# Radiofrequency Telemetry and Immunologic Correlates as Predictors of Acute Inhalational Alphavirus Infection in a Nonhuman Primate Model

by

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University of Pittsburgh, 2020

#### Abstract

Eastern and Venezuelan Equine Encephalitis Virus (EEEV/VEEV) are two neurotropic alphaviruses that can produce acute febrile encephalitis in humans. Though rare, encephalitis caused by either EEEV or VEEV can produce long-term neurological sequelae. The need for development of medical countermeasures (MCM) to prevent EEEV/VEEV encephalitis remains imperative in the face of decades of research to develop VEEV as biological weapons. To this day, no licensed vaccines nor therapeutics exist to prevent or treat EEEV or VEEV encephalitis.

EEEV and some VEEV strains remain registered as Select Agents, and MCM development against EEEV/VEEV must occur in animal models that capture key aspects of human disease pursuant to the US FDA Animal Rule. Infection of cynomolgus macaques (*Macaca fascicularis*) by aerosol reproduces febrile encephalitis, neurological disease, and brain lesions seen in human infection. However, in macaques, encephalitic disease remains poorly characterized by quantitative measures. Improvements in tracking of encephalitis through traditional physiological biomarkers can also expand the data derived from the infected animals. The monitoring of electrical activity in the brain through electroencephalography (EEG) or intracranial pressure (ICP) can provide a more exacting estimate of when encephalitic disease begins and ends.

This study investigates a telemetered cynomolgus macaque model of aerosol-induced equine encephalitis virus infection with a focus on the natural history of disease and to assess the feasibility of the use of such data to delineate the onset and resolution of encephalitis. Macaques infected with EEEV aerosols became febrile three days post infection, and were euthanized at humane study endpoint at 5-6 days post-challenge. EEEV-infected animals demonstrated increases in delta EEG and increased ICP concurrent with fever. VEEV-infected macaques exhibited no fatal illness; all subjects survived the aerosol challenge and displayed characteristic biphasic febrile illnesses that self-resolved. In VEEV disease, macaques experienced a global decrease in EEG power and increased ICP in the second febrile period.

The public health significance of these findings lies within the improved ability to detect the onset and resolution of encephalitic alphavirus disease for the evaluation of MCM efficacy. These methods can aid evaluation of vaccines and therapeutics against other emerging infections.

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#### Preface

From and before the beginning of my doctoral work, I faced a number of challenges that proved obstinately intractable and difficult to overcome. As the years went by, I began to wonder whether the pursuit of scholarly work in the life sciences alongside a career in medicine was perhaps a bridge too far. This work is the culmination of youthful, unbridled optimism, and I have many to thank for their mentorship, companionship, and support.

I commend my advisor Dr. Douglas Reed for his continual mentorship during my PhD training, and for his invaluable support of my endeavors; my gratitude springs eternal. I thank Dr. Amy Hartman, for her steadfast directorship of the Alphavirus NHP project, and for her active role in mentoring me during my PhD training and faculty development. Additionally, the counsel, subject matter expertise, and feedback of Drs. William Klimstra and Tobias Teichert have played integral roles in the completion of this work.

My time as a PhD trainee has given me the freedom and time to reflect upon perhaps some of the most profound lessons learned during early adulthood. Persistence, humility, and the maintenance of strong friendships amid the headwinds of constantly changing circumstances have wrested me time and time again from the jaws of defeat. I thank Jennifer Bowling and Katie Willett for their incomparable friendship and support, as well as the rest of the Reed lab: Emily Olsen, Connor Williams, and Summer Xia. I am also thankful for the close support I received for the work performed for this dissertation from Dr. Cynthia McMillen, and indeed, none of the work in this dissertation would have been possible without the close collaboration between the Hartman, Reed, and Klimstra groups, and the diligent efforts of all hands on the NHP alphavirus project, old and new guard alike: Jeneveve Lundy, Matt Dunn, Emily Cottle, Theron Gilliland, Devin Boyles, Joseph Albe, and Cheng Sun.

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This work has lent me great insight into my practice of medicine. Although I have witnessed a spectrum of human maladies within the limited scope of my clinical training, I have never become so intimately acquainted with the ravages of disease as I have working on so esoteric a project in an animal model. I am continually humbled by the great privilege of training to heal other human beings, and will be forever in debt to our animal subjects' service to our species. Their sacrifice will always serve as an indelible memento of the awesome dignity of all life, and as a perpetual reminder that my patients shall remain my greatest teachers.

#### **1.0 Introduction**

Acute febrile encephalitic diseases share in their etiology the propensity to cause an eponymous encephalitic disease course that occurs before, after, or during the course of a febrile illness. A significant subset of these diseases, approximately 10-40%, owes their etiology to viral pathogens of which include alphaviruses (1-4). In nature, alphaviruses typically rely upon arthropod vector-borne transmission, but accidents in laboratory settings have demonstrated the capacity for human alphavirus infection and encephalitis to occur via the generation and inhalation of infectious aerosols (5-10). This facet of alphavirus infection factored into decisions made by national governments to research and develop alphavirus aerosol-based biological weapons before the advent of arms limitation treaties sought to cease such research (11-13).

Though the introduction of this work begins with a general exposition of alphaviruses, the focus of this work centers upon two equine encephalitis viruses (EEVs): eastern equine encephalitis virus (EEEV), and Venezuelan equine encephalitis virus (VEEV). These two viruses are of particular concern because they remain biological weapons threats. Eastern and Venezuelan were some of the first human viruses discovered. In the early course of research, laboratory personnel would become infected through the generation of infectious aerosols. When it became apparent that such aerosolized alphaviruses were highly infectious, particularly Venezuelan equine encephalitis, biological warfare programs of multiple nations began research and development of these agents as offensive bioweapons. Although such research has thankfully come to an end, the amount of knowledge generated, the low cost and low barriers to culturing alphavirus for such purposes leaves both eastern and Venezuelan equine encephalitis viruses marked as Category B Select Agents by the US government (14-16).

An additional EEV not discussed in this work consists of western equine encephalitis virus (WEEV). Although similar to EEEV in terms of case presentation, the case fatality rate of WEEV is approximately 3-4% and far fewer human cases have been reported throughout the 20<sup>th</sup> and 21<sup>st</sup> centuries relative to disease caused by EEEV or VEEV, and even fewer cases of encephalitic disease arising from the inhalation of infectious aerosols have been reported.

The Department of Defense is concerned about the use of weaponized alphavirus aerosols against soldiers, especially because no licensed vaccines or treatments are available. Although previously discussed, while experimental human vaccines against EEEV and VEEV are available to a very limited subset of people on an investigational new drug basis, their safety, efficacy, and cost remain unsatisfactory for broad licensure. A better vaccine or medical countermeasure should arise to fill the unmet need thus outlined, though efficacy trials cannot take place directly in human subjects due to the Select Agent status of EEEV and VEEV. Animal models remain necessary for continued study, and the nature of such studies demand the most relevant data regarding acute viral encephalitis from said animal models.

Radiofrequency telemetry, or radiotelemetry, denotes the monitoring of physiological data and its electronic transmission to a remote location, abolishing the need for fragile wired setups and mitigating the need for intermittent staff observation. Previous studies with telemetered animal models in the context of infectious disease have illustrated the capacity for the monitoring of temperature, for alerting investigators to the moribund state of animal subjects, and for the determination of efficacy of vaccine candidates by statistical modeling of temperature and quantifying significant deviations (17. 18). Radiotelemetry modalities such as electroencephalography (EEG) measure electrical signals originating from the brain. Additionally, intracranial pressure (ICP) detected through piezoelectric transducer sensors can yield a more

sensitive indicator of encephalitis, as increased intracranial pressure necessarily results from the host response to an infection of the brain parenchyma (19). In addition to aiding decision-making regarding humane study endpoints, telemetry could also portend more specific indications for disease onset and severity, such that investigators and study staff could initiate treatment with experimental medical countermeasures.

The development of a trigger-to-treat in a macaque model requires the interrogation of continuous data obtained through EEG, and ICP, in conjunction with traditional monitoring modalities for disease such as temperature and blood work. To better understand and leverage data from nonhuman primate models, the parallel study of quantitative methods of characterizing EEV encephalitis and of physiological correlates of EEV infection represents an opportunity to better survey alphavirus pathogenesis with respect to acute viral encephalitis and to better evaluate medical countermeasures against alphavirus encephalitis.

#### 1.1 Alphaviruses

#### 1.1.1 Overview of Alphavirus Structure, Function, and Pathogenesis

#### **1.1.1.1 Alphavirus Structure and Function**

Viruses with positive-sense single-strand RNA ((+)ssRNA) genomes encompass a group of pathogens that include flaviviruses (e.g. yellow fever), coronaviruses (e.g. SARS), and picornaviruses (e.g. polio). *Togaviridae*, or togaviruses, comprise a family of (+)ssRNA viruses that include the alphavirus genus. Thought to be of marine evolutionary origin, the roughly thirty known species of alphavirus are divided into numerous classes and clades, and roughly categorized into New World alphaviruses and Old World alphaviruses along putative evolutionary origins. Three distinct subgroups of alphaviruses abound: Semliki Forest Virus, Sindbis Virus, and Equine Encephalitis Virus (EEV) subgroups. While the Semliki Forest and Sindbis subgroups fall into the Old World category, the EEV subgroup falls into the New World category. Notably, EEVs were the first alphaviruses to be isolated in the 1930s, in North America (20-24).

Generally, all alphaviruses share a number of distinct molecular structural characteristics. Alphavirus virions consist of icosahedral capsid structures, enshrouded in a lipid envelope. Altogether, the size of each virus particle can range from 60-70 nanometers in diameter. Each capsid or nucleocapsid contains non-segmented, 5' capped, 3' polyadenylated (+)ssRNA genomes with a hairpin morphology in the middle that divides open reading frames. These genomes, approximately 11-12 kilobase pairs in length, are of a bicistronic nature; they contain two open reading frames translatable into two polyproteins (25-27). The first polyprotein is cleaved into five to six structural proteins, while the second produces four nonstructural proteins. Structural proteins include capsid (C), envelope proteins (E1, E2, E3), the 6K protein, and the TF protein, produced by a truncated structural polyprotein transcript due to ribosomal frameshifting. (26, 28-30).

The contemporary understanding of the physical structure of a competent alphavirus particle envisions an outer shell of E1-E2 trimer glycoprotein stalks that protrude past the lipid envelope of the virus particle for the purposes of attachment to host cells. According to the Caspar and Klug virus symmetry classification system, each virion has a T = 4 icosahedral symmetry composed of 240 monomers, with 80 such E1-E2 trimers (31). While the C protein undergirds the lipid envelope and provides the physical basis for the icosahedral subunit and substructure which anchors the E1-E2 trimers, the E3 glycoprotein may not be incorporated into the mature alphavirus virion. Instead, the E3 glycoprotein plays several vital roles in the production of competent viral

particles; the E3 portion of the structural protein polyprotein sequence hosts the signaling sequence for translocation of the polyprotein into the cellular endoplasmic reticulum, and its furin-mediated cleavage from E2 presages the proper assembly of E1-E2 into its mature spike assembly (30). The 6K protein, so named for its putative encoding of a 6 kDa molecular weight protein, comprises an extremely minor portion of the structure of the finished alphavirus particle, but nonetheless plays an important role in virus assembly and budding; a hydrophobic, cysteine-rich biomolecule, 6K protein exhibits cation-selective ion channel activity (32, 33). The sixth and final structural protein TF (for transframe), produced by a ribosomal frameshift during translation of the 6K protein, shares much structural similarity with the 6K protein, and likewise exists in substoichiometric amounts in the finished virus particle relative to E2, E1, and capsid protein.

Nonstructural proteins, on the other hand, follow a somewhat different pathway to carry out an array of cardinal functions for viral replication in the cell. Highly conserved within each clade of alphavirus, the nonstructural proteins descend from a single polyprotein directly translated from the viral genome following the unpacking of the virion in the cytoplasm in early infection. In order, as translated from the 5' end of the virus genome, these nonstructural proteins consist of nsP1, nsP2, nsP3, and nsP4 (27, 29). Approximately 90% of nonstructural polyproteins contain only nsP1, nsP2, and nsP3 (P123); the suppression of genome-encoded termination enables the translation of the full polyprotein including all four nonstructural proteins (P1234) (27). Briefly, the functions of the nonstructural proteins include the capping of message RNA (nsP1), helicase and protease functions (nsP2), ADP- and stress granule-related protein binding (nsP3), and RNA-dependent RNA polymerase activity (nsP4) (27).

A more expanded view of the collective functions of the structural and nonstructural proteins encompasses the process of alphavirus replication and pathogenesis. Immediately

following inoculation of alphavirus into a host or a cellular environment, the E1-E2 glycoprotein spike triggers receptor-mediated endocytosis (34, 35). The spikes can bind to a number of mammalian cell receptors, for example heparan sulfate (HS), DC-SIGN, and L-SIGN; though E2 facilitates receptor binding, E1 follows through with cell fusion (36-39). A pH-mediated process drives the alphavirus genome into the cytoplasm; direct translation by host machinery of P123 and P1234 from the alphavirus genome results in the generation of both genomic RNA and a subgenomic RNA transcript (26S RNA) (27). The protease function of nsP2 cleaves nsP4 from the P1234 polyprotein; nsP4 and P123 polyproteins synthesize a complementary (-)ssRNA transcript, forming a template from which both progeny (+)ssRNA genomes and subgenomic transcripts are generated. Further cleavage of P123 to nsP1 and a P23 polyprotein by nsP2 tilts the equilibrium of RNA transcription to favor (+)ssRNA synthesis over (-)ssRNA synthesis, and cleavage of the nonstructural polyprotein into discrete nsP1, nsP2, nsP3, and nsP4 proteins forms a viral replicase complex that shifts this equilibrium to favor subgenomic RNA, initiated from an internal promoter in the negative-sense replication intermediate, over genomic RNA synthesis (25, 27, 40, 41). The further this equilibrium shifts towards the production of subgenomic RNA, that is, in late viral infection, the more structural polyprotein is produced. The genomic region that encodes the structural polyprotein remains separated from the region that encodes the nonstructural polyprotein by a hairpin loop (42). As previously discussed, the structural proteins arise from a subgenomic RNA species termed 26S RNA; newly-synthesized viral RNA genomes (vRNA) are packaged into the emerging nucleocapsid assembly (built from E1-E2 trimers and capsid proteins) in the cytoplasm and mature virions form out of the cytoplasmic membrane, aided by membranepotential regulating effects of the 6K and TF proteins (33, 43).

Cytopathic effect in mammalian cell culture produced from alphavirus infection occurs within approximately 24 hours post infection and is thought to stem from either or both nsP2dependent transcriptional downregulation or nsP3-dependent decreases in translation (44). This mechanism varies by virus; in VEEV, for instance, nsP2 inhibits translation of interferon proteins whereas Sindbis virus infection inhibits transcription (45). Moreover, in typically cultured cells, maximum absolute titers of virus are produced between 8-10 hours post infection; virus production ends due to the putative triggering of cell death pathways in infected cells (40). Formation of plaques provides unitary evidence of infectious virus and/or (+)ssRNA genomes making titering for infectious virus by plaque assay the gold standard for the quantitation of alphaviruses (46-48).

#### **1.1.1.2 Alphavirus Pathogenesis**

Alphaviruses are predominantly transmitted by arthropod vectors, placing them into a category known as arboviruses. Insect cells are uniquely disposed to facilitate the spread and replication of alphaviruses in the mosquito vector due to the propensity of virus budding to occur at both the plasma membrane and intracellular membranes (49). In insect vectors, a likely key factor in the sustenance of virus transmission to/between avian or equine hosts consists of alphavirus replication occurring in insect gut epithelium without producing dramatic cytopathology, though virus production eventually plateaus and declines of its own accord (50-53). Subsequently, the carriage of infectious titers of alphavirus in arthropod (likely mosquito) gut and migration to the salivary gland facilitates the infection of numerous vertebrate hosts during blood meals. This host range consists of a large spectrum of avian species and mammals, the latter of which range from small rodents to equid species and humans (5, 54-56).

The zoonotic burden of certain alphaviruses comprises a major link in the chain of infection that promotes natural human alphavirus outbreaks. With respect to New World alphaviruses, and specifically with regards to the two alphaviruses central to the scope of this work: eastern equine encephalitis virus (EEEV) and Venezuelan equine encephalitis virus (VEEV), geographic accretion of large natural or agricultural collections of nonhuman host animals make available large pools of potential amplification hosts to the arthropod vectors. Large populations of equid species in close proximity to human population centers, for example, of donkeys and horses in VEEV outbreaks in South and Central America through the latter half of the 20<sup>th</sup> Century have sickened thousands of humans in VEEV outbreaks following such a pattern (55, 57-60).

Naturally acquired human alphavirus infection can elicit a range of presentations that fall largely into either arthritogenic or encephalitic disease courses. The former group consist of such archetypal viruses as Chikungunya virus, Ross River virus, Sindbis virus, and Mayarovirus; the course of natural infection with such viruses typically manifest bilateral polyarthralgia or polyarthritis, alongside fever, rash, and myalgia (61). However, the key feature of the latter group, encephalitis, remains the mainstay of the core focus of this work, as the accompanying neurological signs of disease lead to a more debilitating, possibly fatal, acute and chronic syndrome, though a larger proportion of humans become infected with arthritogenic viruses (62).

In this context, encephalitis of a viral origin refers to an infection of the brain parenchyma that produces observable changes in brain function (63). The natural course of infection with encephalitic alphaviruses may produce asymptomatic or solely febrile courses of illness, but a variety of neurological signs of disease can arise, including a febrile illness, photophobia, altered mental status, seizures, and coma (5, 64). Not only may these neurological signs of disease arise when the host experiences acute febrile illness, the developments may persist until the host expires, or in the case of host survival, neurological sequelae may persist well after the host is thought to have cleared the virus (42).

The relationship of the neurovirulence and neurotropism of alphaviruses to the encephalitic disease courses that ensue suggest variable approaches for encephalitic alphaviruses' access to the brain. A body of work suggests direct penetration of the central nervous system (CNS) by neurotropic viruses via infection of neuroepithelium and subsequent retrograde axonal transport (65-68). Other means of CNS access include infection and replication of alphavirus in the periphery of the animal, followed by establishment of systemic viremia and subsequent infection and passage through the blood brain barrier (69-71). Though the vast majority of mechanistic work has been performed in animal models, a prodigious amount of evidence suggests that the nature of neuroinvasion, the subsequent immune response, and the compromised integrity of the blood brain barrier precipitate an ill effect on morbidity and mortality (69). Traffic of alphavirus through the neurons and support cells of the neuroepithelium can facilitate alphavirus particle infection of the lamina propria of this layer of tissue. In this manner, EEEV or any other neurotropic prototypical alphavirus can access the vasculature and the lymphatic system. As projections of the cranial nerves transit through the base of the cranial vault and into the brain proper by way of the cribriform plate in the ethmoid bone; cell-to-cell transmission in the CNS can then occur, between neurons, astrocytes, and oligodendrocytes (72, 73). This process is further addressed in the section



Aerosol FlowVirus Deposition

Figure 1. Olfactory Hypothesis of Inhalational Alphavirus Infection.

A nonhuman primate model of inhalational infection by a prototypical aerosolized alphavirus. Deposition of virus can occur in the shallow and deep lung, the gastrointestinal tract, as well as the neuroepithelium, leading to infection of the CNS through cranial nerve I.

regarding animal models of aerosolized EEEV and VEEV (Subchapter 1.1.4.1).

Further work remains necessary for the development of an animal model that can not only capture the human disease phenotype engendered by inhalational EEEV or VEEV encephalitis, but also provide information regarding neurological health and on the onset/resolution of encephalitic disease.

#### 1.1.2 Eastern Equine Encephalitis

#### **1.1.2.1 Human EEE**

Eastern equine encephalitis (EEE) is so named because it is typically found in the eastern half of the United States, with cases reported from the eastern seaboard to as far inland as the Mississippi river valley. The virus is principally borne by the C. melanura as an enzootic mosquito vector and natural human infections peak in summer months (62). In humans, disease caused by EEEV typically manifests after an incubation period that can range between 4 to 15 days, and is characterized by a viral prodrome of nausea, vomiting, fever, general malaise, and/or headache that occurs before or concomitant with signs of neurological disease (62, 74, 75). Viremia can occur during this viral prodrome, but is thought to decline before clinical signs of neurological disease manifest (76). EEEV remains notable among alphaviruses for its high case fatality rate (CFR), which ranges from 30-70% in human cases (56, 75). The rates of encephalitis caused by EEEV in humans remain low, with roughly 2% of adults and 6% of children infected with EEEV affected by encephalitis in the course of natural infection, though these finding are based on serology and may not represent true exposure rates (77, 78). However, the onset of neurological signs of disease correlates with poor patient outcomes, and fulminant CNS disease with manifestations such as focal cranial nerve deficits, seizures, or coma can result; other neurological signs of disease detectable through neuroimaging include generalized slowing of brain electrical activity on electroencephalography (EEG), and brain parenchyma lesions in the basal ganglia, thalamus, and cerebral cortex visible by computed tomography (CT) and magnetic resonance imaging (MRI) (62, 75, 78-84).

An accurate patient history, and the presence of immune serum (e.g. IgM) or evidence of IgM conversion to IgG in the cerebrospinal fluid (CSF) can indicate that EEEV infection has

occurred (75, 77, 78, 80). Confirmation of infection can also proceed by polymerase chain reaction from blood samples or from CSF (75). A granulocytic, predominantly neutrophilic pleocytosis is also observed in the CSF during EEEV infection, alongside elevated protein (77, 78). The clinically observable phase of encephalitis comes with a number of laboratory findings that include hemagglutinin inhibition, the presence of neutralizing antibodies, as well as electrolyte imbalances such as hyponatremia (76, 83). Both hyponatremia and CSF pleocytosis have documented associations with poor outcomes (83). Clinical procedures such as lumbar puncture (not recommended during acute infection due to deleterious effects on patient outcomes) can detect elevated CSF pressures; the elevated total protein, with the presence of neutralizing antibodies against EEEV, suggests the pathological disruption or erosion of the blood brain barrier (78, 84).

Neuropathology indicative of encephalitic disease also helps to characterize EEEV infection; previously mentioned lesions observable by neuroradiography in the deep brain: the thalamus and the basal ganglia show signs of hypoperfusion, possibly due to edema or ischemia (78). For obvious reasons, diagnostic methods for encephalitic EEEV disease through histological examination of prepared slides for inclusion bodies, lymphocytic infiltrates, or direct staining for EEEV cannot be employed during the therapeutic window (1). However, in survivors of EEEV disease, behavioral or clinically observable evidence of neuronal/CNS damage persist after recovery, despite the presence of circulating antibodies, suggesting either or both that the immune response does not adequately protect against neurological sequelae and/or that the immune response cannot adequately extirpate competent virus from the brain parenchyma and may actually contribute to pathology (51, 85, 86). Up to 30% of such survivors may suffer from neurological sequelae such as cranial nerve deficits, intermittent focal paralysis, and seizures, with possible

altered mental status (1, 5, 63, 65). Signs and symptoms of human EEEV disease are illustrated in Figure 2.



Figure 2. Human Eastern Equine Encephalitis Infection.

Effects of Eastern Equine Encephalitis in Homo sapiens, listed and organized by organ system.

The strain of EEEV specifically considered for the basis of this work, designated Strain V105, arose from the brain parenchyma of a fatal human case of EEEV during a 2005 epidemic in New England (87). After three independent courses of investigation by the state health departments of Massachusetts and New Hampshire, in addition to the Centers of Disease Control and Prevention, the clinical case definition consisted of meningitis or encephalitis that was confirmable by anti-EEEV IgM antibody in the cerebrospinal fluid or serum of the patient. The 2005 epidemic witnessed a case fatality rate (CFR) of 4/11 = 36%; five out of the eleven patients, inclusive of the four fatal cases, visited healthcare providers for nonspecific complaints before hospitalization for

encephalitis (87). Although the vast majority of the 11 patients had documented altered mental status, only three of them had acute neurological complaints of sufficient severity to warrant sameday hospitalization. Of the 10/11 patients with CSF samples taken, all manifested pleocytosis (87).

#### 1.1.2.2 Treatment of EEE

No unitary doctrine governs the treatment of EEE; as recently as the 2019 US EEEV epidemic, the most frequently encountered recommendation for treatment comprises supportive care, with respect to the maintenance of fluids and mitigation of neurological signs of disease, in an intensive care unit with ventilatory support, represents the best standard of care at this time (88). Although certain experimental therapies, including monoclonal and polyclonal antibodies and small-molecule drugs have shown promise in the recent past, they still require additional work to become licensed therapies in humans.

#### 1.1.2.3 Human Vaccines against EEEV

As previously mentioned, a formalin-inactivated vaccine exists for the treatment of EEEV (PE-6), though the formalin-inactivated, lyophilized vaccine retains its experimental investigational new drug (IND) designation and is not licensed for widespread human use (86). The inactivated vaccine is administered with 0.5mL diluent in two doses spaced four weeks apart, with smaller 0.1mL doses given to maintain antibody titers. Reactogenicity and immunogenicity have been documented in trials of this study, and the less than 60% of those vaccinated generate antibodies against the vaccine, with a <sup>1</sup>/<sub>4</sub> chance of non-maintenance of antibody titers for over a year (79). The side effect profile and the difficulty in mounting a sustained antibody titer in a large minority of PE-6 vaccine recipients provides a strong argument for the development of an improved vaccine.

#### **1.1.3 Venezuelan Equine Encephalitis**

#### **1.1.3.1 Human VEE**

Venezuelan Equine Encephalitis (VEE) remains the most well-studied equine encephalitis virus to date, and is known for its high infectivity via aerosol, as previously described (89). In contrast to EEEV, VEEV disease in humans espouses a significantly decreased CFR of less than 1% (35, 77, 79). As with EEEV, close human proximity to large populations of equid species in addition to critical populations of arthropod vectors such as *Culex*, *Aedes*, and *Psorophora* mosquito species can drive human epidemics (5, 55, 57, 62, 64, 90-93). Numerous subtypes of VEEV exist (including but not limited to: IA/B, IC, ID, IE, II, VI, IF) and are divided into epizootic strains (IA/B and IC) and enzootic strains (ID, IE, II, VI, and IF); epizootic strains are more of a concern for human disease, as they produce higher titers of viremia and have a higher propensity to cause encephalitis (5, 62, 64, 94).

Human VEEV disease can take on the clinical appearance of a febrile illness subsequent to a latency period lasting from one to several days (54, 77, 95). The onset of the febrile illness brings about, the possibility of encephalitic disease; approximately 1% of adults and 4% of children achieve a diagnosis of encephalitis during the course of natural infection though it is estimated that as many as 14% of all patients may experience encephalitis (5, 62, 64, 95, 96). Mortality in cases of VEEV showing signs of neurological disease are elevated to 10% in adults and up to 35% in affected children (66). During the time of the febrile illness, patients may test positive for VEEV through either enzyme-linked immunosorbent assays for antibodies generated against the virus or polymerase chain reaction based assays with samples collected by throat swabs (54, 77, 95). Observable signs of neurological disease include nuchal rigidity, confusion, stupor, and aphasia/mutism, as well as involuntary movement of the muscles; more severe cases can include nuchal rigidity, ataxia, aphasia, mutism, nystagmus, convulsions, and cranial nerve dysfunction (including facial nerve weakness, ocular palsies, photophobia) (97). The physiological basis that undergirds the development of encephalitic VEEV disease can include a loss of neurons to apoptosis, neutrophil penetration into the CNS, and vasculitides in the CNS (77). Additionally, some persons infected by VEEV can present with a macular rash or arthritic disease, bilaterally and in the distal extremities; this appears more consistent with natural infection and not with inhalational aerosol disease (35, 77, 98). Regardless of disease etiology, acute VEEV typically resolves after one to two weeks, though neurological sequelae, which include cranial nerve deficits, and involuntary movement can persist afterward for up to a year or longer (5, 64, 77).

Severe cases of human VEEV disease also encompasses signs of disease in other organ systems as well; pulmonary findings can include hemorrhage in the lungs, alongside congestion and edema associated with interstitial pneumonia, and fulminant hepatocellular degeneration may also occur (50, 90). Human cases of VEEV present with clinical laboratory findings of marked lymphopenia or lymphocyte depletion; a number of studies have noted that in both human and veterinary cases, VEEV replicates extensively in lymphoid tissues for replication, in direct contrast to EEEV, as the reduced lymphotropism and heparan sulfate binding of EEEV was linked to reduced prodrome, an effect recapitulated also in humans and macaques (41, 96, 99, 100). This reinforces findings of follicular necrosis in lymphoid tissue (50, 90). Figure 2 summarizes signs and symptoms of human VEEV disease.



Figure 3. Human Venezuelan Equine Encephalitis Infection.

Effects of Venezuelan Equine Encephalitis in Homo sapiens, listed and organized by organ system.

The last major outbreak of VEEV occurred in the late summer of 1995, at the border between Colombia and Venezuela (58). Between the time that the outbreak was first reported and the end of September (a month's time), more than 1,000 cases were reported with the eventual total reaching the tens of thousands. Given that clinical cases, in this outbreak, were estimated to occur in only 11-20% of the exposed population, the number of cases may have numbered higher than 10,000 cases (55, 57, 58, 62, 93). Because vector control measures were not tightly or regularly implemented until recent decades, spillover events manifested as large-scale VEEV outbreaks in the 1950s, 1960s, and 1970s throughout the Caribbean (50, 58, 92, 93). A similar virus strain isolated from mosquitoes from Venezuela in 1983 suggests the existence of a serotype IC epizootic transmission cycle in northern Venezuela (57, 58). VEEV subtype IC strain INH9813, the strain used in this work, was isolated from a human serum sample from this outbreak (89, 92).

