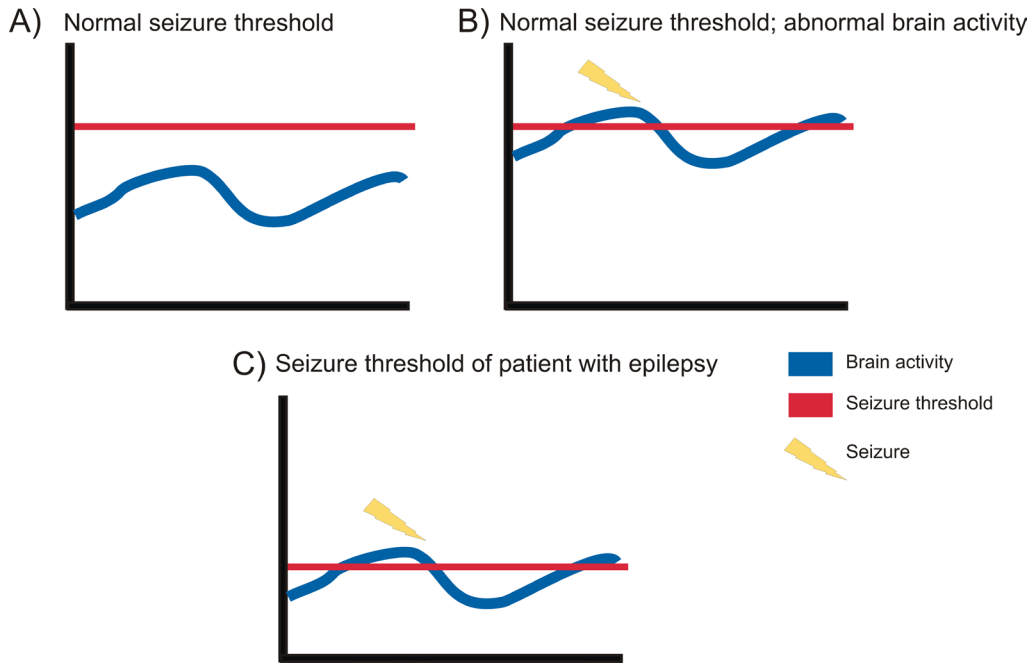

Figure 1.2: Graphical depiction of the concept of seizure threshold. In **A,** a normal seizure threshold and brain activity are depicted. In **B,** a normal seizure threshold is depicted with abnormal CNS activity resulting in a seizure. This abnormal activity can be cause by drugs (illicit or prescription), withdrawal from alcohol or head injury. All of these, however, may result in a seizure but not necessarily epilepsy. In **C,** the seizure threshold of a patient with epilepsy is depicted. Any number of factors can lead to a permanently lowered seizure threshold ("leaky" BBB, disruptions in cortical architecture, etc.). Under these conditions even minor alterations in CNS activity can trigger a seizure (e.g.,strobe lights).

result in a seizure. However, an acute seizure after a traumatic brain injury does not constitute epilepsy **(Figure 1.2 B)**. If this same patient suffers a severe enough traumatic brain injury as to lead to prolonged BBB damage, prolonged CNS inflammation and/or permanent changes in cortical architecture, this may lower the patients overall seizure threshold. Future insults or triggers will more readily produce seizures than before the injury. Unfortunately, some individuals

have such a severely lowered seizure threshold (e.g., patients with Lennox-Gastaut syndrome) that activities of daily living and/or minute metabolic changes can trigger a seizure **(Figure 1.2 C)**.

Over the following pages the role of the blood-brain barrier (BBB) and the interplay between BBB disruption, inflammation and autoimmunity will be discussed. Furthermore, the mechanisms by which they modify seizure threshold and lead to epilepsy will also be detailed.

1.5 A brief introduction to the blood-brain barrier

Prior to a discussion of how the BBB contributes to epilepsy, a short preamble on the function and components of the BBB that maintain homeostatic ion gradients and provide immunological privilege in the brain is necessary.

The BBB lines the cerebral microvasculature and is composed of, among other cellular components, differentiated endothelial cells and the tight junctions (TJs) that link them together.[101] Endothelial cells of the BBB are differentiated in that they display less pinocytic activity, lack fenestrations and have an increased number of mitochondria compared to endothelial cells in the peripheral vasculature. Exposure to luminal flow is a key factor in endothelial cell differentiation.[102] This functional differentiation is most likely due to the tight regulation of transcellular tansport into the brain. While there are a number of molecules that can freely cross the BBB (e.g., ethanol), most substances,

particularly those that are large and/or hydrophilic, must cross the BBB via primary or secondary active transport (transport requiring ATP) or by virtue of existing concentration gradients. Energy dependent transport across the BBB provides a mechanism by which movement of substances into the brain can be regulated based on the requirements of the cerebral environment. For instance, glucose transporters are upregulated on the luminal side of the membrane when cerebral nutrient supply becomes low.[103]

Of particular importance to the maintenance of ion homeostasis and integral to any discussion of the BBB are tight junctions **(Figure 1.3)**. These structures provide a means by which endothelial cells can be physically linked together, creating a continuous impermeable barrier and forcing the movement of ions and macromolecules to occur across the endothelial membrane. TJs are comprised of a number of proteins including the integral membrane proteins occludin and claudins-3,-5 and -12. These proteins serve to form the characteristic paracellular seal of the BBB. In the cytoplasmic compartment, occludin and claudins are linked to the zonula occludens (ZO) family of adaptor proteins. ZO-1,-2 and -3 bind to claudins and ZO-1 binds to occludin. Adaptor proteins are bound to secondary adaptor proteins that anchor the junctional complex to the cytoskeleton. In addition to TJs, a secondary barrier, the adherens junction, is located below the TJ in the paracellular space. Adherens junctions serve to further limit vascular permeability.[104] However, the BBB is not simply an endothelial cell lining, rather it is a unit composed of specifically

differentiated endothelial cells, astrocytes, pericytes and a basement membrane **(Figure 1.3)**. Astrocytes play a particularly important role in the support and formation of the BBB. *In vitro* experiments demonstrate that BBB endothelial cells will not form an adequate barrier in the absence of astrocytes. Further, astrocytes play a major role in shuttling water and ions to and from the area surrounding the cerebral microvasculature ('spatial buffering').[105]

Vital to understanding how compounds are able to cross an intact BBB when a specific transporter is absent is a brief explanation of the log octanol/water partition coefficient and its role in predicting how and if compounds will cross the BBB. The log octanol/water partition coefficient (Log(P))[106] provides an excellent quantitative way to predict how fast and to what extent a compound will cross the BBB. Log(P) is the ratio of concentrations of a given compound in each part of two immiscible solvents. The equation for Log(P) is:

$$Log(P) = \log\left(\frac{[solute]octanol}{[solute]H20}\right)$$

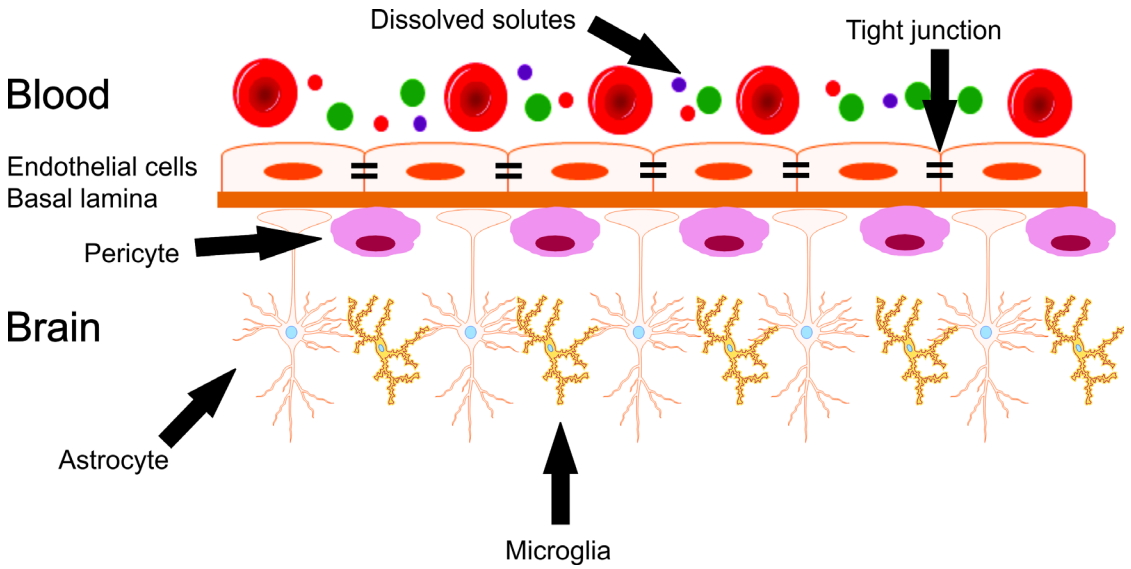

Figure 1.3: 2D depiction of the basic cell types and layout of the blood-brain barrier. It is important to note that each of the cell types illustrated above, as well as tight junctions, are found surrounding the vessels of the cerebral microvasculature forming an encapsulating seal around the vessel. In addition to the cells depicted above, transient "surveillance" cells (e.g.,macrophages) can often be found near the BBB.

Typically this coefficient is determined using an aqueous substance (water) and a hydrophobic substance (octanol). Measurement of Log(P) is achieved by the "shake-flask" method followed by UV/Vis spectroscopy or HPLC.[107] An important concept to keep in mind is that the final result of this equation is a velocity (e.g., ml/g/sec) indicating that while many compounds will cross the BBB eventually, only compounds with appropriate properties will cross rapidly enough to produce an effect.[106] Compounds with a high Log(P) favor hydrophobic compartments and will cross a lipid bilayer while compounds with a low Log(P) will tend to stay in hydrophilic compartments (e.g., serum) and will not cross the BBB.[108] As a

general rule, compounds with a Log(P) > 0 will cross the BBB rapidly with the major limiting factor being supply of the drug. On the other hand, compounds with a Log(P) < -1 are limited in their ability to cross the BBB.[109] It is therefore the coupling of the biophysical properties of cell membranes, facilitated transport mechanisms and receptor specificities that allow the BBB to maintain the normal homeostatic milieu in the brain.

A secondary role of the BBB is the maintenance of CNS immune privilege. In that vein, an abundance of studies have demonstrated the preferential anti-inflammatory environment promoted in the CNS.[110] Evidence for this can be seen in the low levels of MHC-1 molecules and immune-stimulatory molecules found in the normal CNS[111] in addition to the production of anti-inflammatory cytokines in the CNS.[112]

T cell responses are particularly suppressed in the brain parenchyma; the strongest evidence for this comes from transgenic mice that, though programmed to develop CNS autoimmunity, do not when maintained under pathogen-free conditions.[113] This lack of inflammation occurs despite evidence demonstrating the ability of CNS targeted T cells to detect antigens across the intact BBB.[114] Going further, the above information does not mean that T cells are unable to enter the CNS. Many studies have demonstrated that several types of T cells (non-activated CD4+ lymphocytes in particular) readily enter and exit the CNS.[115] However, T cells found in the CNS are rarely observed in the parenchyma but

stay in the perivascular space where their effecter functions may be held at bay.[116]

Protecting the delicate environment in the brain is the lack of direct lymphatic drainage.[117] While some studies have demonstrated that CNS antigens can make their way to the cervical lymphnodes[118], other studies have demonstrated the opposite.[119] Irrespective of lymphatic drainage, it can be said that immunological privilege in the brain is relative. From time to time the CNS interacts with the peripheral immune system usually resulting in no damage either way. Further, transient opening of the BBB due to insult or aging is neither sufficient nor necessary to trigger an autoimmune response. However, the environment produced in the epileptic brain is not typical of other CNS diseases. In the epileptic brain, both transient and chronic opening of the BBB is a regular occurrence and both CNS and peripheral inflammation has been observed. This cycle sets up an ideal environment for the threshold of immune tolerance to be surpassed and a chronic inflammatory/autoimmune response may ensue.[120]

1.6 The blood-brain barrier and epilepsy

The strongest direct evidence for the role of the BBB in seizures and epilepsy comes from a seminal paper demonstrating that opening of the BBB by hyperosmotic mannitol both in humans and pigs led to seizures.[121] There are a number of potential mechanisms by which opening of the BBB could trigger ictal activity acutely. First and foremost is a loss of ion homeostasis across the BBB. Extracellular potassium levels are kept low in the CNS (3 mM), however serum levels are much higher (5 mM). Opening of the barrier will result in a loss of this ion gradient and promote hyperexcitability.[122] Conversely, Magnesium (Mg) is present at high concentration in the CNS and is relatively low in serum. Opening

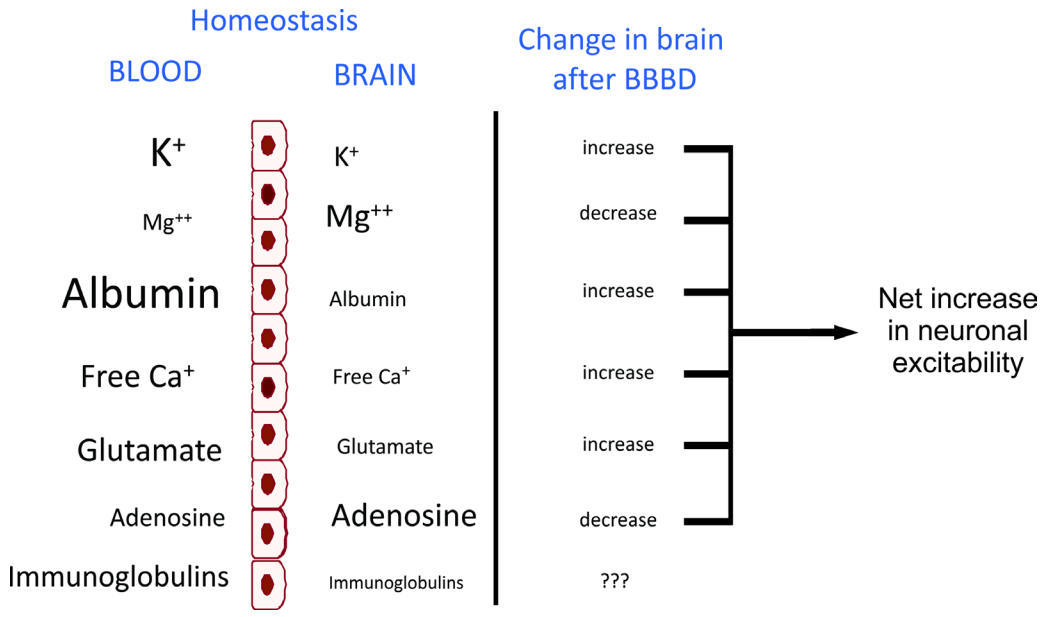

Figure 1.4: Gradients of ions, neurotransmitters and serum proteins across a normal blood-brain barrier and their predicted movement into/out of the brain after the blood-brain barrier is opened during a seizure.

Adapted from Janigro (2012)[94]

of the barrier would promote efflux of Mg from the brain, again, promoting hyperexcitibility.[123] This mechanism is taken advantage of clinically to treat women with preeclampsia in an effort to prevent seizures[124] and some traumatic brain injury patients who are given IV Mg to prevent seizures.[125]

Loss of neurotransmitter homeostasis also plays a role in promoting ictal activity after BBB disruption. The amino acid and excitatory neurotransmitter glutamate is far higher in blood than in brain and therefore opening of the BBB will promote a hyperexcitable environment by providing excess excitatory neurotransmitter.[126] On the other hand, adenosine (an inhibitory neurotransmitter) is present at higher concentrations in brain than blood. Upon opening of the barrier, adenosine will leave the brain further promoting excitability.[127]

A fascinating and particularly well studied modulator of excitability in the brain parenchyma is serum albumin. Abundant evidence demonstrates that serum albumin will enter the brain of most organisms capable of having seizures. Following accumulation in the brain, albumin binds to TGF-β receptors on glial cells triggering down-regulation of the Kir 4.1 potassium channel. This ultimately results in reduced spatial buffering and increased excitability.[128]

The above observations are likely mechanisms for acute and early seizures after traumatic brain injuries, stroke, iatrogenic BBB disruption and other insults that result in short-term BBB opening. Further, it is highly likely that these

mechanisms work in synergy after opening of the BBB to promote an overall excitability in the brain and enhance injury due to excitotoxicity and inflammation.[95] However, seizures and the spectrum disease of epilepsy, though related, are not the same, though it should be noted that the old adage "seizures beget seizures" does hold true.[129;130]

Often, a "neurocentric" approach is taken when studying epilepsy and the neuronal phenomena that contribute to a number of different epilepsies (balloon cells[131], ectopic neurons[132] and dysmorphic neurons[133]) have been well described. Further, many epilepsies are accompanied by dysfunctional cerebral endothelial cells and a malformed BBB.[134,135] These same epilepsies are accompanied by activated glial cells that produce and express inflammatory cytokines and markers.[136] It is therefore likely that a synergy exists between hyperexcitable neurons and inflammation promoting a "leaky" and/or poorly formed BBB that contributes to recurrent seizures in epilepsy. Moreover, frequent seizures lead to more CNS damage and more inflammation that only serves to produce more seizures.[120]

All of the above evidence begs the question: with experimental and clinical evidence thoroughly demonstrating that components from serum can enter the CNS after seizure, and with potential for compromising immune privilege in the CNS after opening of the BBB, does the peripheral immune system interact with

the BBB and CNS in patients with epilepsy? The answer to this is a resounding yes.

1.7 Autoimmunity in epilepsy

It could be said that the majority of epilepsies involve some component of inflammation, which is secondary to the underlying pathology and is produced by recurrent seizures. This can be observed in the extensive microglia activation and astrogliosis observed in many patients with distinct epilepsies.[137] Therefore, it is important to identify the differences between inflammatory epilepsies (infection related epilepsies) and autoimmune epilepsies.

Inflammatory epilepsy syndromes, the most notable of which are febrile seizures, febrile infection related epilepsy syndrome (FIRES) and idopathic hemiconvusive-hemipelegia syndrome (IHHS) are triggered by yet unknown viral infections. While many febrile seizures do not result in life-long epilepsy, FIRES and IHHS often have devastating consequences that lead to permanent intellectual disability and severe intractable epilepsy.[138] Still other epilepsies can result from CNS infection unrelated to fever, most commonly neurocysticercosis. Neurocysticercosis is the most common cause of epilepsy in the developing world. Epilepsy results from the formation of necrotic cysts in the brain parenchyma that often do not produce symptoms for years after infection.[139]

With rare exception, the primary effector mechanisms of autoimmune epilepsies are antibodies. To that end, an ever growing number of antibodies

have been observed in patients with epilepsy (both autoimmune and otherwise).[140]

Much of what is known thus far about autoimmune epilepsies first originated from the studies of paraneoplastic syndromes affecting the CNS. These diseases are caused by tumors in the periphery that express CNS proteins. Antibodies are generated against these perceived foreign proteins and an autoimmune response against the brain ensues. Classic examples of these syndromes are teratomas that cause paraneoplastic anti-NMDA receptor encephalitis and small-cell lung carcinoma that causes limbic encephalitis.[141] However, only a limited number of these antibodies have been shown to be associated with a unique epilepsy syndrome; that is, "pure" autoimmune epilepsies.[142] **Table 1.1** below summarizes data regarding autoantibodies and paraneoplastic syndromes and epilepsy.

Anti-NMDA receptor encephalitis

The most infamous autoimmune epilepsy, due to the recently published New York Times bestselling book *Brain On Fire: My Month of Madness,*[143] is Anti-NMDA (N-methyl-D-aspartate) receptor encephalitis (ANRE). Officially described as its own disease in 2008, ANRE has quickly become the most commonly diagnosed encephalitis in adults with over 300 case reports in the literature.[144] Data suggest that the disease primarily effects women (80% of cases).[145]

Clinically, ANRE is first characterized by flu-like symptoms (nausea, vomiting, fever, fatigue, etc). Within two weeks of the first symptoms, neuropsychiatric symptoms are observed; these can be any number of maladies ranging from confusion to hallucinations to depression and paranoia among others.[146] Additional symptoms include movement disorders and shortened attention span or memory loss.[147] Following neuropsychiatric symptoms patients develop generalized tonic-clonic or complex partial seizures.[148]

Table 1.1 Summary of antibody mediated epilepsies and paraneoplastic neurological syndromes

Antibody Target	Disease association	Prevalence in epilepsy	Response to immunotherapy
VGKC	Limbic encephalitis Paraneoplastic neurological syndrome (small cell lung carcinoma)	6.5% - 11.5%	Good response
NMDA receptor	Anti-NMDA receptor encephalitis Paraneoplastic neurological syndrome (Teratoma)	2.5% - 7%	Good response
AMPA receptor	Paraneoplastic neurological syndrome	NA	Good response
GABA receptor	Idiopathic epilepsies Paraneoplastic stiff-person syndrome	Unknown	Minimal response
GAD	Focal epilepsies, drug refractory epilepsies and limbic encephalitis Paraneoplastic cerebellar ataxia (Hodgkins disease) Paraneoplastic stiff-person syndrome	1.6% - 8.7%	Poor response
ANNA-1	Paraneoplastic syndromes (myasthenia gravis, thymoma)	NA	Poor response
Ma 1 and 2	Paraneoplastic neurologic syndrome (breast cancer)	NA	Moderate response
Histones / Chromatin	Non-autoimmune epilepsies	Unknown	Unknown

VGKC = voltage gated potassium channel, NMDA = N-methyl - D -aspartate, AMPA = , GABA =, GAD = glutamic acid decarboxylase, ANNA-1 = anti-neuronal nuclear antigen 1. Ma 1 and 2 = ribonuclear proteins. NA = not applicable

Adapted from Bien CG, Bauer J (2014)[145]

Patients often seek treatment prior to the onset of seizures and are misdiagnosed as schizophrenic or bipolar and placed on antipsychotic medications, which have little effect under these conditions[149]. As the disease progresses into its later stages, consciousness is impaired and autonomic nervous functions decline resulting in bradycardia or tachycardia, hypersalivation and urinary incontinence. Death results from either SE or severe autonomic depression.[145]

While the clinical manifestations and treatment of ANRE are abundantly clear, little work has been done to understand the pathophysiology of this disease and studies published are often contradictory. It is known, however, that the antibodies do target the NR1 subunit of the NMDA receptor.[150] Whether the origin of the antibody is intrathecal (in cerebrospinal fluid; CSF) or peripheral is still unknown. Anti-NR1 antibodies have been found in serum and in CSF with some studies demonstrating intrathecal production[151] and others demonstrating peripheral production.[152]

Perhaps the more important unanswered question is how the antibody causes the observed symptoms. *In vitro* studies have demonstrated that anti-NR1 antibodies from patients with ANRE can bind to the appropriate receptor subunit and cause the internalization of the receptor and reduction of NMDA currents.[153] Yet another hypothesis is that anti-NR1 antibodies bind to the receptor and activate the complement cascade and membrane attack complex

formation resulting in neuronal death.[144] However, histological examination of brain tissue sections from patients with ANRE shows little to no cell loss.[154]

A final untested, yet surprisingly popular hypothesis is that the antibody binds to and activates the NMDA receptor. Activation of the receptor is consistent with observed symptoms and mimics what is produced by pharmacologic antagonists of the NMDA receptor (e.g., ketamine). No studies have examined how this antibody may be produced to begin with.