One of the peculiarities of VEEV compared to EEEV is the characteristic biphasic febrile curve seen in VEEV but not EEEV infection. While the paper detailing the study of EEEV versus VEEV in the murine model hits upon an important feature of VEEV disease recapitulated in almost all species – the lymphotropic preference of VEEV in early infection – several factors can influence the degree of neurotropism exhibited by any given strain of VEEV (101-103). Several sources have posited that the biphasic nature of the febrile illness induced by VEEV correlates with an initial lymphotropic phase of VEEV infection corresponding to the first febrile phase and the secondary febrile phase corresponding to neurotropic VEEV infection or to encephalitis or inflammation of the brain parenchyma (104-107). In both murine and NHP models, clinical signs of neurological disease are more commonly reported during the second febrile phase. As fever represents a generalized systemic response to infection, this hypothesis regarding the timing of the febrile phases presents fertile ground for continued investigation in the context of encephalitic VEEV infection.

#### 1.1.3.2 Treatment of VEE

Characteristics of neurological signs and symptoms of VEEV are primarily captured and described by compilations of case reports and case series; no definitive doctrine governs the management of the diseases or the aforementioned sequelae, most documentation recommends the implementation of supportive care for the duration of the illness. However, several promising venues of experimental treatments exist for VEEV. For instance, a variety of small molecule nucleoside analogues show promise for broad-spectrum antiviral activity against a number of RNA viruses. A large number of these compounds target RNA-dependent RNA polymerases; putative

candidate compounds include but are not limited to candidate small molecule inhibitors such as favipiravir (also known as T-705) and other small molecule drugs such as BCX4430 or EIDD-2801 (108-113). Preclinical studies have established that the application of polyclonal antibodies within sufficient time and efficacy windows before infection can protect mice against lethal outcomes (114). Similarly, hyperimmune convalescent sera have been used in the past to treat VEEV, and monoclonal antibodies have also shown promise for treatment as well (115-118).

#### **1.1.3.3 Human Vaccines against VEEV**

However, concrete means of prophylaxis against VEEV also exist. Vaccination of equine species in the Americas is either mandatory or strongly recommended; widespread vaccination has reduced the risk of equine species serving as amplification hosts to generate or sustain human epidemics (66, 79). The decades of research invested in VEEV during the mid-20<sup>th</sup> Century bore fruit in the form of the development of two VEEV vaccines: TC-83 (for Tissue Culture, 83 passages), an attenuated strain of VEEV, and C-84, a formalin-inactivated vaccine for VEEV (119). As mentioned previously, neither vaccine is licensed, and are both administered only under the regulatory framework of investigational new drug (IND) rules through the Special Immunization Program at USAMRIID (120). Nevertheless, the vaccines' limited ability to establish immunological memory and/or a persistent immune response, a not insignificant side effect profile, and the high cost of vaccine administration limit the accessibility of the current vaccine regime only to laboratory and veterinary workers most at risk of exposure motivate the development of an improved human vaccine.

#### 1.1.4 Inhalational Alphavirus Encephalitis and the Biosecurity Conundrum

#### 1.1.4.1 Animal Models and the Study of Aerosolized EEVs

A profusion of animal models has accrued through the decades to more closely examine the encephalitic nature of EEEV and VEEV as well as the neuroinvasion of the brain. Studies of EEEV have been done in a number of other animal models, particularly mice, but also hamsters, marmosets, and rhesus macaques towards the evaluation and production of a human disease phenotype.

The advantage to using mice in any laboratory study comes with the intrinsic benefits of diminished space requirements, food, upkeep, and costs relative to the same for nonhuman primate studies. Moreover, inbred mouse models benefit from more genetic homogeneity compared to outbred nonhuman primate models. In mice, aerosol exposure to virus (Strain FL93-939) precipitated the development of high viral titers by 6 hours post infection in the brain, and the manifestation of severe neurological signs by 4 days post infection (DPI) with nearly 100% mortality, though less mortality occurred in animals challenged by a different route (68). Mice infected via aerosol demonstrated a high body temperature at 3 DPI with a steep terminal decline at 3-4 DPI. Research on EEEV in the murine model has shed light on the milieu of biochemical species present during the immune response to EEEV infection; subcutaneous, intranasal, or aerosol exposure to EEEV in mice resulted in an increase of interferon gamma (IFN- $\gamma$ ) at 1-2 DPI, with an increase of granulocyte colony stimulating factor (G-CSF) at 4 DPI subsequent to intranasal or aerosol infection, and at 6-7 DPI following subcutaneous infection (68). This finding supports the observation of a human disease phenotype of a neutrophilic phenotype. The study of EEEV in mice also revealed the tendency of EEEV to exhibit neurotropic qualities depending upon its receptor-binding properties; a study by Gardner et al. demonstrated that natural binding of EEEV due to heparan sulfate facilitated neurovirulence favoring replication in the CNS rather than in lymphoid tissue (36, 38, 39). In fact, the study of EEEV in the mouse model highlighted a major difference between EEEV and VEEV pathogenesis with respect to tissue tropism; translation and replication of EEEV in myeloid tissues was suppressed by the binding of microRNA miR142-3p to sites in the 3' untranslated region, which precipitated the dual effects of limiting Type I interferon response and inhibiting the lymphotropic spread of EEEV (100). However, the mouse model suffers from the lack of full reproduction of neurovascular disease and blood brain barrier leakage, stemming from high mortality before this aspect of the disease is captured.

Murine models of VEEV disease can likewise recapitulate elements of human disease; mice manifest biphasic fevers and exhibit virus replication in primary and secondary lymphoid tissues (90, 121). Although exposure to non-attenuated VEEV is almost universally fatal in immunocompetent mice within 6-9 days, a feature not reflective of human VEEV disease, studies with genetically modified mice with immunity to VEEV have demonstrated high titers of virus in the brain in surviving mice, suggesting that high titers of virus in CNS tissue is not sufficient to cause death, though this outcome is dose- and route-dependent (62). Likewise, related studies demonstrated that T cells were likely to play a critical role in determining the survivorship of mice infected with VEEV though the absence of T cells extended survival only by 1-2 days (62). The murine model of VEEV has also proven instrumental in the elucidation of virus dissemination in infected animals; the mechanism of olfactory bulb-based spread of VEEV into the CNS was determined to play a significant role regardless of whether animals became viremic, a factor important for aerosol infection with both VEEV and EEEV, though this occurs in subcutaneous infection with VEEV as well (99, 122). Likewise, mechanisms of neurovirulence and neurotropism have been studied quite extensively in the murine model, with neurons and astrocytes
in particular outlined as a target of VEEV, although the virus primarily infects neurons. Specific biomolecular species, e.g. cytokines, have been shown to play a prominent role in the murine response to infection (62, 67, 123-125). Finally, a seminal study by Gardner et al. outlines the distinctive features of VEEV infection in the murine model, including not only the recapitulation of the human disease feature of VEEV lymphotropism, but also a preference for dendritic cells and macrophages (101).

A hamster model of EEEV was also constructed; hamsters inoculated subcutaneously with 10<sup>3</sup> plaque forming units (PFUs) of EEEV developed fever in less than 24 hours after infection, with neurological signs of disease appearing at 2-3 DPI, which included head pressing (a sign of increased intracranial pressure), stupor, and coma by 4-5 DPI (126). Histopathology of brain parenchyma on necropsy revealed EEEV in all infected animals; serial sacrifices demonstrated early compromise of the basal ganglia and lesions arising in the cerebellum and brainstem (126). Similarly, guinea pigs subjected to aerosols infection of EEEV showed virus in the CNS within 1 DPI (50, 127).

Hamsters have also been used as a model system in which to study VEEV pathogenesis. The hamster model also recapitulates the olfactory bulb mechanism of VEEV dissemination into the CNS (102). Although hamsters replicated both lymphoid and CNS histopathological features of human VEEV disease, instances of pathology unique to the hamster were also found – for instance, pathology in the pancreas (128). As causes of death can be attributable to gastrointestinal disease, the hamster does not serve as a good model of CNS disease. Live attenuated TC-83 vaccine was found to protect hamsters challenged by aerosol or subcutaneous inoculation of VEEV, while formalinized C-84 vaccine protected hamsters only against subcutaneous infection (129). A disadvantage of the hamster model rests in the propensity of certain strains of VEEV to remain avirulent; the underlying implication that modulation of virulence comprises a prerequisite for a successful reconstruction of human disease does not testify to the robustness of the hamster model (130). However, the more utility-prohibitive feature of the hamster model rests in the finding that hamsters tend to succumb to disease and/or require euthanasia due to humane study endpoint before the onset of CNS disease (66). Guinea pigs have also served as a model for the study of VEEV. Cultured cardiac myocytes from guinea pigs were utilized as the chassis for the culture, passaging, and subsequent attenuation of the Trinidad Donkey (TrD) strain of VEEV that generated the TC-83 vaccine (131). Like hamsters, guinea pigs infected with VEEV also exhibit fulminant infection of the lymphoid tissues and die from gastrointestinal disease (66). The propensity for death from gastrointestinal disease also diminish the value of guinea pigs as a model for the study of VEEV-induced neurological disease although direct intraperitoneal serial passages of VEEV in one study with guinea pigs appeared to augment the neurotropism of the virus (132).

A number of studies of EEEV have been performed in nonhuman primates (NHPs); marmosets have been such a model for the study of EEEV pathogenesis (133). A study by Porter, et al. highlighted that marmosets were exquisitely sensitive to a low infectious dose on the order of approximately 200 PFU of aerosol, with clinical signs of subsequent EEEV disease including fever, piloerection of fur, leukocytosis with a neutrophilic predominance, and generalized signs of meningoencephalitis (133). However, the marmoset model failed to recapitulate seizure activity, and no viremia was detectable by polymerase chain reaction (133). A similar precedent study conducted by Adams, et al. reports brief leukopenia at 1 DPI with a rebound leukocytosis characterized by neutrophilia, lymphocytosis, and monocytosis at 3-4 DPI; similarly, no EEEV viremia was detected in the marmoset model though signs of meningoencephalitis occurred with perivascular hemorrhage in the cerebral cortex (134). Likewise, studies of EEEV have been performed in the rhesus macaque model, with a variety of routes for inoculation, including subcutaneous, intracranial, and intranasal delivery of virus (24, 127, 135). While peripheral subcutaneous inoculation led to febrile disease and death within 1-2 weeks, earlier studies with intracranial injection of the macaques resulted in a faster time to death (24). Viremia was noted in the blood before the onset of encephalitis (21, 24). Passive immunization with hyperimmune sera was found to be protective if given before the onset of fever in previously naïve macaques (24). CNS lesions were found to recapitulate patterns found in human EEEV infection, diffusely throughout the cerebrum and the spinal cord (136).

NHP models of VEEV have also provided a wealth of information regarding VEEV pathogenesis. Although cynomolgus macaques have been the subjects of the vast majority of these nonhuman primate studies, a not insignificant number of studies involving nonhuman primates involved the rhesus macaque. The study of neuropathology in the rhesus macaque comprised some of the first studies in non-murine species that corroborated the olfactory nerve spread of VEEV through the CNS (103). Likewise, early studies of VEEV and CNS pathology saw some of the first electroencephalographic recordings of VEEV-infected macaques; baseline EEG recordings were documented against recordings taken subsequent to infection (102, 131). Such differences, though brief and essentially a window into brain electrical activity over the course of a few seconds to minutes at various intervals, provided some of the first evidence in a human-similar animal model of pathological activity in acute viral encephalitis visualizable by telemetry (102). Any experimental route of virus inoculation except for direct intracranial injection appears to produce remarkably few courses of fatal disease in both rhesus and cynomolgus macaques challenged with VEEV (95, 96, 132, 137, 138). Similar to humans, VEEV-infected macaques often develop a biphasic fever consisting of a primary febrile peak beginning at 1-3 DPI followed by a second

febrile phase at approximately 5 DPI (102). Moreover, a transient viremia occurs in a period from 1-2 DPI. Pathogenesis studies revealed brain lesions originating in the olfactory tract beginning between 2-6 DPI (dependent upon route of infection) with subsequent spread through the thalamus, hypothalamus, and outward through the cerebral cortex (66, 102, 103). Of the several lobes of the cerebral cortex, the occipital lobe and the cerebellum demonstrated the least amount of inflammation; features of human VEEV encephalitis missing in rhesus macaques included frank hemorrhage in the CNS and demyelination, though no direct interrogation of tissues for virus was conducted and data from human case series may suffer from survivorship bias (66, 102). Studies of EEVs in cynomolgus macaques shall be discussed in full in a subsequent subchapter.

It has been hypothesized that inhalation of an encephalitic alphavirus in an aerosol may allow the virus to come into contact with neurons or neural projections from cranial nerve I (the olfactory bulb), thus facilitating neuroinvasion and diffusion/infection through the rest of the CNS (56, 68, 99, 139, 140). Although data that supports this hypothesis has been at times equivocal, studies in mice have endorsed this hypothesis by providing evidence of viral infection of the olfactory nerves (66). However, lesioning of the olfactory tracts in rhesus macaques only delayed, but did not prevent the penetration of VEEV into the CNS (103). In guinea pigs, the aerosol delivery of EEEV via large particle aerosol altered neither the lethal dose nor the time to death (141). Finally, the delay between infection and fever suggests that peripheral EEEV replication occurs prior to the penetration of the CNS.

This brief review of animal models of disease does not cover some of the more exotic models of EEEV such as pheasants, chukars, emus, or other avian species that can serve as reservoirs or amplification hosts, or more esoteric nonhuman primate models such as owl monkeys (14, 79, 133, 142, 143). However, a theme of these various animal models of EEEV is that the

mere presence of neutralizing antibody did not constitute a sufficient bulwark against mortality nor protection against neuropathology or neurological sequelae; the timing of the antibody response may determine the outcome of infection. In aerosol infection with EEEV, inhalation appears to engender a more severe, neuropathological course of disease. Despite the rarity of more severe features of neurological disease such as seizures or coma in VEEV, VEEV remains relevant not only because it has affected more patients (in Central/South America) over the previous decades but because of the concern that it could be used as a bioweapon. Additional indicators of infection by VEEV apart from its propensity to cause a biphasic feature and a general influenzalike illness isolated through the study of a human-like VEEV disease phenotype in an animal model may well yield critical information regarding the development of neurological disease as well as elucidate time-bound findings regarding the onset and resolution of VEEV encephalitic disease. This is especially important for VEEV, since a therapeutic window exists for VEEV that may not exist for EEEV as EEEV infection signs are very closely associated with neuroinvasion and encephalitis.

# 1.1.4.2 Aerosolized EEV Infection and Biosecurity

In contrast to natural human infection and epidemics propagated by arthropod vectors and amplification hosts, cases of human infection by aerosolized EEEV have been exceedingly rare, with only five documented cases of human EEEV infection resulting from aerosol exposure in the 20<sup>th</sup> Century (105, 144, 145). Of these several cases, only the two cases in 1939 and 1942 occurred in persons not previously vaccinated against EEEV. These earlier cases remain of interest due to the severe manifestation of disease in naïve individuals infected by aerosol as well as to their role in spurring recommendations for the development of vaccine countermeasures to prevent human disease. Although neither of the two cases proved fatal, documentation of clinical signs of disease

produced by human aerosolized EEEV infection encompassed febrile illness, myalgia, and focal neurological deficits. Both cases occurred in laboratory workers in close contact with large quantities of virus (146, 147). To date, EEEV remains the most lethal equine encephalitis virus in humans, however the true effect on morbidity and mortality by infection with aerosol-based EEEV in relation to naturally acquired infection is not known.

Inhalational alphavirus disease has been best documented with VEEV. In fact, VEEV is the leading cause of laboratory-acquired disease among arboviruses with as many as 150 reported infections (120). Cases of VEEV acquired by inhalation of infectious aerosols were also documented throughout the 1940s and 1950s, in groups of researchers working with the virus on mice (6-10, 54, 105, 146). Retrospective reviews of the cases suggested that the bedding of the infected animals was heavily contaminated; the researchers documented visible clouds of dust generated during the removal of deceased animal carcasses (8, 9, 105, 146). Signs of disease were first mistaken for courses of influenza, after a latency period of approximately three to four days. Of particular note was the symptom of a severe frontal headache. This contrasts with later cases of inhalational disease arising in the Soviet Union in which containers holding lyophilized virus were broken and dropped in a stairwell, in which disease manifested in slightly over 24 hours (148, 149).

While these accidents inspired safety recommendations that currently constitute the minimum standard of laboratory safety (i.e. no direct mouth pipetting of infectious or toxic fluids, plugging of pipettes with cotton, and covered centrifuge buckets) and made a lasting impact on safe laboratory design and ventilation practices, the propensity of alphaviruses, and specifically EEVs, to cause incapacitating inhalational disease also made a lasting impression on offensive biological weapons development (8-12, 150, 151). During the 1940s, a number of offensive

biological warfare programs considered characteristics of EEVs to yield acceptable risk-benefit ratios for weapons development: large quantities of virus could be cultured with relatively unsophisticated and inexpensive systems, the virus particles themselves were relatively stable in storage and highly infectious to humans when aerosolized, strains of varying degrees of incapacitation and lethality were putatively available, and no treatments or vaccines then existed that could effectively treat aerosol exposure or prevent the development of illness (11, 13, 150, 151).

Now, as then, no licensed vaccines or therapeutic treatments exist for either naturallyacquired or inhalational aerosol alphavirus infection. However, significant progress in research and development has occurred in the intervening period between the mid-20<sup>th</sup> Century and the 21<sup>st</sup> Century. In 1969, the Nixon Administration, by executive order, dismantled the United States offensive biological warfare program and shifted focus to defensive measures such as biosafety and immunization research (12, 150). The fruits of these new directions had borne out both a liveattenuated vaccine and formalin inactivated vaccine for Venezuelan Equine Encephalitis (TC-83 and C84, respectively), and a formalin-inactivated vaccine for Eastern Equine Encephalitis (Inactivated PE-6) (86, 152). As stated, these vaccines currently remain unlicensed for widespread use by the general public. These vaccines are available only as Investigational New Drugs (IND) to a limited number of at-risk laboratory personnel through clinical trials run by the Special Immunization Program at the US Army Medical Research Institute of Infectious Diseases (USAMRIID) (120). The IND vaccines' limited effectiveness and the inability to consistently establish an anamnestic immune response in vaccine recipients result in the requirement of repeat vaccine administrations (152, 153). Moreover, side effect profile of TC83 included transient clinical reactions such as fever, malaise, and chills in approximately 20-25% of vaccine recipients

serious enough to warrant bed rest (91, 154). Finally, the costs of administration of the IND vaccines remain out of reach of the general public: the cost of an annual vaccine series for a single laboratory or healthcare worker enrolled in the Special Immunizations Program numbers several tens of thousands of dollars per annum (155, 156).

The presence of decades' worth of research into applications of alphaviruses and EEVs as biological weapons poses a continuing threat to national and international security (156, 157). An intentional release of weaponized alphavirus could take the form of a small particle aerosol expected to infect a high percentage of individuals, depending upon prevailing environmental conditions, within an area of 10,000 km<sup>2</sup> (79). An aerosolized alphavirus formulated to induce encephalitic disease could cause disease of a more severe phenotype than that encountered in natural infection, as suggested by high-dose aerosol challenges by macaques (104). In fact, two of the cases of human laboratory-acquired VEEV infections from aerosolized virus discussed just previously that occurred in the Soviet Union were likely caused by weaponized strains (148, 149).

In the case of a pathogen optimized for the incapacitation of humans by the onset of encephalitic disease, the costs associated with the treatment of a single individual based off an assessment for the related western equine encephalitis virus range from tens of thousands of dollars to millions of (1988) dollars (158). The costs of recognizing, containing, and mitigating even a limited release would unleash a staggering economic cost upon an unsuspecting civilian populace. Consequently, there exists an urgent, unmet need for a safe, efficacious, lower-cost vaccine protective against alphavirus encephalitis.

### **1.2 The Telemetered Cynomolgus Macaque Model**

In this section I will expand on the cynomolgus macaque as a model for EEEV and VEEV infection and how my work has helped to develop the model.

# 1.2.1 The FDA Animal Rule

The "FDA Animal Rule" comprises shorthand for the Food and Drug Administration Animal Efficacy Rule, 21 CFR Part 314 / 601, as applicable. The seriousness of disease caused by EEEV and VEEV, the lack of natural outbreaks of sufficient size to support clinical trials, and the severity of disease preclude human efficacy studies for medical countermeasures.

In the wake of the 9/11 terrorist attacks and the Amerithrax anthrax attacks on journalists and public officials, the federal government of the United States promulgated the FDA Animal Efficacy Rule to expedite the development of medical countermeasures against previously untreatable, incurable, or otherwise unpreventable hazards to human health. Specifically, the Animal Rule outlines procedures for the development of medical countermeasures for treatment or prevention of serious or life-threatening conditions from exposure to lethal or permanently disabling toxic, biological, chemical, radiological, or nuclear substances (159-161). The pathophysiological mechanism must be understood and mitigated by the medical countermeasure candidate, and at least two animal models are required for the work, some of which were discussed in the previous subchapter. For diseases with "biological" etiologies, animal models that effectively recapitulate human disease must be used to provide safety and efficacy data for humanuse medical countermeasure candidates. To wit, EEEV strain V105 and VEEV strain INH 9813 were selected for their relevance to human disease, as both strains were isolated from human cases of the respective diseases.

In addition to the Select Agent status that belies the severity of EEEV and VEEV disease, the limited incidence of natural cases of EEEV and VEEV, and the fact that aerosols do not comprise a natural route of transmission, the evaluation of efficacy for potential medical countermeasures against both naturally acquired and inhalational EEEV and VEEV encephalitis must proceed in animals in concordance with the Animal Rule.

# 1.2.2 Inhalational Alphavirus Encephalitis in the Cynomolgus Macaque

Despite findings from review of the background literature pertaining to EEEV or VEEV in mice that demonstrate the feasibility of the murine model in the reconstruction of human-like VEEV disease phenotypes, aerosol exposures of mice typically produce more virulent, deadly disease; the increased mortality limits the utility of the mouse model for its generalizability to human disease in the context of the onset and resolution of encephalitic disease (67, 68, 99, 134, 162, 163). Other rodent models discussed previously in this work: hamster, guinea pig, or lagomorph models such as rabbits, suffer from the same problem, and other models such as hamsters and guinea pigs suffer from mortality from gastrointestinal disease (102).

Macaque species of nonhuman primates, such as rhesus (*Macaca mulatta*) and cynomolgus macaques (*Macaca fascicularis*), benefit from almost a century's worth of characterization studies not only encompassing anatomical and physiological features, but also in the context of immune responses to infectious diseases (18, 95). The earliest studies of EEEV and VEEV in the macaque models demonstrated that intracranial inoculation or intranasal inoculation with EEEV could cause fatal encephalitis in these subjects (15, 18, 72, 73, 95). The encephalitic disease produced by

aerosolized EEEV in cynomolgus macaques demonstrated a dose-dependent response in severity of encephalitic disease; febrile encephalitis ensued at approximately 3-4 DPI with almost uniform morbidity that would eventually require euthanasia (18, 138). Similarly, infection of cynomolgus macaques with VEEV demonstrates a mild febrile illness of a characteristically biphasic morphology, thought to reflect lymphotropic spread and invasion of the CNS by the virus, respectively (95, 138). The cynomolgus macaque model is not without its disadvantages, however. High doses of virus are required to establish disease by aerosol route relative to human infection, and the high cost of procuring and maintaining nonhuman primates in general, make work with cynomolgus macaques inherently challenging.

# **1.2.2.1 The Cynomolgus Macaque Model of EEV Infection**

Although most early nonhuman primate studies of EEVs occurred in the rhesus macaque model, a natural history study involving adult cynomolgus macaques challenged with aerosolized EEEV resulted in the development of a severe febrile illness with neurological signs of disease; the risk of lethality was increased with the administration of a higher dose of EEEV in the aerosol exposure (18, 66). A short litany of clinical laboratory findings in the same cohort of macaques revealed leukocytosis, increased electrolytes, and elevated blood urea nitrogen and alkaline phosphatase; likewise, histopathology was positive for lesions in the spinal cord, midbrain, and cerebral cortex (18, 66). Viral load of EEEV in the CNS was reported to attain titers of between  $10^8$ - $10^9$  pfu/g (164). Notably, the release of liver enzyme, or any documentation of any signs of hepatic dysfunction, remains absent from records of human EEEV disease. In the cynomolgus macaque model, extensive vasculitis was also seen to affect the brain parenchyma as well as microthrombi in certain cases (18).

The fevers manifested by cynomolgus macaques infected with aerosolized EEEV became apparent between 2-3 DPI, occurring concurrently with increase heart rate and blood pressure. Approximately two to three days after fever onset, at approximately 4-5 DPI, the fever would peak and plateau prior to the animal becoming moribund and requiring euthanasia by approximately 6 DPI (18). Plasma viremia in the EEEV-infected animals appeared between 2-4 DPI; virus was also found in the throats of whereas macaques infected with high doses of EEEV (18). Moreover, clinical observations of signs of neurological disease included documentation during the febrile period of the severe EEEV disease included involuntary tremor, a sign of encephalitis, in addition to more severe findings such as comatose states. The nature of neuronal tropism, damage, and outward signs of febrile viral encephalitis attest to the strength of the cynomolgus macaque as an animal model for EEEV disease. It is worth noting that in this prior study, the authors used the FL91-4679 isolate, not the V105 isolate used in this study.

In contrast, experimental studies of VEEV in the cynomolgus macaque model have included various routes of infection; as mentioned previously, as in other nonhuman primate models, VEEV causes a relatively mild, febrile illness in cynomolgus macaques (66). Infected macaques developed a biphasic fever and viremia, with signs of encephalitis presenting with gliosis upon examination during necropsy. Most helpfully, however, serial sacrifice studies have been conducted in the cynomolgus macaque model in the context of aerosol infection; at 1-4 DPI and subsequent pathology studies found widespread, fulminant infection of lymphoid tissues consistent with human VEEV disease and other models of VEEV disease (66, 95). Clinical laboratory findings also confirmed leukopenia with an average of 60% decrease of peripheral blood lymphocytes between 0-2 DPI (95). Pathology examination of brain parenchyma found little evidence of virus infection of the brain until 4 DPI, characterized by neuronal apoptosis/necrosis,

evidence of hemorrhage, and perivascular cuffing; locations positive for VEEV at the 4 DPI time point included the olfactory tubercule and the precommisure bundle of axons linking the two temporal lobes of the brain (66).

Fever in the VEEV-infected macaques developed approximately 1-2 DPI in the context of aerosol exposure to VEEV subtype IE, with the first febrile peak occurring during this time of onset, and a second febrile peak occurring 1-2 days after initial fever onset at approximately 3-5 DPI; Resolution of fevers typically occurred at 7-8 DPI with a reinstatement of baseline body temperatures, with some macaques experiencing bouts of hypothermia as well (95). In the cynomolgus macaque, plasma viremia of VEEV appeared to peak between 0-4 DPI, with a narrower window of between 1-3 DPI more characteristic of VEEV subtype IE (14, 15, 95). Clinical observations of VEEV-infected cynomolgus macaques found signs of encephalitis during the post-febrile period, particularly with the manifestation of involuntary muscle movements, diminished food intake (suppression of appetite), alongside less-tangible observations such as depression (14, 95). The cynomolgus macaque model of VEEV infection also warrants distinction as one of few large animal species, let alone nonhuman primate species, to have undergone vaccine trials for countermeasure candidates against VEEV that run the gamut of modified strains of TC-83 or C-84 to DNA and subunit vaccines (14, 96, 165), and inactivated recombinant viruses (Klimstra, unpublished).

The study of immune species present in the cynomolgus macaque during VEEV infection has also made an indelible contribution to the corpus of knowledge regarding the inflammatory and immune response to aerosolized VEEV infection. In a study of the brains of infected macaques, antigen-processing genes such as those related to MHC Class I were highly upregulated, likely in response to the propensity of VEEV to replicate promiscuously in the brain parenchyma following the onset of neuroinvasion (14, 166). Likewise, examination of brain tissue found increased expression of neural cell proliferation marker S100B in the brain parenchyma, as well as markers of inflammatory response, immune response, neurogenesis, and nerve-to-nerve synaptic transmission (14, 166, 167). The content of these studies extends into current investigative work and carries significant implications for the hypotheses that drive this work, and shall be discussed in further detail in the next subchapter.

#### **1.2.2.2 Current EEV Research in the Cynomolgus Macaque Model**

At the time of this writing, work has steadily continued in the investigation of EEEV and VEEV in the cynomolgus macaque model; for my master's thesis, I conducted a preliminary investigation into the utility of electrocardiography (ECG) data collected by radiotelemetry as a biomarker of CNS disease (138, 168). Although earlier studies of the cynomolgus macaque model employed telemetric monitoring of rudimentary aspects of cardiovascular function in the context of VEEV infection such as heart rate, my more recent work examined a large number of ECG metrics (14, 17, 18, 95, 138).

In aerosolized EEEV infection of cynomolgus macaques, the duration of the QRS complex was increased over its baseline values during the febrile period of disease from 3 DPI onward; this occurred despite the increase in heart rate during the febrile period, which typically induces a contraction or decrease in the duration of the QRS complex. The resultant increase, then, suggested autonomic dysregulation affecting the sinoatrial and atrioventricular pacemaker nodes of the macaque hearts in my previous study (138, 168). Similarly, in VEEV aerosol infection of cynomolgus macaques, recent work performed with ECG analyzed the RR-interval, the reciprocal of the heart rate; the RR-interval shows diminished variation during the biphasic fever that persists for at least a week after the resolution of the fever, suggesting that the function of the autonomic

nervous system is affected persistently by VEEV in the cynomolgus macaque; perhaps this effect might also appear in the case of cynomolgus macaques with severe courses of EEEV, but the lack of surviving individuals and by extension the lack of a recovery period made that investigation impossible (138, 168).

### **1.2.2.3** Inflammation and Immune Response in EEE and VEE

Studies of traditional biomarkers of infectious disease were also conducted contemporaneously with the work performed with electrocardiography. As addressed in the previous subchapter, cytokines, chemokines, and other biomolecules associated with inflammation and the immune response to alphavirus infection, demonstrate increased and variegated expression patterns depending upon the tissue under examination. Work performed on blood draws, i.e. blood chemistry and cell counts, revealed results consistent with previously-observed events in other animal models, for instance: increased blood urea nitrogen, alkaline phosphatase, and electrolytes such as sodium, (18, 66). However, in the cynomolgus macaque model of EEEV, little work has been performed specifically on the kinetics of cytokine release during the immune response.

The literature endorses elevated cytokines in blood and cerebrospinal fluid during the first four days' response to alphavirus infection, as seen in mouse models, of increased interferon gamma (IFN $\gamma$ , a Type II interferon), interleukin (IL)-1 $\beta$ , and IL-6 upregulation; minor players in the immune response also included IL-10, IL-12, tumor necrosis factor (TNF)- $\alpha$ , tissue growth factor (TGF)- $\beta$ , IFN $\alpha$  and IFN $\beta$  (Type I interferons), and matrix metalloproteinase (MMP)-9 (169, 170). Type I and II interferons, in addition to cytokines such as IL-1, IL-6, and MCP-1 comprise elements of the innate immune response to alphavirus infection (171, 172). In a cynomolgus macaque model of Chikungunya Virus (CHIKV), a disparate alphavirus with putative neurotropic characteristics, similar increases in Type I interferons, MCP-1 and IL-6 likewise occur, suggestive of conserved similarities in responses to alphavirus infection (173).

Differences in the interferon response, notably the Type I interferon response provided by IFN $\alpha$  and IFN $\beta$  in the earliest stages of infection may help determine the extent and severity of neuroinvasive disease (101). Specific to the CNS, EEEV and other alphavirus infection of neurons stimulates the production of IFN $\beta$  and IFN $\gamma$  in neurons and surrounding glial cells respectively, the latter interferon being a Type II interferon (71, 171, 174). Cytokines and chemokines produce the febrile response in response to direct infection of neurons by EEEV. The production of such cytokines and chemokines serves to recruit macrophages and activate glial cells; these events initiate and maintain a positive feedback cycle wherein upregulation of major histocompatibility complexes makes the CNS more permissive to the recruitment and diapedesis of additional inflammatory cells such as macrophages, natural killer cells, and neutrophils (175-177).

The latter finding of increased production of cytokines leading to increased morbidity and mortality falls in line with the paradigm of T helper 1 (Th1) cluster of differentiation (CD)4<sup>+</sup> T cell-based responses to infection. While Th1 cells produce IFN $\gamma$  and very broadly stimulates phagocytosis and intracellular killing, the IFN $\gamma$  also has the farther-reaching effect of inducing downstream secretion of proinflammatory cytokines/chemokines by nearby cells (38, 60, 100, 178-180). Characteristics of the host response surrounding alphavirus infection remain key to the understanding of viral pathogenesis as well as to the development of vaccines that confer protection from not only mortality from disease but also from features of severe alphavirus encephalitis.

# 1.2.2.3.1 Traumatic Brain Injury Species in EEE and VEE

The subject of neurodegeneration in the EEEV, and to a smaller extent, VEEV disease course in encephalitic disease motivates the investigation of the expression of genes and proteins typically active during or considered biomarkers of traumatic brain injury (TBI). A number of mechanisms belie TBI, including, at the cellular level: astrocytosis, gliosis, neuronal death, excitotoxicity, and extracellular membrane breakdown. Glial fibrillary acidic protein (GFAP) is an intermediate filament that is upregulated following traumatic brain injury and features prominently in concurrence with astrogliosis (181). Immunoblots as well as microvesicle and exome analysis have found increased GFAP concentrations in CSF in particular following injury (172). Leukemia inhibitory factor (LIF), represents yet another biomolecule produced in the brain during the course of alphavirus encephalitis (169). When neurons become damaged in the course of an encephalitic infection, surrounding support cells such as astrocytes or oligodendrocytes upregulate the expression of LIF in response to species such as TNF- $\alpha$  or IL-6 (182-184). The production of LIF stimulates neurogenesis in response to injury; LIF also activates the JAK/STAT pathway in a similar manner to interferons, and may thus also play a role in modulating the immune response to infection in the context of alphavirus encephalitis (184, 185). MMP-9, discussed previously, is a collagenase that demonstrates activity in a number of neurodegenerative disease processes (172, 186-188). On neuronal regeneration, MMP-9 is often found in developing axonal cones; however, during degenerative processes, it is responsible in part for breaking down Collagen IV and basement membrane and appears to reach peak expression at day seven postinjury (188). Transcription factors that encourage MMP-9 expression include IL-1 $\beta$ , NF-kB, and AP-1 in both neurons and glial cells (186, 189). The current collection of information regarding

the biomolecular milieu produced in encephalitic EEEV infection in macaques does not address the pattern of gene expression for cytokines or genes associated with TBI, and this investigation seeks to expand the pool of knowledge on that topic.

Regarding the milieu of biomacromolecules produced during the course of VEEV infection, least-squares geometric mean of samples taken at 1-4 DPI showed, in addition to MHC Class I upregulation, the upregulation by more than 4-log<sub>10</sub>-fold of IFN $\gamma$  responsive genes such as IFITM1, IFITM3, MX1, and STAT1 (166). The examination of peripheral blood mononuclear cells (PBMCs) revealed upregulated expression of apoptosis-related genes as well as upregulation of IL-1, IL-6, and TNF- $\alpha$  at days 3-4 DPI (167). These macaques achieved a peak viremia, as had been achieved in other studies of cynomolgus macaques, at 1-4 DPI (14, 54, 167, 190).

The body of knowledge pertaining to TBI for the cynomolgus macaque model of VEEV is similar to that for EEEV subsequent to neuroinvasion. VEEV most likely invades and infects peripheral lymphoid tissue prior to the infection of the CNS and subsequent neurodegeneration (101). While phenotypically, VEEV in cynomolgus macaques presents with a less severe encephalitic disease than for EEEV, the involvement of interferon-responsive genes in the macaque brain in response to VEEV infection several days after infection points to the possibility of the neurodegenerative aspects of the VEEV encephalitic disease being rooted in immune-based origins rather than in direct infection of brain tissue and virus replication though both mechanisms remain quite possible (166). The question of whether upregulation of MMP-9 and other genes associated with aerosol exposure of macaques occurs in the CNS remains to be answered.

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# **1.2.2.3.2** Circadian Disruption in EEE and VEE

The less severe course of neurological disease seen in VEEV as compared to EEEV neurological disease in the cynomolgus macaque also allows for the longer-term assessment of disruption of circadian patterns in the nonhuman primate model. Of particular significance to such studies lies the observation that cytokines such as TNF- $\alpha$  and IL-6 have the potential to modulate circadian patterns (191-193). IL-6, for example, tends to peak in serum concentration in the evening before an animal sleeps, and again before the animal wakes, with a transient peak at approximately midnight; the transient peak is thought to arise from peripheral blood mononuclear cells (191). Although whether circadian patterns are modulated by cytokines or cytokines are modulated by circadian patterns remains the subject of debate, the preponderance of studies surrounding cytokines and circadian regulation attest to the strength of the association. The impact of VEEV upon the health of the CNS has been shown to be deleterious in the macaque model, but no direct associations have been made between the biomolecules released during the immune response and/or i) the mechanisms that govern the circadian rhythm or ii) the circadian rhythm itself in this model. The scope of this work seeks to document circadian patterns as they arise in the cynomolgus macaque model and to determine as best as possible the disposition of the relationships that make up the neuroimmunology of VEEV infection. Perhaps circadian patterns themselves can offer an additional, qualitative means of gauging the onset and resolution of encephalitic disease and/or the efficacy of prophylactic or therapeutic candidates.

Of particular interest in the context of the development of medical countermeasures or prophylactic candidates against alphavirus encephalitis, specifically VEEV, are critical periods in the course of alphavirus pathogenesis wherein mitigation of the disease course can suppress the development of severe neurological disease and/or sequelae.

#### **1.2.2.4** Advanced Development of the Telemetered Macaque Model of Alphavirus

# Encephalitis

The most recent developments for the study of alphavirus encephalitis in the cynomolgus macaque model lie in the minimally invasive surgical implantation of the cynomolgus macaque with EEG- and ICP-monitoring modalities. While current research and ECG telemetry studies in the cynomolgus macaque model have provided important insights into the realm of EEEV and VEEV encephalitis, technical limitations abound with respect to the determination of the onset and resolution of encephalitic disease. In this vein, apart from telemetric fever measurements (by no means a specific measure of encephalitic disease), the determination of encephalitis often depends on intermittent and subjective veterinary staff observations of sickened animals rather than upon a continuous, objective, and/or quantitative measurement of key variables that can capture the complex and multi-modal manifestation of encephalitic EEEV or VEEV disease. To address this limitation and to extract more data from costly NHP studies, Dr. Tobias Teichert set out to develop a surgical method for the implantation of a radiotelemetry device for continuous monitoring of EEG and ICP in awake, conscious macaques, and with his assistance, I have established methods for the collection and analysis of EEG and ICP data suitable for detecting significant changes indicative of CNS disease. The data presented in this work comprise the largest such collection of continuously-collected EEG and ICP recordings in cynomolgus macaques exposed to aerosolized alphavirus.

This project seeks to evaluate the fidelity of a telemetered nonhuman primate model of inhalational encephalitic alphavirus disease caused by select agent pathogens where potential therapies cannot be evaluated in humans. Previous studies explored the utility of fever and cardiac function in monitoring the response in cynomolgus macaques to EEEV or VEEV. Here I will further extend the cynomolgus macaque model to evaluate the utility of telemetry modalities such as EEG which continuously measure electrical activity of the brain or ICP which measures pressure on the dura mater in the context of CNS diseases caused by EEEV or VEEV. EEG, ICP, thermometry, and activity (accelerometer) sensors facilitate remote, continuous monitoring of physiological status by lab staff and could prognosticate the onset of disease or alert investigators to changes in vital signs (17, 18, 95, 138). Although changes in EEG and ICP have been reported in human viral encephalitis, these parameters have not been explored using telemetry in a nonhuman primate model (19, 194). The use of telemetry could add new dimensions to the assessment of the severity of disease. The ability to i) distinguish between severe and nonsevere courses of disease and to ii) detect the onset and resolution of encephalitis in real time with biomarkers other than fever remain unexplored but are critical for the development of efficacious human EEEV and VEEV countermeasures if quantitative assessment of neurological status is to be considered.

## **1.2.2.5 Circadian Rhythm Disruption**

The circadian patterns of the cynomolgus macaque closely resemble those of humans; a potential application of the telemetered macaque model lies in its capacity to inform upon variegated circadian patterns, not only of EEG and ICP, but also expression of genes of interest (195). All cells have an internal circadian clock, regulated by a litany of transcription factors and biomolecules (196-199). Particular nodes of nervous tissues, colloquially termed "pacemakers," such as the suprachiasmatic nucleus (SCN) of the hypothalamus or the paraventricular nucleus (PVT) of the thalamus in the brain facilitate the entrainment of a 24-hour circadian rhythm dictated

by internal genetic circuits acting as biomolecular oscillators, modulated by external stimuli (196, 200, 201).

The timing, synchronicity, and circadian rhythm of the pacemakers can be altered by various stimuli termed "zeitgebers," which include light, food/meals, sleep, and biomolecular stimuli such as melatonin (202). The entrainment of circadian patterns, or the synchronization of cells from the peripheral tissues of the body to the central master clock, may be carried out through direct synaptic communication between pacemakers, or through endocrine communication via biomolecules dispersed to peripheral locations. The former means of entrainment typically serves to coordinate between pacemakers of the central nervous system; neural projections from the SCN to the PVT, for example, can extend as far as the cerebellar cortex and their coordination remains critical for sleep onset (203-206). Communication between central pacemakers and the periphery, however, can comprise a mixture of both methods. For example, regarding the regulation of a fundamental characteristic such as heart rate, homeostasis is maintained through the regulation of the autonomic nervous system by means of vasoconstriction, glandular secretion, and sinoatrial (SA) and atrioventricular (AV) pacemaker node modulation (207, 208). The impact of encephalitic disease upon the autonomic nervous system via hypothalamic dysregulation has been documented in numerous instances (35, 54, 56, 207, 208). Although regulation of these heart pacemakers receive input from the autonomic nervous system, the SA and AV nodes are themselves autonomous and generate synchronized electrical pulses independent of sustained input from the brain. A more concrete example of the endocrine regulation/coordination of peripheral tissues exists in the form of the hypothalamus-pituitary-adrenal (HPA) axis, which contains multiple levels of negative feedback control systems governing the release of catecholamines and glucocorticoids (209). The endocrine nature of the latter substance both provides feedback to the

hypothalamus as well as provides molecular inputs to cells in peripheral tissues (201, 209-212). Synaptic and endocrine entrainment of circadian patterns are not mutually exclusive mechanisms of entrainment; the synchronization of individual cellular molecular clocks, though a decentralized process, rely upon input from information relayed from the body's central pacemakers.

The bearing of circadian rhythmicity on health and disease branches into the study of neuroimmunology and holds important implications for research on EEEV and VEEV encephalitic disease in the cynomolgus macaque model. The effects of the cytokine IL-6 had previously been mentioned as a potential modulator of circadian rhythm (191). A number of cellular circadian regulators, expressed in most cells of the body but of particular importance in central nervous system tissues, have demonstrated variation in expression during courses of infectious diseases. The phrase "clock genes" encapsulates a wide variety of genes whose expression varies in a circadian fashion and whose expression levels in CNS are critical for maintaining the circadian rhythm of various biological metrics such as heart rate. A clock gene is usually defined as one whose product is required for the generation and/or maintenance of the circadian clock (200). Central regulation of the circadian clock occurs in the SCN, and gene expression of clock genes varies as a function of external zeitgebers such as light or food availability (197, 213). Genes that serve these circadian functions include Per1, Per2, Per3, Bmal1, Clock, CLK, CRBP1, CYC, CKI, Dec1, Cry1, Cry2, and Dec1 (198, 200, 202, 214, 215). Peripheral clock gene expression can also be attuned to internal parameters, however, and expression of such genes can vary from tissue to tissue, and from cell to cell (200, 202, 213, 215). Much regarding the relationship of the regulation of such genes to EEG and ICP in health and disease remains unknown, though studies in mice have demonstrated alterations in EEG delta power correlated with clock gene expression (197,

216, 217). This work shall be among the first to study circadian dysregulation in the cynomolgus macaque model of EEEV and VEEV.

### 1.2.2.5.1 Video Monitoring

The ability to continuously and remotely survey the health status of animal subjects via closed-circuit video recording affords a benefit to researchers and veterinary staff responsible for the monitoring of cynomolgus macaque behavior and health status. The value of such monitoring becomes especially evident as the course of severe EEEV encephalitic disease in the cynomolgus macaque has the documented epileptogenic features (18, 138). The ability to visually observe macaque behavior on a continuous basis, in an archivable fashion, offers a means of accounting for seizures that would otherwise not have been observed incidentally by laboratory or veterinary staff.

The cynomolgus macaque has seen application recently in models of epileptogenesis and induced epileptogenesis (218, 219). The onset of seizures in the cynomolgus macaque encompass numerous forms of seizures: tonic-clonic seizures, simple partial seizures, or complex partial seizures (218). While seizures associated with neurological diseases of non-infectious origins may show a greater breadth of such phenotypes, seizures most commonly associated with acute viral encephalitis, generally and specifically EEE or VEE, consist of generalized tonic-clonic seizures (74, 78, 81, 82, 220). Earlier studies on a cohort of telemetered cynomolgus macaques exposed to EEEV corroborated these observations from the literature (138, 168).

Recently, advances in computer vision and image processing techniques in combination with EEG examination in real time have augmented the ability of researchers to confirm the confluence of abnormal EEG in conjunction with abnormal behavioral or neurological signs of disease and measure the impact of treatments upon seizures (221). In this manner, raw EEG trace was assessed by researchers through computerized analysis for the purposes of pharmacology investigations (222, 223). For the purposes of this work, the telemetered macaque model with EEG, ICP, and video-capable technology allows for association studies to be performed relating signs of EEEV or VEEV disease to recordable epileptic events.

# 1.2.2.5.2 Pharmacological Studies in the Telemetered Macaque Model

Finally, one must remember the motivation for the use of the cynomolgus macaque as an animal model for the study of EEEV and VEEV: as a platform for the evaluation of potential medical countermeasure candidates. In the previous discussion of cynomolgus macaques as a model of EEEV and VEEV disease, pharmacological studies with cynomolgus macaques as platforms of study were only briefly mentioned (222, 223). Apart from applications for studying epileptogenesis, other studies including trials of antibiotics or pharmaceutical reformulations thereof also took place in the cynomolgus macaque model (224). Typical considerations for testing drugs for off-target effects, such as cardiovascular monitoring for arrhythmia in the form of QT-interval prolongation, was also carried out for the antibiotic moxifloxacin in a similar rhesus macaque study (225).

As previously mentioned, a historical study with rhesus macaques exposed to VEEV, though not implanted with EEG leads, documented deviations from normal EEG during the sick state; few quantitative differences were described and instead the authors relied on morphological differences between EEG traces (102). In the decades-long interim between that study and the present, a large corpus of basic science research pertaining to medical countermeasures against EEEV and VEEV has accrued, with therapeutics such as small molecule nucleoside analog drugs and antibody therapies, and virus-like particles or replicons suggested as vaccine candidates (112, 114, 226, 227). Study in the telemetered cynomolgus macaque model should allow for a more exacting characterization of disease and determination of differences by EEG and ICP in conscious and awake animals alongside the traditional documentation via physiological biomarkers. The study of viral pathogenesis and disease courses of EEEV and VEEV could demonstrate a

resolution or mitigation of febrile encephalitic disease either by a candidate therapeutic, or spontaneously in mild disease, making great strides in laying the groundwork for safety and efficacy trials.

# **1.2.3 Radiofrequency Telemetry**

The telemetered components of the telemetered macaque model rely on the culmination of several minimally-invasive implanted technologies: temperature sensors, EEG leads, ICP pressure transducers, and a triple-axis accelerometer geared towards the collection of activity data. With a focus on the health status of telemetered cynomolgus macaques subsequent to either EEEV or VEEV infection, EEG and ICP monitoring of the macaque in states of disease and health allow for the comparison and contrast of aberrant post-infection behavior to baseline, pre-infection data within the same subject. The EEG, in particular, emerges as a versatile and potent gauge of global brain electrical activity in the wake of alphavirus infection, while changes in intracranial pressure have been correlated with infection of the central nervous system, more specific to encephalitis than increased temperature, e.g. fever (19).

This subchapter constitutes a brief exposition that expands on some of the history and background behind the technology and instrumentation of radiofrequency telemetry whereas Chapter 3 will delve into some more technical aspects of the analysis.

# **1.2.3.1** Thermometry

The basis of the use of body temperature, as a core or axillary measurement, as a means of diagnosing disease was first detailed in a seminal publication by German physician Carl Wunderlich in 1870 (228). Contemporary replications of his work have expanded upon his work,

highlighting the capacity of the empirical observation of body temperature to represent a function of multiple variables such as age, gender, and time of day, in addition to state of health (229, 230). With modern forecasting and statistical methods, researchers and clinicians now have the power to assess the clinical state of a patient or subject with a much finer degree of accuracy and precision (229).

The technology behind digital temperature measurement rests in a temperature-sensitive element called a negative temperature coefficient (NTC) thermistor (231). As opposed to an instrument which produces an analog measurement of temperature, such as a mercury or alcohol thermometer, in which deformation of a fluid column relays thermal information to the researcher, the construction of an NTC thermistor instead relies on the conveyance of changes in the electrical resistance of a material in response to small changes in temperature. The term "negative" in NTC denotes that electrical resistance varies inversely and proportionally to temperature change. Comprised of a sintered ceramic semiconductor material, the thermistor is ideal for applications involving small temperature deviations of a few degrees or less, such as the measurement of body temperature.

The clinical significance of fever in the diagnosis of viral encephalitis cannot be overstated. Numerous studies in the recent past have documented the prominence of fever as a sign of alphavirus encephalitis, either as an accompanying sign of disease or as part of the prodrome of prognosticators that herald incipient neurological disease and also more generally with the vertebrate host response to infectious disease (17, 18, 95, 138, 232). Body temperature, as discussed previously, can vary collinearly with electrocardiographic metrics (138). Published work by Reed, et al. broached the use of telemetry in the cynomolgus macaque model by taking measurements of temperature every 15 minutes to record subjects' body temperature over a course of viral encephalitis (18). Therefore, the continuous monitoring of temperature in a cynomolgus macaque model of febrile alphavirus encephalitis remains intuitively cardinal to the study of the disease, such that a profile of the fever itself may be generated and analyzed for each animal infected and to facilitate group analyses. Several articles have documented that in the case of EEEV, VEEV, and WEEV infections, marked temperature elevations occur, ranging from 0.5-2.0°C depending upon the virus and the dose given (18, 64, 75, 95). Moreover, temperature provides insight into whether or not subjects have entered a state of terminal decline, as hypothermia afflicts moribund individuals (18, 138). The regularity of temperature lends itself to forecasting models and can be used to determine and quantify significant deviations from expected patterns to gauge the efficacy of medical countermeasures; the measurement of temperature thus provides a standard against which newer measures such as ECG, EEG, or ICP can be compared. The prime importance of temperature lies in its ease of observation, status as a cardinal clinical vital sign, and its ability to define the period of frank illness in the NHP model.

# **1.2.3.2 Electroencephalography**

As with many clinically-relevant technologies, the development of electroencephalography in humans was presaged by the research and development of the technological component of electrophysiology measurements in animals, including in nonhuman primates. Richard Caton, developed the fundamentals of electroencephalography in animals while his contemporary Adolf Beck performed craniotomies to place electrodes directly on the surface of the brain in animals, and also described spontaneous fluctuations and evoked electrical potentials (233-235).

The human electroencephalogram was first described in the modern biomedical literature by researcher Hans Berger in 1929, and whose work comprised a seminal contribution to the study of brain electrophysiology (236). Berger had originally taken his measurements in 1924, but his progress was impeded by his lack of background in either electrical or mechanical engineering. However, after independent validations of his work, the practical aspect of his work was recognized approximately a decade later, the key finding being that this method proved to be a noninvasive measurement of brain activity, with the utilization of only electrodes on the scalp.

The underlying premise being that the brain emitted electrical activity. His other works included the characterization of the alpha wave rhythm (the wave band between 7.8 and 13.28 Hz, as originally described), and the description of the beta blockade, or enhanced beta waveband activity, in association with saccadic eye movement (236-239).

His methodology consisted of inserting subcutaneous silver electrodes, one attached rostrally and one attached caudally, eventually replaced by the placement of silver foil electrodes that were eventually able to resolve voltages in the microvolt range (236). Were it not for the fact that the cathode-ray-tube oscilloscope was a contemporaneous development with the research and development of practical applications for the cathode ray tube, Berger's research may have progressed at a faster pace. As it was, Berger was unable to visualize his findings in real time due to the limitations of the technology.

The intervening time between Berger's first characterization of the human EEG has seen the development of standards and practices associated with the measurement and interpretation of EEG. For instance, the international 10-20 system of EEG electrode placement so named for the proportional distances between invariant anatomical poles relative to which the electrodes are placed (240-242). These locations include the naison (front of head), inion (rear of head), the auricle and tragus of the ear, and the mastoid process of the temporal bone (242). From these electrodes, raw EEG signal is collected and relayed for analysis. As mentioned briefly in an earlier subchapter, the signal processing minutiae pertinent to EEG analysis shall be described *in extremis* in Chapter 3, but the minimum set of information required for this explanation resides in the Fast Fourier Transform (FFT) (223, 243). For any given time series or signal in the time domain f(t), a set of frequencies ( $\omega$ ) exists such that a linear combination of basic trigonometric functions such as cosine or sine waves can reconstruct the original signal. The phase of each individual trigonometric function, in other words, the frequency, can range from infinitesimally close to zero, to infinity. To find the set of frequencies, as well as the magnitude or degree to which each frequency comprises a constituent of the original time series f(t), we may pass the time series signal through the following integral (Equation 1) in which  $F(\omega)$ 

$$F(\omega) = \int_{-\infty}^{\infty} f(t)e^{-i2\pi\omega t} dt$$
(1)

represents the aforementioned magnitudes as a function of frequency. Figure 4 describes this process of isolating specific frequencies; once isolated, ranges of frequencies can be re-transformed and resolved back into time-domain signals. In the context of EEG analysis, certain events, such as sleep spindles or muscle artifacts, occur on different wave bands and can thus be isolated for the examination of only the events of interest (244-246). The processing and decomposition of raw



Figure 4. Discrete Fast Fourier Transform.

This graphic illustrates the process of transforming a signal f(t) in the time domain, in this example, an EEG signal, into discrete frequency wave bands of neurophysiological significance. Modified from (247).

EEG data according to several pre-defined frequency wave bands corresponding to different aspects of brain function has a storied history that goes back to Berger's initial experiments (237). Frequency wave bands are enumerated, in ascending order of frequency: delta: [0.5 - 4Hz], theta: [4 - 8Hz], alpha: [8-12Hz], and beta: [12-30Hz], within the scope of this work (244, 248-251). Some sources define separately sigma wavebands [12-16Hz] and gamma wavebands [30-40+Hz] (243, 245, 246). The beta and gamma band can overlap with frequencies encountered in the range of electromyographic detection of motor unit firing, and can inadvertently cloud the pool of data with muscle-associated artifact (252-255).

The significance of slow-wave frequencies such as those covered by the delta and theta ranges, lies in their association with non-rapid eye movement sleep (NREM), during which delta wave signal peaks (223, 243, 256). Slow-wave frequencies are associated, as depicted in Figure 4, with restful states with decreased conscious thought and focus. In contrast, higher frequency waves are associated with higher brain functions, voluntary movement, feedback, and muscular coordination, as previously discussed (252-255). As these aspects of brain electrophysiology tend

to vary with a circadian or diurnal pattern, this feature of the EEG frequency spectrum attests to its relevance for the characterization of circadian cycles within the cynomolgus macaque model.

Finally, abnormal EEG has been documented in a number of acute viral encephalitic disease, not only in alphavirus encephalitis. As mentioned previously, a series of researches regarding seizures appeared to manifest generalized or tonic-clonic seizures in humans and a variety of animal models of viral encephalitis as a common feature (74, 78, 81, 82, 220). Similarly, a number of findings, including the initial foray into electroencephalography in a large cohort of rhesus macaques, documented in the studies that commented upon specific pathology evident on EEG "generalized slowing" of electrical activity in the brain, in addition to aberrant delta wave band activity (62, 63, 66, 78, 102, 106). EEG detects the electrical activities in across large numbers of cells in the brain, and can be used to assess brain activity in both healthy subjects and in pathological subjects. A cardinal goal for this dissertation and work related thereto shall lie in outlining and investigating a specific and clearly defined set of EEG parameters in the context of acute alphavirus encephalitis.

# **1.2.3.3 Intracranial Pressure**

The monitoring of ICP has intuitive applications in the context of the assessment of acute viral encephalitis; studies in adult humans have produced demonstrable, replicable increases in ICP in encephalitis of viral origins (19). In humans, pathological elevation of ICP comprises one of many forms of response to acute viral encephalitis. The Monro-Kellie hypothesis posits that the skull and spinal column make up a relatively fixed volume comprised of three main components: blood, CSF, and brain (257, 258). If the fluid in any one of these compartments increases in volume beyond the ability of homeostasis to correct it, ICP will increase. In order to measure intracranial pressure on a living animal, a clinician can conduct a lumbar puncture or perform an ophthalmic

examination to measure optic cup-to-disk ratio for evidence of papilledema, though the latter represents an indirect measure of raised ICP. The direct measurement of ICP requires the insertion of a pressure transducer into or over the brain. Epidural transducers provide a minimally-invasive method of doing so, as the dura mater that encloses the brain is not breached, minimizing the risks of infection and/or hemorrhage. In EEEV or VEEV infection, elevations in ICP are expected, though pressures are generally not measured either in the clinic or during the course of animal subject research due to lumbar punctures being ill-advised for patient care and due to animal husbandry best practices (19, 78, 83, 259).

Assuming a rough conservation of fluid in the body during a finite time period and the need to maintain sufficient oxygen perfusion to the brain, the following equation (Equation 2) can be applied to ICP:

$$ICP = MAP - CPP \tag{2}$$

wherein ICP represents intracranial pressure, MAP denotes mean arterial pressure, and CPP denotes cerebral perfusion pressure, or the net pressure gradient sufficient to perfuse the brain with oxygen, typically presented in units of mm Hg (260). The equation implies that measured ICP derives a substantial constituency from a vascular component, herein termed "vascular ICP." Although the equation describes mean arterial pressure as the key component of the vascular ICP, one may surmise that vascular ICP does not remain constant – as might be expected, individual pulsations, driven by heartbeat, can be detected and plotted as a function of time. This measurement of ICP has its own morphology, known as the vascular ICP wave. The wave consists of the slight trimodal shape of each pulsation that reflects the arterial pulse from the carotid (P1),

the reverberation of this pulse off the brain parenchyma, or the tidal wave (P2), with the final segment recording the closure of the aortic valve of the heart (P3) (261, 262).