Immunotherapy is the treatment of choice for ANRE. In particular, steroids, IVIg and plasmapharesis have been effective in halting progress of the disease. Whether other immunomodulatory drugs also halt the disease, in particular rituximab, remains to be fully determined. After disease progression is stopped, recovery is slow and the patient may remain in the hospital for three to four months. Full recovery has been achieved but many patients will have permanent neurological disability and may need to take anti-seizure medications.[144]

Limbic encephalitis

Limbic encephalitis (LE) is an extremely rare autoimmune epilepsy syndrome that has yet to be characterized by a complete epidemiological study. Males appear to be more commonly affected than females and patients are usually older than 50 years.[155] Symptoms can be similar to those observed in ANRE, though neuropsychiatric symptoms are skewed towards depression and

mood instability.[156] Because of this, LE is often misdiagnosed prior to the development of seizures. As the limbic system is involved patients have severe and often sudden memory loss.[157] A symptom that differentiates LE from ANRE is faciobrachial dystonic seizures. These seizures often develop into generalized tonic-clonic seizures originating in the temporal lobe.[158]

Many studies have demonstrated that LE is caused by antibodies against the leucine-rich glioma inactivated 1 (LGI1) and contactin associated protein 2 (CASPR2) subunits of the voltage-gated potassium channel (VGKC).[159] Serum concentrations of the antibody are far higher than cerebrospinal fluid concentrations, suggesting peripheral rather than intrathecal production of the antibodies[156]. The specific mechanism of action of the antibody remains unknown, though it has been suggested that symptoms are a result of receptor internalization.[160] Although, studies of VGKC LE have demonstrated that there is compliment activation in the brain with associated cell death, suggesting that an alternative mechanism is likely.[161]

Treatment of LE is the same as ANRE with the best outcomes seen in patients treated with steroids and IVIg therapy. Recovery is typically favorable for patients with LE if diagnosed and treated early. Improvement is observed in neuropsychiatric outcomes though some deficits may persist with verbal and memory deficits most common. Hippocampal sclerosis is observed in patients

with LE and the changes persist after disease progression is halted. Patients may therefore need to remain on anti-seizure medication.[162]

Emerging evidence for an "anti-GAD encephalitis"

Antibodies against glutamic acid decarboxylase (GAD), the enzyme that converts glutamic acid to GABA, have been found in a number of autoimmune diseases including autoimmune cerebellar ataxia, stiff-person syndrome, type-1 diabetes and some cases of limbic encephalitis.[163] Emerging evidence demonstrates a role for anti-GAD antibodies in epilepsy that produce symptoms similar to VGKC encephalitis.[164] Reports thus far have shown that antibodies are higher in serum than in CSF, indicating peripheral production.[165] Studies have failed to identify how or why one antibody is able to cause such a wide array of autoimmune diseases, even after examining different epitopes on GAD.[151] Patients with anti-GAD antibodies and seizures are resistant to anti-seizure medications and do not respond well to immunotherapy, which may indicate that anti-GAD antibodies are an epiphenomenon in epilepsy rather than the cause.[165] However, a 2008 case report demonstrated that plasma exchange therapy was effective in reducing seizures in a patient with these antibodies.[166]

The rare exception: Rasmussen's encephalitis

Rasmussen's encephalitis (RE) is a very rare neurological disease effecting approximately 2 per 10 million people aged 16 years or under. This extremely devastating neurological disease usually presents in children around 6

years of age.[167] Though generally not fatal with current treatments, lack of fatality betrays how devastating this disease really is. Patients with RE show a progression of symptoms of increasing severity. Symptoms include hemiparesis, encephalitis, hemianopia, dementia, dysphasia, loss of motor skills and severe intractable seizures. Seizure types include generalized tonic-clonic seizures, status epilepticus and epilepsia partialis continua.[168] Epilepsia partialis continua is frequent, with 50% of patients presenting with this symptom that involves continuous "twitching" in the extremities and face on one side of the body.[169] It is worth noting that symptoms affect only one side of the body and that RE affects only one side of the brain in almost all cases.[168]

The exact mechanism of disease in RE remains elusive. Evidence for antibody mediated disease,[170] T cell mediated disease[171] and microglial mediated disease[172] are all supported in the literature. Perhaps the least likely cause of RE are antibodies. While antibodies targeted to glutamate receptors, GABA receptors, acetylcholine receptors and others do occur in patients with RE, they are found in relatively few cases.[173]

Microglial and astrocyte activation is observed in brain samples from patients with RE.[174] Activation of these cells follows patterns of inflammation in the cortex of RE patients and is often observed in areas where T cells are present.[171] In addition, albumin has been observed in brain tissue from these patients indicating that astrocytic function may be impaired.[175] However,

activated microglia and astrocytes are not unique to RE or epilepsy.[176] It is therefore likely that activation of these cells is the result of inflammation and/or seizure and not *vice versa.*

High levels of cytotoxic T cells are found in the brains of patients with RE and some patients have granzyme-B positive cells as well. Further, these T cells have been shown to have expanded from the same clone indicating a specific T cell response.[177] It has therefore been hypothesized that RE is caused by a yet unknown virus.[167] Thus far, none of the "usual suspects" (herpes, enterovirus, Epstein-Barr virus and cytomegalovirus) have been detected in RE patients brains[178] indicating that an autoimmune disease (perhaps through molecular mimicry) remains an option for the etiology of the disease.

Treatment of RE patients is currently focused on alleviating symptoms. Many immunotherapy drugs have been tried, including corticosteroids, IVIgs, plasmapheresis and the T cell inactivating pharmaceuticals tacrolimus and azathioprine. All have shown some efficacy but none are able to permanently alleviate the patient's symptoms.[167] Currently, immunotherapies are being tested in different combinations and timings in an attempt to improve effectiveness. Unfortunately, patients with RE do not benefit from the use of anti-seizure medications and often the only course of action to stop seizures is a functional or complete hemispherectomy.[179]

Other antibodies in epilepsy

As mentioned above, a number of antibodies have been found in epilepsy but have not (yet) been conclusively linked to a specific autoimmune response or encephalitis. These antibodies are targeted towards anti-AMPA receptor,[180] $GABA_B$ receptor,[181] GluR5 subunit of the NMDA receptor,[134] antineuronal nuclear antigen-1,[182] cardiolipin[183] and the classical antinuclear antibodies against histones and chromatin.[184] These antibodies have been found in various combinations (or alone) in the autoimmune epilepsies discussed above or in the serum of patients with non-autoimmune epilepsies. How each of these does or does not contribute to pathophysiology has not been determined.

1.8 The use of IVIg in epilepsy

A unifying feature of the autoimmune epilepsies detailed above is the effectiveness of intravenous immunoglobulin (IVIg) in stopping the course of disease.[120] Surprisingly, IVIg is also effective in epilepsies where inflammation is not a primary cause.[185,186] A partial meta-analysis published in 1994 demonstrated that 52% of patients with intractable epilepsy given IVIg for up to 12 months experienced a reduction in seizure frequency, and 42% of these patients had noticeable interictal EEG improvement. Further, 23% of patients were able to achieve complete freedom from seizure.[187] This is of particular importance because these patients are able to achieve seizure freedom without

the deleterious effects of many anti-seizure medications.[188,189] It is interesting to note the paradoxical effect of antibodies - they can both cause and treat the same disease.

Presumably, the use of IVIg in patients with autoimmune epilepsy causes depletion of antibodies and may promote an anti-inflammatory environment and thus cessation of seizures,[185] though this scenario has been contested.[190] In epilepsies of non-autoimmune origin, reduction in seizure burden likely occurs through other mechanisms.[191] **Table 1.2** summarizes the effects of long term IVIg treatment on non-autoimmune epilepsies.

The specific mechanism(s) of action of IVIg remains elusive irrespective of disease. Many hypotheses have been tested regarding the peripheral action of IVIg but no definitive conclusions have been reached. Hypotheses include removal of antibodies in bulk by activation of the complement system,[192] or blockade of receptors on inflammatory cells (T cells, B cells and monocytes specifically)[193] and binding to inactivating fragment crystalliable (Fc) receptors that promote an anti-inflammatory response.[192]

In CNS diseases, the primary hypothesis is that IVIg binds to inhibitory Fc receptors that halt the production of inflammatory cytokines resulting in a reduction of inflammation over time[194] **(Figure 1.5)**. Alternatively, when IVIg is administered to patients with epilepsy an increase in IL-1ra is observed in the serum.[195] Prior reports have demonstrated the anti-seizure effect of IL-1ra in the

brain and drugs that mimic IL-1ra have also been successful in reducing seizure frequency in animals. This effect is likely to could occur both peripherally and in the CNS.[195] Alternatively, animal models of stroke with IVIg treatment have shown IVIg in the brain with accumulation in neurons and glia. Therefore, the uptake of IVIg by these cells may also play a role.[191] **Figure 1.4** summaries the proposed hypotheses for the action of IVIg on the CNS (including cerebral microvasculature endothelial cells).

Table 1.2: Summary of literature demonstrating the efficacy of IVIg in non-autoimmune epilepsies

Study type	Study duration	n	Epilepsy type	Seizures Types	Outcomes	Reference
Open-label	3-6 months	11	WS	None specified	EEG improvement, one cessation of seizures, two with transient improvement	Ariizumi, et al (1987)
Open-label	3 months	15	LGS and WS	IS, T, AA, AT	40% improvement in EEG, 70% improvement in seizures	Van Engelen, et al (1994)
Double-blind	6 months	61	LGS, WS, and Localization-related epilepsy	P, G	50% reduction of seizure in patients with partial epilepsy only, improvement in 2 placebo	Van Rijckevorsel-Harmant, et al (1994)
Open-label		6	Various	None Specified	Improvement in EEG and clinical seizures in 66% of patients	Turkay, et al (1996)
Single-blind	½ month	10	LGS	None Specified	Reduction of seizures in 2 children, improvement in intellectual ability	Illum, et al (1998)
Open-label	14 months	5	WS and LGS		IVIg used as an add-on therapy, reduced seizure frequency	Espinosa-Zacarias, et al (2002)
Open-label	12 months	5	LGS	IS, T, MC, A, G, CP	Reduction of seizure in 4 patients (up to 92% reduction)	Bingel, et al (2003)
Open-label	3-6 weeks	13	Various	IS, TC, MC, A,AA, C, T	7 patients experienced up to 50% reduction in seizures	Billau, et al (2007)
Open-label	15 months	37	WS, LGS and localization related epilepsy	P, G	43% had 50% reduction of seizures, 15% seizure free	Mikati et. Al (2009)

WS = West syndrome, LGS = Lennox-Gastaut syndrome, IS = infantile spasms, T = tonic, AA = atypical absence, AT = absence - tonic, P = partial, G = generalized, MC = myoclonic, C = clonic, A = absence

Adapted from Mikati, MA *et al.* (2010)[185]

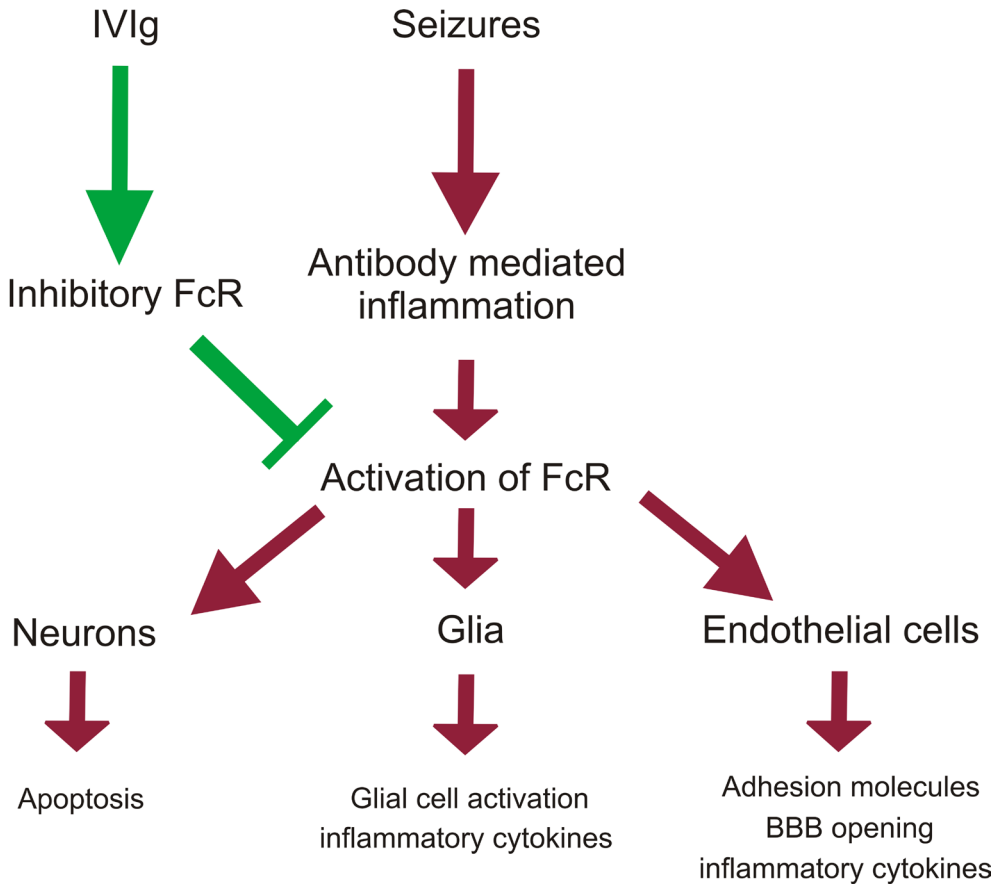

Figure 1.5: Proposed hypotheses for the action of IVIg in the CNS. After insult, natural antibodies released in the peripheral circulation act on neurons, glial and endothelial cells near the site of injury. Activating Fc receptors on these cells trigger an inflammatory response that leads to inflammation, BBB disruption and cell death. IVIg has been proposed (in *green*) to act on the inhibitory Fc receptors found on the same cells to negate the activation of pro-inflammatory Fc receptors.

Adapted from Okun et al. (2010)[193]

1.9 The overarching goals of this research

Taking all of the above into consideration, there were two primary aims of the research detailed in the following chapters:

1) Animal models demonstrate that IgGs accumulate inside neurons after SE using both inflammatory and non-inflammatory models of seizure. It was therefore the goal of this research to determine whether IgGs accumulate inside neurons of patients with non-autoimmune epilepsies. Further, it is an additional goal of this research to determine what these antibodies were specifically targeted to and what their function(s) may be.

2) It remains unclear how IVIg reduces seizure burden in patients and in animal models of epilepsy. Therefore, the goal of the research detailed below was to determine the way(s) by which IVIg reduces seizure burden in epilepsy and in what model(s) it is most appropriate to study IVIg treatment.

CHAPTER 2:

Aim 1:

Discovery of intracellular and circulating antinuclear antibodies in epilepsy

2.1 Introduction

Studies on inflammatory mechanisms in epilepsy have been burgeoning, with a 300% increase in published articles on PubMed from 1993-2003 compared to the previous decade. It is thus not surprising that new models of seizures have emerged. These models take into account the knowledge gained from clinical studies and are based on mechanisms, receptors, and pathways that were formerly reserved for the immunologist.[94,196-198] Evidence to support a role for inflammation and autoimmunity in epilepsy has come from indirect and direct sources. For example, the anti-seizure activity of steroids in some epilepsies (indirect[199]), together with the presence of inflammatory signs and markers in serum or cerebrospinal fluid (CSF) of patients (direct[138])

have been interpreted as clues suggestive of a role for the immune response. In addition, well-established models of seizures which were developed to specifically target neurons have been re-examined to reveal an underlying inflammatory etiology. For example, research has shown that a putative muscarinic convulsant, pilocarpine, acts by immune activation and not, as previously suspected, by a CNS exclusive action on muscarinic receptors.[197,198,200,201] The role of inflammation in seizure disorders has therefore been recognized as an etiologic reality and as an important target for therapy.[190,198,202,203]

There are three groups of "inflammation-related seizures" (IRS): 1) Seizures caused by the presence of a pathogen. These are perhaps the least studied cluster of IRS and include seizures due to meningitis, neurotropic pathogens, etc. In developing countries, pathogens are considered the highest risk factor for acute seizure and increase the risk of epilepsy by eleven fold.[204] 2) A large family of IRS encompasses autoimmune epilepsy syndromes, where one of the etiological mechanisms is believed to be the presence of anti-neuronal autoantibodies typically targeting either ion channels, intracellular epitopes or neurotransmitter receptors.[205-207] 3) A number of seizure disorders lacking either of these features (pathogen or autoantibodies) can be classified as IRS based on a therapeutic response to immunomodulators,[208,209] vascular changes consistent with an ongoing inflammatory process (e.g., BBB disruption; for a review see[95]),

or concomitant brain changes that mimic some, but not all, signs of inflammation.[138,209,210]

As mentioned above, the third type of IRS may be linked to BBB disruption. The BBB is the gatekeeper of immune privilege in the CNS.[110,211] The BBB maintains ionic homeostasis which, in turn, controls neuronal excitability[95,203,212,213]. Thus, BBB disruption (BBBD) not only causes loss of immune privilege but may also directly result in seizures.[121] A reporter of BBB failure, extravasated albumin levels in CSF, has also been used to detect focal BBBD by immunohistochemistry. Interestingly, after diffusion through the CNS extracellular space, albumin accumulates in neurons and glia.[213-215] Regions of focal BBBD can also be measured by detection of extravasated IgGs. Whether extravasated IgGs also enter into brain cells has not been fully elucidated.[216]

The presence of IgGs in brain from patients with epilepsy, together with our understanding of the pathophysiology of multiple sclerosis (MS), has been used to propose autoimmunity as an etiologic factor in seizure disorders. Autoantibodies to the NMDA, $GABA_B$ and α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptors, as well as the voltage-gated potassium channel and its components LGI1 and CASPR2 have been detected in CSF or serum of patients with seizures.[206] In addition, autoimmune diseases such as systemic lupus erythematosus (SLE) greatly influence seizure susceptibility.[217] Thus, seizure threshold can be lowered by direct action on CNS targets (e.g., glutamate receptors), by exposure to endotoxin[218] or by

autoimmune targeting of a specific antigen, such as nuclear components. A recent paper has shown that even in absence of autoimmune disease, IgG can be found in brain of mice after lithium/pilocarpine-induced seizures.[216] This is also consistent with previous work showing that BBBD, as seen in regions of seizure generation in human brain, is characterized by large deposits of extravasated IgG.[216] However, to date, the significance or consequences of IgG extravasation into the CNS has not been fully elucidated.

The CNS of patients with epilepsy provides a unique environment where the coupling of seizure with inflammation, loss of immune privilege and cell death may provide a mechanism for the generation and uptake of autoantibodies against intracellular proteins. Therefore, we examined whether or not autoantibodies against intracellular proteins existed in the CNS and serum of patients with epilepsy where an autoimmune or infectious etiology was ruled out. By using a number of techniques and an approach based on comparison of different pathologies all characterized by BBBD, we isolated autoantigens from subcellular fractions of brain from patients with epilepsy. MS was used as a comparative “neuro-autoimmune” disease, and brain resections derived from cerebrovascular malformations as a means to study BBBD independent of seizures. Our results demonstrate the presence of antinuclear antibodies in brain and serum from patients with epilepsy, and the accumulation of autoantibodies in neuronal nuclei.

2.2 Materials and methods

The multimodal approach used for the experiments detailed in this section is depicted graphically in **Figure 2.1**.

Patient selection

Brain tissue specimens were obtained from patients conforming to the guidelines of the Declaration of Helsinki. All patients signed an informed consent according to institutional review protocols at the Cleveland Clinic Foundation. Patient information and experimental use of patient samples is summarized in **Table 2.1**. All brain tissue samples were obtained from surgical resections with the exception of *post-mortem* MS brain. *Post-mortem* samples were a generous gift of Dr. Bruce Trapp's laboratory at the Cleveland Clinic Foundation Lerner Research Institute. Inclusion criteria were willingness to participate in the study and lack of positive diagnosis for an autoimmune disease. One patient was identified as RA *post-facto* and is considered a positive control **(Figure 2.6)**.

Detection and discovery

Brain tissue was mounted using Tissue-Tek OCT compound (Sakura Finetek Europe B.V., The Netherlands) and sectioned at approximately 25 μm on a Leica CM3050 cryostat (Leica Microsystems Inc, Buffalo Grove, IL, USA). Nine

patients with epilepsy, four multiple sclerosis patients and three arteriovenous malformation (AVM) patients were included in these experiments.

Immunofluorescent detection of IgG and albumin in neurons, glia and brain parenchyma

Free-floating sections were stained for IgG and albumin. Non-specific binding was minimized by incubation in a 3% goat serum blocking solution at room temperature for one hour. Sections of brain tissue were incubated with monoclonal mouse anti-human albumin antibody (1:1000; Sigma-Aldrich, St. Louis, MO, USA). Fluorescently-labeled secondary antibodies used were as follows: Alexa Fluor 594 polyclonal donkey anti-mouse IgG (1:100; Jackson Immunoresearch, West Grove, PA, USA), and fluorescein conjugated polyclonal goat anti-human IgG (1:200, Vector Labs, Bulingame, CA, USA).