As intracranial pressure is intrinsically an analog measurement that varies temporally and positionally, one may rightly imagine that the transduction and digitization of this signal in real time may present several technical challenges. Writ large, the fields of neurosurgery and clinical neurology have a vast array of techniques at their disposal for the measurement of intracranial pressure, spanning invasive and noninvasive techniques such as ventriculostomy or transcranial doppler, respectively (263). Microtransducers of the Spiegelberg sensor type demonstrated no risk of hemorrhage in human patients and minimal risk of infection (264). Though invasive techniques do carry higher risk profiles for hemorrhage and infection, microtransducers of the type used in this study demonstrate a high degree of measurement accuracy (263). However, all types of ICP instrumentation remain potentially vulnerable to the problem of "zero-drift," that is, the difference between the displayed ICP value between the time the sensor is calibrated and the time the sensor is removed. For the purposes of this study, a pioneering technique was developed through the supervision of talented faculty and veterinary staff to re-purpose a dual-modality telemetry implant for dual-function EEG and ICP monitoring of the type used in cardiovascular studies, with an arterial blood pressure transducer utilized for ICP monitoring.

A perhaps more direct, intuitive link exists between the disease state of encephalitis and intracranial pressure measurement than between the analogy of encephalitis and electroencephalography. As infection of the brain sets in, inflammation and the immune response to alphavirus infection will putatively drive an increase in ICP (19, 98, 194, 262). The utility of the cynomolgus macaque model of inhalational alphavirus encephalitis shall lie then, in the
charting and characterization of the relationship of ICP increase to fever and the onset of signs of alphavirus encephalitis.

## 2.0 Hypothesis and Specific Aims

As the FDA Animal Rule prioritizes the recapitulation of human disease phenotypes in relevant animal models, the dichotomy of the severity of EEEV and VEEV alphavirus disease raises a serendipitous experimental lens under which to consider severe and nonsevere disease phenotypes in the macaque model. Practically speaking, macaques with severe EEEV do not outlive the human study endpoint; therefore, the longer lifespans of macaques infected with milder VEEV offer a prolonged period of study under which differences in circadian or diurnal regulation may be more closely scrutinized. Similarly, macaques with severe EEEV can present with seizures or other signs of severe neurological disease, while VEEV-infected macaques do not exhibit quite so severe manifestations of disease. Finally, long-term survivorship as seen in VEEV-infected macaques also offers the opportunity to study the relationship of viremia to signs of neurological disease visualizable by EEG or ICP monitoring. These aspects of the EEEV/VEEV model of alphavirus encephalitis in the cynomolgus macaque show promise for the utility of telemetric surveillance of the disease course as indicators of encephalitis and disease outcome.

The overarching goal of this project lies in the further characterization and interrogation of a cynomolgus macaque model of alphavirus encephalitis, to study the natural history of alphavirus encephalitic disease and to fulfill the unmet need for determinants of and/or guidelines for therapeutic intervention. A number of questions remain unaddressed insofar as the relationship between changes detectable by telemetry and alphavirus disease pathogenesis. Patterns apparent in EEG and ICP change alongside the febrile disease course, but relationships of these signs to temperature and traditional biomarkers remain unexplored and/or unelucidated. The relationship between radiofrequency telemetry and the current mechanistic understanding of CNS infection by EEEV and VEEV remains similarly poorly understood. Based on these unmet needs, the cardinal goals for this dissertation include independent investigation of the role of EEG and ICP telemetry modalities as a trigger for treatment or intervention during a course of acute febrile aphavirus encephalitis and the role of telemetry as an indicator of the onset and/or resolution of alphavirus encephalitis.

I hypothesize that in cynomolgus macaques (Macaca fascicularis) exposed to EEEV and VEEV, changes in fever, EEG, and ICP detectable by radiotelemetry will correlate with neurological signs of disease and be useful in evaluating potential medical countermeasures against EEEV/VEEV in the cynomolgus macaque model. I will test this hypothesis with the following specific aims:

# 2.1 Specific Aim 1: Determine Whether Telemetric Features of EEEV Disease Course Provide Means of Predicting the Onset of Febrile Encephalitis

Severe EEEV manifests with neurological signs such as seizures. Based on data from human cases, I hypothesize that in the cynomolgus macaque, slow-frequency-predominant EEG and increased ICP related to febrile onset will forecast severe courses of EEEV in comparison to mock and sublethal infection. To evaluate this hypothesis, I will:

a. Conduct power-band analysis of the raw EEG traces from each animal to determine if characteristic signs of EEEV encephalitis are observable before or during febrile encephalitis.b. Conduct a logistic regression to determine the relationship between seizures or other signs of severe disease and the appearance of EEG/ICP deviations from baseline.

c. Characterize the relationship between measured ICP and the fever profile for each animal.

d. Confirm courses of severe and nonsevere disease through transcriptional analysis of cytokine genes, genes of circadian regulation, and genes putatively responsive to traumatic brain injury.

# 2.2 Specific Aim 2: Determine Whether Telemetric Features of the VEEV Disease Course Provide Means of Predicting the Onset of Febrile Encephalitis

VEEV produces a less-lethal course of disease than does EEEV with ample time for the study of disrupted circadian rhythm. Based on reports from human cases, I hypothesize that VEEV infection produces radiotelemetry signals: EEG and ICP changes in macaques that reflect changes similar to those in human disease. Pursuant to this aim, I sought to:

a. Conduct power-band analyses of the raw EEG traces from each animal to determine if characteristic signs of VEEV encephalitis are observable before or during febrile encephalitis.b. Characterize the degree of circadian rhythm disruption during VEEV disease as captured by EEG/ICP deviations from baseline.

c. Characterize the relationship between measured ICP and the fever profile for each animal.

d. Determine whether EEG and/or ICP changes correlate to bloodborne or CSF viremia.

# 2.3 Specific Aim 3: Utilize the Telemetered Macaque Model as a Means Of Evaluating the Efficacy Of Medical Countermeasure Candidates

Vaccine and therapeutic candidates against EEEV and VEEV consist of numerous species that include both small molecule inhibitors as well as monoclonal or polyclonal antibodies. I hypothesize that EEG and ICP signals will be useful in determining the efficacy of a medical countermeasure against VEEV in conjunction with temperature, lymphopenia, and viremia. To investigate this, I sought to:

a. Optimize telemetry windowing periods for disease detection.

b. Implement pre-clinical studies with medical countermeasure intervention and determine whether resolution of VEEV encephalitis in treatment groups is identifiable by telemetry.

### **3.0 Materials and Methods**

Large sections of this chapter are incorporated into the manuscript: "Applications of minimally invasive multimodal telemetry for continuous monitoring of brain function and intracranial pressure in macaques with acute viral encephalitis," by Ma, et al. currently in preparation for publication in a peer-reviewed journal at the time of this writing. With respect to biological safety, all virus manipulations occurred with PPE, and in animal suites within the RBL, which contained holding rooms for macaques as well as the physical locations for telemetry hardware, powered air-purifying respirators (PAPRs) were worn over Tyvek<sup>®</sup> high-density polyethylene (HDPE) hooded coveralls, rubber boots, and double layers of nitrile gloves as personal protective equipment, with the previously mentioned 1:128 Vesphene IIse (Steris Corporation, Catalog No. 646101) to water dilution for disinfection when exiting suites.

# 3.1 Statement on Animal Care and Use

This work was approved by the University of Pittsburgh Institutional Animal Care and Use Committee (IACUC) under protocol numbers 16026773, 17100664, and 18113802, respectively, and progressed in complete concordance with Animal Welfare Act Regulations and the Guide for the Care and Use of Laboratory Animals (265). The University of Pittsburgh is fully accredited by the Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC). The care and treatment of the macaques also proceeded in full accordance with the guidelines set forth by the National Institutes of Health (US Department of Health and Human Services) for the care

and use of laboratory animals. All surgical procedures were performed under isoflurane gas anesthesia, and all efforts were made to minimize suffering; surgical procedures will be covered in fuller detail in a subsequent subchapter.

Housing and husbandry of the cynomolgus macaques proceeded as follows: macaques were singly housed in quad-occupancy metal cage banks with absorbent pellet bedding and food forage in a facility for these studies with excreta and food/fluid intake monitored daily. Environmental conditions prevailed at a constant 22°C, with a weight-based diet formulation of Purina Monkey Chow and water *ad libitum*. The conditions under which the macaques were housed provided a binary day-night duty cycle, during which room lights were powered on for 12 hours beginning at 6:00AM each day, and powered off for 12 hours at 6:00PM. Enrichment consisted of food enrichment, puzzles and toys, as well as audio-visual stimulation in the form of several hours of television per day.

For clinical observations following exposure to virus or mock aerosols, macaque welfare was assessed by a series of ordinal scales for: neurology, activity, and temperature (17, 18, 95). All scoring scales ran from 1-6, with the scoring system provided in Appendix C. The neurology scale accounted for signs of neurological disease such as involuntary tremor, gait imbalance, nystagmus, head pressing, seizures, and coma. The activity scale accounted for posture, facial expressions, responses to stimuli, and interactions with observers. Finally, the temperature scale accounted for core temperature set at standard deviations above or below mean core temperature to detect fever or hypothermia. The cumulative score determined by summing all three scales' scores was used to determine whether a macaque required more frequent observation or was at risk of imminent death which triggered humane study endpoint.

#### **3.2 Preparation of the Telemetered Macaque Model**

This subchapter and its subsections address the technical aspect of the implantation of and data collection with the telemetry system used, as well as the processing and analysis of the data collected. Several prerequisite questions required answers before substantial analyses could be performed with the telemetered macaque model. Were the EEG and ICP data stable within the same macaque, and if so, did they remain stable over the course of many days? Did systematic circadian variation of EEG and ICP signals exist? How consistent were EEG and ICP responses between macaques? These questions guided the course of study and played a critical role in the testing and determination of whether EEG and ICP changes could provide investigators a reliable biomarker with which to probe the health status of macaques experiencing alphavirus encephalitis. The subsequent discourse focuses on the description of typical frequency spectra for EEG recordings and baseline ICP measurements and evaluation of deviations from EEG and ICP baseline and the nature of these deviations.

# **3.2.1 Radiofrequency Telemetry**

Table 1 provides a comprehensive survey of all 41 cynomolgus macaques with singlemode and dual-mode telemetry implants described in this work. Certain macaques were implanted with thermometry-only implants and were used for other experiments and shall be described in the periphery of applicable chapters. Baseline data collection periods consisted of at least 3 days of continuous data collection with a minimal amount of interruption (<20-30 minutes/day of stopped recording for data transfer). This data from the baseline period served as the basis for characterization of normal cynomolgus macaque EEG and ICP. Data from the two mock infected macaques served as the basis to evaluate the stability and consistency of the relevant measures in the absence of an infection.

Macaque	Age (yr)	Weight (kg)	Virus	Inhaled Dose (PFU)	Neurologic al Disease (Y/N)	Implant Model/Type
M115-16 ♂	6	7.8	Mock	-	N	M11: EEG/ICP
M116-16 🖒	5	7.2	Mock	-	N	M11: EEG/ICP
M120-16 ♀	6	3.8	EEEV V105	1.08 x 10 <sup>7</sup>	Y	M01: EEG
M123-16 ♀	5	4.8	EEEV V105	1.50 x 10 <sup>7</sup>	N	M01: EEG
M57-17 ♂	5	6.0	EEEV V105	3.71 x 10 <sup>8</sup>	Y	M01: EEG
M58-17 👌	5	5.5	EEEV V105	1.02 x 10 <sup>9</sup>	Y	M01: EEG
M160-17 👌	6	6.6	EEEV V105	1.37 x 10 <sup>9</sup>	Y	M11: EEG/ICP
M163-17 👌	5	8.1	EEEV V105	2.50 x 10 <sup>10</sup>	Y	M01: EEG
M108-18 ♀	6	3.4	EEEV V105	1.69 x 10 <sup>7</sup>	Ν	M11: EEG/ICP
M110-18 ♀	6	3.5	EEEV V105	1.50 x 10 <sup>7</sup>	Ν	M11: EEG/ICP
M113-18 ♀	7	6.3	EEEV V105	6.34 x 10 <sup>6</sup>	Y	M11: EEG/ICP
M114-18 ♀	5	3.1	EEEV V105	7.64 x 10 <sup>6</sup>	Y	M11: EEG/ICP
M118-18 👌	8	7.5	EEEV V105	9.87 x 10 <sup>6</sup>	Y	M11: EEG/ICP
M1-19 ♀	2	2.3	EEEV V105	1.03 x 10 <sup>8</sup>	Y	M11: EEG/ICP
M2-19 ♀	2	2.3	EEEV V105	1.92 x 10 <sup>8</sup>	Y	M11: EEG/ICP
M3-19 ♂	2	2.7	EEEV V105	3.76 x 10 <sup>8</sup>	Y	M11: EEG/ICP
M4-19 ♂	2	2.7	EEEV V105	6.70 x 10 <sup>7</sup>	Y	M11: EEG/ICP
M121-16 ♀	6	3.6	VEEV INH-9813	5.93 x 10 <sup>6</sup>	Y	M01: EEG

 Table 1. Macaque Cohort Characteristics.

Table 1 (cont'd)						
M122-16 ♀	5	3.2	VEEV INH-9813	1.49 x 10 <sup>7</sup>	Y	M01: EEG
M53-17 ්	6	5.4	VEEV INH-9813	1.90 x 10 <sup>8</sup>	Y	M01: EEG
M54-17 ්	5	6.4	VEEV INH-9813	2.48 x 10 <sup>8</sup>	Y	M01: EEG
M111-18 👌	6	7.8	VEEV INH-9813	3.16 x 10 <sup>7</sup>	Y	M11: EEG/ICP
M112-18 👌	6	8.4	VEEV INH-9813	2.00 x 10 <sup>7</sup>	Y	M11: EEG/ICP
M115-18 👌	5	6.7	VEEV INH-9813	1.58 x 10 <sup>7</sup>	Y	M11: EEG/ICP
M116-18 👌	9	6.9	VEEV INH-9813	6.31 x 10 <sup>7</sup>	Y	M11: EEG/ICP
M107-18 ♀	5	3.5	VEEV INH-9813	2.90 x 10 <sup>6</sup>	Y	M11: EEG/ICP
M109-18 ♀	5	3.7	VEEV INH-9813	9.51 x 10 <sup>6</sup>	Y	M11: EEG/ICP
M96-19 👌	2	2.5	VEEV INH-9813	1.35 x 10 <sup>7</sup>	Y	M01: EEG
M97-19 ♂	2	2.4	VEEV INH-9813	3.47 x 10 <sup>7</sup>	Y	M01: EEG
M98-19 👌	2	2.4	VEEV INH-9813	5.13 x 10 <sup>6</sup>	Y	M01: EEG
M99-19 👌	2	2.6	VEEV INH-9813	3.98 x 10 <sup>6</sup>	Y	M01: EEG
M100-19 👌	2	2.8	VEEV INH-9813	1.74 x 10 <sup>7</sup>	Y	M01: EEG
M101-19 ♀	2	2.6	VEEV INH-9813	1.82 x 10 <sup>7</sup>	Y	M01: EEG
M102-19 👌	3	2.6	VEEV INH-9813	3.09 x 10 <sup>6</sup>	Y	M01: EEG
M103-19 👌	2	2.5	VEEV INH-9813	6.17 x 10 <sup>6</sup>	Y	M01: EEG
M104-19 ♀	3	2.9	VEEV INH-9813	4.47 x 10 <sup>6</sup>	Y	M01: EEG
M106-19 ♀	2	2.6	VEEV INH-9813	6.61 x 10 <sup>6</sup>	Y	M01: EEG
M107-19 ♀	2	2.8	VEEV INH-9813	2.51 x 10 <sup>6</sup>	Y	M01: EEG
M108-19 ♀	3	2.7	VEEV INH-9813	1.70 x 10 <sup>7</sup>	Y	M01: EEG
M109-19 ♀	3	2.2	VEEV INH-9813	1.35 x 10 <sup>7</sup>	Y	M01: EEG
M110-19 ♀	2	2.4	VEEV INH-9813	9.33 x 10 <sup>6</sup>	Y	M01: EEG

The surgical procedure for the implantation of all Physiotel Digital Telemetry implants is detailed in the subsequent paragraphs; Table 2 represents a parts list specifically for the surgical procedures with details of manufacturers, part numbers, and specifications. Implantation surgeries were performed by Dr. Tobias Teichert and Dr. Anita Trichel, as well as CVR and DLAR veterinary technicians. In preparation on the day of surgery, each macaque was given 0.04 mg/kg atropine intramuscularly (IM) to reduce mucus secretion. Anesthesia was induced with 20 mg/kg ketamine hydrochloride, IM. In preparation for surgery, an intravenous (IV) catheter for 0.09% normal saline was inserted into the greater saphenous vein, and the macaques were intubated with a tracheal tube for oxygen (1.0–1.5 L/min) and gas anesthesia. The macaque's head, neck and upper back were shaved and the macaque was transferred to the surgical suite and maintained on gas anesthesia (Isoflurane, 0.5–3.0%) throughout surgery. The macaque was positioned in a fivepoint stereotax to ensure positional stability during surgery. The incision sites were aseptically prepared with betadine and chlorhexidine scrubs, and draped with sterile drapes. Heart rate, respiratory rate, pulse oximetry, and core temperature were monitored continuously until the end of surgery. Incisions were made to expose the surgical sites on the scalp and on the upper back between the scapulae slightly off midline.

Table 2. Surgical Materials List.

Part	Manufacturer	Model/Catalog Number	Notes
Stereotaxic Frame Assembly: Cat/Monkey	David Kopf Instruments	Model 1430-B	
Upright Post and Clamp Assembly	David Kopf Instruments	Model 1725	
Intracellular Base Plate Assembly	David Kopf Instruments	Model 1711	

Table 2 (cont'd)					
Copalite Varnish	Temrex Corporation	200-400001V	0.50z Bottle		
Titanium Bone Screws	Crist Instruments Co., Inc.	6-YXC-035	EEEV V105		
Gold Plated Amphenol Pin	DIGI-KEY	609-2814-ND	1mm Diameter		
Dental Acrylic	Lang Dental Manufacturing	1230FIB			
Drill	Veterinary Orthopedic Implants	20020	Hand Drill, Quick- Coupling Driver Handle		
Coupling Bits	Veterinary Orthopedic Implants	10006	1.5mm to 2.0mm Quick-Coupling Bits		

A ~7cm long incision was made down the midline of the head from ~5mm posterior of the ocular ridge to the occipital ridge. Superficial fascia and temporalis muscle were separated from the calvaria using sharp surgical spoons. Residual tissue was thoroughly scraped from the bone, and the calvaria itself was dried with sterile gauze. A 5cm long incision was subsequently made between the scapulae on the macaque's upper back. With blunt dissection, a subdermal pocket superficial to the trapezius muscle was formed of sufficient size to contain the telemetry transmitter (Data Sciences International (DSI) Model No. M01, M11, or L11). As mentioned previously, implanted transmitters contain a temperature sensor and an accelerometer designed to track body movement. A tunneling rod was inserted in the caudal aspect of the cranial cut and tunneled to the pocket between the scapula. The two EEG lead wires and the ICP pressure sensor were threaded from the transmitter into the tunneling rod and pulled underneath the skin rostrally to the cranial cut.

The locations for the EEG screws were identified relative to landmarks on the skull. The position of the anterior EEG screw ( $F_4$ ) was identified just anterior of the coronal suture, approximately 0.5cm to the right of midline. The position of the posterior EEG screw ( $O_1$ ) was identified just anterior of the occipital ridge, approximately 0.5cm to the left of the midline.  $F_4$  and

 $O_1$  refer to positions on the 10-20 EEG map (240). The location of the intracranial pressure (ICP) sensor was identified over the right hemisphere between the anterior EEG electrode and the occipital ridge. To reduce the likelihood of subsequent tissue growth or granulation underneath the implant, circular regions of ~1cm diameter around these locations on the skull were sealed with three layers of copalite varnish (Temrex Corporation).

Holes for the EEG screws were drilled using a 1.2mm surgical hand-operated drill. The self-tapping titanium EEG bone screws (Crist Instruments Co, Inc. 6-YXC-035) were fastened in place at a depth that would allow them to touch but not dimple the underlying dura. A 1mmdiameter, 2mm-deep hole had been drilled in the center of the internal hex-head of the screw prior to the surgery. The lead wires of the DSI transmitter were affixed to the EEG screws by means of a 1mm amphenol pin that was subsequently soldered to the EEG leads. After protruding portions of the amphenol pin and excess solder were clipped with pliers, the screws and pins were covered with dental acrylic to prevent damage and to preserve the integrity of the lead junctions. The access hole for the ICP transducer was drilled using a 2.5mm surgical drill. An electric drill with a dental drill-bit was then used to file down the ridge of the posterior aspect of the hole and to provide an approach parallel to the dura for the ICP sensor wire. A small plastic probe was inserted to carefully loosen the attachment of the dura to the skull. After removing the protective sleeve, the ICP sensor was carefully inserted into the space between dura and skull. The hole and the ICP apparatus were likewise sealed and covered with dental acrylic. The sites of instrumentation placement on the macaque cranium are illustrated in Figure 5. Post-operative care consisted of 3 days' administration (PRN) of IM buprenorphine for analgesia and 5 days' administration (BID) of IM cefazolin for infection control.



Figure 5. Telemetry Instrumentation Placement Sites.

A) EEG biopotential electrodes; locations marked on macaque skull, top-down view. Anterior/cranial aspects face the right of the page, Posterior/caudal aspects face the left of the page. Pink outlines illustrate profile of dental acrylic cone used to encase sensors. B) Same as previous, but for EEG-ICP dual biopotential electrodes. C) Location of implanted telemetry transmitter over macaque scapulae. (CREATIVE COMMONS LICENCE Servier Medical Art by Servier is licensed under a Creative Commons Attribution 3.0 Unported License) Figure 6 provides an illustration of the radiofrequency telemetry implants used in the experiments.



Figure 6. Radiofrequency Telemetry Implants.

A) M01/M11 Physiotel Digital Telemetry implant. The two model numbers have the same physical profile. B) L11 Physiotel Digital Telemetry Implant. The L11 has an approximately three-fold longer battery life than the M01/M11.

The telemetry computer, hardware, and receivers were positioned adjacent to the macaque housing. The core collection hardware consisted of a desktop workstation connected via ethernet cable to a communication link controller (CLC) to transceiver units (TRX-1) mounted on the cage banks. The transceivers facilitated the reception of signal from the implanted M01/M11/L11 transmitters, to be relayed and logged in real time to the Ponemah software package (v.5.20 SP8; DSI). Likewise, the Noldus Media Recorder (Noldus, v.2.6) video interface facilitated the recording of macaque behavior via closed circuit cameras (Axis Model No. M-1144/M-1145) placed at fixed positions in front of the singly-housed macaques. Although video was not recorded for every macaque, the review of video, as well as in-person observations of food/water intake, excreta, activity, signs of neurological aberrations, and general appearance, played an integral part of clinical scoring as described for macaque welfare checks. Data recorded in Ponemah could be

viewed in real time through a graphical user interface with a multichannel display of biopotential modalities including EEG and ICP, alongside temperature and activity. However, for analysis purposes, raw data was transferred through a secure intranet to an analysis desktop outside of BSL-3 space. From the raw data, users can specify sampling rates for exported data; additionally, the export of raw data to the NeuroScore software platform (v.3.0.0, DSI) allowed for the rendering of the data *in toto* via batch export into the European Data Format (.edf) file extension, readable in MATLAB via the EEGLab suite and associated plugins (250). Further processing in MATLAB consisted of frequency decomposition of the raw EEG signal into delta, alpha, and beta wave band channels and reconstitution into traces in the time domain.

Although every effort was made to keep recording continuous, significant signal drop-offs occurred for some macaques when the desktop computer crashed. In some cases, it was possible to salvage data, but for other macaques, the loss of data was significant enough such that their results required censoring from group-wide analyses. Table 3 provides a materials list for all hardware and software components required for telemetry data collection and data processing.

Part	Manufacturer	Model/Catalog Number	Notes
PhysioTel Digital RF Transmitter Model No. M01	Data Sciences International	M01	EEG & Temperature Only, ~40 day battery charge
PhysioTel Digital RF Transmitter Model No. M11	Data Sciences International	M11	EEG/ICP, and Temperature ~40 day battery charge
PhysioTel Digital RF Transmitter Model No. L11	Data Sciences International	L11	EEG/ICP, and Temperature ~105 day battery charge
Ponemah Software Package	Data Sciences International	v.5.20 SP 8	
NeuroScore	Data Sciences International	v.3.0.0	

Table 3. Telemetry Materials List.

Table 3 (cont'd)			
Communication Link Controller	Data Sciences International	272-8000-001	CLC Firmware version 0.1.28
Radiofrequency Telemetry Transceiver	Data Sciences International	272-9000-001	Includes proprietary TRX-1 to Ethernet Cables
Ambient Pressure Reference (APR-1) Module	Data Sciences International	275-0020-001	Measures ambient air pressure
E2S-1 Module	Data Sciences International	275-0248-001	Communicates between Ethernet switch and APR-1
Telemetry Acquisition Computer	Lenovo	Thinkstation Desktop	64-bit CPU
Ethernet Switch/Router	Cisco	SF100D-16P	
Cat6E Ethernet Cables	-	-	Color-coded if purchased from Data Sciences International
Axis Closed Circuit IP Camera	Axis	0436-001-01 / 0591-001-02	Models M1144-L / M1155-L
Noldus Media Recorder	Noldus	v.2.6	
MATLAB	Mathworks	R2018b	
PRISM	GraphPad	v.8.0.0	

# **3.2.1.1 Thermometry Methods**

The methods for determination of significant temperature deviations was described in previous work (168). Briefly, the temperature modality in the implants are sampled at 10 Hz, and as with other modalities, a baseline recording period commenced at least three days prior to each individual macaque's exposure to either mock or virus-containing aerosol. Values for body temperature outside two standard deviations of mean baseline temperature were considered indicative of febrile or hypothermic status. As described above, the data were transferred to MATLAB from the raw data, and missing or unphysiological values (i.e. 0°C or >41.5°C) were censored out.

It was in the context of temperature forecasting for the purposes of establishing deviations from baseline that autoregressive integrated moving average (ARIMA) modeling, derived from Box-Jenkins, found the most utility (266-268). The ARIMA model generates a forecast of likely future values based off its own past values; for a metric such as body temperature in primates, which varies with circadian regularity, the ARIMA process lends itself to the characterization of temperatures that are decidedly outside of expected values (195, 207). As a function with userdetermined inputs, ARIMA is denoted by ARIMA(*P*, *D*, *Q*), wherein input arguments denoted by *P*, *D*, and *Q* refer to the orders of the autoregressive, differencing, and moving average terms, respectively, displayed in Equation 3 below. The term  $\Delta^D y_t$  refers to a differenced time series of

$$\Delta^{D} y_{t} = c + \phi_{1} \Delta^{D} y_{t-1} + \dots + \phi_{P} \Delta^{D} y_{t-P} + \epsilon_{t} + \theta_{1} \epsilon_{t-1} \dots + \theta_{P} \epsilon_{t-P}$$
(3)

order *D*,  $\varepsilon_t$  denotes an innovation process,  $\phi$  denotes a slope coefficient,  $\theta$  refers to a moving average parameter, and *c* denotes an offset by a constant value. With contracted lag operator notation, in which the lag operator *L* represents a term that operates on a current element of a time series to produce the previous element, the equation can be reformulated as that displayed in Equation 4 below, wherein L<sup>i</sup>y<sub>t</sub> = y<sub>t-i</sub>. Different values of *P*, *D*, and *Q* produce models with

$$\phi * (L)y_t = \phi(L)(1-L)^D y_t = c + \theta(L)\epsilon_t$$
(4)

a range of corrected Akaike Information Criterion (AICc) values, a metric that ranks the optimization of over/underfitting of the model with respect to the original baseline dataset.

To aid with the construction of a model of febrile encephalitic disease for the cohort of macaques exposed to each virus group, individual macaque clinical score profiles (addressed in subchapter 3.4) were plotted as ordinal variables on a day-by-day basis. When juxtaposed with individual temperature curves, disease profiles remained largely similar across multiple macaques in each virus cohort such that a consistent model disease course severable into distinct segments could be produced. Subsequent statistical analyses comparing metrics associated with baseline (pre-infection), incubation (post-infection, pre-disease-onset), febrile, and recovery periods, if applicable. For EEEV and VEEV febrile encephalitic disease, the models of disease shall be discussed and illustrated in Chapter 4 and Chapter 5, respectively.

### **3.2.1.2 Electroencephalography Methods**

Variations in EEG naturally arise on a daily basis, due to innumerable factors such as agitation, appetite, variant daily enrichment, and veterinary care personnel that enter the room at irregular intervals: a cacophony of conflicting external stimuli. Raw EEG traces were therefore subjected to frequency spectrum analysis to extract interpretable data. Both EEG and ICP data were analyzed for each macaque using modified MATLAB scripts graciously provided by Dr. Teichert that built on routines from the MATLAB toolbox EEGLAB can be seen in Appendix B (250). First, the 500Hz raw data was imported into MATLAB using the *edfread* function and converted to the EEGLAB data format. The data was then filtered using a 256-point noncausal digital low-pass finite input response (FIR) filter (*firws* function from EEGLAB toolbox, Blackman window, high-frequency cutoff 50Hz, transition bandwidth 21Hz). A windowed FFT was calculated using a cosine taper (400ms-long flanks) for sliding 4s windows stepped in increments of 4s.

The introduction to the Fourier Transform in Chapter 1 considered a continuous signal. In practice, the data, as mentioned just above, was sampled at 500Hz, or 500 samples per second. The sampling rate is dictated by the technical and physical capabilities of software and hardware respectively, but more importantly, the sampling rate imposes a limit on the recoverable Fourier components of the raw EEG signal that is half the highest waveform frequency – this limit is known as the Nyquist frequency (269). That is, in this case, the Nyquist frequency at which the original signal may be reconstructed with fidelity is 250 Hz. As the range of EEG wave bands relevant to clinical neurology (delta, theta, alpha, gamma, etc.) lies between 0.5 to ~40 Hz, the collection apparatus is more than capable of reconstructing the wave bands of interest (243, 245, 246). The 4s windows specified for the *edfread* function have the practical effect of extracting a 4-second segment of the raw EEG time series. Mathematically, this is equivalent to multiplying the time series with a rectangular window. Consider, for example, an example function f(t) in Figure 7; the function represents a cosine function of time t with a frequency phase of value  $\omega_{0}$ .



Figure 7. Rectangular Window Sample of Trigonometric Function.

The multiplication of trigonometric function f(t) by rectangular function g(t) results in the function  $f_u(t)$ , a unitary representation of the function f(t) from time [-T/2, T/2].

If the ideal function f(t) were passed through the Fourier Transform, as Equation 1 in Chapter 1:

$$F(\omega) = \int_{-\infty}^{\infty} f(t)e^{-i2\pi\omega t} dt$$
(1)

The following frequency spectrum would result, illustrated in Figure 8, with frequency components at  $-\omega_0$  and  $\omega_0$ . Because the objective consists of the examination of examine a raw



Figure 8. Continuous Fourier Transform Result.

The Fourier transform of continuous signal  $f(t) = \cos \omega_0 t$  results in a singular frequency component of  $\omega_0$ .

EEG signal composed of a linear combination of trigonometric functions in four second windows, the corresponding mathematical operation in the frequency domain, represented in the time domain, is called the convolution integral, exhibited in Equation 5, wherein  $x_1(t)$  and  $x_2(t)$  represent f(t) and g(t) from the former example from Figure 7. For the FFT specified in the *edfread* function,

$$y(t) = x_1(t) * x_2(t) = \int_{-\infty}^{\infty} x_1(\tau) x_2(t-\tau) d\tau$$
 (5)

the Blackman window refers to Equation 6 shown below, instead of a rectangular window. This

$$g(t) = 0.42 + 0.5 \cos\left(\frac{2\pi t}{T}\right) + 0.08 \cos\left(\frac{4\pi}{T}\right)$$
 (6)

window was chosen to reduce artifact generated by the mathematical transform. Integration underneath the curve of the frequency spectrum plot generated from the FFT yields the energy spectral density between the two given frequencies; because the raw signal is a stationary process, and integration occurs over a finite time interval, the result instead consists of the power spectral density (270). The following paragraphs shall illustrate this concept.

After excluding 4s bins with EEG artifacts or excessive motion, the remaining spectra were then averaged within 15-minute long bins. As previously discussed, four distinct frequency bands were defined (delta: [0.5 - 4Hz); theta: [4 - 8Hz); alpha: [8-12Hz); beta: [12-30Hz]) and the power estimated in each band as the average power of all frequencies in the corresponding range. This resulted in four distinct time-series that represented power in each enumerated band sampled at a rate of 1 sample per 15 minutes. Initially, a frequency spectrum analysis was done to assess the normal pattern for EEG data across the four power bands. The average EEG power spectrum of a representative example macaque is shown in Figure 9A. The approximately linear decline of EEG power with frequency (when plotted on a log-log scale) is expected and suggests that the leads were implanted correctly and record physiologically plausible EEG activity. While there is some variability in the daily averages, there is no systematic drift over the entire recording period. This speaks to the utility of the spectral analysis as a tool to identify pathological changes of brain function. Fig 9B shows the 12 average spectra for all macaques during the baseline period. Note the high similarity of power-spectra across the entire population. This suggests that the surgical procedure was successful at providing replicable electrode positions and electrical contacts for all macaques. Note that only three macaques show a peak at specific frequencies at baseline. The presence of such idiosyncrasies would suggest that pre-exposure baseline data from each macaque, if available, can further enhance ability to detect pathological changes by serving as its own control (18, 95, 138).



Figure 9. EEG Baseline Frequency Spectral Analysis in Normal Macaques.

A) A power spectral density (PSD) plot from the entire time course of a mock-infected macaque. Highlighted by different background colors are the delta( $\delta$ ) (0-4Hz), theta( $\theta$ ) (4-8Hz), alpha( $\alpha$ ) (8-12Hz), and beta( $\beta$ ) (12-30Hz) bands. Daily averages are indicated by thin gray lines. B) Pooled baseline data from all macaques in the study. Individual macaques are indicated by thin gray lines.

To visualize the circadian modulation of EEG power bands, a circadian index was constructed by normalizing each of the power band time-series to a mean of 0 and a standard deviation of 1. The circadian index was then defined as the difference between the normalized delta and beta power. Since delta power peaks during slow-wave sleep and beta power during alert wakefulness, the circadian index would putatively exhibit large positive values during night time and negative values during day time. To test for the presence of circadian EEG rhythms, EEG spectra were averaged for twelve macaques during the baseline period over the light and dark conditions of the animal holding room in Figure 10. This initial analysis suggested a high degree of circadian variability in the beta band (p<0.0001).



Figure 10. Power Spectral Density Circadian Differences.

Population spectral density plots at day (red) and night (black). Note the increased beta activity at day (p<0.0001).

To better understand the temporal dynamics of these circadian variations, time-series data were extracted for all four EEG power bands in bins of 15 minutes. Figure 11A documents the averaged circadian modulation of these power bands in a single mock-infected macaque, the same

macaque as in Fig 9A. As expected, beta activity coincides roughly with activity and body temperature and is highest during daytime while delta peaks at night when activity and body temperature are lowest. Similar patterns are seen for pooled, averaged baseline data for all macaques in the cohort across a similar 24-hour span is shown in Figure 11B. At the group level, a number of details of the circadian pattern emerge. Specifically, the group data show a transition during the night from a delta peak in the first half of the night (1800 to 2400) to a theta and an alpha peak in the second half of the night (0000 to 0600). It is also interesting to note that beta power starts ramping up a few hours before the day (facility lights-on). These transitions of the EEG power spectrum during the night suggest diurnal arousal because they cannot be explained by motion artifacts; monitoring by the accelerometers registers little activity during the night.



Figure 11. EEG, Temperature, and Activity Circadian Variation.

A) Circadian modulation of alpha, beta, theta, and delta power for one mock-infected macaque (M115-16). Gray boxes denote night, or dark periods, i.e. no lighting in the holding room. B) Pre-infection population average of all days' circadian modulation. Beta-band activity is elevated throughout the day; Delta-band activity peaks in the first half of the night; alpha- and theta- band activity peaks in the second half of the night.

The natural circadian variation of the EEG power bands is largest for the beta band, and is also present in the other power bands. The detection of pathological changes thereby requires discrimination of anomalous peaks and troughs against a constantly varying background. Such discrimination of pathological changes from natural circadian variation depends not only on the power bands' absolute magnitude, but on the reliability of the time series data on a day-by-day basis. Figure 12A illustrates daily circadian rhythms over an 18-day period in an example mock-infected macaque. Note that the circadian rhythms were stable over the course of the entire experiment. The high predictability of these circadian rhythms should facilitate the detection of pathologic changes. The data in the figure represent the envelope surrounding the filtered EEG data, and convey less noisy traces while preserving trends of increase and decrease in the wave band magnitude. The PSD plot (Figure 12B) demonstrated no significant deviations between pre-exposure and post-exposure spectra in this mock-infected animal.



Figure 12. EEG Processed Time Series in the Uninfected Macaque.

A) EEG time course and accompanying B) PSD plots from Mock-infected macaque (M115-16). Solid vertical black bar on Day 0 indicates time of aerosol exposure, gray boxes indicate night.

#### **3.2.1.3 Intracranial Pressure Methods**

Increased ICP during alphavirus encephalitis has been reported in humans (82, 271-274). The raised ICP may reflect increased brain parenchymal volume due to inflammatory and immune responses such as brain swelling. Hence, the monitoring of ICP serves as an intuitive, proximate indicator of the response to infection of the brain, and therefore the status of acute encephalitis. To measure ICP in macaques, we inserted a blood-pressure sensor from the M11 or L11 EEG/ICP implant between the dura and the skull. Because blood pressure typically ranges much higher values of mm Hg than intracranial pressure, blood-pressure sensors are tuned to a different range of pressures. Hence, to verify that we could detect meaningful changes of ICP using this bloodpressure sensor a frequency decomposition of the raw ICP was performed as a validation step in determining whether the implant could successfully detect pulsations from each heartbeat. As discussed in Chapter 1, it is well known that ICP fluctuates with individual heartbeats, known as the vascular ICP, essentially, cardiac pulsations (261, 262). If the blood pressure sensor were indeed sensitive to physiological variations of ICP, the collected telemetry data would presumably register an ICP pulsation corresponding to the cardiac pulsation, within the range of physiological values of the macaque heart rate (here defined as between 1.5 and 3.5 Hz, corresponding to 90 to 210 bpm). Figure 13A shows a segment of raw ICP trace for one macaque. Note the clearly identifiable cardiac pulsations. This demonstrates that the blood pressure sensors of the M11 and L11 implants were exquisitely sensitive to the cardiac pulsation of ICP within the expected physiological range. In humans, normal values of intracranial pressure range from 0-15 mmHg, varying according to postural changes; though circadian variation in ICP has been documented, such variation may be due in large part to expected changes in postural preferences during awake and asleep states (275, 276). Though few studies have comprehensively studied the subject, reports

in the literature have demonstrated that ICP values in adult cynomolgus macaques fall into a similar range of normal pressures. A combination of observations, the activity traces seen in Figures 11A, 11B, and 12A, and documented reports of postural preferences in awake and asleep macaques suggest the possibility of diurnal variation of ICP in cynomolgus macaques due to associated variation in posture and movement (277, 278). Deviation from a circadian pattern can provide an additional piece of evidence for the onset of a pathological process in a monitored macaque. Figure 13B demonstrates the circadian variation of the intensity of the ICP. Comparisons of daytime and nighttime values of the data obtained through frequency spectrum analysis indicated that measured pressures tended to display increased values at night (p<0.01). This finding, a group measure, can reflect the general preference for a seated, head-down posture during the night (278). Alongside the understanding that supine or seated postures can produce increased ICP relative to standing, upright postures, the output of increased nighttime intracranial pressure in this set of macaques represented a fairly reasonable outcome (279).



Figure 13. Intracranial Pressure Raw Trace and Circadian Variation.

A) The raw ICP trace in NeuroScore from a single example macaque exhibits individual heartbeat pulsations (red arrows). B) ICP exhibits significant diurnal differences (p<0.01), from the day and night averaged ICP values of a mock-infected macaque.

ICP measurements can be contaminated by "zero-drift," a slow artificial change in the measurements caused by a gradual de-calibration of the sensor or device (263). The fast changes of ICP observed in the infected animals are not consistent with such gradual loss of calibration. The potential problem of zero-drift was addressed by examining the frequency spectrogram averages out to day 33 post implant activation. The manufacturer of the implant / DSI reports that pressure sensing systems exhibited drift rates of <2-3 mm Hg/month depending on the implant model. The M11 and L11 implants, in the current iteration, used for the animals discussed here have a factory specification of an average deviation of ~0.25 mm Hg/month (280).

Frequency spectral analysis can provide a secondary measure putatively unaffected by potential zero-drift to test whether the amplitudes of cardiac ICP pulsations were elevated during periods of increased ICP. This would be a strong independent confirmation that the observed increases of ICP during acute encephalitis are real, and not caused by zero-drift. Representative data from one example animal infected by EEEV shows that the amplitude of cardiac pulsations indeed increase in line with mean ICP during encephalitic disease (Fig 14A).

The frequency spectra of data collected for a total of 33 days (Figure 14B) in a mockinfected macaque demonstrated no statistically significant changes in the pressures taken continuously before exposure to a control media aerosol and through 27 DPI. Furthermore, the amplitude of cardiac pulsations and mean daily ICP are linearly correlated (Figure 14C); these analyses were performed with the post-exposure data of mock-infected macaques as an invariant control alongside temporally-matched EEEV post-exposure data, before the humane study endpoint. What the latter panel (Figure 14A) in particular suggests is that the daily grand averages of ICP measurements correlate to a high degree to the magnitudes observed in the frequency spectrum analysis, implying a limited equivalency, the full extent of which was not investigated. Taken together, these additional analyses provide strong evidence that an observed increase of ICP would be reflective of disease. The data below suggests that even if drift from calibrated zero occurred, the impact on the statistical analysis was minimal at best and reflected actual symptomatic behavior. Although some anomalous pressures did occur, grand averages taken of the ICP measurements minimized that impact, and the frequency spectra show little variation.



Figure 14. ICP Measurement Stability and Correlation to Time Series Grand Averages.

A) ICP power spectral density plots from representative EEEV-infected macaque (M3-19) demonstrate not only a rise in pressure intensity visualized as local maxima, but also a rise in heart rate as the maxima shift right from baseline to the febrile period between -2 DPI and 4 DPI (red box marks a truncated physiological range of heart rate from 1.5-3.5Hz). B) B) No statistically significant changes in ICP occur over the study course from -5 days through 27 DPI in the frequency spectrum. C) The local maximum magnitude scales proportionally with aggregate daily ICP values measured from the raw ICP trace.

# 3.3 Virus Culture

The selection of virus strains in these studies was made by the Department of Defense in conjunction with the Food & Drug Administration; these strains: EEEV V105 and VEEV INH 9813 (IC epizootic subtype) were chosen, as discussed in Chapter 1, because of their human isolate lineage. To satisfy guidelines in the FDA Animal Efficacy rule, human isolates of virulent pathogens take precedence in application to animal models versus non-human isolates. However, due to the Select Agent status of "wild-type" EEEV and VEEV, laboratory work and culture of virus for the most part occurred within BSL-3 conditions, and/or in the University of Pittsburgh Regional Biocontainment Laboratory (RBL), a facility with the appropriate registration and oversight from the Centers for Disease Control's Division of Select Agents and Toxins (DSAT). However, once virus stocks or samples containing or exposed to virus were inactivated with verified procedures, work with said specimens could proceed in BSL-2 conditions.

Alphavirus stocks were generously generated and maintained by Dr. William Klimstra's laboratory. Because of the tendency of RNA viruses' propensity to accumulate genomic mutations over successive passages in cell culture (thereby concluding experiments with potentially vastly different viruses than the initial human isolate) and to minimize the associated risk of the attenuation of virus virulence through cell culture, the alphavirus stocks were derived from cDNA clones. All cells for cell culture were cultured at 37°C with 5% CO<sub>2</sub>. For stock production, viral RNAs (vRNA) were generated through *in vitro* RNA synthesis from the linearized cDNA plasmid template; these capped, infectious vRNAs were electroporated into baby hamster kidney cells (BHK-21, ATCC), and virus stocks were harvested at approximately 24 hours post electroporation.

Electroporation supernatants were centrifuged and virus-containing supernatant clarified through centrifugation at 1000 RCF for 30 minutes. Single-use aliquots of this supernatant were

stored at -80°C and considered zero-passage (p0) stocks. The p0 stocks were titered for plaqueforming units (PFUs) by standard plaque assays with BHK-21 cells; the same stocks were then used to infect roller bottles plated with African Green Monkey (AGM) kidney cells (VERO cells, ATCC CCL-81) at a multiplicity of infection (MoI) of 10. One day subsequent to roller bottle infection, the supernatant from the roller bottle was collected and clarified by centrifugation; subsequently, the supernatant was purified by sucrose layer purification: the supernatant was layered over a 60/20% sucrose cushion and subjected to ultracentrifugation. The interface between the 60/20% cushion was collected via pipette and diluted in 10mM Tris, 1mM EDTA, 100mM NaCl-STE 10X NaCl (TNE) buffer. The diluted collection was layered over 20% sucrose and again subjected to ultracentrifugation to collect the virus as a pellet. The virus pellet was resuspended in Opti-Mem<sup>®</sup> Reduced Serum Medium (ThermoFisher, Catalog No. 31985-070), and single-use aliquots were stored at -80°C. The virus stock was also titered using the standard plaque assays mentioned above with BHK-21 cells, with standard LD<sub>50</sub> doses confirmed in a murine model before use in macaque studies. Descriptions of this procedure can also be found in preceding manuscripts (138, 168).

### **3.4 Aerosol Exposures**

Aerosol exposures of cynomolgus macaques to cultured virus or media aerosol (for mockinfection) were performed in a modified Class III biosafety cabinet optimized for aerobiological work within the Aerobiology Suite of the RBL. When prepared for aerosol exposure, the macaques were transported a mobile transfer cart (MTC) to and from the Aerobiology Suite. Each macaque was anesthetized prior to aerosol with 6 mg/kg of Telazol<sup>®</sup>. Contemporaneously, a blood sample was taken for complete blood count (CBC) and clinical chemistry with the Abaxis HM2 and Abaxis VetScan VS2, respectively. Veterinary technicians facilitated the transfer of the macaque from the holding room/suite to the Class III space in the Aerobiology Suite via a MTC with a selfcontained air filtration system for containment. Upon transfer through an airlock to the modified Class III biosafety cabinet, each macaque was fitted with Jacketed External Telemetry Respiratory Inductive Plethysmography (JET-RIP) belts (DSI) around chest and abdomen for the recording and transmission of respiratory function (for minute volume calculation) via the Ponemah software suite (DSI). Macaques were exposed inside an acrylic head-only chamber to an EEEV, VEEV, or mock aerosol generated by an Aeroneb vibrating mesh nebulizer (Aerogen, Chicago, IL) Aerosol exposures were coordinated and monitored with the AeroMP aerosol management platform (Biaera Technologies, Hagerstown, MD). Ancillary variables such as humidity and barometric pressure within the cabinet were monitored alongside macaque plethysmography. The monitoring of plethysmography allowed for the termination of the aerosol at a desired inhaled volume and thereby a finer degree of control over the inhaled dose, with each aerosol followed with a 5-minute duration air wash to purge the chamber of virus before the macaque was transported back to the holding room to recover from anesthesia under veterinary staff oversight.

For virus dose determination for each macaque, standard plaque assays with BHK-21 cells were performed on samples collected from the all-glass impinger (AGI; Ace Glass, Vineland, NJ) during the aerosol to determine the virus concentration in the aerosol and the dose presented to the macaque. Equation 7 represents the aerosol concentration in which *[Virus]*<sub>AGI</sub> denotes the virus

$$[Virus]_{Aerosol} = \frac{[Virus]_{AGI} * V_{AGI}}{Q * t_{exposure}}$$
(7)

concentration in the AGI,  $V_{AGI}$  denotes the volume of virus-containing fluid in the AGI, Q represents the flow rate through the AGI (a constant 6 liters per minute), and  $t_{exposure}$  and the duration of the exposure.

Presented dose (i.e. inhaled dose) is calculated in Equation 8 below, which represents the

$$[Virus]_{Inhaled} = \frac{[Virus]_{Aerosol} * MV}{t_{exposure}}$$
(8)

product of  $[Virus]_{AEROSOL}$ , described previously, MV, the minute volume of breaths taken by the macaque, and  $t_{exposure}$ , again, the time duration in minutes of the exposure. For the calculation of the  $[Virus]_{Aerosol}$  variable from the previous subchapter, samples of aerosol were taken through the AGI, depicted in Figure 15.



Figure 15. Schematic of AeroMP Aerosol Apparatus with Head-Only Chamber.

A) Depiction of similar Class III Biosafety Cabinet in Aerobiology Suite. B) Apparatus of AeroMP System: 1.
Aerodynamic Particle Sizer (APS); 2. All-Glass Impinger; 3. Aeroneb Nebulizer and External Control; 4. HEPA
filters; 5. Network and Power; 6. Data Output; 7. Vacuum Out and Air Intake; 8. Head-Only Chamber; 9. Aerosol
Mixing Tube. Reproduced with permission from, and also referenced in Lackemeyer, et al and Bohannon et al.
(281, 282).

### 3.5 Polymerase Chain Reaction Assays

Both the quantitation of viral genome copies and the quantitation of upregulation or downregulation of expression of host genes of interest in the cynomolgus macaque argued for the use of real-time polymerase chain reaction (PCR) assays. The following methods were specifically designed for QuantStudio 6 Flex qPCR Machine in the 96-well format with TaqMan FAST reagent sets. Macaque-derived specimens sampled for Quantitative Real-Time PCR (q-RT-PCR) included plasma, CSF, and nasal swabs. Solid tissue samples included in the scope of this work consisted of thalamic and cerebellar sections from necropsied macaque brain. Samples of CSF were collected at necropsy, alongside solid tissue samples. However, during the baseline period and post-infection periods, 3mL plasma and nasal swabs were obtained intermittently on a bidaily basis, in addition to being taken during necropsy. Figure 16 consists of an illustrated schemata detailing when samples were isolated.





Blood samples were taken at bidaily intervals, while solid tissues and CSF were harvested at necropsy of the macaque.
Plasma samples totaled 3mL per collection, while CSF samples totaled 4mL, under aseptic conditions. Solid tissues were suspended in 500uL DMEM + 2mM Glutamine + 5% Fetal Bovine Serum (FBS) cell culture medium immediately following harvest, and subsequently homogenized using an Omni tissue homogenizer (Omni International) for a target tissue mass to volume ratio of 200pg/µL for all tissue homogenates. In preparation for removal from BSL-3 conditions, liquidated samples (homogenized tissue, plasma, or CSF) were inactivated in a 1:9 volume to volume ratio of sample to TriZol<sup>®</sup> guanidine isothiocyanate Tri-Reagent mixture and allowed to settle for 10 minutes. Subsequently, storage at -80°C or removal of samples from the RBL could proceed under BSL-2+ conditions.

In BSL-2+ space, the extraction of RNA from the macaque-derived samples proceeded through a modified Invitrogen PureLink Viral RNA/DNA kit protocol. In brief, 200µL of chloroform was added to the inactivated specimen/Tri-Reagent mixture, followed by vigorous inversion/agitation for 30 seconds and centrifugation of samples at 4°C and 12,000 RCF for 15 minutes. Centrifugation separates the organic phase from the RNA-containing aqueous phase. The aqueous phase was collected and mixed with an equivalent volume of 70% RNAse-free ethanol, and the mixture applied to a PureLink spin column. For the remainder of the RNA extraction, the PureLink RNA/DNA manufacturer's instructions were followed, including the enzymatic deoxyribonuclease inactivation step. Eluted RNA was assessed via a NanoDrop spectrophotometer and stored at -80°C until further processing into cDNA.

The cDNA synthesis was performed using the First-Strand cDNA Synthesis M-MLV Reverse Transcriptase Kit (Invitrogen) with RNaseOUT (Invitrogen). 100ng of total RNA from tissue homogenates or 5uL of undiluted plasma or CSF was used during cDNA synthesis. For vRNA amplification, the following cDNA synthesis forward primers (IDT) shown in Table 4 and a reverse primer with a T7 promoter tag (bolded) targeting the positive strand were used (101). Primers targeted the EEEV and the VEEV NSP2 coding regions. For the purposes of amplification of message RNA for gene expression analyses, 100ng of total RNA from tissue homogenates were used in combination with cDNA synthesis random oligonucleotide primers (ThermoFisher Catalog No. 48190011) to amplify all macaque mRNA transcripts. Thermocycler parameters recommended by the manufacturer were utilized for cDNA synthesis. cDNA was stored at -80°C until further analysis via q-RT-PCR.

Table 4. First-Strand cDNA Synthesis Forward Primers

Virus Strain	Forward Primer	
EEEV V105	5'- GCGTAAT ACGACTCACTATACACCGGCCAAAGTCTTCCATACTAT-3'	
VEEV INH 9813 (IC)	5'-GCGTAATACGA CTCACTATAGTCTTCTGTTCACAGGTACTAGAT-3'	

For quantitation of EEEV- and VEEV-specific vRNA by q-RT-PCR, procedure consisted of utilizing the 2x Fast Taqman Universal PCR Master Mix and kit, No AmpErase UNG (Applied Biosystems), following the manufacturer's instructions. For this second q-RT-PCR step, forward primer probe targeting EEEV comprised: forward primer 5'and GCGCTACAAGGTCAATGAGA-3' and probe 5'-ACGCACAGACATCTGAGCATGTGAA-3'. Forward primer and probe targeting VEEV comprised: forward primer 5'-CCGGAAGAGTCTATGACATGAA-3' and probe 5'-CTGGCACGCTGCGCAATTATGATC-3'. Probes were labeled at the 5' end with the reporter molecule 6-carboxyfluorescein (6-FAM) and quenched internally at a modified "T" residue with BHQ1 (Black Hole Quencher 1), with a modified 3' end to prevent probe extension by Taq polymerase. Reverse primers for EEEV and VEEV targeted the T7 promoter tag generated during first strand cDNA synthesis: 5'-GCGTAATACGACTCACTATA-3'. Thermocycling parameters included the following: initial denaturing, 95°C for 20 seconds, and cycling PCR amplification, 95°C for 3 seconds and 60°C for 20 seconds (45 cycles). Quantitation of virus was determined by comparing the cycle threshold (CT) values from unknown samples to CT values from positive-sense EEEV or VEEV vRNA standard curve in 1:10 serial dilution. Positive-sense vRNA was developed in-house by in vitro transcription, using the mMessage mMachine T7 kit (Ambion) and following the manufacturer's instructions (101). LOD & LOQ Determination was carried out in accordance with the Minimum Information for the Publication of Quantitative Real-Time PCR Experiments (MIQE) guidelines (283).

The established procedure above for quantitation of vRNA lent itself for adaptation to a 96-well plate format for the q-RT-PCR analysis of mRNA expression and its up/down-regulation. The TaqMan procedure offered improved specificity compared to a SYBR Green intercalating dye based q-RT-PCR method, and the logistical burden for sample processing was minimized, with few changes to the q-RT-PCR protocol for vRNA quantitation. TaqMan Gene Expression Assay kits were used to target genes of interest (Table 5). Probes were labeled at the 5' end with the reporter molecule 6-carbocyfluorescein (6-FAM) and quenched internally at a modified "T" residue with BHQ1 (Black Hole Quencher), with a modified 3' end to prevent probe extension by Taq polymerase. Thermocycling parameters comprised the following: hold step, 50°C for 120 seconds; initial denaturing, 95°C for 120 seconds; and cycling PCR amplification, 95°C for 1 second and 60°C for 20 seconds (40 cycles).

Analyte	Gene	Category	Part Number
Clock	CLOCK		Mf_04392546_g1
Cryptochrome Circadian Regulator 1	CRY1		Mf02847438_m1
Cryptochrome Circadian Regulator 2	CRY2	Circadian Regulator Genes	Mf02878746_m1
Period 3	PER3		Mf00997917_m1
Macrophage Chemoattractant Protein 1	CCL2		Mf02787889_g1
Interferon Gamma Induced Protein 10	CXCL10		Mf02788358_g1
Interferon Gamma	IFNG	Cetabing Course	Mf02788577_m1
Interleukin-1 Beta	IL1B	Cytokine Genes	Mf02789775_m1
Interleukin-6	IL6		Mf02789322_m1
Interleukin-8	IL8		Mf02789706_g1
Glial Fibrillary Acid Protein	GFAP		Rh00909240_m1
Matrix Metalloproteinase 9	MMP9	TBI Injury Species Genes	Mf02856273_g1
Leukemia Inhibitory Factor	LIF		Mf04365516_m1
G3P Dehydrogenase	GAPDH	Housekeeping Genes	Mf04392546_g1
18S rRNA	18SrRNA	(Endogenous Controls)	Hs99999901_s1

Table 5. q-RT-PCR Primer-Probe Kits.

All kits, with two exceptions, were targeted to cynomolgus macaque-specific, highlyconserved amplicons that spanned one or more exons to guarantee desired mRNA transcript specificity. Endogenous controls comprised TaqMan Gene Expression Assay kits for GAPDH in the investigation of cytokine gene and traumatic injury species gene expression, with thalamus tissue from a mock-infected macaque serving as the reference tissue; endogenous controls for genes of circadian regulation employed 18S rRNA as a time-invariant housekeeping gene. Interrun calibration to normalize inter-run/inter-plate variability was carried out according to best practices (283, 284). In cases where macaque-specific kits were unavailable, rhesus macaque or human-specific research-grade kits were used, in the cases of GFAP and 18S rRNA, respectively. Figure 17 summarizes and illustrates the procedure governing cDNA synthesis and q-RT-PCR.



Figure 17. q-RT-PCR for vRNA Quantitation and mRNA Gene Expression Analysis.

Quantitative real-time PCR was used both for assaying viral genome copy numbers in plasma and CSF viremia, and for gene expression assays in tissues using the  $\Delta\Delta$ CT method. Shown here is an illustration of the gene expression assay pathway, which differs from the viral genome copy number assays in the primers and probes used for qPCR, but most importantly for the linear primers used for the initial reverse transcription step, wherein virus-specific primers to amplify the highly conserved nonstructural protein were used to ensure specificity. For all solid tissues, 100 nanograms of RNA was used to begin the reaction and to normalize between samples.