3,3'-Diaminobenzidine staining of AVM patient brain tissue

3,3'-diaminobenzidine (DAB) staining of brain tissue sections was achieved using the method from Marchi, *et al (2010)*.[202]

Immunofluorescent detection of IgGs in neurons and astrocytes

Free-floating brain sections were stained for IgG and microtubule-associated protein 2 (MAP2). Adjacent sections were stained for IgG and glial fibrillary acidic protein (GFAP). Non-specific binding was minimized by incubation in a 3% goat serum

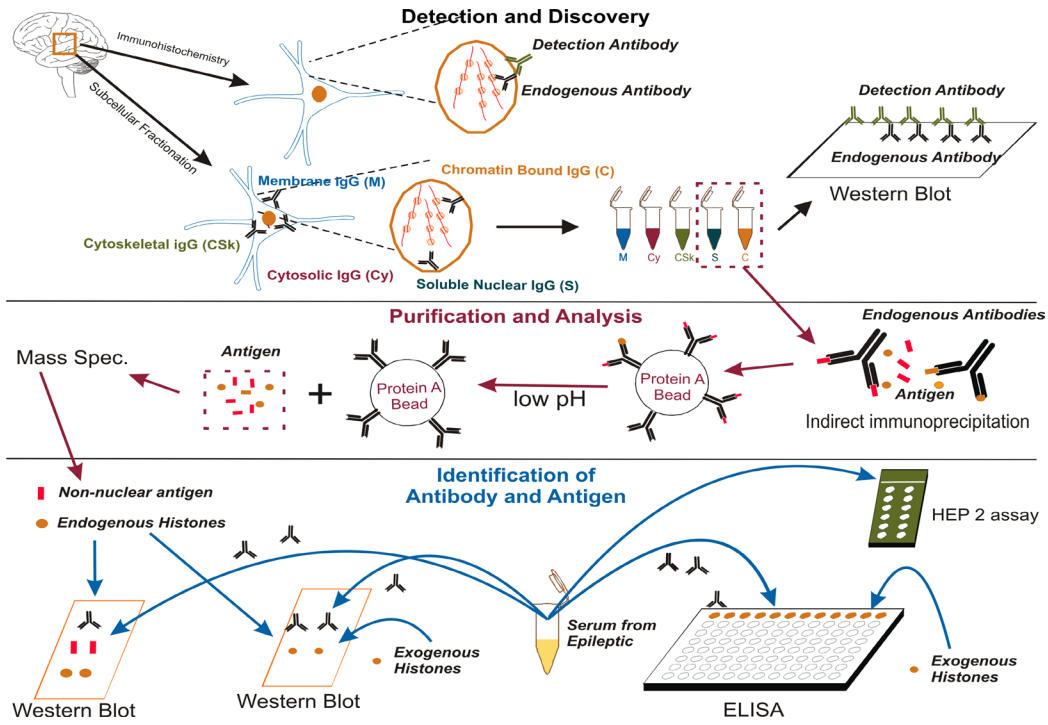

Figure 2.1: Schematic representation of the experimental design used for different aspects of the research presented herein. The figure is divided in sections to highlight the three different phases of this research effort. The phase labeled as **Detection and Discovery** describes the process of isolation of brain samples from surgically resected specimens and their use to unveil antinuclear antibodies. The subcellular fractionation (see **Figure 4)** allows segregating antigen-antibody complexes from the membrane (M), cytoskeletal (CSk), cytosolic (Cy) and nuclear fractions. The nuclear fraction was further divided into chromatin-bound (C) and soluble (S). These fractions were analyzed by Western blotting. In parallel experiments, formalin-fixed tissue was used to produce the results shown in **Figures 2.2 and 2.3**. Exogenous IgG antibodies were used to unveil endogenous IgG content in nuclear and subcellular fractions. Next, a Second phase **(purification and analysis)** was undertaken. The first step consisted of an indirect immunoprecipitation process where antigen-IgG complexes were dissociated to obtain free antigen fractions. These fractions were isolated from the brain. The antigens isolated by these means were further analyzed by mass spec. to unveil **(identification of antibody and antigen)** the nature of IgG targets. These putative autoantigens were divided into groups, endogenous histones or non-nuclear protein content. To confirm these findings, two separate means of investigations were used. In the first step, brain-derived putative autoantigens were probed by Western blotting to confirm or refute their suspected nature (histones, see **Figure 2.5**). In addition, serum samples from patients with epilepsy and controls were isolated to confirm the presence of antinuclear IgG in these samples. Again, the specificities for histones of these antibodies were tested by Western blotting and also by an *ad hoc* ELISA. An additional set of non-epilepsy patient controls consisted of using samples taken from brain from patients with multiple sclerosis (e.g., **Figures 2.2 and 2.3**) or brain samples isolated during surgery to relieve arteriovenous malformation (AVM) **(Figure 2.4)**.

blocking solution at room temperature for one hour. The following primary antibodies were used to stain the tissue sections: mouse monoclonal anti-human MAP2 (1:1000; Covance, Princeton, NJ, USA), mouse monoclonal anti-human GFAP (1:500; Sigma-Aldrich, St. Louis, MO, USA). The following secondary antibodies were used: goat anti-mouse polyclonal Alexa Fluor 594 (1:400, Jackson Immunoresearch, West Grove, PA, USA), fluorescein goat polyclonal anti-human IgG (1: 200; Vector Labs, Burlingame, CA, USA). Auto-fluorescence was minimized using Sudan Black B. Finally, tissue slices were placed on glass slides and mounted using a glass coverslip and Vectorshield mounting medium containing 4',6-diamidino-2-phenylindole (DAPI) to visualize nuclei (Vector Labs, Bulingame, CA, USA). Images were obtained using a Leica Leitz fluorescent microscope and a Leica Microsystems upright confocal microscope with attached cameras (Leica Microsystems, Allendale, NJ, USA). Fluorescence intensity and co-localization were measured using Q-Capture software (Q-Capture, Surrey, BC, Canada). Three-dimensional reconstruction of confocal images was performed using Velocity (PerkinElmer, Waltman,MA, USA).

Subcellular fractionation of brain tissue resections

Snap frozen tissue stored at -80°C was processed according to the protocol provided with the Thermo Scientific Subcellular Protein Fractionation Kit for Tissue (Thermo Fisher Scientific, Rockford, IL, USA). 200mg of tissue was

used for each fractionation. Ten patients with epilepsy and four AVM patients were used for these experiments

Western blots for IgG from subcellular fractions

Proteins from subcellular fractions were separated via SDS-PAGE electrophoresis under non-denaturing conditions and transferred onto a polyvinylidene difluroride (PVDF) membrane (Millipore Corporation, Billerica, MA). Membranes were incubated overnight at 4°C with horseradish peroxidase (HRP) conjugated anti-human IgG (1:2,000; Calbiochem-Novabiochem Corporation, CA, USA). Proteins were visualized using Western Lightning Plus ECL (PerkinElmer, Waltman, MA, USA) and developed on Kodak Biomax MR Film (Eastman Kodak Company, Rochester, NY, USA). IgG volume was quantified using Phoretix 2D software (Nonlinear USA Inc., Durham, NC, USA).

Purification and analysis

Indirect immunoprecipitation

Nuclear fractions were divided into soluble nuclear and chromatin-bound samples. Typically, indirect immunoprecipitation requires a step to allow the antibody-antigen complex to form. However, as antibody-antigen complexes were already present in cell nuclei, no antibody-antigen complex forming step was required. Nuclear fractions were incubated with protein-A coated agarose beads (Santa Cruz Biotechnology Inc., Dallas, TX, USA) for three hours at 4°C

with gentle mixing. Beads were washed and centrifuged four times in 20% tween-PBS to remove as much unbound protein as possible. Samples were then placed in microcentrifuge tubes and exposed to a low pH (2.6) glycine-HCL solution for 3, 15 or 30 minutes. After centrifugation and removal of supernatant (containing antigen), the pH of the supernatant was neutralized with 1M Tris at pH 8.5.

Liquid chromatography - mass spectrometry analysis

Antigen samples, obtained as described above, were analyzed by SDS-Page electrophoresis. Bands were cut out of the gel, washed and dehydrated in acetonitrile. Bands were reduced using dithiothreitol and alkylated with iodoacetimide. Proteins were digested in gel overnight at room temperature using 10 ng/µl trypsin in 50 mM ammonium bicarbonate. Proteins were extracted from the polyacrylamide with acetonitrile (50%) and formic acid (5%). Extracts were evaporated in a Speedvac and resuspended in acetic acid (1%). Five µl volumes of extract were injected on a Dionex 15 cm x 75 µm id Acclain Pepmap C18, 2 µm, 100 angstrom, reversed-phase capillary chromatography column for liquid chromatography separation (Thermo Fisher Scientific Inc, Rockford, IL, USA). Peptides were eluted from the column by acetonitrile/formic acid (0.1%) gradient at a flow rate of 0.25 µl/min and introduced to the mass spectrometry source on-line. For mass spectrometry analysis, a Finnigan LTQ-Orbitrap Elite hybrid mass spectrometer system (Thermo Fisher Scientific Inc, Rockford, IL, USA) was used. The microelectrospray ion source was operated at 2.5 kV. The peptide digest

was analyzed using the data-dependent multitask capability of the instrument acquiring full scan mass spectra to determine peptide molecular weights and ion spectra to determine the amino acid sequence. See **Appendix I** for a description of the underlying principles of mass spec. and HPLC

Data from this experiment were analyzed using all collisionally-induced dissociation spectra collected. These were used to search the NCBI Mascot program with a human taxonomy filter. Manual interpretation, Sequest and Blast were used to verify Mascot matches.

Identification of potential antigens from mass spectrometry data

In our patient sample, time-dependent change in total antigen content was evaluated. The procedure (see above) was used to study both soluble nuclear and chromatin-bound fractions. Before collection, columns were washed four times to remove unbound proteins. Thus, the remaining proteins were considered putative autoantigens; weakly-bound non-specific IgGs were also present in this sample. Qualitative analysis was achieved by examining the change in putative autoantigen count observed over time. A positive increase in a given autoantigen abundance (spectral count) was interpreted as elution of antigen tightly bound to IgGs. In contrast, a negligible increase or decrease was interpreted as non-specific binding of antigen to IgG. A transient increase or decrease at 15 minutes followed by a return to levels less than or equal to that observed at 3 minutes were not further analyzed. In short, putative autoantigen were further analyzed

only if they were not obvious contaminants (e.g., keratins) and if their kinetic dissociation was indicative of specific antibody-antigen binding.

An additional validation step was used to unveil autoantigen. We compared the kinetics of soluble nuclear (presumably non-specific) versus chromatin-bound (specific) complexes. This analysis revealed that, in the soluble nuclear fraction, the dissociation of protein from IgG was not time-dependent. In fact, increase, decrease or no change in total protein content were equally common. In contrast, the chromatin-bound IgG-antigen dissociation was observed by a definite time-dependent increase of dissociated autoantigen. The comparisons of these two behaviors for each putative autoantigen (see **Figure 2.5**) together with the observations above were merged into the following:

[(30 min CB – 15 min CB) – (15min CB – 3min CB)] – [(30 min SN – 15min SN) – (15min SN – 3 min SN)] = Q

where Q is a coefficient and where each value equals the normalized spectral count of a given autoantigen. CB = chromatin bound fraction, SN = soluble nuclear fraction. Q was used to discern between soluble unbound IgG, and chromatin-IgG or histone-IgG complexes.

All proteins associated with negative coefficients were considered to be likely autoantigen specifically bound to antibodies. Proteins were then ranked from most negative to least negative and any non-nuclear proteins were

eliminated. The nuclear proteins with the most negative number were considered the primary targets of the autoantibodies **(Table 2.2).**

Identification of antibody and antigen

Western blots using serum to detect isolated nuclear antigen

Protein from one patient with epilepsy, serum from eight patients with epilepsy, two control patients and one positive control patient with rheumatoid arthritis (RA) were used in this experiment. Isolated antigens from the immunoprecipitation experiment were separated via SDS-PAGE electrophoresis under non-denaturing conditions and transferred onto a PVDF membrane. Membranes were incubated at room temperature for one hour with serum from patients with epilepsy (1:1000). After repeated washing, membranes were incubated at room temperature for two hours with HRP-conjugated goat anti-human IgG (1:2,000; Calbiochem-Novabiochem Corporation, CA, USA). Proteins were visualized using Western Lightning Plus ECL and developed on Kodak Biomax MR Film.

Western blots using serum to detect purified histones

Total histones were separated by SDS-PAGE electrophoresis under non-denaturing conditions and transferred onto a PVDF membrane. Membranes were incubated at room temperature for one hour with serum from patients with epilepsy (1:500). After repeated washing, membranes were incubated at room

temperature for two hours with HRP-conjugated goat anti-human IgG (1:2,000). Proteins were visualized using Western Lightning Plus ECL and developed on Kodak Biomax MR Film.

Anti-histone and anti-chromatin ELISA using serum

Serum obtained from human patients with epilepsy was diluted 1:300 in serum diluent (sterile filtered 0.5% bovine gamma-globulin, 5% gelatin, 0.05 mM Tween in 1x PBS) and analyzed for levels of anti-chromatin and anti-histone IgG autoantibodies. Microtiter plates (Immulon 2HB) were coated with purified chromatin or total histones over night at 4°C, blocked in 5% gelatin/PBS for 2hrs, and incubated with serum samples for 2 hours. Secondary HRP-conjugated anti-human IgG antibodies were added for 1.5 hours and plates were developed using 10mg/ml 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS) in McIlwain's buffer (0.09 M Na_2HPO_4, 0.06 M citric acid, pH 4.6). Samples were read on a spectrophotometer at 405 nm.

Human epithelial type 2 (HEp-2) cell assay

Slides provided by the manufacturer (Bio-Rad, Hercules, CA, USA) were placed into a humidity chamber. Positive and negative controls provided with the manufacturers' kit were added (1:64 dilution) to two wells of the slides in addition to three human serum controls and serum from eight patients with epilepsy (1:10 dilution). Slides were incubated for 20 minutes and then washed for 10 minutes in PBS. 25 µl of fluorescein conjugate was added to each well of the slide and

incubated in a humidity chamber for 20 minutes. Images were obtained on a Leica Leitz fluorescent microscope with attached camera.

2.3 Results

For the results presented here we used a total of 32 subjects (including control). 21 brain samples and blood from 11 donors were analyzed. Of brain samples, 13 were obtained from patients affected by multiple drug resistant seizures, four were from patients treated by a neurosurgeon to repair AVMs and four were *post-mortem* brain samples from patients affected by MS. Brain samples were used for Western blotting, immunohistochemistry and general morphology. Eight patients with epilepsy, two healthy volunteers and one patient with epilepsy and RA were enrolled to donate blood samples used for ELISA, HEp-2 and Western blot data. The AVM samples were obtained from one patient with seizures responding to treatment, one patient with multiple drug resistant seizures and two with no seizure history. None of the MS patients had a history of epilepsy or seizures. Data obtained from the medical records of patients used, including volunteers, are shown in **Table 2.1**.

Our experimental design also encompassed another layer of distribution, namely presence or absence of recurring seizures. Thus, one AVM patient where seizures were present at time of surgery was grouped together with resected brain from patients with epilepsy where focal electrophysiological properties

Table 2.1a Patient Information

Patient ID	Age	Gender	Seizures Y/N	Epilepsy Y/N	Diagnosis
1	45	F	Y	Y	Frontal lobe epilepsy
2	18	M	Y	Y	Temporal lobe epilepsy
3	2 m	M	Y	Y	Temporal lobe epilepsy
4	23	M	Y	Y	Temporal lobe epilepsy
5	19	M	Y	Y	Occipital lobe epilepsy
6	1	M	Y	Y	Temporal lobe epilepsy
7	12	F	Y	Y	Frontal lobe epilepsy
8A	14	M	Y	Y	Temporal lobe epilepsy
8B	14	M	N	Y	Temporal lobe epilepsy
9	7	M	Y	Y	Parieto-occipital lobe epilepsy
10	27	M	Y	Y	Temporal lobe epilepsy
11	46	F	Y	Y	Temporal lobe epilepsy
12	48	M	Y	Y	Temporal lobe epilepsy
13	14	F	Y	Y	Temporal lobe epilepsy
14	36	M	Y	Y	Temporal lobe epilepsy
15	29	F	Y	Y	Temporal lobe epilepsy
16	32	M	Y	Y	Temporal lobe epilepsy
17	20	M	Y	Y	Epilepsy
18	21	F	Y	Y	Temporal lobe epilepsy
19	33	M	Y	Y	Temporal lobe epilepsy
20	30	F	Y	Y	Temporal lobe epilepsy
21	38	M	Y	Y	Temporal lobe epilepsy
22	U	U	N	N	Multiple Sclerosis**
23	U	U	N	N	Multiple Sclerosis**
24	U	U	N	N	Multiple Sclerosis**
25	U	U	N	N	Multiple Sclerosis**
26	48	M	N	N	AVM w/ med. controlled seizure
27	23	M	N	N	AVM w/ no history of seizure
28	37	F	Y	N	AVM w/ epilepsy
29	28	F	N	N	AVM
C1	26	M	N	N	Healthy control
C2	33	M	N	N	Healthy control
Pos. C 1	19	F	Y	Y	Epilepsy

Table 2.1b Patient information (continued)

Patient ID	Pathology	Autoimmune /Inflammatory Disease	Previous path./ surgery
1	Cortical Atrophy and WM degeneration	N	Meningioma
2	Neuronomegaly and loss of cortical architecture	N	
3	Cortical Dysplasia, neuronal cytomegaly /dysmprphia, gliosis and microcalcifications	N	
4	Neuron loss and gliosis	N	
5	Cortical dysplasia	N	VNS
6	Cortical dysplasia, WM atrophy and gliosis	N	VNS
7	Cortical Dysplasia and gliosis	N	
8A	Cortical dysplasia, fibrotic leptomeninges	N	
8B	Cortical dysplasia, fibrotic leptomeninges	N	
9	Ganglioglioma w/ cortical dysplasia	N	
10	U	N	U
11	U	N	U
12	U	N	U
13	Cortical malformation	N	
14	Cavernous angioma	N	
15	U	N	Ant temporal lobectomy
16	U	N	
17	Perisylvian polymicrogyria	N	VNS
18	U	N	
19	U	N	
20	U	N	
21	U	N	
22	U	Y	U
23	U	Y	U
24	U	Y	U
25	U	Y	U
26	AVM	N	Embolization
27	AVM	N	Embolization
28	AVM	N	Embolization
29		N	
C1		N	
C2		N	
Pos. C 1	U	RA	

Table 2.1c Patient Information (continued)

Patient ID	Current AEDs	Sample Type	Experimental use
1	Carb, Lev	Brain	WB, IHC, IP, MS,
2	Lam, Lev, Pheno, Clor	Brain	WB
3	Lev, Lam, Oxcarb, Pheno	Brain	WB
4	Lam, Lev	Brain	WB, IHC
5	Oxcarb, Clon	Brain	WB
6	Topi, Pheno	Brain	WB, IHC
7	Val, Zon, Lev	Brain	WB, IHC
8A	Topi, Lev, Clon	Brain	WB
8B	Topi, Lev, Clon	Brain	WB
9	Carb, Lev	Brain	WB, IHC
10	Unknown	Brain	IHC
11	Laco, Preg	Brain	IHC
12	Unknown	Brain	IHC
13	Carb, Pheny, Val, Gabap, Lam, Pheno, Top, Oxcarb, Lev	Brain	IHC
14	Lev	Serum	WB-IP,WB-H, ELISA, HEp-2
15	Carb	Serum	WB-IP, WB-H, ELISA, HEp-2
16	Lam, Lev	Serum	WB-IP,WB-H, ELISA, HEp-2
17	Laco, Val	Serum	WB-IP, WB-H, ELISA, HEp-2
18	Topi	Serum	ELISA, HEp-2
19	Lev	Serum	ELISA, HEp-2
20	Oxcarb, Clon	Serum	ELISA, HEp-2
21	Val	Serum	ELISA, HEp-2
22		Brain	IHC
23		Brain	IHC
24		Brain	IHC
25		Brain	IHC
26	Val, Zon, Lev	Brain	WB, IHC
27		Brain	WB, IHC
28	Lev, Oxcarb, Pheny, Clon	Brain	WB, IHC
29		Brain	MRI
C1		Serum	ELISA, WB-IP, WB-H, HEp-2
C2		Serum	ELISA, WB-IP, WB-H, HEp-2
Pos. C 1	Carb, Lam	Serum	WB-IP, ELISA, HEp-2

**indicates *post-mortem* brain tissue samples. All other brain tissue specimens were obtained from surgical resections.

AED abbreviations: Carb = carbamazepine, Lev = levetiracetam, Lam = lamotrigine, Pheno = phenobarbital, Clor = clorazepate, Oxcarb = oxcarbazepine, Clon = clonazepam, Topi = topiramate, Val = valproate, Zon = zonisamide, Pheny = Phenytoin, Laco = lacosamide, Preg = pregabalin,

Experimental use abbreviations: WB = Western blot, IHC = immunohistochemistry, IP = immunoprecipitation, MS = mass spectrometry, WB-IP = Western blot using immunoprecipitation samples, WB-H = Western blot using purified histones, ELISA = enzyme linked immunosorbent assay, HEp-2 = human epithelial cell assay.

Other Abbreviations: U = unknown, Y = yes, N = No, VNS = vagal nerve stimulation, AVM = arteriovenous malformation, F = female, M = male, Ant = anterior, RA = rheumatoid arthritis

("spiking cortex", see [219]) were observed, while AVM patients with well-controlled or no history of seizures were grouped with resections from "non-spiking" areas of brain from patients with epilepsy. These criteria were only used for **Figure 2.4**.

BBB disruption in multiple sclerosis, epilepsy, and AVM brain

One of the goals of this study was to detect and localize immunoglobulins in the CNS. We first wished to understand the mechanisms by which these macromolecular complexes gain entry into the brain. There are two known mechanisms for IgG CNS ingress, namely passage across the BBB or synthesis by CNS B lymphocytes.[220] Our results suggest that the former was the predominant source of CNS IgGs in brain from patients with epilepsy. **Figures 2.2A-B** demonstrate the topographic overlap of extravasated immunoglobulins and albumin. Extravasated IgGs and albumin were found in the extracellular as

well as the intracellular compartments **(Figure 2.2 B1-B3)**. The arrows in **B1-3** points to a neuron filled with IgGs and albumin.

In brain tissue from patients with epilepsy, grey matter neurons were filled with IgGs **(Figure 2.2 C1)**, while white matter extravasation of IgGs was restricted to the extracellular space **(Figure 2.2 C2)**. Note the concentric feature of IgG extravasation; a large venule (indicated by an *asterisk*) gave rise to a circular pattern of leakage. Two types of control were used to confirm or refute the hypothesis that the fate of extravasated IgGs differs across pathologies. We used MS brain samples **(Figure 2.2 D1** and **D2)** as well as cortical samples isolated during repair of AVM **(Figure 2.2 E)**. In addition to differences between pathologies we also detected topographic segregation of intracellular uptake patterns in brain from patients with epilepsy *vs.* MS brain (compare **Figure C** and **D**). Unlike in grey matter from patients with epilepsy **(Figure 2.2 C1)**, MS brain neuronal cell bodies were devoid of IgG content **(Figure 2.2 D1)**. The arrows in **C1** indicate parenchymal cells while in **C2** they indicate ectopic neurons in white matter. In white matter of MS patients, extravasated IgGs were found in the extracellular compartment; in contrast to what was observed in neurons from patients with epilepsy, no nuclear IgGs were present (*asterisk* in **D2**). The *arrow* in **D2** points to a glial cell.

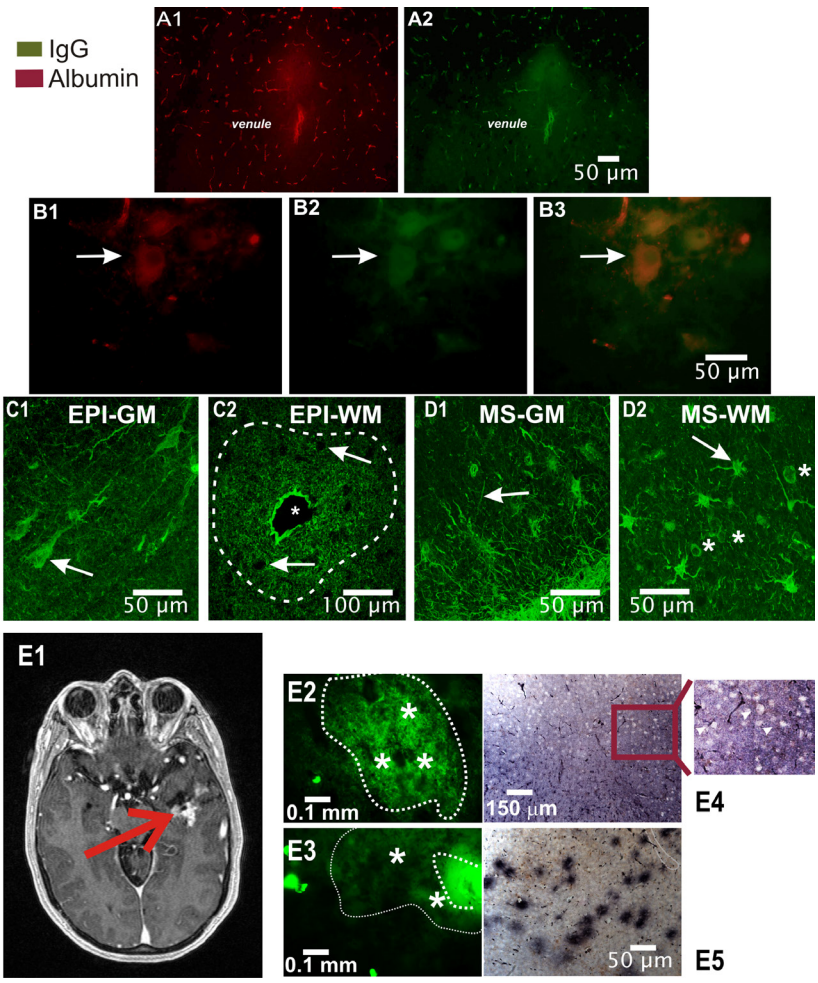

Figure 2.2: Extravasation of IgGs in brain of patients with epilepsy coincides with regions of albumin extravasation. A–B) Similar pattern of IgG and albumin extravasation. The micrographs show the presence of extravasated albumin and IgGs in tissue samples resected from the brain of a patient with epilepsy. Note the patterns of albumin and IgG extravasation. Intravascular presence of both albumin and IgG reveal the capillary network. **B1-B3) Intracellular accumulation of albumin differs from intracellular localization of IgGs**. Unlike albumin, IgGs were detected extracellular space, cytosol and the nucleus of neuronal cells. **Different patterns of IgG in brain from patients with epilepsy (C1-C2) compared to multiple sclerosis brain (D1-D2).** In brain from patients with epilepsy (*arrow* in **C1),** IgG accumulation was restricted to neuronal cells. Large regions of extravasation were seen around blood vessels as indicated by the dotted line in **C2**. The *asterisk* in C2 indicates the lumen of a large venule. The *arrows* point to cell bodies which, in regions of white matter from the brain of patients with epilepsy, reveal cells with no intracellular accumulation of IgG in spite of the abundant presence of extracellular. In multiple sclerosis **(D1 and D2)**, extravasated IgGs were inversely correlated to gray matter extravasation patterns **(D1 vs. D2)**. In addition, note that in MS brain, cells identified as astrocytes revealed an uptake of IgGs in the cytosol but not the nucleus (asterisk **D2**). **E** shows the same results in other tissue sections where DAB was used in lieu of immunofluorescence. Note the patchy profile of IgG extravasation **(E5)** and lack of intracellular staining in **E4**. EPI-GM = gray matter of patient with epilepsy, EPI-WM = white matter of patient with epilepsy, MS-GM = multiple sclerosis gray matter, MS-WM = multiple sclerosis gray matter

An additional “control” for human brain with epilepsy and BBBD is the use of resections obtained during AVM repair. **Figure 2.2 E1** shows the MRI of a typical AVM patient; the *red arrow* points to a region of extravasated contrast agent (gadolinium). The micrographs in **Figure 2.2 E2** and **Figure 2.2 E3** depict a pattern of IgG extravasation in a specimen obtained from another AVM patient. Note the lack of visible IgG accumulation in cell bodies (*asterisks* in **E2**), as well as the gradient of IgG extravasation (shown in **E3**). The *dashed line* indicates regions of maximum extravasation while the *dotted line* in **E3** refers to a broader region of extravasation characterized by weak IgG signals. These results were confirmed by DAB-stained brain slices **(Figure 2.2 E4** and **E5)** where variable extent of IgG extravasation as well as the absence of intracellular IgG was evident (enlargement in **Figure E4**).

Detection and discovery

Figure 2.3 shows a typical outcome of experiments where frozen sections from human brain with epilepsy or MS brain were stained for the presence of neuronal or astrocytic markers as well as for DNA. In brain tissue resections from patients with epilepsy **(Figure 2.3 A-B)**, intracellular and nuclear staining of IgG was readily revealed. However, intra-nuclear accumulation of immunoglobulin was restricted to neurons while astrocytes lacked intra-nuclear accumulation of IgGs despite robust uptake in the cytosol (*arrows* in **A1** and **B1**). The right side panels **(Figure 2.3 A3-B2)** show a quantitative analysis of immunofluorescence.

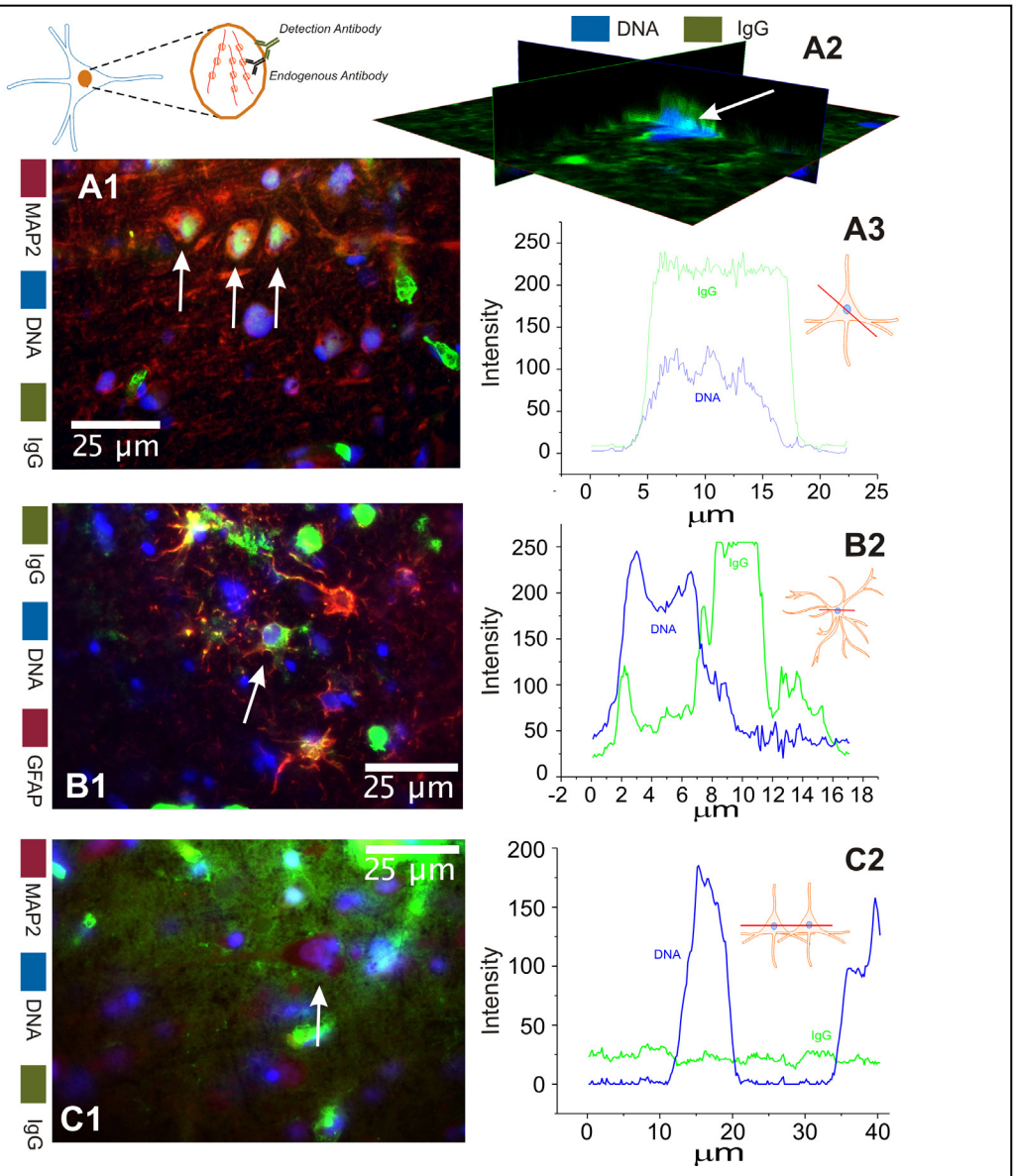

Figure 2.3: Nuclear IgGs are exclusive to neurons in brain of patients with epilepsy. These figures compare results obtained in brain of patients with epilepsy **(A-B)** to multiple sclerosis tissue samples **(C)**. The left panel shows the actual micrographs while the right panel shows a schematic representation of the values of IgGs (in *green*) or nuclear DNA (in *blue*). Note that in neurons, labeled by the neuronal marker MAP2, nuclear accumulation of IgGs was observed whereas in GFAP+ astrocytes from brain of patients with epilepsy **(B)** the content of intracellular IgG was limited to the cytosol and 2 processes. Nuclear co-localization is also observed in a confocal 3D reconstruction **(A2)** of brain tissue from a patient with epilepsy. In contrast, grey matter regions in MS brain (temporal lobe; **C**) revealed widespread extracellular leakage with neuronal cells (indicated by *arrows*) failed to reveal any significant intracellular accumulation of IgGs.

A computer-drawn line was used to detect the profile of nuclear (*blue*) or immunoglobulin-related (*green*) fluorescence in different cells. These results show the co-localization of IgG and DAPI in neurons **(Figure 2.3 A3)** but not in astrocytes **(Figure 2.3 B2)**. To further demonstrate the co-localization of DAPI with IgGs, we used a three dimensional reconstruction of confocal images **(Figure 2.3 A2)**. Regardless of the methods used, the results demonstrate the presence of fluorescently labeled immunoglobulin in neuronal nuclei. In comparison, although extravasation of IgG was widespread in MS brain, intracellular accumulation of IgG was absent (arrow in **Figure 2.3 C1**). The profile lines in **Figure 2.3 C2** show two continuous neurons to demonstrate the lack of any IgG content in these cells.

In order to determine the specific location of IgGs, subcellular fractionation was performed. The Western blot in **Figure 2.4A** shows the presence of intracellular IgGs in each subcellular fraction. The 75 kDa band in the cytoskeletal fraction represents an IgG fragment corresponding to the heavy chain regions. **Figure 2.4B, C and D** show the difference between pathologies with BBB leakage with no seizures *versus* BBB leakage in patients affected by seizures. AVM patients' brain displayed abundant IgGs in soluble nuclear fractions but a less remarkable level in the chromatin-bound fraction. In samples

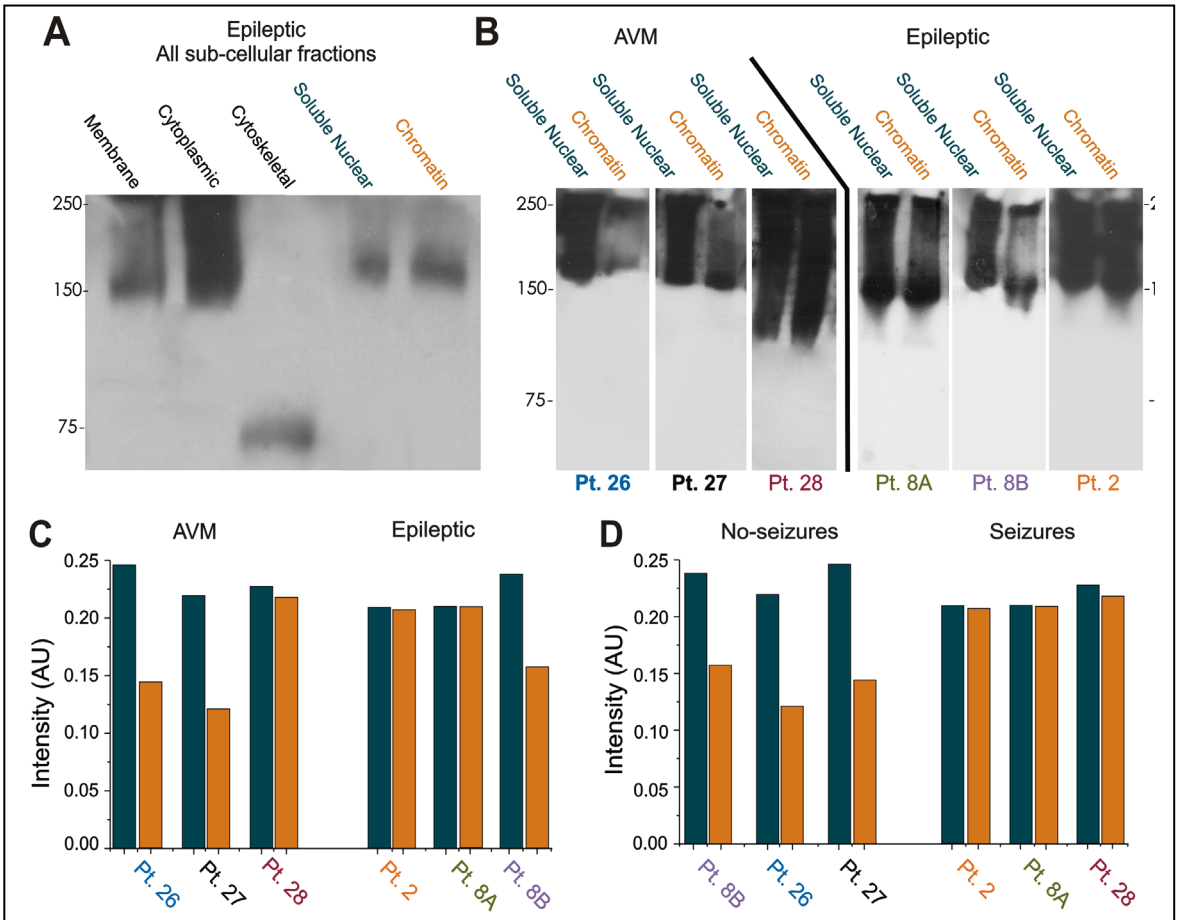

Figure 2.4: Subcellular fractionation and comparison of AVM and brain tissue from patients with epilepsy reveal that BBBD alone is not sufficient to cause antibodies to bind to chromatin. (A) Sub-fractioned brain of patients with epilepsy which was probed with HRP-conjugated anti-human IgG reveals abundant presence of immunoglobulin in all fractions tested. **(B)** Notably, chromatin-bound IgGs were quantitatively similar to levels in the soluble nuclear fraction of patients with epilepsy. Results were compared across several patients and against patients affected by AVM, a disease also characterized by widespread leakage of the BBB, revealing that brain from patients with epilepsy, but not brain from AVM patients, accumulates significant amounts of IgGs in the chromatin-bound fraction. The bar graph in **(C)** shows the quantitative relationship between measured content in the 2 fractions based on pathology. The bar graph in **(D)** shows the same quantitative relationship between IgG in both fractions according to whether or not a patient had seizures. Note that regardless of pathology, BBBD with seizure produces larger amounts of IgG bound to chromatin than with BBBD alone.

from patients with epilepsy, results demonstrated comparable amounts of IgGs in both soluble nuclear and chromatin-bound fractions. In addition, the AVM patient who also experienced seizures had similar levels of chromatin-bound IgGs as patients with epilepsy **(Figure 2.4B, C and D** patient #28**)**. "Non-spiking" brain from patients with epilepsy displayed chromatin-bound IgG levels similar to those found in AVM patients without seizure **(Figure 2.4B, C and D** patient #8B**)**.

Purification and analysis

A second major goal was to determine the molecular profiles of putative autoantigen in the nuclei of neurons from patients with epilepsy. The strategy used to unveil the nature of these autoantigens is described in detail in the methods section. **Figure 2.5** summarizes the experimental design and results. After disassociation of antigen from the antibodies by low pH the soluble nuclear fraction displayed a dramatic drop in protein content over time **(Figure 2.5A)**; this was the opposite of what was observed in the chromatin-bound fraction where a dramatic increase in protein content was observed **(Figure 2.5A)**. This was

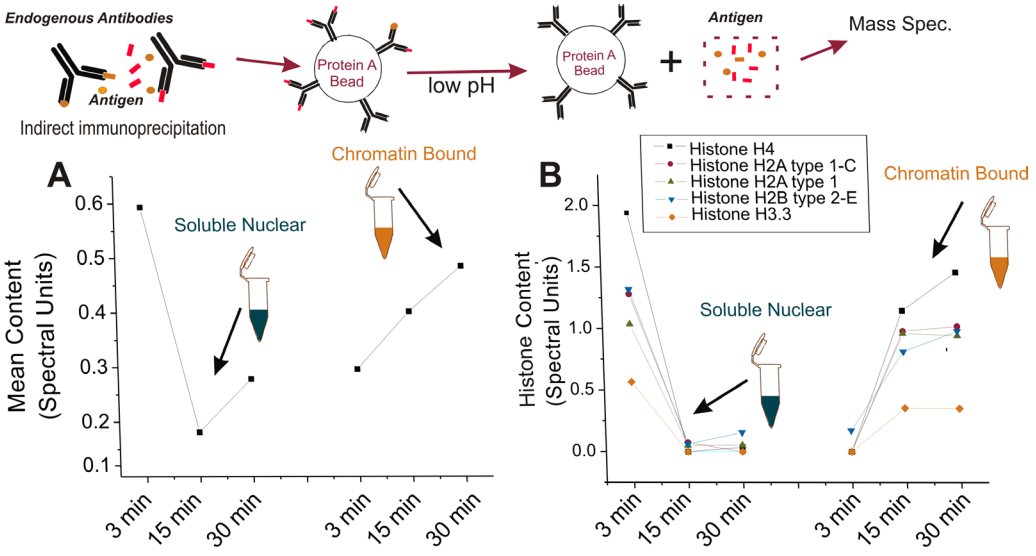

Figure 2.5: Mass spec. analysis revels histones are a likely target of autoantibodies in brain from patients with epilepsy. (A) The average change in antigen concentration in a sample obtained from brain of a patient with epilepsy. After exposure to low pH, there is a dramatic decrease in protein concentration between 3 and 15 minutes with a moderate increase after 30 minutes in the soluble nuclear fraction. In the chromatin-bound fraction, there is a constant increase in protein concentration over time. These kinetic differences suggest the presence of high affinity antibodies in the chromatin- bound fraction and low affinity antibodies (non-specific) in the soluble nuclear fraction. **(B)** When examining the dissociation kinetics for each protein found by mass spec., histones most closely matched the average dissociation profile seen in **(A)** suggesting that there was specific binding of antibodies to histones in the chromatin bound fraction. Refer to the legend for the dissociation kinetics of each histone observed. The insert at the top corresponds to the experimental steps in **Figure 2.1.**

interpreted as presence of specific binding of IgG to nuclear proteins and chromatin in comparison to free-floating or non-specifically bound protein-IgG complexes in the soluble fraction. When mass spectrometry analysis was performed, a number of non-nuclear proteins were found and discarded **(Table 2.3)**. When narrowing down likely nuclear autoantigen candidates, the most prominent family of putative autoantigen were histones. The legend in **Figure 2.5B** highlights the molecular descriptions of the histones found to be significantly associated with specific binding to IgG in the chromatin fraction.

Table 2.2 Candidate autoantigens

Protein	CB-SN	Accession #	Mass (kDa)	Autoimmune Association
Histone H4	-2.83	4504301	11	**SLE** (Bulingame, *et al.*, 1994)
Histone H2A type 1-C	-2.07	4504245	14	**SLE** (Imoka, *et a*l., 1990)
Histone H2A type 1	-1.96	4504239	14	**SLE** (Gu, *et al.*, 2013)
Histone H2B type 2-E	-1.82	4504277	13	
Histone H3.3	-0.92	4504279	15	**SLE** (Van bavel, *et al.*, 2011)
Histone H1.3	-0.74	4885377	22	
Histone H2A.Z	-0.70	4504255	13	
Core histone macro-H2A.1 isoform 2	-0/47	4758496	39	
Heterogeneous nuclear ribonucleoproteins C1/C2 isoform a	-0.18	117189975	33	
Heterogeneous nuclear ribonucleoprotein A3	-0.16	34740329	39	**SLE, Scleroderma** (Siapka, *et al.,* 2007)
Histone H1.0	-0.07	4885371	20	
Nucleoside diphosphate' kinase B isoform a	-0.03	4505409	17	

SLE = systemic lupus erythematosus

Table 2.3: Additional putative autoantigens identified by mass spec. analysis (excluding those in Figure 2.5)

Protein	CB-SN	Accession #	Cellular Localization	Mass (kDa)
Calcium/calmodulin-dependent protein kinase type II subunit alpha isoform 1	-0.78	25952114	Cytoplasm, Membrane	55
Guanine nucleotide-binding protein	-0.49	11321585	Membrane	38
G(I)/G(S)/G(T) subunit beta-1				
2~,3~-cyclic-nucleotide 3~-phosphodiesterase	-0.48	94721261	Membrane	47
Transforming protein RhoA precursor	-0.42	10835049	Cytoplasm, Membrane	22
Calcium/calmodulin-dependent protein kinase type II subunit beta isoform 1	-0.33	26051204	Cytoplasm, Membrane	73
Thy-1 membrane glycoprotein preproprotein	-0.33	19923362	E.R. , Golgi	18
Brain acid soluble protein 1	-0.32	30795231	Membrane	22
Guanine nucleotide-binding protein G(I)/G(S)/G(O) subunit gamma-2 precursor	-0.29	54114974	E.R., Golgi	7
Gelsolin isoform a precursor	-0.22	4504165	E.R., Golgi	86
Septin-7 isoform 2	-0.19	148352329	Cytoplasm, Cytoskeleton	50
Guanine nucleotide-binding protein G(i) subunit alpha-1	-0.13	33946324	Membrane	40
Tubulin alpha-4A chain	-0.04	17921989	Cytoplasm, Cytoskeleton	50
Guanine nucleotide-binding protein G(I)/G(S)/G(O) subunit gamma-7 precursor	-0.02	32698769	E.R., Golgi	7
Cysteine and glycine-rich protein 1 isoform 1	-0.01	4758086		21

All histones tested behaved as predicted by the equation shown in the methods section and according to the time-dependent association of IgG and antigen shown in **Figure 5A**. This also implies that IgG-histone binding displayed high affinity owing to the fact that the kinetic profile was consistent with tight binding antibodies. This is in contrast to what was observed in the soluble fraction where histones were rapidly dissociated from antibodies. A summary of the molecular properties and quantitative analysis of histone and other putative autoantigens is shown in **Table 2.2**.

Identification of antigen and antibody

While the results in **Figure 2.5** and **Table 2.2** show an indirect approach to determine the molecular nature of putative autoantigens found in brain from patients with epilepsy, these results heavily rely on statistical analysis of mass spectrometry data. We performed three additional immunodetection experiments to confirm or disprove the presence of histone-specific IgGs in patients with epilepsy. We first used Western blotting to qualitatively analyze histones by molecular weight. To this end we prepared Western blots using protein extracts from the immunoprecipitation experiments shown in **Figure 2.5**. In other words, we used a mixture of nuclear protein that contained histones and other nuclear proteins obtained from brain tissue resections of patients with epilepsy as a target for patients' serum IgGs. In doing so we wished to test the hypothesis that the putative autoantigens isolated from the brain were also targets of IgGs

present in serum of patients with epilepsy. The results are shown in **Figure 2.6A**. Note that, when control serum was used, no significant signal was detected, whereas with serum from patients with epilepsy a distinct band was apparent. This band reflects the binding of IgGs isolated from serum of patients with epilepsy to autoantigen isolated from IgG present inside cells of human brain with epilepsy. This band also corresponds to the molecular weight of histones (kDa). To confirm that these bands indeed reflected the presence of histones, we ran a similar gel (**Figure 2.6A;** patient 17*) to demonstrate that total histones are recognized by the same serum used in the previous experiment. These results show that the molecular target of IgG extracted from neuronal nuclei of brain tissue resections of patients with epilepsy consists of histones.

While Western blots used to screen and confirm the presence of specific nuclear protein are useful for a few experiments, screening of a larger number of subjects is less convenient. To this end we used ELISA to quantitatively detect and quantify the presence of anti-histone IgGs **(Figure 2.6B)**. Note that in five of eight patients with epilepsy a signal suggestive of autoimmunity against histones was present. Also note that in control subjects (labeled as **C1**) the signal was not present. These results confirm the Western blot findings shown in **Figure 2.6A** but also underscore the fact that these autoantibodies are not present in all patients with epilepsy. However, some patients with epilepsy have higher levels of antinuclear antibodies than the positive control rheumatoid arthritis patient (labeled **Pos. C 1**)

The presence of anti-histone antibodies can be indicative of a broader autoimmune response against multiple nuclear components. That is, many antinuclear antibodies recognize not just histones, but the histone-DNA complex (chromatin). Therefore, we tested the hypothesis that in addition to anti-histone antibodies, serum from patients with epilepsy also contains autoantibodies targeting chromatin **(Figure 2.6C).** Using the HEp-2 assay, a distinct pattern of staining was discovered. Serum from patients with epilepsy, but not serum from control subjects **(Figure 2.6C C1)** displayed various types of antinuclear staining. The *insets* in the figures show specific binding to nuclei or other sub-nuclear fractions (specifically nucleoli **(Pt. 14)** and centromeres **(Pt. 16))**. Note the similarity between the manufacturer's negative control and the serum control provided by the volunteer. The HEp-2 assay results demonstrate that a clinical tool for the diagnosis of autoimmune disease is able to detect the antinuclear antibodies in the serum of patients with epilepsy.