#### **3.6 Statistical Analysis**

For the totality of the work, with an expected incidence of 0% encephalitis or fever detectable by radiofrequency telemetry in mock-infected macaques and a >99% expected incidence of fever or encephalitis in macaques exposed to alphavirus, a 1:1 enrollment ratio, and Type I and II error rates of 0.05 and 0.80, respectively, the minimum sample size required for adequate power and comparison between groups stipulated a minimum of two macaques per group. As can be gathered from Table 1, more macaques than the minimum number were used in the study, to augment the effect size of the findings.

By and large, statistical analyses were performed through the use of MATLAB (Mathworks; Natick, MA) in addition to Graphpad PRISM 8. The statistical test with the most farreaching effects consisted of variable iterations (dependent upon test conditions) the analysis of variance, or ANOVA, with *post hoc* pair-wise t-tests performed to probe for differences within groups, between groups, and/or between different pools of data in the same macaque. ANOVA was used to provide for a robust differentiation between conditions belying discrete time periods (sick vs. well) between infected macaques. Analyses of power spectral density data were conducted to compare differences between different time periods, most commonly between days post-infection and baseline, with respect to disease onset. Significance levels utilized comprised  $\alpha$ <0.05. Both EEG and ICP data underwent ANOVA and post-hoc tests, when applicable of data from power spectral density plots. Linear regression and curve fitting were applied to ICP data.

The analysis of seizure data surfaced with idiosyncratic analytical challenges. The only macaques which manifested seizures in this study consisted of the EEEV-infected macaques. NeuroScore contained a native algorithm, with user-definable voltage and frequency parameters, for the flagging of suspected seizure activity. As described in Chapter 1, the seizures that occurred

in infected macaques mostly represented generalized or tonic-clonic seizures, with less if any representation of other seizure types such as absence seizures. Spike detection at times registered these seizures, but were exquisitely sensitive to noise, to be discussed in the subsequent chapter. The determination of false positives and true positives thereby required visual/behavioral confirmation of seizure occurrence through viewership of closed-circuit television recordings.

The conflation of abnormal EEG, defined as a statistical deviation either above or below baseline, on any given day, with the occurrence of seizures, both considered as ordinal variables, required the assumption of a standard nonlinear model, depicted in Equation 9, wherein p, the probability of the outcome variable, is a function of the independent variable x. The question: *how* 

$$p = \frac{1}{1 + e^{-(b_0 + b_1 x)}} \tag{9}$$

*do seizures contribute to abnormal EEG, or ICP, and vice versa?* assesses the impact on how changes in the dependent variable affect the outcome of interest as well as facilitates a method of making predictions regarding the variables.

That latter aspect of the data, gives consideration to the use of the receiver operating characteristic (ROC) curve. The ROC curve plots the true positive rate against the false positive rate, or alternately: the sensitivity of the predictor against 1-specificity of the predictor. Integration of the curve, or area under the curve (AUC) indicates how good the predictor is at predicting the outcome. In this context, a straight line with a slope of 1 through the origin (zero-intercept) would have an AUC of 0.5, which essentially equates to a coin flip. An AUC approaching 1.0 would

imply a perfect predictor, and a near-zero AUC means that the predictor predicts the opposite of the intended outcome. Ancillary methods used to probe the relationship of seizures to abnormal EEG/ICP included methods such as Fisher exact tests of independence, wherein two proportions are compared; the  $\chi^2$  test, or Wald z test, is nearly identical to the Fisher exact test with respect to comparisons between two proportions to determine what differences exist between the two aforementioned proportions.

Finally, with respect to comparing differences between elevated ICP grand averages in light of the need to compare macaques' ICP to baseline ICP measurements, percentage change from baseline was computed to determine what group effects existed, in parallel with ANOVA. Because the output of the ICP modality exists as a relative measure of ICP rather than an absolute measure of ICP in mm Hg, it was not an unheard-of outcome for baseline ICP measurements to yield negative values. Although at first glance, the display of negative ICP measurements may raise confusion, as physiologically, a vacuum cannot exist within the intrathecal space nor exert negative pressure against the dura, the value of the ICP measurement was computed against the ambient room pressure given by the APR-1 module. Changes in ICP still registered, although these changes occurred in relation to a negative baseline. That is, positive deflections in ICP relative to a negative deflection in ICP relative to the same negative baseline value would imply a decrease in ICP. Thus, in concordance with established practices in the medical literature, absolute percentage change in ICP was computed from which to conduct statistical analyses (285-288).

# 4.0 Severe EEE In Cynomolgus Macaques

To begin this first chapter of results, a reminder of the Aim, associated sub-aims, and pertinent background shall follow. The overall aim associated with this chapter seeks to *determine whether telemetric features of the EEEV disease course provide means of predicting the onset of febrile encephalitis.* The cynomolgus macaque model of severe EEEV manifests with febrile encephalitis and neurological signs of disease such as seizures and coma. The central hypotheses undergirding the content of this chapter postulates that slow-frequency EEG and ICP increases related to febrile onset shall forecast severe courses of EEEV in comparison to mock infection, which should show few if any changes from baseline. The onset of encephalitis of viral origin can cause disruptions to electrical activity in the brain; similarly, the same circumstances have induced documented increases in ICP (19, 78, 84, 102).

Preliminary analyses detailed in Chapter 3 showcased EEG under normal circumstances in mock-infected macaques and in macaques during baseline periods. Macaques infected with EEEV produced either a severe course of disease which manifested a febrile illness with neurological signs of disease or an afebrile post-infection course, termed nonsevere disease. Macaques that received an inhaled dose of more than 2.22\*10<sup>7</sup> PFU of EEEV typically displayed that severe disease; these macaques typically became moribund a couple of days following the onset of fever, while animals that received below that stated dose almost uniformly survived without any signs of disease. Table 6 lists all macaques pertinent to Chapter 4; "time to fever onset" and "day euthanized" refer to days from the day of exposure.

Macaque	Age (yr)	Virus	Inhaled Dose (PFU)	Severe / Nonsevere Disease (S/NS)	Time to Fever Onset (d)	Day Euthanized (d)	Implant Model/Type
M114-16 👌	5	Mock	-	-	-	28	-
M115-16 👌	6	Mock	-	-	-	30	M11: EEG/ICP
M116-16 👌	5	Mock	-	-	-	28	M11: EEG/ICP
M117-16 ♂	6	Mock	-	-	-	30	-
M160-16 👌	7	EEEV V105	$1.10*10^{7}$	NS	-	60	M01: ECG
M161-16 ♂	7	EEEV V105	1.60*10 <sup>7</sup>	S	3.3	6	M01: ECG
M162-16 ♂	7	EEEV V105	4.64*10 <sup>5</sup>	NS	-	31	M01: ECG
M163-16 👌	7	EEEV V105	3.39*10 <sup>7</sup>	S	2.8	6	M01: ECG
M120-16 ♀	6	EEEV V105	1.08 x 10 <sup>7</sup>	S	1.5	5	M01: EEG
M123-16 ♀	5	EEEV V105	1.50 x 10 <sup>7</sup>	NS	-	26	M01: EEG
M57-17 ♂	5	EEEV V105	3.71 x 10 <sup>8</sup>	S	2.3	5	M01: EEG
M58-17 ♂	5	EEEV V105	1.02 x 10 <sup>9</sup>	S	2.3	6	M01: EEG
M160-17 👌	6	EEEV V105	1.37 x 10 <sup>9</sup>	S	3.5	6	M11: EEG/ICP
M163-17 👌	5	EEEV V105	2.50 x 10 <sup>10</sup>	S	5.3	9	M01: EEG
M108-18 ♀	6	EEEV V105	1.69 x 10 <sup>7</sup>	NS	-	34	M11: EEG/ICP
M110-18 ♀	6	EEEV V105	1.50 x 10 <sup>7</sup>	NS	-	34	M11: EEG/ICP
M113-18 ♀	7	EEEV V105	6.34 x 10 <sup>6</sup>	NS	-	31	M11: EEG/ICP
M114-18 ♀	5	EEEV V105	7.64 x 10 <sup>6</sup>	NS	-	33	M11: EEG/ICP
M118-18 👌	8	EEEV V105	9.87 x 10 <sup>6</sup>	NS	-	33	M11: EEG/ICP
M1-19 ♀	2	EEEV V105	1.03 x 10 <sup>8</sup>	S	2.5	6	M11: EEG/ICP
M2-19 ♀	2	EEEV V105	1.92 x 10 <sup>8</sup>	S	1.3	6	M11: EEG/ICP

 Table 6. Aim 1 Macaque Cohort Characteristics.

Table 6 (cont'd)							
M3-19 🖒	2	EEEV V105	3.76 x 10 <sup>8</sup>	S	2.5	6	M11: EEG/ICP
M4-19 🖒	2	EEEV V105	6.70 x 10 <sup>7</sup>	NS	-	28	M11: EEG/ICP
M5-19 ♂	2	VEEV INH- 9813	9.94 x 10 <sup>6</sup>	NS	0.5	6	M01 – Temperature Only
M6-19 ♂	2	VEEV INH- 9813	8.51 x 10 <sup>6</sup>	NS	0.5	6	M01 – Temperature Only

Of the macaques that exhibited signs of severe neurological disease, the febrile course of disease was replicated with fairly high fidelity between such subjects; following exposure to EEEV by aerosol, these macaques became febrile following a mean time of 2.73 DPI, compiled from data from Table 6. Commensurate increases in neurological scores are likewise detectable during the adjacent times; for the purpose of model construction, days 0-2 post-aerosol, inclusive, shall be construed as the incubation period, and the period from the beginning of 3 days post-aerosol onward, denotes the febrile period. In macaques with severe disease, a febrile plateau of approximately 40-41°C is reached, as illustrated in Figure 18A, at or after 3 DPI. Subsequently, a terminal decline in temperature (so-called because of the rapidly-deteriorating health status that often accompanies the decrease in temperature in severe EEEV infection), often incurred the humane study endpoint typically by the first observational check of 6 DPI. Macaques with nonsevere disease exhibited neither severe signs of neurological disease such as seizure, coma, or tremor, nor did they manifest any fever over the period of typical febrile onset and terminal decline for macaques with severe disease. This is reflected in the models of disease displayed in Figure 18, which contrasts the two disease phenotypes. Because all macaques with severe disease triggered the humane study endpoint, a recovery period was not considered appropriate for

definition in macaques infected with EEEV. However, for the purposes of EEG analysis, "recovery" denotes the period of time following 6 DPI in macaques with nonsevere EEEV, as an analogue to the time period following resolution of fever in VEEV-infected macaques. This definition was critical for the comparison of EEG data during like temporal periods between the cohort of macaques with severe and nonsevere disease, and also to test whether the period of time following 6 DPI in infected macaques.



Figure 18. Severe and Nonsevere EEEV in Cynomolgus Macaques.

A) Temperature traces (averages of temperature traces from all macaques grouped by disease phenotype) of EEEVinfected and mock-infected cynomolgus macaques implanted with EEG or EEG/ICP implants investigated for Aim 1. B) Overall model of EEEV disease course in the telemetered macaque. The pre-infection period consists of the duration of time before the aerosol challenge, which varies between macaques; following aerosol exposure, the postinfection period for macaques that experience nonsevere disease can be taken as simply the entire duration following infection, unless otherwise specified. For macaques that experienced severe disease, the febrile period was consistently seen at/after three days post-infection, and a incubation period was defined as 0-2 days (inclusive).

# 4.1 Pathological EEG Changes in Febrile EEE

The initial analysis of EEG data from the cohort of EEEV-infected macaques produced a bevy of time-series data and frequency spectrum data. For example, Figure 19A shows data from a macaque that developed severe disease after EEEV infection. Prior to fever onset, the time series analysis shows a regular, robust circadian modulation much like the mock-infected macaques. However, beginning on 3 DPI, a number of increasingly dramatic changes are evident that diverge from the normal circadian cycle. An increase of the alpha power band on 3 DPI coincided with fever onset; a PSD plot confirmed the increase in alpha power band magnitude on day 3 was significant (Fig 19B and 19C). In the subsequent night, abnormalities started developing in the delta power band. While the early night delta peak still emerges, it is cut short and followed by an unusual drop in delta power that extends into 4 DPI and reaches a dramatic trough during the early morning (before lights-on) of 5 DPI, contemporaneous with peak fever. Concurrently, power dropped in all other frequency bands; however, the drop in delta power is particularly noteworthy since delta power in this macaque typically shows a clearly-delineated early night-time peak. The morning of 6 DPI brought another dramatic change that manifested as an increase of power across all bands. Both the uniform drop of power on 5 DPI as well as the uniform increase of power on 6 DPI are highly unusual, given the normally inverse relationship of delta power on the one hand and theta-, alpha-, and beta- power on the other. The pattern of uniformly increased power holds for the entirety of 6DPI as the fever begins its terminal decline. The unusual coupling of the power bands can also be observed as a decrease in the amplitude of the circadian index on days 5 and 6.



Figure 19. Processed EEG data in the EEEV-infected Macaque.

A) EEG time course and accompanying B) PSD plot from a single EEEV-infected macaque (M1-19), dotted lines represent upper and lower bounds as standard deviation. Blue circle indicates increases in alpha activity on 3DPI, visualized on the PSD plot (red trace) emphasized with corresponding blue arrow in comparison to baseline spectra for that macaque, note also taupe ellipse marking suppression of sleep index from 4-5 DPI. C) T-test of alpha power between 3 DPI and baseline data demonstrates statistically significant increase (p<0.0001). Febrile period for severe EEEV begins at 3 DPI and ends at euthanasia (~5-6DPI). The solid magenta bar on Day 0 indicates time of aerosol exposure, while gray boxes indicate night.

Certain macaques with significant gaps in the time series course from both the baseline and post-infection period were excluded from EEG analysis, as were macaques with idiosyncratic baseline data, such as the case of a macaque with positive delta deflection during the day, compared to the usual negative deflection (Figure 20). The difficulty associated with quantifying or validating EEG changes with severe disease with significant data loss becomes apparent from this data; the macaques surveyed for EEG changes represented a group of macaques with minimal to no data loss (n = 4).



Figure 20. Idiosyncratic EEG Time Series in EEEV-infected Macaques.

Time series EEG traces of A) M57-17, B) M58-17, two macaques with severe EEEV with significant data loss in both baseline and post-infection collection periods. C) M160-17 was the case of a macaque with severe EEEV with both baseline and post-infection data that showed an idiosyncratic increase in delta activity during the daytime.

To test the hypothesis that in relation to fever onset, EEG activity would decrease as a whole or increase in favor of slow-frequency EEG band activity such as the delta or theta band, the EEG power spectral density magnitudes for each macaque were converted to percent change from baseline values across the whole of the specified periods: baseline, incubation (0-2 DPI, inclusive), febrile (3-6 DPI, inclusive), and recovery (6+ DPI). Group averages of percent change were taken for severe and nonsevere cohorts of macaques and compared against baseline by repeated measures ANOVA, as illustrated in Figure 21. This was done because a large degree of



Figure 21. Group Averages of EEEV PSD Plot Magnitude as Percentage Change from Baseline.

Power spectral density plotted as percent change from baseline in cohorts of EEEV-infected macaques that displayed A) severe and B) nonsevere disease. This plot illustrates traces from individual macaques which show the disparate power spectral density curves in each disease period rather than showing box plots with error bars. Repeated measures ANOVA and *post hoc* pairwise comparisons at each discrete frequency showed a statistically significant increase in delta band activity during the febrile period for EEEV-infected macaques with severe disease. Although different degrees of significance were present from the wave band between 1.25 and 3.25 Hz, one asterisk is shown for brevity.

variability existed in the data of individual macaques in the context of day-to-day changes in EEG, as mentioned earlier in the subchapter and also as illustrated in Figure 20; therefore, the group analysis sought only to assess what information could be conclusively determined across the group of macaques with severe and/or nonsevere EEEV disease. From these data, the observation arises that a significant amount of EEG variability occurs following infection in the cohort of EEEV-infected macaques with severe disease. The frequencies sampled for the statistical testing were taken at increments of 0.25 Hz, for instance: 0, 0.25, 0.50, 0.75, ... and so on. Repeated measures ANOVA with pairwise comparisons (Dunnett *post hoc* comparisons) of the same frequency during different periods of disease revealed statistically significant increases in between 1.25 - 3.25 Hz, corresponding to the delta band during the febrile period; the most significant increase in delta occurred at 1.25 Hz, with a 22.4% increase in delta band activity (p<0.001).

The upward percentage change of delta band activity that occurs contemporaneously with the febrile period in EEEV-infected macaques with severe disease supports the hypothesis that slow-wave EEG activity might increase with the onset of encephalitis. While other trends appear to exist, such as increases or decreases in alpha or theta activity for individual macaques, these effects did not elicit statistical significance at the group level. Incidences of significant elevations emerge on the day-to-day power spectral density plots for specific macaques; towards this point, the wide range of the box plots in Figure 21A in both the theta and alpha bands that straddle both positive and negative values attest to the notion that disparate effects emerge during the febrile period. On closer scrutiny, an observer can also note that a large positive deflection in the alpha band during the incubation period in Figure 21A; however, this change is not statistically significant (p > 0.1082). In a similar vein, there appears to be a trend of decrease in percentage change of beta band activity during the febrile period in Figure 21A as well, but the magnitude of this percentage change was not sufficient to be statistically significant (p > 0.4826) either.

In contrast, with respect to EEEV-infected macaques with nonsevere courses of disease, as depicted in Figure 21B, no statistically significant effects on percentage change from baseline power spectral density emerge throughout the entirety of the frequency band of neurophysiological significance between the post-infection period and the baseline period. To reiterate the overall group result, a unilateral increase (as opposed to bidirectional positive and negative variation relative to baseline) of delta activity was seen in the cohort of EEEV-infected macaques with severe disease.

#### 4.2 Seizures in EEE Correlate with EEG Pathology

The measurement, or detection of seizures and their relationship to EEG and/or ICP abnormalities first required the verification or confirmation that a seizure had in fact, occurred. Apart from direct observation of a seizure by a veterinarian or veterinary technician, visual assessment of seizure activity required the perusal of hundreds of hours of closed-circuit video recordings that accompanied EEG and ICP recordings. These video recordings were time-stamped such that examination of contemporaneous EEG recordings could be flagged for seizure activity through NeuroScore. Previously, other researchers had observed in cynomolgus macaques, using the NeuroScore platform (v.1.1) that abnormal EEG could be detected at least 4 minutes prior to generalized seizures; they witnessed increased beta activity with visual stimulation, and noted that delta activity was predominant in normal awake EEG as well in all sleep stages (223).

Though a number of parameters were issued to test for seizures detected by the software against to those documented by either veterinary staff or by video recording, the quality of flagged seizures suffered from a propensity of the software to tag eye movements, spikes of beta activity, or other nonspecific events not associated with seizure activity. Though the algorithm proved useful for flagging possible seizure activity on days during which seizures were known to occur, false positive rates ran as high as 95% due to flagged activity on days during which no seizures occurred. Figure 22 illustrates the propensity of the native software to exhibit such false positives.

However, to reiterate the question that undergirded this subchapter: what relationship, if any, exists between seizure activity and EEG/ICP abnormalities? Thus, it fell to the direct review



Figure 22. EEG-Based Algorithmic Seizure Detection.

Native NeuroScore algorithmic detection of seizures. Shown are two plots of EEG traces taken during periods of macaque physical activity and saccadic eye movement, confirmed via reference to time-stamped closed-circuit video. A) shows a five second recording of a true negative, whereas B) shows a five second recording from a minute previous, during the same episode of movement, that was labeled a (false positive) seizure.

of the closed circuit video for any episodes suffered by the EEEV-infected macaques that could be considered a confirmed seizure, putatively termed "confirmed paroxysmal episodes." These episodes were counted and stratified temporally relative to the day of aerosol exposure. Due to time constraints, a total of 3 macaques were assessed for seizure activity, which resulted in a number of seizures tallied that occurred mostly during the febrile period (3-6 DPI) of severe EEEV. Figure 23 charts the number of seizures for this group against the times at which these seizures occurred. All three macaques assessed came from the group of macaques with severe disease, so



Confirmed Paroxysmal Episodes (EEEV)

Figure 23. Confirmed Paroxysmal Episodes (Seizures) During EEEV Infection.

Seizures assessed by visual review of closed circuit video recordings and tallied by day post infection.

any conclusions from said data may only be generalized to EEEV-infected macaques that exhibit severe disease. Thereafter, EEG abnormalities, denoted by statistically significant delta, theta, alpha, or beta increase or decrease, were tallied by day post infection and assigned values: 0 = no

abnormalities, 1 = any abnormalities, in preparation for a logistic regression with the independent variable consisting of the day-matched number of seizures.

Logistic regression tests whether or not a given dataset fits a nonlinear curve; this curvilinear model is defined by a binary dependent variable and an ordinal (or binary) independent variable, and the reported p-value delineates whether or not to reject the null hypothesis that the slope of the relationship is significantly nonzero. Figure 24 illustrates the relationship between the number of seizures and the number of abnormal EEG event-days. M160-17 was the only macaque with ICP measurements, and the sample size was insufficient to perform a logistic regression with



Figure 24. Logistic Regression of Abnormal EEG vs Seizures.

The probability of finding abnormal EEGs increases as the number of seizures counted increases (p<0.05).

an acceptable degree of internal or external generalizability. However, to return to the relationship of abnormal EEG signatures and the prevalence of seizures, it appears that a weak correlation exists between the probability of finding abnormal EEG, defined as an abnormality in any of the EEG wave bands: delta, theta, alpha, and/or beta. Viewed under this lens, the data suggest a positive relationship between the probability that abnormal EEGs occur and the number of seizures associated with abnormal EEG, in EEEV-infected macaques with severe disease.

To gauge how well the appearance of seizures predicts abnormal EEG, a receiver operating characteristic (ROC) curve was constructed. As alluded to in Chapter 3, the ROC curve is a graphical rendering of the diagnostic potential of a binary variable for a specific outcome along its discrimination threshold; simply put, the curve itself is the sensitivity, or true positive rate, as the dependent variable plotted against 1-specificity, or the false positive rate. The area under the curve (AUC) denotes to a degree how successful the discriminator (seizures predict EEG, in this case), is at forecasting the outcome. An AUC = 0.50 implies that the discriminator has a 50-50 chance of predicting the outcome, and an AUC of 1.00 means the discriminator can predict the outcome with near or absolute certainty. Figure 25 shows the associated ROC curve from the logistic regression plotted in Figure 24. The reported AUC value for seizures as a predictor of abnormal EEG is 0.779,



**Figure 25.** Receiver Operating Curve of Seizures as a Discriminator for Abnormal EEG. Seizures are a fair predictor of finding abnormal EEG (p<0.01). The red dotted line denotes a curve an AUC = 0.50.

a fair, but not good or strong predictive value. However, the notion of predicting the finding of abnormal EEG by counting the number of seizures may seem slightly counterintuitive. To consider the opposite, and more intuitive perspective: that abnormal EEG may predict the seizure incidence, a slightly different approach to classification was taken with the logistic regression: with seizures defined as the dependent variable (0 = no seizures, 1 = one or more seizures), and abnormal EEG as the independent variable (X = 1,2,3, or 4 as the total number of abnormal wave bands) of wave bands delta, theta, alpha, or beta showing a statistically significant increase *or* decrease, Figure 26 is the resultant logistic regression curve with a slightly higher cut point than the logistic regression



Figure 26. Logistic Regression of Seizures vs Abnormal EEG.

The probability of finding seizures increases as the number of EEG wave bands with statistically significant differences increases (p<0.01).

depicted in Figure 24. Although no scenarios were encountered in which EEEV-infected macaques in this limited cohort of macaques experiencing severe EEEV disease experienced statistically significant differences in more than two of any given wave bands; delta, theta, alpha, or beta, the result of the logistic regression of the data showed a statistically significant nonzero slope (p = 0.0045).

In a similar vein, the ROC curve (Figure 27) for the propensity of abnormal EEG wave bands as a discriminator for the prevalence of seizures showed an improved ability for abnormal EEG to predict seizure prevalence than for the reciprocal relationship, with the AUC = 0.929 (p = 0.0016) compared to the previous AUC = 0.779 suggests that the use of abnormal EEG as a method



Figure 27. Receiver Operating Curve of Abnormal EEG as a Discriminator for Seizures. Abnormal EEGs appear to be an excellent predictor of finding seizures (p<0.01). The red dotted line denotes a curve an AUC = 0.50

of discriminating seizure activity merits additional consideration over the reciprocal definition and logistic regression that conceptualizes abnormal EEG as a predictor of seizure activity. However, given the small sample size of the macaque cohort studied for seizure detection (n=3), these results suffer from potential overfitting and positive result bias due to the selection of macaques screened for seizures emerging only from the group of EEEV-infected macaques exhibiting signs of severe disease. To address this limitation, a contingency table (Table 7) was constructed to compare the proportions of macaques with/without abnormal EEG by the latter definition of EEG abnormality

(cumulative number of wave bands demonstrating statistically significant increases *or* decreases compared to baseline) against the presence or absence of seizures. The contingency table was

	Seizure(s)	No Seizures
Abnormal EEG	5	3
Normal EEG	2	17

**Table 7.** 2x2 Contingency Table for Seizures and Abnormal EEG.

1

subjected to a  $\chi^2$  test with the Yates Correction for Continuity (to prevent the overestimation of statistical significance), which yielded a statistically significant  $\chi^2$  statistic of 5.44 (p=0.0196,  $\alpha$ =0.05). This leads to a rejection of the null hypothesis that the two variables are independent and suggests that the two variables: seizure incidence/prevalence and abnormal EEG findings are indeed correlated. The relative risk of manifesting seizures with the finding of abnormal EEG in EEEV-infected macaques with severe disease , is RR = 5.94, 95% CI : (1.61, 22.8).

# 4.3 Intracranial Pressure Increases in Febrile EEE

The raw traces of ICP were first aggregated and plotted against time, shown in Figure 28.



Figure 28. Raw EEEV ICP Time Series.

Time series traces of ICP from individual macaques, separated by severe and nonsevere disease, or mock-infection.

To ascertain what, if any group effects exist in the context of the onset of febrile illness or the appearance of signs of neurological disease, the data from the raw ICP traces for each individual macaque was aggregated into mean daily averages (Figure 29) which spanned days; for example, a data point at t = 5.5 DPI corresponds to the mean daily average of data from 5 DPI to 6 DPI, then converted to absolute percentage change from baseline (average ICP over the entire baseline period). In aggregate, the percentage change from baseline for mean intracranial pressure



Figure 29. ICP Absolute Percentage Change from Baseline in EEEV.

Repeated measures ANOVA with post-hoc pairwise comparisons found significant increases in ICP in the cohort of A) EEEV-infected macaques with severe disease over baseline in contrast to B) EEEV-infected macaques with nonsevere disease. Percentage change in mean ICP (daily mean +/- SEM) in EEEV-infected macaques in severe disease (n=4) and nonsevere disease (n=6), were each superimposed upon aggregate temperature data (crimson line) for the same cohort. Gray box indicates febrile period.

for the group of EEEV-infected macaques with severe disease rose to 143% of baseline and was statistically significantly increased compared to baseline at 6 DPI, though the apparent trend of increase of ICP begins before 6 DPI and closer to the time of febrile onset. In contrast, the percentage change in ICP stayed below 50% on any given day post infection for the group of surviving EEEV-infected macaques in the six-day period typical of survival for lethal macaques and never differed significantly compared to mock-infected macaques. Interestingly, with respect

to the group of macaques with severe EEEV, there exists a correlation (Pearson R = 0.593, p=0.0353) between the ICP and the increased temperature during the first four days of infection, noninclusive of the terminal decline in temperature seen in lethal cases. No correlation exists (Pearson R = -0.113) between the ICP of survivor macaques infected with EEEV and their temperature data (p = 0.6257).

#### 4.4 EEEV Infection Increases Biomarker Expression

Two core hypotheses provided the basis for the investigation detailed in this subchapter: i) higher levels of cytokine / TBI gene expression abound in brain sections of macaques with severe disease, and ii) genes of circadian rhythm regulation shall have more similar expression profiles in mock-infected macaques than in EEEV-infected macaques. These two claims underscore the idea that neurodegeneration features prominently in the course of viral encephalitic infections.

To address the first hypothesis, q-RT-PCR-based assays were performed to assess the status of gene expression in a group of EEEV-infected macaques euthanized at 6 DPI. As no sacrifices of EEEV-infected macaques with nonsevere disease occurred at 6 DPI, two VEEV-infected macaques sacrificed at 6 DPI served as substitutes for a "mild" disease phenotype and also to serve as a time-bound control for the expression of cytokines responsive to alphavirus infection. Finally, mock-infected macaques would serve as a reference control for the putative absence of any changes in gene expression due to lack of exposure to an infectious pathogen.

In the course of the overarching study of EEEV and VEEV infection of cynomolgus macaques, several cytokines had been positively identified by Luminex assays at higher than baseline concentrations in the blood and in the brain: MCP-1, IP-10, IFNγ, IL-1β, IL-6, and IL-8.

The literature has documented upregulation in IFN $\gamma$ , IL-1 $\beta$ , and IL-6, as well as MCP-1, leaving the question open as to the disposition of gene expression in disparate areas of the brain (169, 170). Similarly, the literature has alluded to increased upregulation by 100-fold or more of gene regulation of cytokines such as IL-1 or IL-6, and IFN $\gamma$ -responsive genes in lymphoid tissues and PBMCs during 1-4 DPI in VEEV.