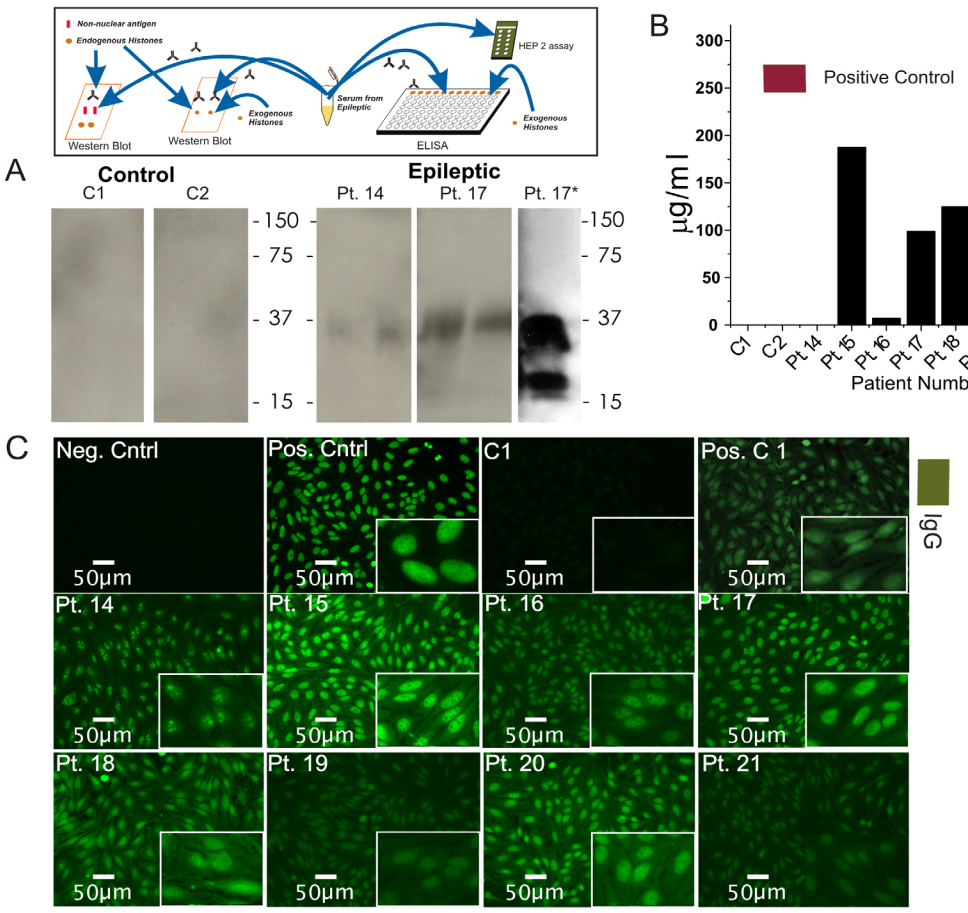

Figure 2.6: A multimodal analysis of serum from patients with epilepsy reveals the presence of specific anti-histone and anti-chromatin antibodies. (A) Immunoprecipitation isolated nuclear antigen taken from the brain tissue of a patient with epilepsy was run on a gel and probed with serum from patients with epilepsy and healthy controls. While control serum produces no signal, serum from patients with epilepsy produces a signal corresponding to histone complexes. Additionally, serum from Pt. 26 was used to probe a gel containing only purified histones **(A Pt. 17*)**. Note the similar binding pattern between both gels using serum from patient 17. **(B)** ELISA quantifying anti-histone antibodies using the serum of patients with epilepsy and control patients. The ELISA did not detect anti-histone antibodies in control serum but did detect them in 5 of 8 patients with epilepsy. Going further, a HEp-2 assay using the same serum detected the presence of anti-chromatin antibodies **(C)**. Patient 15 shows the speckled pattern of general antinuclear staining, patient 14 and 16 show the specific binding pattern of anti-nucleolar and anti-centromere antibodies, respectively. The insert at the top of the figure refers to the phase of experimental design discussed in **Figure 2.1.**

2.4 Discussion

The main finding of this research is the presence of antinuclear antibodies in brain and serum from patients with epilepsy. These findings were obtained in patients who did not present any signs, symptoms or clinical diagnoses of a typical autoimmune disease (such as SLE). Serum from a patient with epilepsy also diagnosed with RA was used as a positive control to underscore that the levels of autoimmune response in subjects with epilepsy were comparable to *bona fide* patients with autoimmune disease.

Nuclear antibodies were predominately found in neurons and appeared to spare glia. Nuclear targets included histones and chromatin. Autoimmune IgGs are derived from the systemic circulation as their presence overlapped with areas of albumin leakage across the BBB. An alternative interpretation may support an indirect role of B lymphocytes in BBBD; this hypothesis needs to be tested by *ad hoc* experiments.

Significance

Many clinical studies suggest that aberrations of the immune system may be associated with seizures. Our results suggest the existence of a new category of autoimmune epilepsy. Additionally, while a number of studies have demonstrated the presence of antinuclear antibodies in sera of patients with epilepsy, we have for the first time shown that these antibodies are able to enter the nuclei of neurons. We focused on a population where antibodies against

traditional “epilepsy related autoantigen” were not present and discovered that the misguided immune response in this population targeted the nuclear antigens histones and chromatin. A previous report by others concluded that subjects affected by epilepsy had levels of antinuclear antibodies similar to non-epilepsy controls.[221] In these studies no attempt was made to characterize these autoantigen as histones or chromatin. When the study was repeated in a controlled fashion, the authors reported a significant difference between prevalence of autoantibodies in patients with epilepsy compared to control.[222,223] Furthermore, these results were exclusively based on the HEp-2 assay, which is qualitative, and not with a clinical-grade ELISA test. In addition, the same group recently reported that antinuclear antibodies are associated with recurrent seizures in patients with refractory focal epilepsy.[223] Our results show a convergence of positive findings, since both qualitative (HEp-2) and quantitative tests (ELISA) demonstrated the presence of antinuclear antibodies in patients with epilepsy. We also used an extracted nuclear antigen fraction to confirm the presence of IgG-bound autoantigen which was found to be histones or chromatin.

Given the fact that areas of IgG leakage corresponded to areas of albumin extravasation, we concluded that ingress of IgGs in neurons occurred by a trans-endothelial route either across altered tight junctions or by other trans-cellular means. We found no evidence of local production of these immune molecules. Others also confirm the lack of significant B cell presence in brain tissue

resections of patients with epilepsy where an infectious trigger is not suspected.[224]

In typical autoimmune epilepsy the presence of a pathogen is often suspected but not always demonstrated.[138] Both bacterial and viral mechanisms have been proposed. The onset and development of the autoimmune response may be due to molecular mimicry or breaking of a barrier maintaining immunological privilege. The latter is typical for CNS disorders owing to the presence of a BBB. The question, however, remains of why an autoimmune disease should cause seizures which are characterized by neuronal hyperexcitability and synchronization, or alternatively do seizures trigger an autoimmune disease? There are several lines of evidence pointing to the devastating effect of antibodies targeting ion channels,[225] glutamate receptors, voltage gated calcium and potassium channels, etc.,[205,226] In these pathologies the proposed and accepted cascade of evidence/events is as follows: 1) The immune system mounts a response against a pathogen or against a self-molecule perceived as such; 2) Antibodies are produced to target these proteins or a specific sequence; 3) Upon binding to its target the antibody promotes loss of function or excessive function of one of the several crucial components that regulate neuronal resting potential or synaptic function (see[227] for details). We did not directly investigate whether autoantibodies may have an influence on and/or produce a downstream event that may impact comorbidities such as cognitive decline. Antinuclear IgGs may be yet another mechanism of seizure-induced

neuronal cell loss originally described in hippocampal sclerosis but nowadays recognized also for neocortical seizure disorders. However, further experiments are needed to determine the pathological mechanisms involves.

Consequences of intranuclear brain IgGs

How IgGs pass through a breached BBB is not known, nor is it understood how once they enter the cellular compartment IgGs may act as "neurotoxins."[228] There are many possible mechanisms for their "toxic" actions, including cell-induced death by an immune response, altered transcription owing to binding of IgGs to DNA, altered mechanisms of cell cycle or apoptosis, etc. Our results show no evidence of widespread neuronal cell death in the regions of albumin and IgG extravasation. In fact, the cells displaying the most intranuclear IgG content (for example **Figure 2.3A**) were characterized by healthy-appearing chromatin and nuclear content.[229] Others have suggested that IgGs are "toxic" and that they promote neuronal cell death in the lithium/pilocarpine model of status epilepticus or in human cortex.[216,228]

Alternative hypotheses to speculate on the consequences of nuclear IgGs in human brain from patients with epilepsy may focus on comorbidities rather than seizures themselves. It is well known that complex sequelae of pathologies follow the onset and progression of epilepsy. This is particularly true for pediatric populations where developmental delays, mental illness and other noxious consequences of prolonged seizures are often encountered. It is possible that

given a similar family history, seizure severity and age/gender, patients deteriorate more rapidly when neuronal cells are exposed to nuclear IgGs. It is also possible that IgGs have a neuroprotective effect.

A role for the blood-brain barrier?

Seizures are characterized by widespread vascular changes that span from hyperemia to vascular leakage.[95,199,230-235] We and others have shown that BBB disruption precedes seizures in patients with epilepsy and that BBBD causes seizures in humans and animals even in absence of a prior history of epilepsy (for review see[203]). There is therefore strong evidence that the BBB is impaired in patients with epilepsy, at least at time of seizure onset. BBB leakage results in extravasation of IgGs which is the first step towards their ingress into the nuclear compartment of neurons.[236] Thus, either BBBD, seizures, or both may be necessary for this to happen. To address this hypothesis, we performed experiments using two human pathologies also characterized by BBB leakage but not seizures; we used *post-mortem* samples of MS brain as well as brain samples isolated during surgeries to repair AVM. We also studied samples from patients with BBBD and seizures and compared those to BBBD but no ongoing seizures or negative seizure history. To this end, in addition to resections of brain from patients with epilepsy, we used a “non-spiking” region of resected temporal lobe (as a BBBD+ but no seizure sample) and AVM tissue from a patient presenting with multiple drug resistant seizures (as BBBD+ and seizure+) (see

Table 2.1). The data in **Figure 2.4** show that chromatin-bound autoantibodies were elevated in all samples from “spiking” cortex, regardless of its origin. This group contained all tissue isolated from foci of patients with epilepsy as well as AVM brain associated with seizures. The group with the least chromatin-bound IgGs encompassed brain samples from non-epilepsy patients and the brain tissue resected from “non-spiking” brain of patients with epilepsy. These preliminary results led us to conclude that leakage of the barrier is necessary for entry of IgG into the parenchyma but is not sufficient to allow binding of antibodies to chromatin.

If BBB dysfunction is not sufficient to cause accumulation of IgGs into the nucleus of neurons, what is the likely mechanism? We propose a scenario where seizures themselves are responsible for uptake of IgGs into the nuclear compartment of neurons. According to this scenario, prolonged excitation of neurons and other cellular elements occurring during a seizure, and the subsequent metabolic mismatch, acts synergistically to decrease selective permeability of the cell membrane. According to this hypothesis, a specific receptor is not necessary, but rather, this uptake occurs due to a non-specific spreading depression-like episode. However, this scenario explains how IgGs enter into the *cells* but do not account for the presence in the *nucleus*. In fact, spreading depression affects all cells in a certain region but nuclear uptake was only present in neurons. How IgGs may migrate from cytosol to nucleus remains unknown. A recent paper has shown that electrical stimulation characterized by

low intensity (μA) and a frequency comparable to neuronal firing during an ictal event (50Hz) causes translocation of membrane-bound protein to the nucleus.[237] It is thus possible that field potential changes alone are sufficient to cause subcellular redistribution of macromolecules.

Limitations

One of the potential confounders in this study are the autoimmune side-effects of certain anti-seizure medications. For example, carbamazepine is known to induce lupus-like symptoms or full-blown disease; this has been shown to occur primarily in female patients.[238] This does not appear to be a significant factor in our study because of all the patients enrolled, only five were under carbamazepine therapy. In addition to carbamazepine, anecdotal reports have shown that lamotrigine and valproic acid can produce lupus-like symptoms.[239;240] However, this was unlikely to be a factor in antinuclear antibody generation in our patients as none of them presented with symptoms of a drug-induced lupus. The probability of finding antinuclear antibodies was, in other words, unrelated to drug regimen. Others also found cohorts of patients with epilepsy undergoing carbamazepine therapy with no symptoms or signs suggestive of SLE.[221] It has to be noted, however, that in this study the presence of antinuclear antibodies was more common in female patients compared to male. Of the three patients with undetectable levels of auto-IgG, all were male while 60% of the patients with detectable levels of anti-histone antibodies were female. This finding is in

agreement with numerous findings linking autoimmune disease to gender differences.[186]

Another possible limitation of this study is the fact that we did not use CSF to test for autoantibodies. CSF analysis is a routine clinical approach to diagnosis infectious or autoimmune diseases. We did not have access to CSF samples for the patient population whose data is shown.

2.5 Conclusions

Our results point to a sterile inflammation mechanism[241] by which failure of the BBB promotes neuronal dysfunction. We focused on three neurological conditions (epilepsy, multiple sclerosis, AVM) all characterized by leakage of the BBB but found nucleus specific IgGs only in brain from patients with epilepsy and within these samples only in a population of neurons. IgGs directed towards histones and chromatin were subsequently found in sera from other patients with epilepsy suggesting that both compartments (circulatory and CNS) contain these antibodies and that antibodies found in serum are able to bind nuclear protein extracted from brain samples from patients with epilepsy.

2.6 Acknowledgements

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CHAPTER 3:

Aim 2:
The protective effect of
IgGs in an animal model of status epilepticus

3.1 Introduction

Evidence has demonstrated a role of neuroinflammation in epilepsy. Support for the "autoimmune/inflammatory hypothesis" comes from all levels of scientific inquiry (see[120]). Evidence can be divided into three broad mechanistic categories: 1) Central nervous system immune dysregulation in seizure models,[175] 2) Peripheral cellular responses in models[233] or patients[120,199,242] and 3) Humoral mediators.[243]

It has been demonstrated that the humoral immune response (i.e., autoantibodies) plays a role in seizure disorders. Autoantibodies have been found in patients with epilepsy with specificities towards voltage-gated potassium channels (VGKC),[244] N-methyl-D-aspartate (NMDA) receptors,[245]

glutamic acid decarboxylase (GAD)[246] and, most recently, nuclear components.[184] The latter were found inside neurons and in sera of patients with epilepsy. These antibodies have been demonstrated or hypothesized to enter the brain via pre-ictal or ictal BBB disruption rather than intrathecal production.[247] Regardless of origin, autoimmne IgGs exert a deleterious effect.

While the pathogenic role of autoantibodies in patients with epilepsy is well established, it remains unclear what role non-autoimmune antibodies may have in seizures. Further, clinical evidence shows that administration of non-specific, serum antibodies (intravenous immunoglobulin's; IVIg) is a powerful tool for the treatment of seizures,[248] especially when auto-IgGs are an etiologic mechanism. Treatment with IVIg decreases seizures in patients with anti-NMDA receptor autoimmune encephalitis,[148] Rasmussen's encephalitis,[168] and drug-refractory seizures,[249] among others. In each of the aforementioned studies, administration of IVIg was either the primary or an add-on treatment given over time, though IVIg has also been effective acutely in treating SE.[90] The efficacy of IVIg has been established for certain types of epilepsy but the mechanism of action and whether that action occurs in the CNS or in the periphery remains unknown.[185] In autoimmune epilepsies it has been hypothesized that IVIg works to deplete antibodies and promote an anti-inflammatory environment that produces a reduction in symptoms.[191] This mechanism is likely effective for long term use but does not explain its efficacy for acute therapy.

In non-autoimmune epilepsies the primary mechanism of IVIg is likely different. For CNS diseases, it has been proposed that IVIg works through an Fc receptor-dependent mechanism either in the periphery or the CNS. By this mechanism, IVIg antibodies bind to inhibitory Fc receptors on cell membranes that compete with antibodies binding to activating Fc receptors on the same cell. IVIg antibodies therefore swing the inflammatory/anti-inflammatory balance in favor of anti-inflammatory.[192] While it has been demonstrated that neurons and glia have Fc receptors,[250] it remains unknown whether the anti-seizure effect occurs on these cells or elsewhere.

It is also not known whether the efficacy of IVIg is specific to exogenous antibodies or whether the effect also applies to endogenous antibodies at high levels. It is unlikely this question could be answered using humans, as endogenous levels of antibodies cannot be manipulated; therefore, animal models are necessary to answer this question. However, there is strong evidence for naturally occurring therapeutic antibodies in humans.[251] These antibodies are produced by a subset of B cells and are implicated in removing cellular debris,[252] amyloid plaques[253] and in remyelination in MS models.[254] The effect of natural antibodies is not associated with high total levels but rather target specificity.[251]

Taking all of the above into consideration, the overarching hypotheses are 1) High levels of serum antibodies, either endogenous or exogenous, will limit or prevent seizures; 2) The seizure mitigating effect of IVIg lies in the periphery

rather than directly on the CNS and 3) The action of IVIg does not proceed through Fc receptors.

3.2 Materials and methods

Animal Care and Compliance

Mice were housed in a clean and controlled environment at the Cleveland Clinic Foundation (21°± 1°C, 60% humidity, 12 hour light/dark cycle, food and water *ad libitum*). Procedures involving these animals and their care were conducted conforming to the institutional guidelines and are in compliance with their laws and policies (Guide for the Care and Use of Laboratory Animals, U.S. National Research Council, 1996) and were approved by the Cleveland Clinic Foundation Institutional Animal Care and Usage Committee. Experiments are outlined in **Figure 3.1**.

Mouse model selection

In order to dissect the temporal and causal relationship between seizures and the antibody response in the epileptic brain, we generated an animal model where seizures and antibodies can be manipulated. To this end we adapted a genetic mouse model of lupus to a model of *status epilepticus* (SE; pilocarpine model). The genetic lupus model adapted for these experiments are NZBWF1/J mice, the F1 cross of New Zealand Black (NZB) and

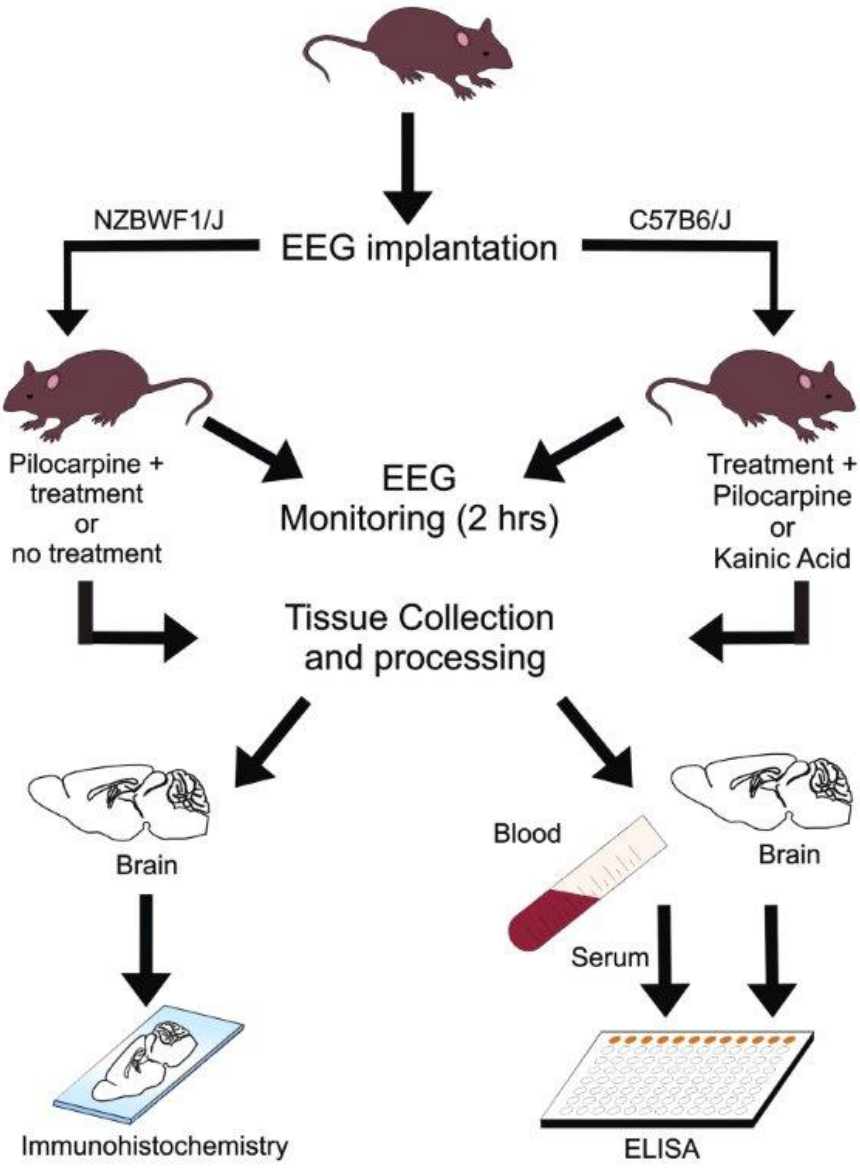

Figure 3.1: Schematic representation of animal experiments performed. In brief, mice were implanted with EEGs and allowed to recover for 7 days and divided into two groups: NZBWF1/J mice and C57B6/J mice. NZBWF1/J mice were divided into control or IgG treatment groups. C57B6/J mice were divided into pilocarpine and kainic acid groups. Pilocarpine mice were divided into control, IV IgG and IV denatured IgG treatment groups. Kainic acid mice were divided into control, IV IgG at time of KA and IP IgG 12 hours prior to KA. All IgG injections were 200 µl of 10 mg/kg whole rat IgGs. All mice were monitored using EEG for two hours after baseline. Mice were sacrificed and tissue collected. Brains were processed for immunohistochemistry and blood was collected to isolate serum for ELISA.

New Zealand White (NZW) mice. This model was chosen because the mice display a predictable presence of antinuclear antibodies in serum that has been carefully characterized throughout the literature since the late 1960s.[255] Older animals (15-24 weeks; as we have used here) are characterized, in part, by hypergammaglobulinemia and antinuclear antibody production. C57B6/J mice (B6; approximately 12 week old males) were used to determine the effects of intravenous injection of antibodies on both pilocarpine and kainic acid (KA) induced SE in the absence of antinuclear antibodies.

EEG implantation and recording

EEG implantation and recording was performed using Pinnacle Technology 3 channel EEG system (Lawrence, KS, USA). Implantation was performed using aseptic technique, under Ketamine/Xylazine (100mg/kg and 15 mg/kg respectively) general anesthesia injected intraperitoneally (IP). A rostral-caudal incision was made in the scalp and membranous tissue located under the scalp was cleared away. The skull was dried with ethanol and the implant was secured onto the skull using cynoacrylate 3 cm behind the bregma. A 23-gauge needle was used to create pilot holes for screws. Each screw was advanced into the skull after silver epoxy was applied to the threads.