Figure 30 documents the gene expression profiles of the cytokine panel in cerebellum and thalamus tissue in heat map format. At a glance, it appears that, referenced to GAPDH expression in mock-infected thalamus, that thalamic tissue in EEEV-infected macaques with severe disease experiences in general more upregulation of cytokine genes than does cerebellar tissue, with the exceptions of IL-1 $\beta$  and IL-8. This trend of increased expression in the thalamus relative to the cerebellum is also observed for the VEEV-infected macaques. However, in VEEV-infected macaques, though increases in gene expression of the selected cytokines also occur, they occur in fewer genes and also to a lesser degree of fold-change compared to the same in EEEV. An example of this phenomenon exists in cerebellar expression of IFN $\gamma$ ; while more than a geometric mean  $3\log_{10}$ -fold occurs in EEEV-infected macaques, the same in the VEEV-infected macaques registers at less than a 5-fold change. Finally, in the mock-infected macaques, no amplification occurred for IL-6, which can signal either muted expression due to the lack of pathogenic stimulus or the fact that circadian expression of IL-6 reaches a trough in the early light cycle and therefore rendered the levels of IL-6 in the mock-infected macaque below the level of detection.



Figure 30. Cytokine Gene Expression Increases in EEEV- and VEEV- infected Macaques.

Heat maps plot geometric means of fold-change in cerebellar and thalamic gene expression of cytokines in response to EEEV or VEEV infection relative to GAPDH expression in mock-infected thalamus. Fold-changes less than x5.0 not enumerated on heat map. Number of macaques under each group with samples performed in duplicate.

Likewise, the investigation of genes expressed in the aftermath of traumatic brain injury (TBI) comprised MMP-9, a biomarker of blood-brain barrier damage, GFAP, a gene expressed in

the circumstance of astrogliosis, and LIF, a marker of neuronal damage expressed by astrocytes or oligodendrocytes upregulate expression. Figure 31 illustrates in a similar manner to Figure 30 the



Figure 31. TBI Gene Expression Increases in EEEV- and VEEV-infected Macaques.

Heat maps of geometric means of fold-change in cerebellar and thalamic gene expression of traumatic brain injury species in response to EEEV or VEEV infection relative to GAPDH expression in mock-infected thalamus. Fold-changes less than x5.0 not enumerated. Number of macaques under each group with samples performed in duplicate.

gene expression profiles in heat-map format of the TBI panel. Surprisingly, there were no significant changes in the expression of GFAP between mock-infected, VEEV-infected, and EEEV-infected macaques; notably, this gene expression assay kit was one of the few that was not specifically tailored to the cynomolgus macaque. MMP-9 expression is upregulated in EEEV-macaques more so than in VEEV-infected macaques, and LIF follows the same pattern. Indeed, the pattern of increased gene expression in the thalamus relative to the cerebellum reflects a trend similar to that of the cytokine gene panel.

In both cerebellum and thalamus of EEEV-infected macaques, MMP-9 and LIF are significantly elevated over the expression in mock-infected macaques, with a larger effect size in the thalamus. In VEEV-infected macaques, only the expression of LIF is significantly increased in the thalamus relative to that of uninfected macaques.

With respect to cytokine expression in EEEV-infected macaques, IFN $\gamma$ , IP-10, and MCP-1 are significantly upregulated in the cerebellum when compared to mock-infected animals. In VEEV-infected macaques, both IP-10 and MCP-1 display increased upregulation in comparison to expression in mock-infected macaques. In the thalamus, statistically significant upregulation of IFN $\gamma$  and IL-6 was seen in EEEV-infected macaques; although IL-1 $\beta$  and MCP-1 also demonstrated a trend of increase in gene expression in EEEV-infected macaques, variation in the pool of mock-infected macaques for these analytes precluded the achievement of statistical significance for these species. Similarly, though IFN $\gamma$ , IP-10, MCP-1, and IL-6 demonstrated increased upregulation in VEEV-infected macaques, only IP-10 was statistically significantly increased in comparison to mock-infected macaques. Although no conclusions should be drawn from the following, informal comparisons with respect to the fold-upregulation of MMP-9 and LIF in EEEV-infected and VEEV-infected macaques reveal at a glance that upregulation of MMP-9 and LIF are increased at least an order of magnitude in EEEV-infected macaques relative to VEEV-infected macaques, in both the thalamus and the cerebellum.

The results for the expression of genes of circadian pattern regulation warrant a short reintroduction: a large number of genes govern the regulation of circadian rhythms in the body. One particular genetic circuit consists of the CRY1/2 – PER3 – CLOCK complex. CLOCK is a transcription factor that, as part of a complex of other proteins, regulates enhancer box or E-box genes. E-box genes regulate gene expression in a number of terminally differentiated tissues, with tissue-specific patterns of downstream gene regulation. As one may surmise, CLOCK activity varies with circadian rhythm, by means of negative feedback control by proteins CRY1, CRY2, and PER3. Therefore, when cellular concentrations of CRY1, CRY2, and PER3 are increased, CLOCK gene activity, and therefore the downstream expression of E-box genes is suppressed. As the concentrations of CRY1, CRY2, and PER3 decline, CLOCK transcriptional activity of E-box genes increases and drives the downstream synthesis of more CRY1, CRY2, and PER3. This cycle occurs in a manner such that CLOCK transcription is most active during periods of natural light or daytime, and conversely, that CRY1, CRY2, and PER3 concentration and activity become most active during periods of darkness or nighttime. Mechanisms of circadian entrainment in lightsensitive tissues, or other modes of reception of environmental input can alter these circadian patterns of expression, and pathological processes such as the physiological milieu brought about by alphavirus infection have the potential to desynchronize or disrupt the normal, homeostatic rhythms of circadian regulation. At a glance, Figure 32 shows that geometric means of genes of circadian regulation remained apparently unaffected by EEEV- or VEEV-infection with the exception of PER3, which experienced downregulation in both cerebellum and thalamic tissue samples in EEEV-infected macaques that displayed severe disease.

#### Circadian Panel: GM Fold Change Relative to 18S rRNA in Mock Thalamus CEREBELLUM Circadian Panel - Cerebellum 100 Fold-Upregulation PER3 ×0.05 80 e CLOCK 60 40 RO 20 CRY2 0.00 EEEV VEEV

-2)



Figure 32. Expression of PER3 Decreases in EEEV- infected Macaques.

Heat maps plot geometric means of fold-change in cerebellar and thalamic gene expression of clock genes in response to EEEV or VEEV infection relative to 18S rRNA expression in mock-infected thalamus. Fold-changes greater than x5.0 not enumerated on heat map. Number of macaques under each group with samples performed in duplicate.

in the context of EEEV- or VEEV-infected macaques. Because of the nature of circadian gene expression, the choice of an appropriate endogenous control was critical in ensuring that the design of the experiment did not introduce bias into the experiment. 18S rRNA was chosen as the endogenous control because its constitutive expression and centrality for all cellular processes involving protein expression minimized the putative cyclical effects of circadian expression.

# 5.0 VEE in Cynomolgus Macaques

The VEEV-infected macaque cohort, in contrast to the EEEV-infected macaque cohort, was investigated for its relatively mild presentation of signs of neurological disease, compared to EEEV-infected macaques with severe disease. To wit, the aim associated with this chapter sought to determine whether telemetric features of the VEEV disease course provide means of predicting the onset of febrile encephalitis. Do EEG and/or ICP present a useful set of biomarkers to signal when the virus reaches the CNS, and if so, how do the EEG and ICP data compare with findings in the literature regarding human disease? The cynomolgus macaque model of VEEV presents as an almost uniformly mild febrile illness with a biphasic febrile period, with limited signs of neurological disease and no signs of severe encephalitic disease such as seizure or coma. In contrast to the cohort of EEEV-infected macaques with severe disease, VEEV-infected macaques discussed in this chapter were not expected to achieve a moribund state, allowing for a relatively longer period for analysis of a milder encephalitic disease phenotype, including a dedicated study of the disruption of circadian patterns in response to encephalitic disease. Similar to the effects observed in EEEV-infected macaques with severe disease, VEEV-infected macaques were expected to demonstrate either or both increases in slow-frequency EEG and global decreases in EEG power spectral density; likewise, ICP was expected to increase in close temporal proximity to febrile onset. Given the biphasic nature of the fever associated with VEEV in infected macaques, investigation of the timing of increase in ICP in VEEV-infected macaques proved fertile ground for investigation. Characterization of bloodborne and/or CSF viremia was a core facet of contextualizing the relationship of EEG and ICP changes to CNS disease. Table 8 lists all macaques pertinent to Chapter 5; and "day euthanized" refer to days from the day of exposure.
Macaque	Age (yr)	Virus	Inhaled Dose (PFU)	Day Euthanized (d)	Implant Model/Type
M114-16 🖒	5	Mock	-	28	-
M115-16 🖒	6	Mock	-	30	M11: EEG/ICP
M116-16 🖒	5	Mock	-	28	M11: EEG/ICP
M117-16 🖒	6	Mock	-	30	-
M121-16 ♀	6	VEEV INH-9813	5.93 x 10 <sup>6</sup>	35	M01: EEG
M122-16 ♀	5	VEEV INH-9813	1.49 x 10 <sup>7</sup>	33	M01: EEG
M53-17 👌	6	VEEV INH-9813	1.90 x 10 <sup>8</sup>	28	M01: EEG
M54-17 ♂	5	VEEV INH-9813	2.48x 10 <sup>8</sup>	29	M01: EEG
M111-18 👌	6	VEEV INH-9813	3.16 x 10 <sup>7</sup>	52	L11: EEG/ICP
M112-18 👌	9	VEEV INH-9813	2.00 x 10 <sup>7</sup>	54	L11: EEG/ICP
M115-18 🖒	5	VEEV INH-9813	1.58 x 10 <sup>7</sup>	48	L11: EEG/ICP
M116-18 👌	9	VEEV INH-9813	6.31 x 10 <sup>7</sup>	50	L11: EEG/ICP
M107-18 ♀	5	VEEV INH-9813	2.90 x 10 <sup>6</sup>	29	M11: EEG/ICP
M109-18 ♀	5	VEEV INH-9813	9.51 x 10 <sup>6</sup>	29	M11: EEG/ICP

 Table 8. Aim 2 Macaque Cohort Characteristics.

The biphasic fever of the VEEV-infected macaques is illustrated in Figure 33; compared to the mock-infected macaques, a definite first febrile peak (F1: 0.5 - 2.0 DPI) appears during the day following infection, before a temporary decline in temperatures that lasts for approximately half a day, followed by a second febrile peak (F2: 2.5 - 8.0 DPI). The fever then resolves after approximately a week. During this time, signs of neurological disease manifest, such as involuntary tremor or muscular twitch,



Figure 33. VEEV in Cynomolgus Macaques.

A) Temperature traces (averages of temperature traces from all macaques grouped by disease phenotype) of VEEVinfected and mock-infected cynomolgus macaques implanted with EEG/ICP implants investigated for Aim 2. "P" denotes "Post-Infection," "F1" denotes first febrile peak, while "F2" denotes B) Overall model of VEEV disease course in the telemetered macaque. The pre-infection period consists of the duration of time before the aerosol challenge, which varies between macaques; following aerosol exposure, all macaques experience a biphasic febrile phase following an immediate post-infection period, followed by a recovery – defined by the resolution of fever.

nystagmus, photophobia, lethargy, and most commonly, stress yawning. For the purposes of model construction, as depicted in Figure 33B, the entire biphasic fever was considered the "febrile period, encompassing F1, F2, and the time in between. However, to gauge for distinctive EEG or ICP effects during each period, F1 and F2 were treated as discrete periods for analytical purposes.

### 5.1 Pathological EEG Manifests During Febrile VEE

Investigation of characteristic signs of VEEV encephalitis by EEG was a more complicated process than accounting for changes in EEG in EEEV-infected macaques. Preliminary analysis of a macaque with disease following VEEV infection as an archetype of encephalitic VEEV infection in macaques is illustrated in Figure 34A alongside activity and power band traces from one macaque that developed disease following VEEV infection. Note also slight dips every other day in the temperature trace due to anesthesia administered for blood draws. Initially, the time series analysis also shows a regular and robust circadian modulation much like the mock-infected macaques. However, on 7 DPI, there was a reduction in both beta power and delta power, with the latter finding being part of a trend ranging from 5-10 DPI, and a suppressed circadian index. These findings were also visible in the PSD plots and subsequent statistical analyses (Figure 34B, 34C, and 34D). It is notable that both of these changes are seen during the second febrile period, which has been hypothesized to be a period in which the virus penetrates the CNS (62). Note the massive increase in delta waveband intensity in night 12 post-infection. This may correspond to a rebound of slow-wave sleep after several days without discernable activity in the same during the night. With respect to whole-group effects across the cohort of VEEV-infected macaques with EEG and EEG/ICP implants examined in the context of Aim 2, two main findings arose. The first finding was clearer and more substantiated by a simple overview of the daily power spectral density plots of the VEEV-infected macaques: all macaques in the cohort appear to exhibit a global drop in all EEG waveband magnitude from approximately 4-7 DPI, depicted in PSD plots in Figure 39. The second finding lies in the apparent increase in delta waveband magnitude during 1-2 DPI in some, but not all, macaques in the VEEV-infected macaque cohort; this phenomenon is showcased in Figure 40. When subjected to increased scrutiny for group-wide effects, by an identical process of



Figure 34. EEG Processed Time Series in the VEEV-infected Macaque.

A) EEG time course and accompanying B) PSD plot from VEEV-infected macaque (M115-18); dotted lines represent one standard deviation as upper and lower bounds. Black circles indicate decreased delta power and beta power on 7 DPI, visualized on the PSD plot with corresponding arrows in comparison to baseline spectra for that macaque. Note also taupe ellipse marking suppression of sleep index from 5-8 DPI. C) T-test of delta power and D) beta power between 7 DPI and baseline data demonstrate statistically significant decreases (p<0.0001). The febrile period for VEEV is biphasic with two fever periods from 0.5-2.0 DPI and



from 2.5-8 DPI. Solid magenta bar on Day 0 indicates time of aerosol exposure, gray boxes indicate night.

Figure 35. EEG Magnitude Trends Downward in F2 of VEEV.



VEEV-infected macaques exhibit an apparent global trend of decrease in all EEG wavebands from 4-7 DPI.

Figure 36. Delta EEG Magnitude Trends Upward in F1 of VEEV.

Some but not all animals display an increase in delta waveband activity on 1-2 DPI.

conversion to percent change from baseline values across several periods: baseline, post-infection (0-1 DPI, non-inclusive), fever peak 1 (F1; 1-2 DPI, inclusive), fever peak 2 (F2; 3-8, inclusive), and recovery (9+ DPI). Group averages of percent change, as in EEEV-infected macaques shown in Figure 21, were taken for the VEEV-infected cohort of macaques and compared against baseline by repeated measures ANOVA in Figure 37. From this rigorous statistical analysis, it appears that



Figure 37. Group Averages of VEEV PSD Plot Magnitude as Percentage Change from Baseline.

Power spectral density plotted as percent change from baseline in cohorts of VEEV-infected macaques. This plot illustrates traces from individual macaques which show the disparate PSD curves in each disease period. Wave bands are color coded by shaded boxes; the box plots at each discrete frequency segment are compared between baseline, incubation, and febrile periods. Repeated measures ANOVA and *post hoc* pairwise comparisons at each discrete frequency showed a statistically significant decrease in all wavebands during F2 (p<0.05).

only the first assertion: global decline in EEG PSD magnitude during the second febrile period prevails as a distinctive group-wide effect occurs. Although certain exceptions apply, such as small segments of discrete frequencies from 10 - 11 Hz and 22.5 - 26.75 Hz, each previously defined wave band: delta, theta, alpha, and beta all experience an approximate 5% decrease from baseline (p<0.05).

Substantial variation between macaques with regard to this finding occurs, although a trend upward in percent change from baseline occurred in certain wave bands (e.g. delta) during the first febrile period of the VEEV-infected macaque cohort, the effect was not pronounced enough to warrant statistical significance (p > 0.1557). In contrast, the downward change in all bands of EEG activity that occurs in concordance over the second febrile period, was statistically significant despite a relatively small effect size (p < 0.0483). Curiously, as with the EEEV-infected macaques, other trends such as increases in percent change from baseline of alpha band EEG magnitude also occurred during the entire duration of fever, though these were again not statistically significant at the group level (p>0.0502). The implications of a global decline in EEG activity in the second febrile period of VEEV suggest pathological neurological activity though the finding does not directly support the condition of increased slow-wave EEG in the context of encephalitic onset. The data suggest that the decrease in EEG magnitude during the second febrile period of VEEV is real and not artifactual. However, a trial of experimental anti-VEEV antibodies in Chapter 6 offered additional opportunities to document by EEG the onset of neurological disease in VEEVinfected macaques.

### 5.2 Circadian Disruption Occurs During Second VEE Fever

Sub-aim 2B sought to characterize what, if any, circadian disruption occurs via EEG or ICP indicators. The circadian index was discussed in Chapter 3 and was constructed by subtracting delta band activity from beta band activity. This is held to represent diurnal variation in brain activity between sleep and waking states; while peaks in the circadian index may indicate rapid eye movement (REM) versus non-REM sleep, this is a level of nuance that is not necessarily resolvable for the scope of this work. Rather, the focus lay in the determination of whether significant pathology suggestive of circadian disruption in the CNS could be detected by the telemetric means, e.g. EEG, available. Results from gene expression assays in Chapter 4.4 showed no statistically significant changes in the expression of genetic regulators of circadian rhythm, although the data encapsulated therein represented a cross-sectional time point taken at 6 DPI. The analysis of the EEG data, due to its quasi-continuous nature, allowed for additional insight into vEEV infection of the CNS.

The time-series traces of the circadian indices revealed a visually appreciable suppression of circadian indices, as showcased in Figure 34 in the previous subchapter, across all macaques in the VEEV-infected cohort, empaneled in Figure 38. Between approximately days 5-8, across all VEEV-infected macaques, manifests a suppression in the amplitude of the circadian index not seen in mock-infected animals. Although this presaged the existence of a pattern of circadian disruption in these macaques, the analysis of the periodicity of the circadian index was hampered by segments of missing data; to overcome this limitation, the Lomb-Scargle methodology of least-squares frequency analysis was employed to account for the gaps by adopting a model to "fill in" the missing periodic data as a practical means of constructing accurate periodograms for circadian analyses (289, 290). Circadian indices were compared via repeated measures ANOVA of their fundamental frequencies, or the lowest frequency of the associated waveform. This corresponds to the lowest frequency with the highest magnitude corresponding to previously outlined baseline,



Figure 38. Time Series Circadian Indices of Mock- and VEEV-infected Macaques.

Time series traces of A) Mock-infected macaques and B) VEEV-infected macaques. Note the decrease of amplitude in the circadian index during approximately 5-8 DPI, of variable magnitudes in different macaques during F2.

post-infection, F1, F2, and recovery periods. Should no significant disruption occur, the fundamental frequencies should remain unchanged from pre- to post-infection.

The Lomb-Scargle methodology of examining changes in circadian patterns was first piloted in the cohort of EEEV-infected macaques with and without severe disease, as well as in the mock-infected macaques. Figure 39 demonstrates the differences seen in EEEV-infected and mock-infected macaques between the febrile period and the post-infection period. This method of analysis demonstrates that there indeed exists a distinguishable, statistically significant difference



**EEEV Circadian Indices** 



Repeated measures ANOVA of uninfected macaques, macaques with nonsevere courses of EEEV (no discernable neurological disease), and macaques with severe EEEV disease show the suppression of circadian variation in macaques with severe EEEV (p < 0.0001) and virtually no changes in the former two groups.

between the circadian indices during the time period between 3-6 DPI for the EEEV infected macaques versus a time period of equal length during the baseline period.

To return to VEEV specifically, the suppression of circadian indices as illustrated by Figure 38B suggested the coincidence and/or contemporaneous onset of disrupted circadian patterns with the occurrence of the second febrile period, and moreover seemed in most cases to have been driven by the decrease in delta band magnitude. The drop in delta band magnitude was part and parcel of the global drop in EEG magnitude during the second febrile period discussed in Chapter 5.1; although decreases in beta band magnitudes also occurred, the effect size of the delta band decrease appears to have overshadowed that of the beta band decrease and can perhaps explain the differential degrees of circadian index suppression illustrated in Figure 38B. Figure 40 showcases







Repeated measures ANOVA of uninfected macaques and macaques infected with nonsevere courses of VEEV show the suppression of circadian variation during the second febrile peaks compared to baseline in VEEV-infected (p < 0.0001) and also a significant decrease compared to the same period in uninfected macaques (p<0.05). the changes in fundamental frequencies during the different outlined periods. For all periods except for the second febrile period, no statistically significant changes occurred between either the VEEV-infected and mock-infected cohorts, or within the disparate segments of the VEEV-infected cohorts. However, the data for the second febrile period in the VEEV-infected macaque cohort exhibits a loss of higher-frequency range fundamental frequencies, which reinforces the visually distinctive suppression of the circadian index: should the variability of the post-infection period resemble the pre-infection period, the thing should be unchanged.

Although the ANOVA for Figure 40 was not significant overall and thus signals a failure to reject the null hypothesis that the means are all equal, the individual means for the fundamental frequencies in the unaffected segments are all roughly similar, so this result for the overall ANOVA should not be surprising. However, the data for the pairwise comparisons shown in Figure 40 reinforce the data shown in Figure 38, wherein the fundamental frequency decrease reflects the suppression of the amplitude of the circadian index. The lack of significance for the ANOVA between the outlined periods should not preclude the interpretation of the results (291).

Across the population of macaques exposed to alphaviruses EEEV or VEEV, the blunting of the circadian rhythms was the most common pathological change visually appraisable on firstpass assessments of the processed time-resolved EEG. The duration of suppression of the circadian index for VEEV-infected macaques is typically longer than for the EEEV-infected macaques. Intuitively, the heightened severity of disease in EEEV-infected macaques, whose neurological signs of disease warrant euthanasia during the febrile period, may mask this effect. Additionally, the EEG spectra of macaques infected by VEEV demonstrated less intense deviations from baseline magnitudes (as assessed by PSD plots) compared to EEEV-infected macaques.

# **5.3 Intracranial Pressure Increases in VEE**

To characterize the relationship between ICP and fever, the raw traces of ICP in VEEVinfected macaques were aggregated and plotted as time series, as illustrated by Figure 41. In this



Figure 41. Raw VEEV ICP Time Series.

Time series traces of ICP from individual macaques, separated by A) VEEV-, or B) mock-infection.

cohort, changes in ICP were initially less visible from baseline than for EEEV-infected macaques. However, visually apparent increases did seem to occur between days 5-10 DPI. To determine whether these changes were statistically significant across the group of VEEV-infected macaques, the data from these raw traces was again aggregated into mean daily averages spanning whole days and converted to absolute percentage change from baseline, shown in Figure 42. In aggregate, the



Figure 42. ICP Absolute Percentage Change from Baseline in VEEV.

Repeated measures ANOVA with post-hoc pairwise comparisons found a significant increase in ICP on 7-8 DPI (p<0.05) in the cohort of VEEV-infected macaques with severe disease over baseline ICP. Absolute percentage change in mean ICP was superimposed upon aggregate temperature data (crimson line) for the same cohort. Gray boxes indicate febrile periods of the biphasic fever, F1 and F2; magenta line indicates time of aerosol challenge.

percentage change from baseline for VEEV-infected macaques saw over 50% increases from baseline between days 6-9 DPI, noninclusive. However, only the increase in ICP from the

beginning to the end of 7 DPI, a 66.5% increase over baseline, was statistically significant (p=0.0483).

This time point falls into the second febrile period; as can be seen in Figure 42, the trend of increase in ICP appears to begin during the second febrile period, as well; this suggests that the elevation of ICP occurs either contemporaneously with the infection of the CNS or subsequent to it. This implication, in conjunction with the persistence of elevated ICP after the resolution of fever suggests that as in EEEV-infected macaques, the increase in ICP in VEEV-infected macaques succeeds that of temperature. To wit, an inverse correlation (Pearson R = -0.9878) between ICP and temperature between 4-8 DPI in mean daily averages (p = 0.0122) that corresponds to the increase of ICP in VEEV-infected macaques during the decline of the second febrile peak.

## 5.4 Plasma and CSF Viremia In VEE

The purpose of sub-aim 2D lay in the examination of the relationship between viremia and EEG/ICP changes. Presumably, the viral titer, with viral genome copy number as determined by q-RT-PCR as a surrogate measure, would see a positive correlation with respect to EEG or ICP changes suggestive of virus penetration/infection of the CNS. Figure 43 illustrates the peak times of plasma viremia from longitudinal blood samples in the VEEV-infected macaque cohort (schema of blood draws documented in Figure 16). The plasma viremia peaks between 0-4 DPI; although Figure 48 demonstrates unequivocal *prima facie* peaks at 2 DPI, this could represent an outcome



Figure 43. Plasma Viral Genome Copy Numbers in VEEV-Infected Macaques.

Plasma viremia appears to peak at 2 DPI then declines to non-detectable levels after 4 DPI in this sample of macaques.

driven by the fact that blood was drawn immediately preceding aerosol challenge then every other day afterwards, leaving a gap of a day between the data points portrayed. Parallel viral titers in plaque assay corroborate a peak viremia at 2 DPI with infectious virus, although the lack of positive results after 2 DPI suggest that q-RT-PCR may constitute a more sensitive assay or that the viral genomes detected by the q-RT-PCR did not represent replication-competent or infectious virus. While data discussed in Chapter 5.1 suggested that a putative increase in delta band activity might occur during the first febrile peak in VEEV, the peak viremia does in fact coincide with the first febrile period, presumably coinciding with the lymphotropic aspect of VEEV. However, no significant increases in plasma titers occurs during the second febrile period or peak of VEEV disease in this cohort of macaques studied, the same period during which both significant changes in EEG and suppression of the circadian indices were detected. Therefore, based off the available information, it appears that plasma viremia precedes changes in EEG reflective of neurological disease. A similar temporal relationship occurs between the plasma viremia and the increase in ICP documented in the preceding subchapter.

Likewise, Figure 44 documents the viral genome copy numbers/concentrations found in solid tissue specimens at necropsy, including the lobes of the brain, lymph nodes, and CSF from



Figure 44. Solid Tissue and CSF Viral Genome Copy Numbers in VEEV-Infected Macaques.

vRNA copies were isolated in varying concentrations in VEEV-infected macaques necropsied at differing time points. Virtually all macaques infected with VEEV showed vRNA in lymphoid tissue, at least one lobe of brain, and in CSF at necropsy. Notably, macaques sacrificed at 6 DPI following VEEV infection did not yield vRNA in the CSF. the VEEV-infected macaques offer cross-sectional examinations at varying time points following infection. Apart from the macaques sacrificed at 6 DPI, for which no EEG data was available, most macaques sacrificed between 26-52 DPI yielded viral RNA in the CSF at necropsy. However, as the time period (the second febrile period of VEEV) during which EEG changes and circadian disruption occur span the pair of macaques sacrificed at 6 DPI, the basis for deriving a relationship between EEG pathology and viral RNA in the CSF remains fairly weak. Based on the information available, it appears that no relationship exists between EEG changes and the appearance of vRNA in CSF during the second febrile period wherein EEG changes have appeared in this cohort of VEEV-infected macaques. Likewise, there additionally appears to be no relationship between the appearance of ICP changes to viremia in the plasma or in the CSF. Although preliminary examination of raw ICP traces appeared to reveal a relationship between the absolute value of ICP increase with the CSF titer at necropsy, the effect was not statistically significant, and the different time points at which the necropsy samples were obtained (a 20-day or more period between the L11 samples and the M11 samples) proved problematic for direct comparison.

# 6.0 Windowing, Optimization, and Therapeutic Trials

The goal of the Aim 3, discussed in Chapter 2, metamorphosed from the utilization of the telemetered model as a means of detecting disease by augmenting to it the goal of evaluating the efficacy of medical countermeasure candidates. That is, how did EEG/ICP data collected from macaques in a trial of a therapeutic candidate aid in determining efficacy, if at all? A cohort of 14 macaques, documented in Table 9, were infected with VEEV strain INH 9813, same as in Chapter

Macaque	Age (yr)	Virus	Inhaled Dose (PFU)	Group	Implant Model/Type
M115-16 🖒	6	Mock	-	MOCK	M11: EEG/ICP
M116-16 👌	5	Mock	-	MOCK	M11: EEG/ICP
M96-19 ♂	2	VEEV INH-9813	1.35 x 10 <sup>7</sup>	IN+IV	M01: EEG
M97-19 👌	2	VEEV INH-9813	3.47 x 10 <sup>7</sup>	IN	M01: EEG
M98-19 ♂	2	VEEV INH-9813	5.13 x 10 <sup>6</sup>	IN	M01: EEG
M99-19 ♂	2	VEEV INH-9813	3.98 x 10 <sup>6</sup>	CONTROL Ab	M01: EEG
M100-19 ්	2	VEEV INH-9813	1.74 x 10 <sup>7</sup>	IN+IV	M01: EEG
M101-19 ♀	2	VEEV INH-9813	1.82 x 10 <sup>7</sup>	CONTROL Ab	M01: EEG
M102-19 ්	3	VEEV INH-9813	3.09 x 10 <sup>6</sup>	IN	M01: EEG
M103-19 🖒	2	VEEV INH-9813	6.17 x 10 <sup>6</sup>	IV	M01: EEG
M104-19 ♀	3	VEEV INH-9813	4.47 x 10 <sup>6</sup>	IN	M01: EEG
M106-19 ♀	2	VEEV INH-9813	6.61 x 10 <sup>6</sup>	IV	M01: EEG

Table 9. Anti-VEEV Antibody Trial Macaque Cohort and Groups.