A novel departure from the standard EEG implantation procedure provided by Pinnacle is the use of flowable light-cured dental composite (Benco Dental, Pittston, PA, USA). Liquid composite was placed over each screw and around the base of the implant to insulate the screws and further secure the device to

the skull. Blue light was then used to quickly harden the composite. A light-cured composite, rather than chemically curing resin, was chosen for its biocompatibility, reduction in surgery time and hardening properties that eliminated thermal damage to the mouse from the curing reaction and unnecessary shifts in the EEG implant due to contraction of the composite. Sutures were used to close the incision. Mice were allowed to recover for a minimum of five days. EEG recording is achieved using Pinnacle Technology Seizure Software (Lawrence, KS, USA) and USB-powered EEG connection to the animal. EEGs were recorded for approximately 2.5 hours including a 30 minute baseline. Recorded EEGs were analyzed using pClamp software 9.0 (Axon instruments, Sunnyvale, CA, USA).

Seizure induction, IgG treatment and seizure scale

To study the effects of exogenous IgGs on seizures, animals were given total rat immunoglobulins (200 µl at 10 mg/kg; Jackson Immunoresearch; West Grove, PA, USA) 12 hours prior to or tail vein IV injection at time of pilocarpine or KA injection. Rat IgGs (rather than mouse IgGs) were used to determine where the IgGs localized after SE and to test whether the anti-seizure action of IVIg in epilepsy occurs through the Fc receptor or not; rat IgGs cannot bind mouse Fc receptors (see [256,257]). Additional sets of mice were given denatured total rat immunoglobulins or saline at time of pilocarpine or KA. IgGs were denatured at 70° C for 20 minutes to promote denaturing but not aggregation of the proteins in solution.[258] Seizures were induced using IP injection of pilocarpine at 170 mg/kg

(NZBWF1/J) or 300 mg/kg or KA at 35 mg/kg (black 6 mice). Twenty minutes prior to pilocarpine injection, N-methyl-scopolamine (1 mg/kg) was administered IP to alleviate peripheral side-effects.

A behavioral seizure scale was used to define and quantify the manifestations of seizure in real-time and by EEG review. Seizures were scored as follows: 1) Raised tail and/or abnormal posturing; 2) Myoclonic extension of limbs, favoring one side; 3) Brief tonic-clonic seizures; 4) Tonic-clonic seizures with rearing or jumping; and 5) SE. Seizures were monitored in this way for two hours and mice were subsequently sacrificed.

Total rat IgG ELISA

Blood and brain tissue was collected after sacrifice of the animals. Serum was obtained by centrifugation and diluted 1:10 in serum diluent. Brain tissue was homogenized in radioimmunoprecipitation assay (RIPA) buffer and centrifuged for 45 minutes at 4°C. Supernatant was collected and diluted 1:10 in serum diluent. Samples for total rat IgG were analyzed by ELISA according to manufacturer instructions (Abcam, Cambridge, MA, USA). Samples were read on a spectrophotometer at 490 nm.

Immunohistochemistry

Mouse brain sections were cut on a cryostat at 30 μm. Sections were washed in phosphate-buffered saline (PBS) and placed in blocking solution for one hour at room temperature. Slices were then incubated with a fluorescent anti-mouse (FITC; 1:200) or anti-rat IgG (Alexa fluor 594; 1:200; Jackson

ImmunoResearch, Westgrove, PA, USA) for two hours at room temperature. Slices were washed with PBS and then mounted and coverslipped in VectaShield with DAPI (Vector Labs, Burlingame, CA, USA). Image acquisition was performed on a Leica fluorescent microscope, slide scanner or fluorescent confocal microscope. Image analysis was performed using Image J (National Institutes of Health, Bethesda, MD, USA).

Statistical analysis

Data are expressed as ± SEM and considered significant when $P < 0.05$. Analysis was performed using JMP (SAS Institute Inc, Cary NC, USA) and Origin statistical software (OriginLab, Northampton, MA, USA). ANOVA and pair-wise correlations were used to assess data significance.

3.3 Results

For the results presented here we used a total of 56 mice **(Table 3.1)**. Brains and blood from each of these mice were analyzed. Experimental details can be found in **Table 3.1** and are outlined in **Figure 3.1.** Of the 56 mice, 14 were NZBWF1/J mice. All but one mouse were implanted with EEG and all were given pilocarpine. Half of the NZBWF1/J mice were treated with immunoglobulins (IP) 12 hours prior to pilocarpine administration.

The remaining 42 mice were B6 mice. Of these mice, 29 were given pilocarpine and were divided into the following treatment groups: control (saline injection; n= 8), IVIg treatment at time of pilocarpine (n= 10) and IV denatured

IgGs (n = 10). The remaining B6 mice were injected with KA and divided into the following treatment groups: control (saline injection; n=4), IVIg treatment at time of KA (n=5) and IP IgG treatment 12 hours prior to KA administration (n=5). Details of the experimental use for each animal can be found in **Table 3.1** and an outline of the methods used for these experiments can be found in **Figure 3.1.**

Table 3.1: Mouse data and experimental use

ID	Gender	Treatment	Pilo or Ka dose	Seizure	Mortality	Total IgG (ug)	Exp. Use
				NZBWF1/J			
1	M	Pilo only	170	Yes	Yes	1057	IHC, ELISA, Implant
2	M	Pilo only	170	No	Yes	669	IHC, ELISA, Implant
3	M	Pilo only	170	No	No	958	IHC, ELISA, Implant
4	F	Pilo +preIgG	170	Yes	No	711	IHC, ELISA, Implant
5	F	Pilo +preIgG	170	No	No	1595	IHC, ELISA, Implant
6	M	Pilo only	170	Yes	No	924	IHC, ELISA, Implant
7	M	Pilo only	170	Yes	Yes	707	IHC, ELISA, Implant
8	F	Pilo +preIgG	170	Yes	No	2479	IHC, ELISA, Implant
9	F	Pilo +preIgG	170	No	No	1572	IHC, ELISA, Implant
10	F	Pilo +preIgG	170	Yes	No	821	IHC, ELISA, Implant
11	M	Pilo only	170	No	No	865	IHC, ELISA, Implant
12	M	Pilo only	170	No	No	1057	IHC, ELISA
13	F	Pilo + preIgG	170	Yes	No	791	IHC, ELISA, Implant
14	F	Pilo + preIgG	170	No	No	530	IHC, ELISA, Implant
				C57B6/J			
15	M	Pilo control	300	Yes	Yes	1170	ELISA, Implant, IHC
16	M	Pilo control	300	Yes	Yes	2956	ELISA, Implant
17	M	Pilo control	300	Yes	Yes	595	ELISA, Implant
18	M	Pilo control	300	Yes	Yes	375	ELISA, Implant, IHC
19	M	Pilo control	300	Yes	Yes	782	ELISA, Implant
20	M	Pilo control	300	Yes	Yes	367	ELISA, Implant
21	M	Pilo control	300	Yes	No	611	ELISA, Implant, IHC
22	M	Pilo control	300	Yes	Yes	947	ELISA, Implant, IHC
23	M	Pilo + IgG	300	No	No	611	ELISA, Implant
24	M	Pilo + IgG	300	No	No	231	ELISA, Implant, IHC
25	M	Pilo + IgG	300	Yes	Yes	858	ELISA, Implant, IHC
26	M	Pilo + IgG	300	Yes	Yes	496	ELISA, Implant
27	M	Pilo + IgG	300	Yes	Yes	397	ELISA, Implant
28	M	Pilo + IgG	300	Yes	Yes	422	ELISA, Implant, IHC
29	M	Pilo + IgG	300	No	No	392	ELISA, Implant, IHC
30	M	Pilo + IgG	300	No	No	383	ELISA, Implant
31	M	Pilo + IgG	300	Yes	Yes	1058	ELISA, Implant
32	M	Pilo + IgG	300	Yes	No	667	ELISA, Implant
33	M	KA control	35	Yes	No	477	ELISA, Implant, IHC
34	M	KA control	35	Yes	No	576	ELISA, Implant, IHC
35	M	KA control	35	Yes	No	461	ELISA, Implant

Table 3.1 continued

ID	Gender	Treatment	Pilo or Ka dose	Seizure	Mortality	Total IgG (ug)	Exp. Use
36	M	KA control	35	Yes	No	288	ELISA, Implant
37	M	KA + IgG	35	Yes	No	380	ELISA, Implant, IHC
38	M	KA + IgG	35	Yes	No	265	ELISA, Implant, IHC
39	M	KA + IgG	35	Yes	No	425	ELISA, Implant, IHC
40	M	KA + IgG	35	Yes	No	589	ELISA, Implant
41	M	KA + IgG	35	Yes	No	699	ELISA, Implant
42	M	Pilo + DIgG	300	Yes	Yes	211	ELISA, Implant, IHC
43	M	Pilo + DIgG	300	Yes	No	67	ELISA, Implant,
44	M	Pilo + DIgG	300	Yes	Yes	223	ELISA, Implant
45	M	Pilo + DIgG	300	Yes	Yes	408	ELISA, Implant, IHC
46	M	Pilo + DIgG	300	Yes	Yes	469	ELISA, Implant
47	M	Pilo + DIgG	300	Yes	Yes	2413	ELISA
48	M	Pilo + DIgG	300	Yes	No	965	ELISA
49	M	Pilo + DIgG	300	Yes	No	157	ELISA
50	M	Pilo + DIgG	300	Yes	No	327	ELISA
51	M	Pilo + DIgG	300	Yes	No	477	ELISA
52	M	KA+ preIgG	35	Yes	Yes	299	ELISA, Implant, IHC
53	M	KA+ preIgG	35	Yes	No	152	ELISA, Implant
54	M	KA+ preIgG	35	Yes	No	37	ELISA, Implant
55	M	KA+ preIgG	35	Yes	Yes	174	ELISA, Implant, IHC
56	M	KA+ preIgG	35	Yes	No	65	ELISA, Implant

M = male, F = female, Pilo = pilocarpine, KA = kainic acid, IgG = IV immunoglobulins at time of chemoconvulsant, preIgG = immunoglobulins given IP 12 hours prior to chemoconvulsant, DIgG = denatured immunoglobulins given IV at time of pilocarpine, IHC = immunohistochemistry, ELISA = enzyme-linked immunosorbent assay, Implant = EEG implantation

Discerning the effect of antinuclear antibodies vs. non-specific serum IgGs

A major goal of this study was to determine whether or not antinuclear antibodies had an effect on seizure propensity or severity. To that end, NZBWF1/J mice with anti-chromatin and anti-histone antibodies found in serum were treated with pilocarpine to establish a threshold for SE. A statistically significant negative correlation between anti-chromatin antibodies and seizures was found **(Table 3.2)**. No correlation was found between anti-histone antibodies and seizure parameters measured **(Table 3.2)**. However, a highly significant positive correlation was found between total serum IgG levels and anti-chromatin antibodies **(Table 3.2).** Additionally, total IgG and mortality after SE were negatively correlated **(Figure 3.2 A)**. As both antibody types displayed statistical

Table 3.2 Correlations and statistical significance between measured seizure parameters and antibody levels in serum

Variable	by Variable	Correlation	n	Signif. Prob.
Anti-chromatin Ab (ng)	**Seizure**	**-0.4296**	**14**	**0.0330**
Anti-chromatin Ab (ng)	Latency to seizure	0.2524	14	0.3456
Anti-chromatin Ab (ng)	Seizure Intensity	-0.2369	14	0.3771
Anti-chromatin Ab (ng)	Seizure Duration	-0.1547	14	0.5672
Anti-chromatin Ab (ng)	Mortality	0.122	14	0.6527
Anti-histone Ab (ng)	Seizure	0.2769	14	0.3177
Anti-histone Ab (ng)	Seizure Duration	0.2256	14	0.4189
Anti-histone Ab (ng)	Latency to seizure	0.0291	14	0.9454
Anti-histone Ab (ng)	Seizure Intensity	0.2873	14	0.2991
Total IgG	**Seizure**	**-0.6513**	**14**	**0.0063**
Total IgG	**Anti-chromatin Ab (ng)**	**0.6843**	**14**	**0.0035**
Total IgG	Anti-histone Ab (ng)	0.2068	14	0.4597

** All data in **bold** are statistically significant

significance when measured against seizure parameters, and given that total IgG levels correlated significantly with total anti-chromatin antibody levels **(Table 3.2)**, an experimental (rather than a statistical modeling) approach was required to reveal the true relationship between IgGs and seizure propensity. In other words, we wished to ascertain whether anti-chromatin antibodies have a seizure mitigating effect because of their target specificities or because of their non-specific IgG binding. To this end, non-specific rat IgGs were administered IP 12 hours prior to pilocarpine. In these animals a 75% reduction of seizures was observed compared to control **(Figure 3.2 B, C1 and C2).**

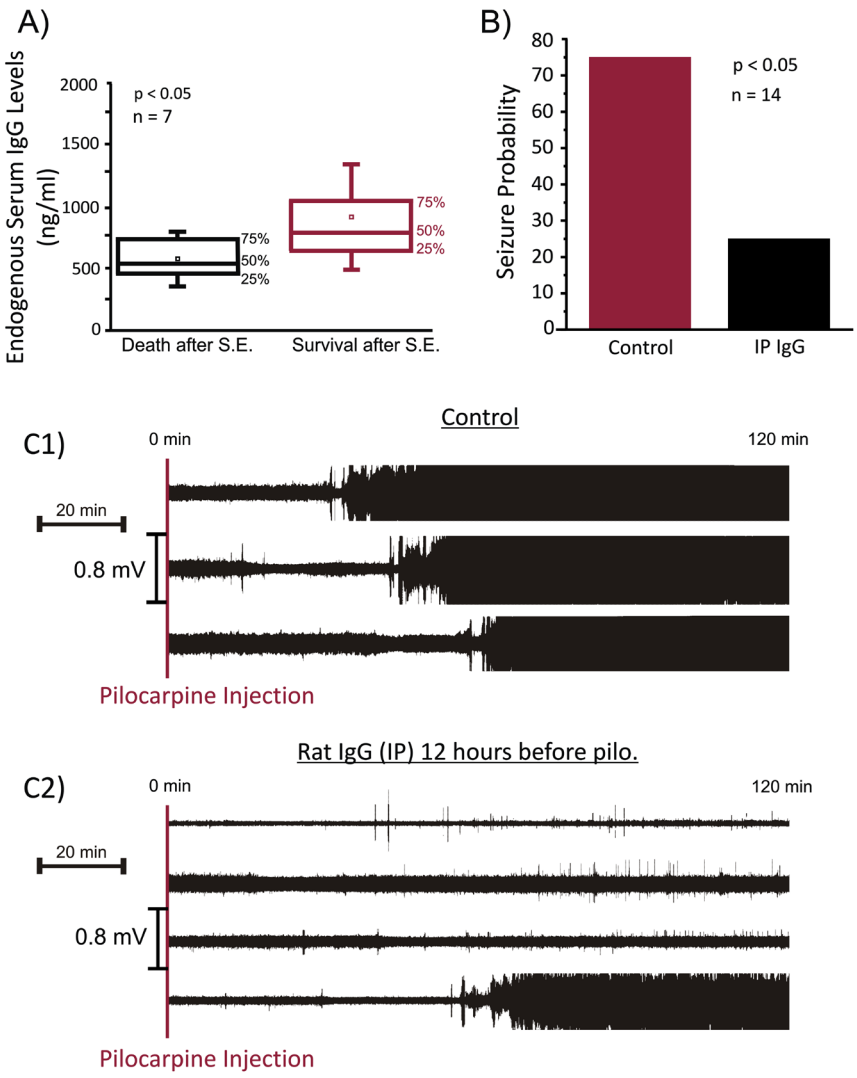

Figure 3.2: High endogenous and prophylactic administration of exogenous non-specific antibodies reduces mortality and mitigates seizures in NZBWF1/J mice. A) Shows endogenous total IgG levels in mice after pilocarpine seizure as measured by ELISA. Mice with higher endogenous total IgGs had higher rates of survival after pilocarpine *status epilepticus* than mice with lower endogenous IgG levels. **B)** Additional rat IgGs were injected IP 12 hours prior to pilocarpine and EEGs were recorded**.** The group of mice given IP IgGs had a much lower seizure probability compared to the control group**. C)** Shows 7 representative EEG recordings from IP IgG injected mice **(C2)** and control mice **(C1)**. Note that all control mice seized while only one mouse treated with IP IgG seized.

Effect and localization of Injected non-specific antibodies in B6 mice given pilocarpine

In B6 mice, rat IgGs had a seizure mitigating effect in the absence of antinuclear antibodies. All control mice given pilocarpine developed SE and 88% died as a result. Similarly, all mice given denatured IgGs IV developed SE and 50% died **(Table 3.3).** Mice administered IV intact IgGs at the time of pilocarpine injection experienced a 40% reduction in seizure compared to control. No difference in mortality was found between denatured and intact IgGs when given at time of pilocarpine **(Table 3.3)**. Mortality was decreased when IgGs were given 12 hours prior to pilocarpine **(Table 3.3)**.

Brain sections of mice in this group revealed interesting patterns of IgG leakage in the cortex **(Figure 3.4)**. Mice treated with saline and denatured IgGs showed no brain staining for rat IgG **(Figure 3.4 A and B)**. However, diffuse and localized patterns of endogenous mouse IgGs can be seen throughout the cortex **(Figure 3.4 A and B, insets)**. Mice injected with rat IgGs either 12 hours prior to pilocarpine **(Figure 3.4 D)** or at time of pilocarpine showed abundant vascular staining for rat IgGs but no extravasation into brain **(Figure 3.5 C and D)**. In addition, these same mice did not show any leakage of endogenous IgGs into the cortex **(Figure 3.4 C and D insets)**. ELISA data in **Figure 3.3 D** shows very little CNS accumulation of IgGs in the brain. Note that **Figure 3.3 D** measures the difference between serum and brain to underscore the net flux directions.

Table 3.3: Statistical data of measured seizure parameters versus treatments

Treatment	Convulsant agent	n	Seizure prob.	Mortality prob.	Avg. latency to seizure (min)	Avg. seizure Severity
Control	Pilo	8	1 ± 0.0	0.88 ± 0.35	18.5 ± 9.7	4.5 ± 1.07
IV IgG	Pilo	10	**0.6 ± 0.52 (0.013)**	0.5 ± 0.52 (>0.05)	21 ± 12.5 (>0.05)	3.7 ± 1.49 (>0.05)
IP IgG, 12 hrs prior	Pilo	7	**0.57± 0.53 (0.009)**	**0 ± 0 (< 0.001)**	**47.6 ± 9.6 (0.004)**	**2.7 ± 1.25 (0.01)**
Denatured IgGs, IV	Pilo	10	1 ± 0.0	0.5 ± (>0.05)	25 ± 12.0 (>0.05)	3.9 ± (>0.05)
Control	KA	4	1 ± 0.0	1 ± 0.0	17.3 ± 5.6	3.6 ± 0.5
IV IgG	KA	5	1 ± 0.0 (>0.05)	1 ± 0.0 (>0.05)	21.8 ± 15.1 (> 0.05)	3.8 ± 0.83 (>0.05)
IP IgG, 12 hrs prior	KA	5	1 ± 0.0 (>0.05)	1 ± 0.54 (>0.05)	31 ± 10.8 (0.09)	**2.6 ± 0.54 (0.01)**

All data in **bold are statistically significant

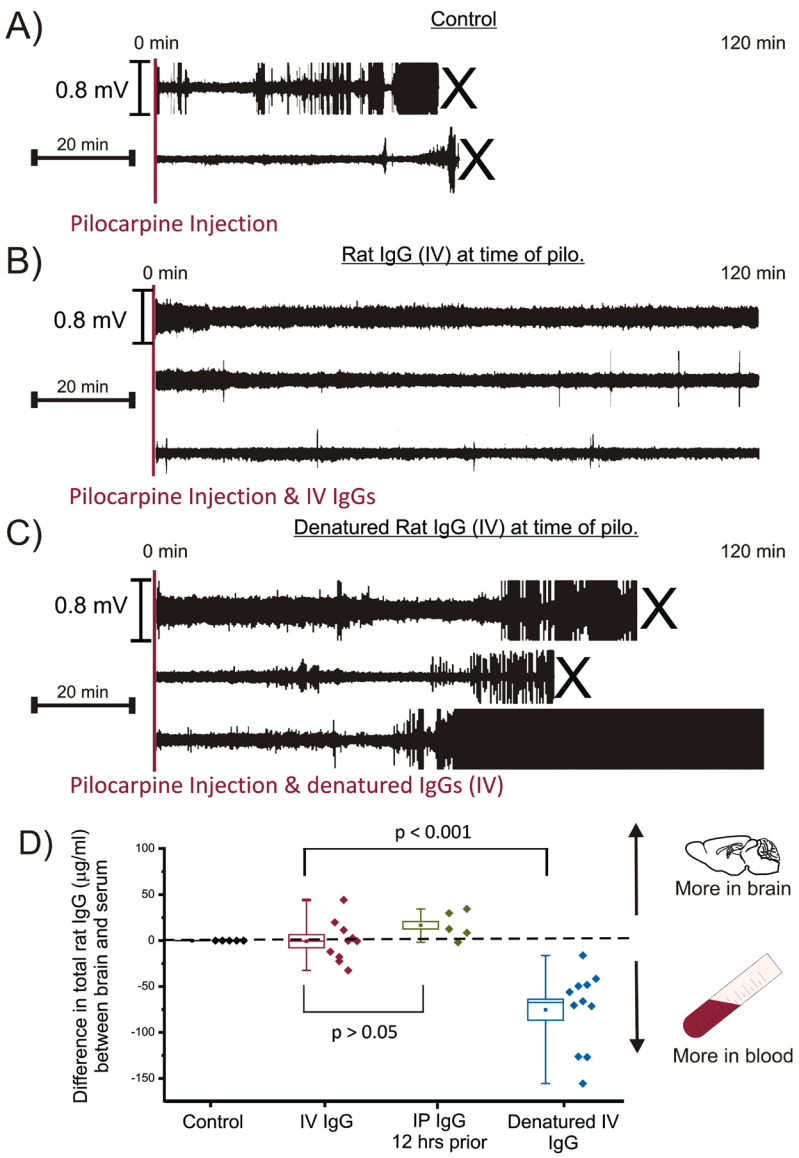

Figure 3.3: Only intact IgGs mitigate seizures when injected IV at time of pilocarpine SE. Control mice treated with pilocarpine **(A)** all developed SE and died as a result. Two representative single channel EEG traces are shown. All mice treated with denatured IgGs at time of pilocarpine developed SE and a 50% mortality rate was observed in these mice **(C and Table 1).** Three representative single-channel EEG traces are shown in **C**. In mice injected IV with native rat IgGs at time of pilocarpine, a 60% reduction in SE was observed. Three representative single channel EEG recordings from these mice are shown in **B**. ELISA data in **D** show that intact IgGs administered 12 hours prior to pilocarpine or at time of pilocarpine rarely enter the brain. Denatured IgGs were found at high levels in serum. For this data, all points greater than zero represent greater amounts of IgGs in brain than in serum and all points less than zero represent greater amounts of IgGs in serum than brain. *X's* at the end of EEG traces indicate mortality.

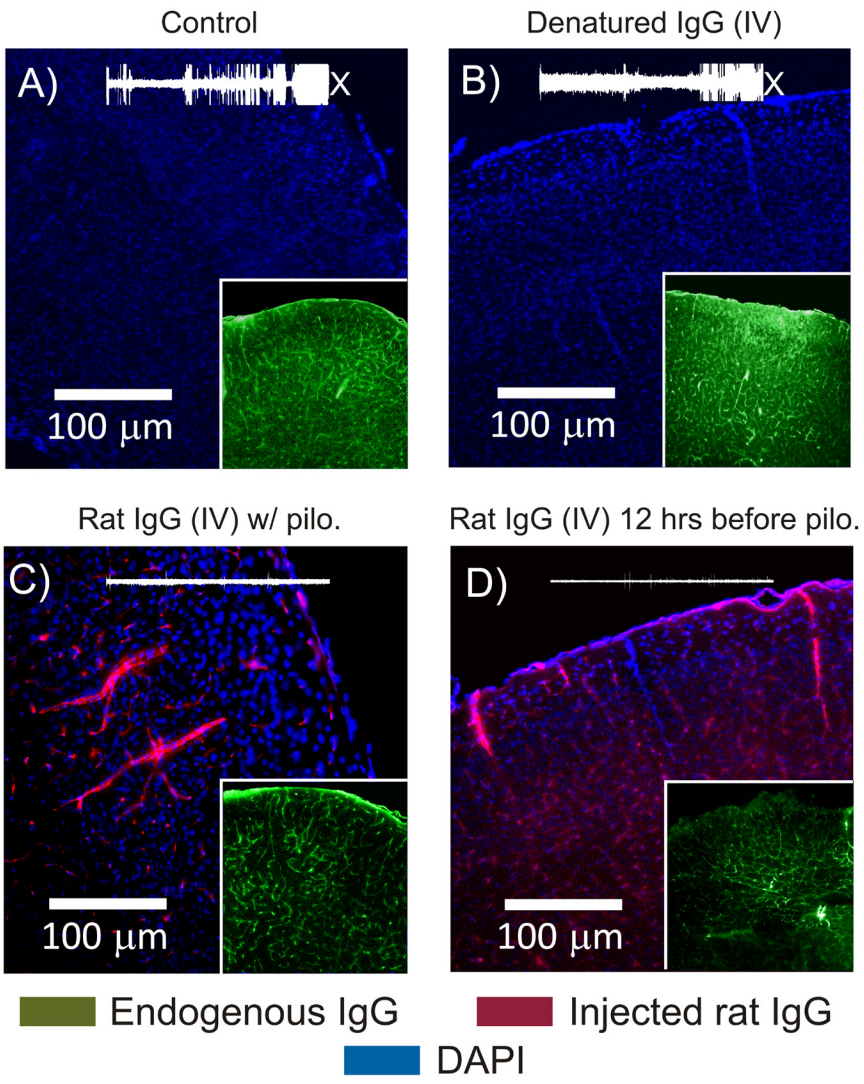

Figure 3.4: In IgG treated mice that did not develop SE, no injected rat IgGs or endogenous mouse IgGs were found in the brain parenchyma but were abundant in the vasculature. Control mice and mice given denatured IgGs at time of pilocarpine displayed no immunoreactivity to rat antibodies **(A and B)**. However, regions of blood-brain barrier leakage were observed in the cortex, as measured by endogenous mouse IgG leakage and halos of IgGs around neurons can be observed **(insets in A and B)**. Immunoreactivity to rat IgGs was only observed in pilocarpine mice where rat IgGs were injected **(*red,* C and D).** However, no endogenous mouse or injected IgGs were observed in the brain parenchyma in mice that did not have seizures **(C and D and insets of both)** though abundant vascular staining can be seen. Representative EEG traces from mice are shown in *white* at the top of each image to demonstrate whether or not the animal developed SE.

Effect and localization of Injected non-specific antibodies in B6 mice given KA

As pilocarpine driven SE is primarily inflammatory and its CNS effect secondary to inflammatory BBB disruption,[197] KA was used to test the effect of injected IgGs on CNS driven SE. Across the three treatment groups examined (control, injected IgG at time of KA and injection of IgG 12 hours prior to KA) no differences were observed **(Figure 3.5 A, B and C; Table 3)** with the exception of a single statistically significant correlation between decreased seizure severity (based on a behavioral seizure score) and IV administration of IgGs at time of KA injection **(Table 3.3)**. Examination of brain tissue sections revealed leakage of mouse IgGs into the parenchyma in control animals **(Figure 3.6 A)**. Further, cortical leakage was observed in mice injected with IgGs at time of KA and 12 hours prior to KA **(Figure 3.6 B and C)**. ELISA data examining rat total IgGs in these mice also supports our findings of leakage of rat IgGs into the CNS with an abundance of IgGs found in the brain of mice treated with IgGs 12 hours prior to KA **(Figure 3.6 D)**.

An intriguing corollary finding illustrated both mouse and rat IgG leakage across the BBB in the hilar/CA3 regions of the hippocampus **(Figure 3.6 C and D)**. A 3D confocal reconstruction of the CA3 region revealed significant cellular uptake of both rat and mouse IgGs **(Figure 3.6 E and F)**. It is worth noting that this same region showed leakage and cellular accumulation of IgGs in a control pilocarpine mouse using both 2D fluorescence imaging and 3D confocal reconstruction **(Figure 3.7)**. While this phenomenon has been observed

previously by us[184] and others[216] in humans and animal models respectively, this is the first time it has been demonstrated to occur in animals where antibodies from a different species are also found inside the cells. Fluorescence quantification in **Figure 3.7** revealed that high levels of IgG are found in hippocampal regions with high DAPI fluorescence (CA3 and hilus) and low IgG fluorescence was associated with low DAPI fluorescence (CA2).

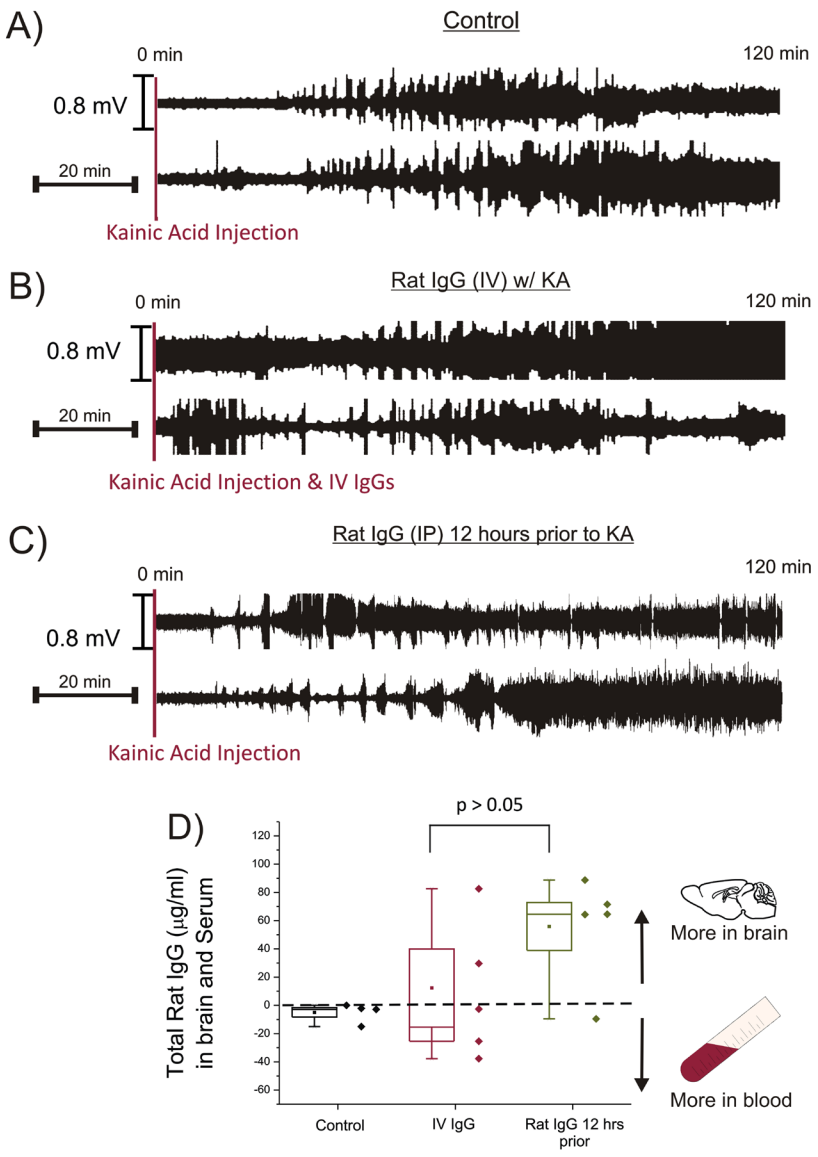

Figure 3.5: Injected IgGs do not have a seizure mitigating effect in the kainic acid (KA) model of SE. No difference was observed between C57B6/J mice given kainic acid and IgGs at time of KA, 12 hours prior to KA or control mice. Two representative single-channel EEG traces for each group are shown in **A, B and C**. Further statistical analysis of EEG traces revealed only one significant correlation between seizure severity and mice injected with rat IgGs 12 hours prior to KA **(Table 3.3)**. In **D** IgGs are frequently observed in the brain parenchyma of mice and often at levels far higher than serum. IgG levels in brain were not statistically different between groups of mice treated with IgGs at time of or 12 hours prior to KA injection. For this data, all points greater than zero indicate more IgGs in brain than blood while all points less than zero indicated more IgGs in blood than brain.

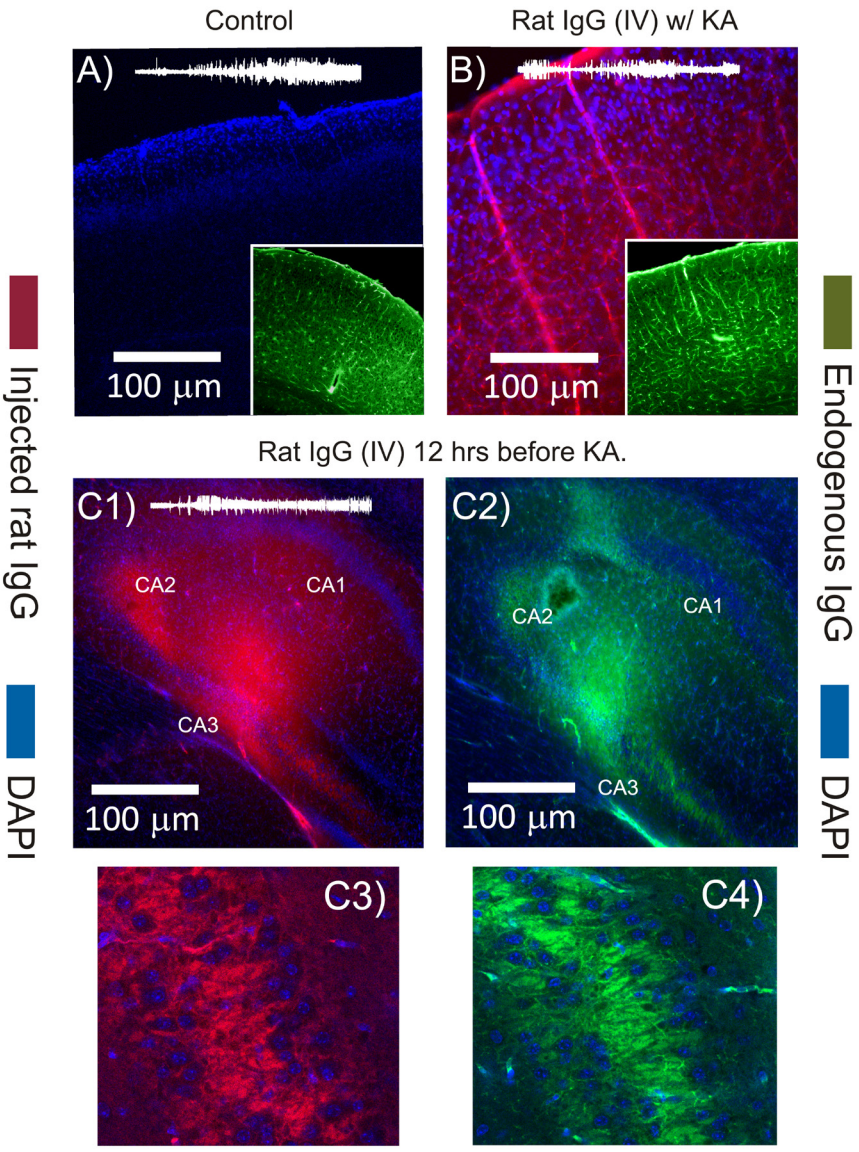

Figure 3.6: Endogenous and exogenous IgGs are observed in brain tissue sections of KA treated mice after SE. In control mice no immunoreactivity to rat IgGs was observed. However, abundant endogenous mouse IgGs can be found in the brain parenchyma and halos of IgG around cells can be observed **(A)**. Mice injected with rat IgGs either 12 hours prior to or at time of KA administration displayed both injected and endogenous IgGs in the parenchyma **(B, C1-C4)**. In **C** IgGs, both injected and endogenous, can be observed in the hilar, CA3 and CA2 regions of the hippocampus with cellular uptake of IgGs observed in the CA3 and hilar regions **(C1 and C2)**. 3D confocal reconstruction of the hilar and CA3 subfields in **C1 and C2** show cellular uptake of both endogenous mouse IgGs **(C4)** and injected exogenous rat IgGs **(C3)**. Representative EEG traces are shown at the top of each image indicating that all mice developed SE **(A-C1)**.

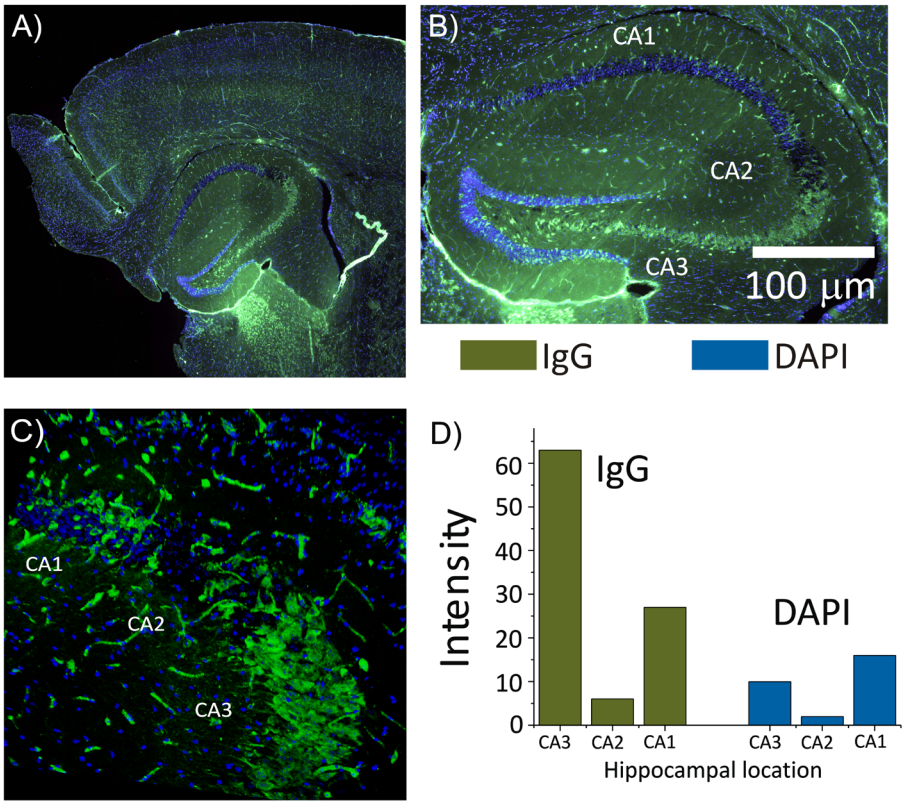

Figure 3.7: BBB leakage and cellular uptake of IgGs in a control pilocarpine mouse after SE. A slidescan in **A** shows leakage of IgGs into the hippocampus and down into thalamic nuclei. A closer view of the hippocampus in **B** reveals abundant cellular uptake in the hilus and CA3 subfields similar to patterns of leakage seen in **Figure 3.6**. A Confocal 3D reconstruction of the CA1-CA2-CA3 junction shows similar cellular uptake as that observed in **Figure 3.6.** Fluorescence quantification of each hippocampal subregion **(D)** reveals that increased IgG fluorescence corresponds to increased DAPI fluorescence suggesting that intracellular uptake of IgGs may be cytoprotective.

3.4 Discussion

The main finding of the research herein is that non-specific IgGs increased survival and latency to seizure after pilocarpine challenge. This phenomenon is not specific to IgGs that are injected but also applies to endogenously high levels of IgGs. Further, the primary action of IgGs does not occur through the Fc receptor and does not act directly on the CNS but rather on peripheral inflammation.

Antinuclear antibodies and seizures

Previously we had reported the presence of antinuclear antibodies in serum and brain of patients with epilepsy.[184] In addition, other serum antibodies were observed in the brain of these patients. To that end we examined whether or not these antibodies had an effect on seizures. While statistically these antibodies appear to correlate with reduced seizures, experimental evidence suggests that this correlation is due to their properties as antibodies rather than their target specificity. Therefore, antinuclear antibodies may be an epiphenomenon in epilepsy that could serve as a useful biomarker for ongoing neurodegenerative processes rather than a target for seizure treatment.[259]

Effects of humoral immune system on seizures

A clear link has been established through numerous studies demonstrating that antibodies targeted to specific neuronal proteins are associated with epilepsy;[243] their exact mechanism of action and whether or not

the antibodies precede or are a consequence of epilepsy remains unknown. To add to the role of antibodies in epilepsy we show that endogenous IgGs not targeted to a specific neuronal proteins prevent seizures when given prior to SE. Support for this comes from studies of natural antibodies which show that antibodies can provide a means to maintain homeostasis by removing debris and modulating cellular metabolism.[251] Further, a set of patients that lack IgG2 production are susceptible to seizures that are alleviated by administration of IVIg.[260]

Targeting endogenous IgG levels for therapy by modulating a patient's endogenous antibody production could, in conjunction with anti-seizure medication, ameliorate the inflammatory milieu observed in patients with epilepsy[120] while reducing the deleterious side effects of anti-seizure medication and long-term immunomodulatory therapies. Support for modifying the immune system of patients with epilepsy comes from others who have demonstrated that administration of IL-1ra antagonists has been shown to prevent seizure and SE.[198,261] Further, it has been demonstrated that IVIg may modulate IL-1ra production.[120,262]

Effect of IgGs at the blood-brain barrier

We have demonstrated in this study for the first time that IgGs can prevent leakage of the BBB. Whether this mechanism of action takes place at the BBB or prevents activation of proinflammatory cells/cytokines that promote BBB leakage

remains unknown. However, the mechanism of action does not occur through the Fc receptor. As rat immunoglobulins were used in these experiments and these antibodies are not capable of binding to mouse Fc receptors,[256,257] the mechanism of action must occur at a different level. Ischemic stroke models have demonstrated that cellular uptake of IVIg may be a mechanism to prevent injury.[191] In the population of mice studied here, only in the KA group and one untreated pilocarpine mouse was cellular uptake observed but no effects on the measured seizure parameters were seen. Neurons that did take up IgGs were more likely to appear healthy after pilocarpine SE compared to those that were devoid of intracellular IgGs. Indeed, throughout the literature it has been reported that cells that accumulate IgG appear healthy.[184,216] In addition, denatured rat IgGs had no effect on pilocarpine SE and were not found in significant concentrations in brain tissue homogenates, but were found in abundance in serum. This indicates that the action of IgGs occurs due to the intrinsic properties of native antibodies rather than through protein binding of chemoconvulsant (as the same volume of native IgGs and denatured IgGs were injected).

The effects of IgGs on pilocarpine vs. kainic acid SE

It has been demonstrated that the primary seizure-inducing effects of pilocarpine occur in the periphery and through an inflammatory mechanism. Further, pilocarpine does not cross the BBB readily prior to SE.[197] As such, the efficacy of injected or endogenous high levels of IgGs in this model is consistent

with its indication to treat inflammatory epilepsies. The degree of reduction in seizures in B6 mice is comparable to seizure reducing effects in patients with autoimmune epilepsy when used as a stand-alone treatment.[263] Therefore, part of the efficacy of IVIg in autoimmune epilepsies may be to physically prevent antibodies from entering the CNS by keeping the BBB "closed" rather than promoting their removal by aggregation and compliment activation.

KA works through a CNS mechanism to provoke SE. Therefore IVIg would not be expected to prevent seizures or SE acutely as, based on the findings here, the mechanism of IVIg lies in the periphery. Yet IgGs injected 12 hours prior to KA did show some efficacy in reducing seizure severity. This may be due to a "priming" effect of these antibodies by reducing any underlying inflammation that may exacerbate SE once it has begun. IVIg has been used to treat non-autoimmune epilepsies and super-refractory SE with some success.[90,263] These epilepsies, while not primarily inflammatory or autoimmune, have been shown to have a component of inflammation and BBB leakage.[264,265] IVIg rarely eliminates seizures entirely in these patients when used as a stand-alone treatment but does reduce their frequency which is suggestive of both a CNS origin and peripheral inflammatory exacerbation of these seizures.[263] As it is unlikely that a pro-inflammatory environment is fully developed in these mice after two hours of SE, IVIg may be effective in KA mice long-term rather than acutely.

Taken as a whole, it may be beneficial to administer IVIg to patients with epilepsy prophylactically and/or in conjunction with anti-seizure medications. This would serve to prevent the cycle of seizures and inflammation that serves to produce more seizures.

Limitations

This study is limited by the concentration of IgGs administered to each mouse, which were far lower than in other animal studies. However, in these models such high concentrations were given that an increase in blood viscosity was observed.[191] This would have posed problems with sample collection and analysis. Further, that IgG administration was effective at much lower concentrations indicates that it may be unnecessary to give high volumes of IVIg to patients.

An additional limitation is that IgGs given 12 hours prior to pilocarpine were administered to NZBWF1/J mice rather than B6 mice. However, the pilocarpine dose in these different strains was adjusted so that seizure parameters were similar irrespective of chemoconvulsant dose. That is, mortality, intensity and duration of SE were the same between strains. Further, both strains of mice have intact BBBs and normal CNS function prior to chemoconvulsant administration and show abundant leakage in the same regions and to a similar degree after untreated SE **(Figures 3.4 and 3.6)**.

3.5 Conclusions

Our results open a number of doors to therapeutic opportunities to enhance or modulate the humoral immune response in patients with epilepsy. This study provides support for further studies regarding the specific action of IVIg treatment, though a mechanism of action through the Fc receptor is unlikely. IgG uptake by neurons has also been ruled out as a mechanism of IVIg as IgG uptake by cells was only observed in animals that had seizures, but it may be a mechanism of cytoprotection. We have demonstrated here for the first time that IVIg may, in part, work by preventing opening of the BBB. An additional finding is that the effect of IVIg is not limited to injected IgGs but extends to endogenously high levels of IgG. Additional research should be performed to determine the specific effect of IVIg on the BBB (direct action on BBB endothelial cells or peripheral inflammation) in addition to mechanisms to enhance the effect of endogenous immunoglobulins and better targeting patients for treatment.

3.6 Acknowledgements

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CHAPTER 4:

Overall significance and future directions

4.1 Summary of findings

Detailed discussions of the individual findings of this research can be found in the previous chapters and these individual findings will not be recapitulated here in detail. Rather, the overall significance of these findings, potential clinical applications and future areas of study will be discussed. In summary, the salient findings of this dissertation are:

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- IgGs associated with BBB leakage are present inside all subcellular compartments of neurons in patients with epilepsy **(Figures 2.2, 2.3 and 2.4)**.
- IgGs inside neuronal nuclei of patients with epilepsy are targeted towards histones and other chromatin components **(Figure 2.5, 2.6 and Table 2.3)**.
- These same antibodies can be found in sera from patients with epilepsy but are absent in control sera samples **(Figure 2.6)**.
- Experimental data demonstrate that endogenous non-specific IgGs have a protective effect in an animal model of SE **(Figure 3.2)**.
- In the pilocarpine mouse model, injected IgGs mitigate SE, but SE is not mitigated by IgGs injected in the kainic acid mouse model **(Figure 3.3 and 3.5)**.
- Data reveal an intact BBB in pilocarpine model animals that did not develop SE (No IgG leakage into the brain) **(Figure 3.5)**.
- Abundant IgG leakage is observed and limited cellular uptake is seen in pilocarpine and kainic acid animals that experienced seizures **(Figure 3.6)**.

4.2 Significance and clinical application of intracellular accumulation of IgGs

Data presented here and by others has demonstrated the ability of neurons and glial cells to take up IgGs under conditions produced in the epileptic or seizing brain. *In vivo* studies have shown that IgGs are taken up specifically by hippocampal neurons in both the pilocarpine and kainic acid model of

epilepsy,[216,266] while the studies contained herein demonstrate both cortical and hippocampal uptake of IgGs by neurons and glial cells in humans[184] and in two models of SE. However, in all of the above studies no readily observable deleterious effect due to IgG accumulation in neurons or glia was observed. Indeed, the data herein suggests that intracellular accumulation of IgGs may be cytoprotective.

Outside of epilepsy, the phenomenon of endogenous IgG uptake by glial cells and neurons is poorly understood. The few studies that have examined IgG uptake by neurons and glia in other CNS diseases have only examined injected IgGs. In this framework, amyotrophic lateral sclerosis (ALS) is the most well studied example. Several studies across different mouse models (normal and ALS models) using serum from patients with ALS have demonstrated that IgGs are taken up by neurons and that they modulate calcium levels and neurotransmitter release of spinal cord motor neurons.[267] The authors suggest that this mechanism proceeds through Fc receptors on these neurons. However, staining with F(ab')2 fragments (antibodies lacking the Fc portion) shows uptake by neurons to a lesser extent. Further examination using Fc receptor knockout mice demonstrated IgG uptake by motor neurons in the spinal cord, again, to a lesser extent. Therefore, while uptake of human IgGs into mouse spinal cord neurons may, in part, proceed through a few evolutionarily conserved Fc receptors, additional mechanisms of uptake are also present.[267] No studies have

examined human spinal cord sections from ALS patients to determine whether endogenously produced antibodies are present inside motor neurons.

IgG uptake by neurons has also been implicated in the clearance of tau protein by exogenous monoclonal antibodies. In this study, mice expressing all forms of humanized tau proteins were used for organotypic slice cultures with addition experiments conducted using mouse primary neuronal cultures. Mouse anti-tau monoclonclonal antibodies were then produced to mimic therapeutic antibodies for the treatment of Alzheimer's disease.[250] Under these experimental conditions, IgGs were taken up into neurons in both slice cultures and primary neuronal cultures and shown to remove tau aggregates by Fc receptor mediated endocytosis. As with the ALS studies, blocking the Fc receptor did not eliminate uptake of IgGs but did decrease uptake. While this study demonstrates that antibodies can be taken up by mouse neurons in culture, the authors made no attempt to replicate these finding *in vivo.*[250] Furthermore, clinical trials have failed to demonstrate efficacy of anti-tau[268] (and anti-amyloid) antibodies[253] in human disease. Whether this failure is due to the inability of the therapeutic antibodies to interact with Fc receptors *in situ* is unknown.

An additional therapeutic example is the use of IVIg in a mouse model of stroke. IVIg was administered after experimental stroke and IgGs were observed crossing the BBB and were present in the cytosol of glial cells.[191] This report concluded that uptake of IgGs by glial cells was cytoprotective and that the

protective mechanism of IVIg is due to the ability of antibodies to bind complement components produced at the site of injury, thereby preventing cell death. An additional finding relevant to the research contained herein is that a decrease in cellular adhesion molecules on the endothelium and decreased levels of microglial activating cytokines were observed.[191]

All of the above evidence demonstrating the uptake of injected and/or therapeutic antibodies in models of CNS disease fails to directly address the findings in patients with epilepsy and findings presented here and by others in animal models.[184,216] A common thread in each of the above diseases as well as epilepsy is the presence of BBB leakage that provides the opportunity for serum IgGs to make contact with CNS cells.[269-271] In epilepsy, repeated and often widespread BBB leakage provides access of IgGs to the brain and may promote uptake by receptor binding or passive uptake by clatherin–coated pits. However, this may only be part of the uptake mechanism in epilepsy. Animal models have demonstrated that IgG uptake by neurons occurs in areas that are prone to seizures, particularly the hilar and CA3 regions of the hippocampus[216] indicating that the seizure itself may play a role in IgG uptake. In support of this, it has been observed that membrane proteins can be internalized when electrical currents mimicking seizures are passed through cells in culture.[237]

There are potential therapeutic benefits to intracellular localization of endogenously produced IgGs. If intracellular localization of non-specific serum

IgGs is neuroprotective in epilepsy, enhancing this effect by some mechanism (perhaps a deep-brain stimulation device) would allow the body's own immune system to reduce seizures. More importantly though, the mechanism of intracellular localization of antibodies could be used to better target therapeutic antibodies to the CNS of patients with other diseases.

Osmotic opening of the BBB by mannitol has been used therapeutically to target chemotherapy drugs to the brain and has also been demonstrated to produce seizures. This mechanism could be used to target therapeutic antibodies into the brain and toward intracellular targets. In this scenario, antibodies could specifically target a protein or act as a carrier for a chemotherapeutic agent (for cancer or otherwise). While further research is needed before this avenue can be pursued, evidence has shown that internalized antibodies can make their way into the cytosol without being degraded in lysosomes thereby allowing their effect to be achieved.[272]

For some time, scientists have been working on the development of therapeutic "intrabodies." That is, using gene therapy to force target cells to produce intracellular antibodies that target their own intracellular proteins.[273] While this mechanism has shown efficacy for a number of diseases (CNS and otherwise),[274,275] modifying the BBB and inducing a seizure may be a short-term solution to enhance uptake of therapeutic antibodies until gene therapy science provides technology to single out target cells in patients.

4.3 Significance and clinical application of antinuclear antibodies in epilepsy

Antinuclear antibodies (ANA) are found in a number of systemic autoimmune diseases. Of particular interest to the research contained herein are anti-chromatin and anti-histone antibodies, both of which are commonly found in patients with systemic lupus erythematosus (SLE).[276] In SLE, ANA exert their pathological effects by forming immune complexes that deposit in the kidney and activate complement leading to lupus nephritis.[277] While a number of hypotheses have been put forward, the exact mechanism by which ANA are generated in SLE remains unknown. Irrespective of mechanism, the source of antigen is likely from apopotic or necrotic cells.[278] It is therefore likely that in epilepsy ANA are produced by a similar cell death mechanism.

Cell death is a common finding in brain tissue resection from patients with epilepsy and in animal models of the same. Studies in animal models have revealed neuronal loss, particularly in the hippocampus, either by apoptosis[279] or necrosis[280] linked to excitotoxic cell death.[281] Repeated rounds of seizures and accompanying BBB leakage may allow release of neuronal proteins into the systemic circulation where an immune response can be elicited. Further rounds of seizure and BBB opening would allow autoantibodies back into the CNS where they can be taken up by neurons. Support for this hypothesis comes from

findings that the astrocytic protein S100B, which is normally sequestered behind the BBB, can cross a disrupted BBB after sub-concussive injury in American football players. In these same football players, autoantibodies against S100B were found in serum.[282]

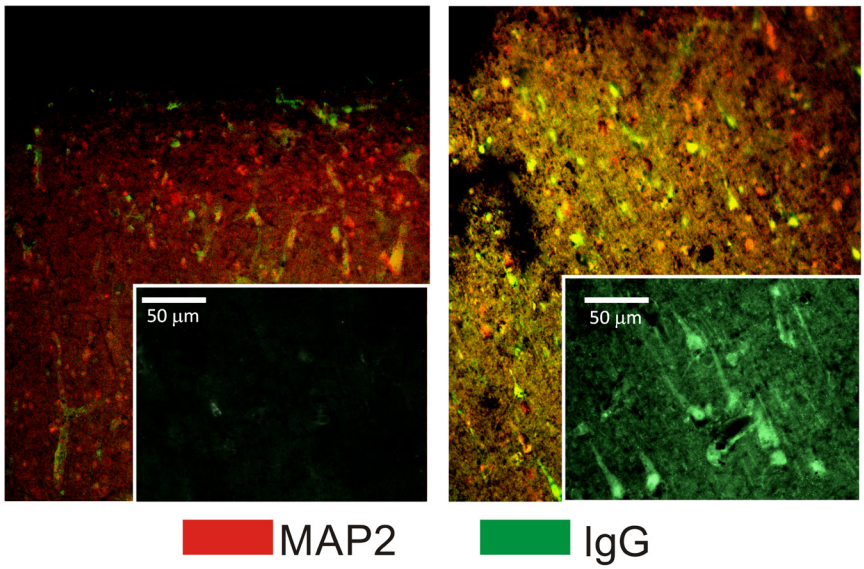
An interesting difference between ANA found in patients with epilepsy and patients with SLE is their presence inside cell nuclei. There are no reports of ANA present inside cells or nuclei in patients with lupus. Further, studies in epilepsy have shown that ANA are present in sera from patients with epilepsy but no study, except the work contained here, has looked for ANA in the CNS. Those studies that have demonstrated ANA in serum from patients with epilepsy did not find a correlation between serum levels and whether the patient was drug resistant or drug respondent, but did correlate with having greater than one seizure per month.[221,283] Moreover, patients with newly-diagnosed (untreated) epilepsy had higher levels of ANA in serum than patients who were treatment respondent. This same study also demonstrated that patients with hippocampal sclerosis had higher levels of ANA than patients with other types of epilepsy.[223] All of these data point towards cell death as a mechanism of ANA generation in epilepsy.

Taking all of the above into consideration, the presence of ANA inside neuronal nuclei may be related to a "leaky" BBB coupled with the intrinsic ability of neurons to take up IgGs, though how they localize to the nucleus remains

unknown. However, based on our findings and similar findings throughout the literature, ANA may be a valuable biomarker to diagnose an ongoing neurodegenerative process in patients with epilepsy. Similarly, ANA may serve as a valuable marker for early diagnosis of hippocampal sclerosis (before an MRI scan can detect it) or for a fast and cost-effective way to grade the degree of hippocampal sclerosis.

The use of ANA as a biomarker for neurodegenerative processes in epilepsy may only be scratching the surface for its use in CNS disease. Preliminary data **(Figure 4.1)** from *post-mortem* brain samples taken from former American football players diagnosed with chronic traumatic encephalopathy (CTE) demonstrates neuronal uptake of IgGs into neurons and neuronal nuclei. Immunoprecipitation of antigens from these intracellular IgGs followed by mass spectrometry analysis revealed antibodies targeted to the same proteins as those observed in **Figure 2.5**. It is unknown whether these ANA were also present in the serum from these players. Pending further analysis, ANA may be a useful biomarker in diseases (in addition to epilepsy) where neurodegeneration and BBB disruption are present.

A)



B)

Protein	MW	AVG. Diff
Histone H2A	**14**	**3.372**
Histone H4	**11**	**2.2**
Cystatin-B	11	1.065
Junction plakoglobin	82	1.015
MAP-1B	14	.573
Synaptophysin	34	.36
Plectin isoform 1	532	.24
Protein S100-B	11	1.04

Figure 4.1: IgGs taken up by neurons in the brain of CTE patients are targets towards the same epitopes as those observed in patients with epilepsy. In **A,** an age-matched control and post-mortem brain sample from a patient with CTE are shown. Abundant cellular uptake of IgG colocalized with the neuronal marker MAP2 is visible in CTE brain but only vascular staining of IgG is present in the control. In **B**, antigens dissociated from neurons and analyzed by mass spectrometry are shown. Quantification (see **Chapter 2**) revealed that the most likely targets for these antibodies were histones H2A and H4 (*bold*).

4.4 Significance and clinical applications of the seizure mitigating effects of IgGs

While the usefulness of IVIg treatments have been known for some time in epilepsy and other diseases, it has been shown here for the first time that high levels of endogenous IgG can have a similar effect. That is to say that it is the intrinsic properties of IgGs, rather than the administration of IV immunoglobulins, that provides the seizure mitigating effect of IgG treatment in epilepsy. This finding opens up opportunities to artificially enhance total non-specific IgG levels in patients with epilepsy that may reduce peripheral inflammatory burden and reduce seizures. While numerous studies are still required to validate the efficacy of endogenously high IgG levels to treat seizures, there are a number of plausible options to produce such an effect.

IVIg therapy is expensive (for the patient and manufacturer) and treatment takes a considerable amount of time. Therefore, developing strategies whereby the patient's endogenous IgG levels could be raised would be of great benefit. Pathologically, increased serum IgGs is called 'hypergammaglobulinemia'. While a search of the literature did not reveal any drugs known to induce hypergammaglobulinemia, there are a variety of articles detailing virus-induced hypergammaglobulinemia.[284,285] Note that a distinction is being made between drug-induced SLE and drug-induced hypergammaglobulinemia. While virus-induced hypergammaglobulinemia occurs under pathological conditions, it may be possible to develop gene therapy strategies to modify the endogenous

production of IgGs to increase total serum IgG levels without raising the level of IgGs so high as to produce pathology.

While the possibility (and plausibility) of using gene therapy to modify endogenous serum IgG levels lies in the distant future, modifying IVIg therapy to benefit patients with epilepsy may have more immediate benefits. The latest advancement in IVIg therapy has come from several studies where pooled serum immunoglobulins were administered subcutaneously (SQIg) rather than intravenously.[286,287] In these studies patients with primary immunodeficiency disease and treatment-resistant polymyositis were used and SQIg was administered at 100mg/kg/week. Not only does this method allow patients to administer their treatments at home, but serum levels of IgG were maintained at a steady state with less variability in serum concentrations compared to IVIg and fewer adverse effects were observed.[286] Maintaining consistent steady state levels of serum IgGs may be the key to increasing the efficacy of IgG treatment in patients with epilepsy, and if used in conjunction with anti-seizure medication could break the cycle of seizures and inflammation. No studies to date have examined SQIg in epilepsy. To stop acute seizure events (e.g., SE) IV administration of immunoglobulins is still the best option when traditional methods (e.g., diazepam) fail.[90]

4.5 Significance and clinical applications of decreased BBB permeability after IVIg administration

The efficacy of IVIg has been demonstrated in a number of CNS diseases. Two key features of many of these diseases are inflammation and BBB disruption. In addition to epilepsy (autoimmune or otherwise), IVIg has shown at least partial efficacy in the treatment of multiple sclerosis[288] and Alzheimer's disease.[289] However, evidence for the use of IVIg to treat these diseases comes from clinical trial data or case reports of off-label use. Few studies have examined the cellular/molecular effect of IVIg on these diseases in animal models, human tissue samples or cells (epilepsy included)[290,291] and in these studies the BBB was not examined.

The data in **Chapter 3** from the pilocarpine model of SE suggests a role for "BBB tightening" by administration of injected IgGs, either through a reduction in peripheral inflammation or direct action on the BBB in epilepsies where inflammation is the primary cause or exacerbating factor. In either instance, this finding provides an additional role of IgGs in CNS disease. Therefore, it may be useful to examine other CNS diseases where BBB leakage is suspected or demonstrated (e.g., CTE, hemorrhagic stroke, small vessel ischemic disease, etc).[292] Irrespective of mechanism, correcting a "leaky" BBB may not cure these diseases, but IVIg may provide a means to slow the progression of disease by supporting CNS homeostasis.

4.6 Significance and putative mechanism of action of IVIg on seizures

The majority of research regarding the action of IVIg in disease has revolved around Fc receptor binding or complement depletion.[192] However, in the experiments contained in **Chapter 3** an Fc receptor binding mechanism is unlikely. A number of non-Fc receptor mechanisms have been demonstrated in the literature and may be more likely scenarios for the action of IVIg in epilepsy.

It has been demonstrated that IVIg is capable of binding to components of the complement cascade. Specifically C1q, C3b and C4b have all been shown to bind IVIg, thereby preventing their assembly and subsequent activation.[293] Further, extensive complement activation has been demonstrated in both patients with epilepsy and in animal models of epilepsy.[294] In the pilocarpine model of SE, complement activation is observed acutely and SE can be mitigated by specifically blocking components of this cascade.[295] However, in the kainic acid model of SE, activation of complement is observed after 48 hours or longer.[296,297] It should be noted that this delayed increase in complement activation may be due to recurrent seizures rather than acute SE. This could explain the difference observed between the two models used in **Chapter 3,** as IVIg is only administered acutely in these experiments. Longer experiments would need to be performed to assess the effect of IVIg in mitigating KA SE through the complement cascade.

Another plausible mechanism for the seizure mitigating effect of IVIg in epilepsy is through reduction in matrix metalloproteinase-9 (MMP-9). MMP-9 has been demonstrated to cause BBB leakage in a number of CNS diseases,[298,299] including epilepsy.[300] The exact mechanism by which MMP-9 causes leakage of the BBB remains unknown. It is thought that MMP-9 directly degrades tight junctions and the basal lamina of the BBB.[301]

MMP-9 levels are increased in sera of patients with epilepsy[302] and in the brains from KA[303] and pilocarpine model mice.[304] To date no studies have examined serum levels of MMP-9 in these models. If serum levels are elevated (as they are after stroke[298]) IVIg administration could play a direct role in protecting the BBB and preventing seizures. It is interesting to note that the action of IVIg on MMP-9 has been shown to occur through the F(ab')2 region of the antibody rather than the Fc portion, which further supports the efficacy of rat IgGs in the experiments in **Chapter 3**.

4.7 Conclusion

The work contained herein demonstrates a paradoxical effect of antibodies in epilepsy. That is, they can both cause disease- or in the case of ANA, be an epiphenomenon of disease - and antibodies can be used to treat the disease. Collectively, this work demonstrates, for the first time, the intracellular/intranuclear localization of antibodies inside the brains of patients

with epilepsy and that these same antibodies, targeting nuclear proteins, can be found in serum. Moreover, this research provides a framework around which scientists can build a better understanding of the mechanism(s) by which IVIg can alleviate seizures and may allow clinicians to better target patients who will receive the greatest benefits from IVIg treatment.

The mechanism(s) of action and purpose of intracellular accumulation of IgGs in epilepsy remains unknown. However, preliminary data suggest that uptake of IgG by neurons may not be limited to epilepsy but may play a role in a broader scope of neurodegenerative diseases **(Figure 4.1)**. It is therefore likely that IgG uptake is due to changes in membrane permeability/integrity associated impaired neuronal function. Whether these changes in permeability are due to upregulation of membrane proteins, increase endocytosis or loss of selective permeability by the lipid bilayer is unknown. However, it does not appear that neuronal uptake of IgG directly causes cell death **(Chapter 3)**.

The majority of drugs developed to treat CNS disease fail either due to lack of efficacy in human studies or an inability to cross the BBB.[305] Therefore, intracellular localization of IgGs after a seizure may prove to be a useful mechanism to deliver chemotherapeutic agents. This is due, in part, to the ease with which antibodies can be "tagged" with small molecules and the demonstrated safety of clinically-induced seizures. Further, studying the mechanism by which IgGs are able to cross the BBB and subsequently enter

neurons may provide an avenue to enhance these mechanisms and deliver therapeutics without accompanying antibodies.

Perhaps the most intriguing finding of this research are antinuclear antibodies that appear in both serum and neuronal nuclei of patients with epilepsy. This finding is particularly interesting because of the apparent lack of autoimmune disease in these patients. As such, antinuclear antibodies may be an epiphenomenon in epilepsy that is related to excitotoxic cell death and repeated BBB “opening.” A mechanism for the generation of these antibodies may be as follows: 1) Seizures promote cell death and opening of the BBB which leads to release of nuclear components into serum; 2) Antibodies are generated against nuclear components in the periphery; 3) Future seizures lead to "opening" of the BBB and entry of antinuclear antibodies back into the CNS where they are taken up by neurons. A similar mechanism could play a role in neurodegenerative diseases where chronic BBB opening and neurodegeneration are present (e.g., CTE).

What remains unknown regarding the antinuclear antibodies observed in patients with epilepsy is the means by which they enter neuronal nuclei. As nuclear transport is highly regulated,[306] entry of IgGs into nuclei may be due to seizure-induce changes in nuclear transport proteins that promote transport of IgGs into the nucleus. That aside, ANA in serum of patients with epilepsy may be a useful biomarker for ongoing neurodegenerative processes in patients with

epilepsy (e.g., hippocampal sclerosis). This may prevent unnecessary MRI scans or allow clinicians to track the progress of disease via serum analysis rather than imaging.

An intriguing finding from the experiments in **Chapter 3** is the lack of BBB disruption (and SE) in pilocarpine model mice treated with IVIg. Whether this action is due to IVIg preventing peripheral inflammation that leads to BBBD or whether IVIg acts directly on the BBB remains unknown. The immediate action of the injected IgGs indicates that IVIg-mediated increase in IL-1ra is an unlikely mechanism in the context of SE, but that does not rule out the role of IL-1ra release in epilepsy. Further, as rat IgGs do not bind the mouse inhibitory Fc receptor readily, this seems an equally unlikely source of the anti-seizure effect of IVIg. Further studies are needed to determine the exact mechanism of IVIg in SE/epilepsy and should include injection of F(ab)'2 fragments in pilocarpine mice to determine whether the proposed action of IVIg on MMP-9 holds true. Moreover, studies in mice with recurrent spontaneous seizures are needed to determine whether IVIg can prevent seizures long-term rather than acutely.

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Appendix

Principles of high-performance liquid chromatography

High-performance liquid chromatography (HPLC) is a technique designed to separate and purify complex mixtures of molecules. In addition to the use of HPLC in industry to ensure product purity, HPLC is used in biomedical research to separate complex mixtures of digested peptides prior to mass spectrometry analysis (as described in **Chapter 2**).

HPLC is differentiated from standard liquid chromatography by the use of high-pressure, rather than gravity, to force the sample through the HPLC device. HPLC is performed as follows: 1) The sample is either loaded manually or injected automatically into a solvent (e.g., acetone); 2) The sample and solvent ('mobile phase') are pumped under high-pressure through a chromatography column containing packing particles ('stationary phase'); 3) As the sample is separated, the isolated compounds pass a detector and a readout ('chromatogram') is generated. The chromatogram can then be analyzed to

determine the compounds in the sample. In addition, isolated compounds can be retrieved and further analyzed.

Principle of Mass Spectroscopy

Similar to HPLC, mass spectrometry (mass spec.) can be used to determine the amount and chemical nature of a given sample. Mass spec. is commonly used in biomedical research to identify proteins, detect post-translational modifications and screen samples for metabolites. Mass spec. is performed as follows: 1) Samples are injected into the mass spectrometer and ionized. In the case of the experiments performed in **Chapter 2** an electrospray device is used to pass a high voltage current through the sample to produce an aerosol. 2) The ionized samples are accelerated by an electrical field and separated by a magnetic field ('deflection') . This process, therefore, separates particles by both mass and charge. 3) The separated ionized particles are passed through a detector. A spectra representing the particles mass-to-charge ratio is produced and can be compared to known spectra of previously identified compounds.