Table 9 (cont'd)								
M107-19 ♀	2	VEEV INH-9813	2.51 x 10 <sup>6</sup>	IN+IV	M01: EEG			
M108-19 ♀	3	VEEV INH-9813	1.70 x 10 <sup>7</sup>	IV	M01: EEG			
M109-19 ♀	3	VEEV INH-9813	1.35 x 10 <sup>7</sup>	CONTROL Ab	M01: EEG			
M110-19 ♀	2	VEEV INH-9813	9.33 x 10 <sup>6</sup>	IN	M01: EEG			

5, for the purposes of testing the efficacy of an experimental therapeutic antibody against VEEV. Preliminary work with thermometry had suggested that macaques infected with VEEV that were administered a monoclonal antibody therapeutic candidate subsequent to the first febrile peak (approximately 1-2 DPI) did not manifest the second febrile peak characteristic of the VEEV biphasic fever in macaques, compared to macaques administered an antibody isotype control, illustrated in Figure 45.

The treatment of an experimental group of cynomolgus macaque with a putative antibody therapy at the onset of the first febrile peak of the biphasic fever saw the suppression of the second febrile peak of the same. A control group of macaques that received a placebo treatment manifested a second febrile peak as might be expected of a typical course of VEEV. The underlying expectation forecasted the reduction of signs of febrile encephalitis detectable by EEG and ICP in the context of the prophylactic administration of virus-specific therapeutic antibodies as a passive immunization method. Specifically, that the mitigation or abolition of the global decrease in EEG PSD plot magnitude during the second febrile period might occur in macaques that receive prophylactic administration of anti-VEEV antibody compared against those administered an anti-Dengue Virus (Anti-DENV) control antibody. As the macaques receiving the experimental antibodies were not equipped with EEG/ICP implants, there was no way within the constraints of this experimental setup to assess the effects of the treatment upon intracranial pressure. The means by which the VEEV-infected macaques in this cohort would be evaluated constituted the same means of evaluation discussed in the previous chapter, with respect to analysis of EEG frequency spectra and the analysis of the disruption of circadian patterns.



Figure 45. Putative Monoclonal Antibody Treatment of VEEV.

Experimental anti-VEEV antibody administered following the first febrile peak of VEEV facilitated the mitigation of the second febrile peak in treated, VEEV-infected macaques.

Finally, the next subchapter shall briefly discuss windowing periods assessed for the majority of the work performed for Chapters 3 through 6; telemetry data collection proceeded in a manner that captured data in real time at a sampling rate of 500 samples per second, but the

implications of window period choice precipitated significant implications for the results generated and the conclusions derived from the results.

## 6.1 Daily Grand Averages Comprise Optimal Windowing Period

Throughout the course of this work, there existed a need to balance the extraction of information from raw EEG and ICP traces from which meaningful conclusions could be interpreted without excessive risk of artifact or noise. The bar for adjusting this signal-to-noise ratio, so to speak, would in part determine the nature of the conclusions reached. The telemetry data was collected, as described, at 500 samples per second. Analysis of telemetry was to occur in real time – in 30-minute, 1-hour, 6-hour, 12-hour, and 24-hour reports, to minimize false positives and false negatives. To this effect, windowing periods of 1 minute, 5 minutes, 15 minutes, 1 hour, 6 hours, 12 hours, and 24 hours were assessed for differences between infected and mock-infected animals. The EEG raw trace underwent frequency spectrum analyses to assess low and high frequency brainwave activity, analyzable by repeated measures ANOVA.

However, one pitfall of utilizing ANOVA lies in the inescapable fact that ANOVA evaluates whether one or more group means differs significantly from the rest. While the use of ANOVA could be useful with respect to the testing for statistically significant differences in time series data, the time-point-by-time-point comparison of EEG waveband data or ICP data could prove computationally burdensome. Figure 46 illustrates patterns exhibited by the examination of the magnitude of delta wave band EEG by five minute intervals and by mean daily averages. While the 5-minute intervals provide the viewer of the data a much more granular view of circadian variation in the EEG at large, the large amount of data and the variability of individual EEG traces

by macaque made this format unwieldy to work with. In contrast, the daily averaged data provide a much more succinct summary of the data, at the risk of losing the visual representation of circadian variation to an overly reductionist approach. Ultimately, neither of these forms of representation or measures of central tendency were chosen, as the best means of comparing each



Figure 46. Putative Monoclonal Antibody Treatment of VEEV.

Examples of differing windowing periods of delta wave band EEG magnitude, sampled at either A) five minute intervals or B) mean daily averages. Both graphs demonstrate increased magnitude during the febrile period of EEEV.

macaque's data to its own baseline period called for the averaging of the absolute percent change of the magnitude from baseline, over the period of 24 hours, after much consideration and referral to reviews of studies of circadian patterns (292). Additional weight was given to conclusions reached that were generalizable regardless of which windowing period was chosen, such as increased delta wave band magnitude during the febrile period of severe EEEV.

The focus of the investigation shifted from prediction to evaluation owing to evidence such as the body of work presented in Chapter 4 and Chapter 5 that suggested EEG and ICP changes occurred either contemporaneously or after the incidence of fever and clinically observable signs of neurological disease. A 24-hour period, in the context of the simultaneous study of EEG, circadian indices, seizures, and ICP, appeared to be the optimal choice of windowing period, due to its utility in segregating results into discrete time segments relative to day post infection, and into pertinent periods of disease, if need be. For example, paroxysmal episodes, or putative seizures, were counted by day and attributed/correlated with abnormal EEG over a 24-hour period. Seizures constituted relatively rare events even in the context of severe EEEV disease, such that grouping them by smaller denominations risked ever greater complexity. Similarly, the resolution of fundamental frequencies resulted in results on the order of miniscule values of cycles per second, such that results were more viably rendered in units of cycles per day (cpd), which intuitively complements the natural variation of circadian patterns.

### 6.2 Anti-VEEV Antibody Mitigates VEE

The investigation of the impact of the therapeutic anti-VEEV antibody proceeded according to the experimental schema illustrated in Figure 47. Macaques were placed in groups of



Figure 47. Antibody Treatment Experimental Schema.

at least 3 macaques each, by route of anti-VEEV antibody administration: intranasal (IN, n = 5), intravenous (IV, n = 3), and combined intranasal and intravenous antibody administration (IN+IV, n = 3). An antibody isotype (CONTROL, n = 3) consisting of anti-DENV antibody was administered intranasally as a negative control, while uninfected control (MOCK, n = 2) data was taken from the previous macaque studies for the sake of comparison for statistical analysis. The inciting set of analyses aside from purely descriptive statistics regarding the behavior of EEG power band data assessed the behavior of EEG during the Baseline, post-infection, F1, F2, and Recovery periods, as outlined in Chapter 5. A 3-way ANOVA (2x2x2) that controlled for the disease period, power band, and treatment showed that significant differences existed between the measures of central tendency (on initial evaluation, the median EEG magnitude rather than mean) for the EEG power bands at disparate periods of the disease (p < 0.01). All else held equal, one would expect that: i) the group of VEEV-infected macaques receiving the control anti-DENV antibody treatment would experience a disease course similar to untreated VEEV-infected macaques, and that ii) one or more groups of VEEV-infected macaques receiving the experimental anti-VEEV antibody, regardless of route of administration, might show EEG activity in their respective wavebands comparable to baseline data, on the premise that the anti-VEEV antibody demonstrates efficacy.

As mentioned, medians were initially reported for each waveband of activity to filter out noise. Each median value was attributed to a single disease period, just as it was later performed for PSD plots and CI plots. Certain characteristic trends were identified in the VEEV-infected macaques from median values that recapitulated features of VEEV disease identified in Chapter 5: while not statistically significant during the analysis of median values, all wave bands, with the exception of beta wave band EEG, experienced decreases in magnitude during the second febrile period. The statistically significant finding of the *post hoc* pairwise comparisons between different disease periods versus baseline associated with the 3-way ANOVA found that median delta wave band EEG magnitude during the post-infection period (0-1 DPI) was increased over baseline in the group of macaques administered anti-DENV control antibodies (p = 0.0233), a result congruent with observed EEG changes in VEEV-infected macaques documented in Chapter 5. While this finding suggested that VEEV-infected macaques in the control group did in fact manifest disease, the nonsignificant findings in the IN+IV group, which showed relatively little, if any changes, across all EEG wave bands intuitively suggested in opposite fashion that abnormal EEG did not present during the post-infection period in any of the wavebands, nor during any of the periods. The results from this preliminary first-pass were somewhat corroborated by a review of the neurological scores assigned to the macaques by veterinary staff on the lookout for signs of clinically apparent VEE such as tremors or involuntary movement, illustrated in Figure 48.



**Figure 48.** Daily Neurological Scores of VEEV-Infected Macaques Given Experimental Antibody. Median neurological scores of VEEV-infected macaques administered A) Anti-DENV control antibody and B) IN+IV route anti-VEEV experimental antibody. Red arrows demarcate the post-infection period prior to the febrile period during which macaques apparently experience fewer signs of neurological disease in the IN+IV group.

Subsequent to this preliminary screening of the antibody-treated macaque cohort, a more comprehensive approach similar to that taken in Chapter 5 was taken to evaluate whether or not the experimental antibodies achieved their desired effects.

Figure 49 represents a panel of EEG PSD plots of the first few days following infection (post-infection and F1), while Figure 55 illustrates EEG PSD plots during F2 and early recovery.



Figure 49. EEG PSD Trends in Early VEEV Infection, by Experimental Antibody Cohort.

Panel of PSD plots from 1-3 DPI in (from left column to right column) IN+IV experimental antibody group, IN antibody group, and anti-DENV control antibody group.

Notice the increase in delta band EEG magnitude in the control cohort of macaques prophylactically exposed to anti-DENV antibodies. This partially corroborates the earlier finding from the 3-way ANOVA that delta activity is pathologically increased during the immediate postinfection period in the group of macaques who were administered antibodies that should theoretically have no impact on mitigating or abrogating the course of VEEV disease in infected macaques. By the same token, in the left column of Figure 49, the cohort of macaques that received anti-VEEV antibodies intranasally and intravenously did not exhibit such an increase, as the 3-way ANOVA had also found. Meanwhile, although the trend did not achieve statistical significance in the 3-way ANOVA, the characteristic global decrease in EEG magnitude during



**Figure 50.** EEG PSD Trends During F2 in VEEV Infection, by Experimental Antibody Cohort. Panel of PSD plots from 1-3 DPI in (from left column to right column) IN+IV experimental antibody group, IN antibody group, and anti-DENV control antibody group.

F2 can clearly be visualized in the right column of Figure 50, suggesting a phenotypic resemblance to VEEV disease in the control cohort of macaques receiving anti-DENV antibody.

When the EEG band magnitudes were assessed by percentage change from baseline, the VEEV-infected macaques administered the anti-DENV antibodies demonstrated a statistically significant increase in the theta and alpha wave bands during the first febrile period of VEEV

disease, as well as statistically significant decreases in EEG power across the 0-30 Hz spectrum during the second febrile period of VEEV disease (p<0.05); Figure 51A showcases this relationship. During these analyses, as can also be seen in Figure 50, the group of VEEV-infected macaques administered anti-VEEV antibodies intranasally likewise demonstrated a statistically significant trend of decreases in EEG power in the second febrile period as an absolute percentage change from baseline (p<0.001). In contrast, the group of VEEV-infected macaques administered



**Figure 51.** Experimental Antibody Effects on VEEV PSD Magnitude as Percentage Change from Baseline. Power spectral density plotted as percent change from baseline in cohorts of VEEV-infected macaques prophylactically administered A) anti-DENV antibodies, and anti-VEEV antibodies B) both intranasally and intravenously. Wave bands are color coded by shaded boxes; the box plots at each discrete frequency segment are compared between baseline, incubation, and febrile periods.

anti-VEEV antibodies IN+IV showed significant changes from baseline during the second febrile peak. Although these percentage changes in IN+IV groups evinced decreases relative to baseline, the lack of statistical significance appears to suggest that perhaps the prophylactic IN+IV administration of anti-VEEV antibody can mitigate some signs of neurological disease detectable by EEG.

In contrast, assessment of circadian indices in with these groups of macaques did not reveal significant differences neither overall in the ANOVA nor in individual pairwise comparisons. Figure 52 documents the disposition of fundamental frequencies in each disease period. While the



Figure 52. EEG PSD Trends During F2 in VEEV Infection, by Experimental Antibody Cohort.

Repeated measures ANOVA of uninfected macaques and macaques infected with nonsevere courses of VEEV show fairly little suppression of circadian variation across different periods of VEEV disease in groups of macaques administered anti-VEEV antibodies, but also in a group of macaques administered a control anti-DENV antibody. Circadian rhythm disruption appears to have occurred only during the post-infection period of the VEEV disease course, with a significant decrease in the fundamental frequencies of macaques treated with IN antibody (p<0.05).

main expectation from the analyses of circadian indices centered about the finding documented in Chapter 5 of a decrease in fundamental frequency during the second febrile period of VEEV disease, such a finding did not occur in the various cohorts of VEEV-infected macaques in the context of this experimental setup. While this was perhaps the desired outcome for the several groups of macaques prophylactically treated with anti-VEEV antibodies, the more problematic aspect of the results arises from the lack of apparent circadian rhythm disruption in the group of VEEV-infected macaques treated with anti-DENV antibodies. The lack of distinct differences in circadian rhythm by Lomb-Scargle analysis with this method in macaques who were ostensibly going to experience a major decrease in fundamental frequencies during the second febrile period makes the drawing of conclusions from this final panel of results a difficult exercise.

### 7.0 Discussion

Pursuant to the goal of establishing biomarkers for endpoint determination in the context of therapeutic or vaccine trials, this study saw the application of radiofrequency telemetry modalities that included continuous electroencephalography (EEG) and intracranial pressure (ICP) monitoring. In addition to continuous monitoring of temperature for the febrile response, the study of EEG and ICP added two dimensions of information relevant to the natural history of inhalational alphavirus infection in the macaque model that have largely remained unexplored in the macaque model. Finally, a major goal outlined in this work sought to address whether EEG or ICP could determine the efficacy of an experimental alphavirus vaccine.

The relationship between radiofrequency telemetry and the current mechanistic understanding of CNS infection by EEEV and VEEV was poorly understood. The hypothesis underlying the whole of this work asked whether in cynomolgus macaques implanted with EEG and ICP radiotelemetry implants, changes in EEG and/or ICP signal, in addition to physiological biomarkers of disease, would correlate with neurological signs of disease indicative of virus penetration into the CNS, instigated by EEEV or VEEV aerosol infection. Holistically, the study of both EEEV and VEEV demonstrated signs of disease detectable by either or both EEG and ICP implant modalities that helped to characterize either a severe or mild phenotype of alphavirus encephalitis modeled by EEEV and VEEV, respectively.

#### 7.1 Main Findings

The main findings of this study, explained in detail in the subsequent subchapters, consist of the following observations.

The study of EEEV-infected macaques revealed that slow wave EEG, particularly delta band EEG (0.5-4 Hz), increased during the febrile period of EEEV (3-6 DPI) over 20% of baseline values of power spectral density. This effect was observed only in macaques that experienced severe courses of EEEV disease and not at all in macaques with nonsevere courses of EEEV disease. The appearance of seizures in macaques with severe EEEV neurological disease was correlated with the same-day appearance of abnormal EEG findings (statistically significant increases or decreases) in any of the four EEG wave bands examined. The intracranial pressure in EEEV-infected macaques with severe disease increased during the febrile period up to or over 100% of baseline values of ICP. This increase began following the onset of fever and is of limited predictive value, though the appearance of increased ICP can constitute a marker of severe and/or fatal EEEV disease. Circadian index suppression occurs in severe EEEV during the febrile period, and a gene of circadian regulation, to wit, PER3, is downregulated by 3-4 log<sub>10</sub>-fold, albeit inconsistently, in EEEV-infected macaques with severe disease at 6 DPI. The expression of cytokine genes and genes of traumatic brain injury are conversely increased over 3-4 log<sub>10</sub>-fold in EEEV-infected macaques over that of mock-infected macaques. This pertains to the expression of LIF and MMP-9 for the TBI panel in both cerebellum and thalamus tissue, suggestive of neuronal injury and blood brain barrier breakdown, and to the expression most significantly of IFNy in both cerebellum and thalamus in EEEV-infected macaques with severe disease.

For VEEV-infected macaques, which represent a milder, more longitudinal model of alphavirus encephalitis, cytokine genes and genes associated with traumatic brain injury were likewise upregulated by approximately 2-3 log<sub>10</sub>-fold in VEEV-infected macaques, but did not achieve statistical significance over the expression of the same analytes in mock-infected macaques in both cerebellum and thalamus. Similarly, there were no statistically significant differences in the expression of genes of circadian regulation in VEEV-infected macaques. A global decrease in EEG wave band magnitude/power over baseline values appears to occur during the second febrile period of VEEV (2.5-8 DPI), and a trend of increase of delta EEG wave band power over baseline values appears to trend briefly in the post-infection period (0-1 DPI); however, the latter finding lacks statistical significance. Circadian index suppression in VEEV appears to occur during the second febrile period of VEEV, and may be attributable to a larger relative decrease in delta EEG wave band power compared to beta EEG wave band power. Intracranial pressure increases significantly during the second febrile period of VEEV as well, trending above 50% of baseline values between 6-9 DPI, but the effect appears to be statistically significant only at 7 DPI. Regardless, this increase in ICP occurs after the onset of the second febrile period and after peak plasma viremia. Although plasma VEEV viremia appears to peak at 2 DPI and decreases to undetectable levels of vRNA at 6 DPI as determined by q-RT-PCR, complementary plaque assay data suggests that the infectivity of circulating vRNA is curtailed at 2 DPI. The peak viremia possibly coincides with a trend of increased delta EEG wave band power, but the results from this work are inconclusive. VEEV viral genomes were also isolated from the CSF, but due to the crosssectional nature of the sampling of CSF, very little can be concluded from the positivity of CSF for VEEV vRNA in most but not all VEEV-infected macaques.

The optimal windowing period for evaluation of EEG data in the context of a trial of a medical countermeasure candidate appears to be a 24-hour period to allow for the simultaneous consideration of circadian patterns, seizures, data from blood draws and necropsies, and of EEG
abnormalities. Finally, the prophylactic (-1 DPI), intravenous administration of an anti-VEEV antibody either alone or in conjunction with intranasal administration of the same antibody, appears to modestly curtail the onset of neurological disease in the context of aerosolized VEEV infection due to the lack of statistically significant global decreases across all EEG power bands though clinically apparent signs of neurological disease still manifested by veterinary staff observation.

#### 7.2 EEE in the Telemetered Macaque

The work conducted with EEEV-infected macaques in particular saw the eventual division of the macaques into three groups: macaques with severe EEEV, macaques with nonsevere EEEV, and mock-infected macaques. The mock-infected macaques and the macaques with nonsevere EEEV demonstrated neither significant changes in EEG nor ICP, and had no substantial changes in gene expression. However, the results obtained from EEEV-infected macaques with severe encephalitic disease warrant particular attention: the resultant increase in slow-wave (delta) EEG during the febrile period, coupled with the suppression of circadian index fundamental frequency and the increase in ICP during the same period of time help to add to the paucity of studies of EEEV encephalitis induced by aerosol infection in the cynomolgus macaque model.

The analysis of the power bands via measuring percentage change in baseline from the EEG power spectral density was purposed to assess whether characteristic slow-wave EEG data, would be increased in the context of EEEV encephalitis. Slow wave, or low frequency band, EEG activity typically ebbs and flows along a circadian pattern wherein delta EEG activity typically peaks at night during the brain's inactive or asleep states, as observed in Chapter 3 during normal

physiological status. The increase in the power spectral density of the EEG waveform in this frequency band is indicative of impaired electrophysiological status due to the appearance of delta EEG waves, and has at once been seen in clinical human EEG in EEEV encephalitis, as well as for other acute viral encephalitides such as herpes simplex encephalitis (78, 82, 84). Of note is that other EEG abnormalities appeared as well, for example: increases in the alpha wave band of the EEG of multiple macaques did occur, though this trend did not prove statistically significant. While the changes in delta wave band EEG were observed during the febrile period, they were of limited predictive utility due to the contemporaneous occurrence of such signs of disease with both the onset and fever as well as the onset of clinically apparent neurological signs of disease.

Likewise, the power-band analysis aided the study of seizure activity in the determination of the relationship between the incidence/prevalence of seizures and emergence of abnormal EEG. While initially, logistic regression and receiver operating curve construction were employed to test whether a predictive relationship existed between seizures and abnormal EEG and vice versa, the small sample size of timestamped seizures, the potential for overfitting to the logistic model, and the potential for positive result bias, render these results ultimately inconclusive without a larger sample size and closer attention to seizure detection, either by algorithm or by laboratory staff. However, the chi-squared results, at least suggest a relationship whose causality cannot be determined from the data but which intuitively can be presumed to arise from seizures arising out of abnormal neurological states rather than the reverse, between abnormal EEG and seizure incidence/prevalence. It is unknown how well these models will accommodate new and/or future data. Reliable indicators are hard to find because persuasive measurements require repeated measurements over multiple studies. Suffice it to say, the macaques that exhibited severe courses of EEEV neurological disease exhibited deviations from the typical frequency spectra in their EEG data. Seizures are described with some frequency in human clinical cases of EEEV encephalitis, though formal correlations with abnormal EEG have not been explored in detail (78, 80, 88).

The assessment of intracranial pressure and its relationship to febrile EEEV illness in the cynomolgus macaque model found that intracranial pressure in EEEV-infected macaques with severe disease increased during the febrile period up to or over 100% of baseline values by 6 DPI. Repeated measures ANOVA found that despite a trend of increase beginning at or before the onset of fever, the only statistically significant elevation of ICP in the group of macaques with severe EEEV occurred at 6 DPI. However, this statistical significance does not necessarily equate with clinical significance. Assessment by LOWESS (LOcally WEighted Scatterplot Smoothing), a method of regression analysis fitted to the aggregate data across all EEEV-infected macaques with severe disease but not used in the results, shows an inflection point from the zero line at approximately 1.74 DPI when the ICP begins its terminal increase. If a 20% increase over baseline were to be considered a threshold beyond which ICP values are to be considered deviant, this places the beginning of the terminal increase of ICP at 3.17 DPI, roughly contemporaneous but occurring slightly after the onset of febrile disease. In humans, raised intracranial pressure is related to poor prognoses and severe neurological damage, and were typically encountered by 4 DPI, though case profiles exhibited large degrees of variation (80, 82, 293).

Finally, the expression of cytokines in EEEV-infected cynomolgus macaques only showed statistically significant upregulation in macaques with severe EEEV disease. While the statistical significance with which each analyte was associated varied, trends of upregulation by at least 3  $log_{10}$ -fold occurred in IFN $\gamma$  and the associated gene IP-10, or C-X-C motif chemokine 10 (CXCL10) in both cerebellum and thalamus. Similarly, upregulation of MCP-1, macrophage chemoattractant protein 1, occurred by approximately 3  $log_{10}$ -fold in both cerebellum and

thalamus, but to a lesser degree in thalamus. Taken together, these results help to drive conjecture regarding which cell types play a role in the response to alphavirus infection; as these specimens all came from 6 DPI necropsies, neither innate nor adaptive immune response species or cell types may be ruled out from consideration. Presumably, at 6 DPI, the innate immune response species are at below-peak concentrations in response to EEEV infection; that is to say, the upregulation of IFN $\gamma$  and IP-10 may have been encountered at even higher fold-expression levels had the necropsies been performed at the peak of febrile illness. With the consideration that a large number of cell types produce IFN $\gamma$ , including natural killer cells, T cells, macrophages, and others, alongside the consideration that IFN $\gamma$  defines the Th1 response, it may seem reasonable to state generally that T cells and recruited macrophages may perhaps comprise the major cell types responding to infection in this context of alphavirus encephalitis (294). The upregulation of both IP-10 and MCP-1 also point towards the recruitment of macrophages or monocytes, in particular, although MCP-1 is also known to be expressed by neurons, astrocytes, and microglia (295, 296).

The expression of LIF and MMP-9 in EEEV-infected macaques with severe disease suggests that neuronal injury begets the expression from astrocytes and oligodendrocytes; in contrast, the lack of significant changes in GFAP expression suggests either poor primer homology for cynomolgus macaque or the absence/obliteration of GFAP-expressing cells in EEEV-infected macaques, among other possibilities. Moreover, the increase expression of LIF could occur in response to other cytokines such as TNF- $\alpha$  and/or IL-6; the expression of markers associated with traumatic brain injury appears upregulated to a higher degree in the thalamus than in the cerebellum, a result corroborating other findings in studies of EEEV in other animal models (182-184). The tie-in to increased gene expression of LIF by TNF- $\alpha$  and/or IL-6 likewise suggests the

modulation of circadian disruption by means of the circadian entrainment properties of those cytokines.

Given the thalamic and cerebellar roles of movement control and coordination, the results have the potential to shed light on i) the disposition of gene expression at a later time point (6 DPI) after infection, ii) where the expression comes from, and iii) whether or not such changes relate to EEG or ICP pathology. While circadian index suppression unequivocally occurs during the febrile period of severe EEEV disease, only the gene PER3 was downregulated significantly albeit in only half the EEEV-infected macaques sampled at 6 DPI. While the other circadian genes of regulation included in the panel, CRY1 and CRY2 tend to peak at night, and are important in the negative regulation of CLOCK, a transcription factor for E-box genes, the gene CLOCK is most actively expressed during the day. A possible explanation for the differential behavior of PER3 compared to the other genes lies in the fact that PER3 is also subject to regulation/expression by D-box genes, a category of genes that are taxonomically similar to E-box genes but subject to functionally dissimilar modes of regulation that sometimes overlap. There is a large chance that the effect of downregulation of PER3 in EEEV-infected macaques may be artifactual owing to the propensity of multiple factors to have affected the results: anesthesia from blood draws, statistical contamination by small sample size and the outsize influence of variation in the mock-infected macaque cohort, and the use of human 18S rRNA as a time-invariant endogenous control could all have contributed to the results obtained. Most importantly, the cross-sectional nature of this analysis precludes the establishment of any definitive conclusions regarding expression of genes of circadian regulation.

Although the literature breaks down expression levels by tissue, these delineations are often categorized at the whole-organ level and do not typically make distinctions between expression

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patterns in different subsections of brain. However, this was theoretically controlled by normalizing the tissue to volume ratio when the things were sampled. Therefore, the expression values should be taken at somewhat face value. The results obtained from this work, however, in conjunction with the fact that particular nodes of nervous tissues, colloquially termed "pacemakers," such as the suprachiasmatic nucleus (SCN) of the hypothalamus or the paraventricular nucleus (PVT) of the thalamus in the brain facilitate the entrainment of a rough 24-hour circadian rhythm dictated by internal genetic circuits, suggest that the circadian oscillation of expression can be affected by infection or the response to infection of the CNS by alphavirus as an external stimulus (198, 202, 203).

#### **7.3 VEE in the Telemetered Macaque**

The study of VEEV in telemetered cynomolgus macaques represented a more longitudinal study of an admittedly milder phenotype of alphavirus encephalitis; the model of VEEV infection in cynomolgus macaques did, however, present unique features not seen in EEEV-infected macaques. Foremost, the biphasic fever, a characteristic feature of VEEV infection in the cynomolgus macaque, manifests two peaks, the first thought to coincide with the lymphotropic and/or viremic phase of VEEV infection, and the second thought to coincide with the infection of the CNS. The study of gene expression profiles of the same cytokine genes and genes associated with traumatic brain injury show a modest increase in gene expression following similar trends in IFN $\gamma$ , IP-10, and MCP-1 as in EEEV-infected macaques in both cerebellum and thalamus, but without the achievement of statistically significant increases compared to mock-infected controls in macaques. Again, this may partially stem from the sampling of necropsy specimens at 6 DPI,

but the results obtained fall neatly into concordance with studies of cytokine gene expression in the 1-4 DPI time period in other animal models (124, 125, 166, 167).

The finding that the expression genes of circadian expression remained unchanged in VEEV-infected macaques proved somewhat surprising in the context of the decrease in fundamental frequencies seen in the second febrile period, although the lack of statistically significant results in the gene expression panels may be in small part attributable to the generally milder change in gene expression seen in all VEEV-infected macaque tissue samples. Multiple reports in the literature comment on the loss of circadian variability in various segments of the EEG frequency spectrum during viral infection, with various empirical associations outlined that implicate both systemic and neurogenic origins of circadian disruption (199, 201, 297-302). Acute phase reactants such as tumor necrosis factor (TNF- $\alpha$ ) have been tied to slow-wave EEG activity, including the delta power band; TNFa is thought to directly or indirectly regulate somnolence (302). Endogenous fluctuations of inflammatory or immune species in response to infection may thereby produce monumental shifts in changes detectable by EEG. Our findings hint at a neurogenic origin of disrupted circadian rhythmicity. For instance, the first febrile peak in VEEV is associated with virus dissemination through the lymphatic system, while the second febrile period is proposed to be associated with infection of the CNS (62, 95). Circadian disruptions of EEG in VEEV infection occur largely during the second febrile peak, suggesting that infection of the brain parenchyma and/or the subsequent immune response cascade precipitates the disruption.

While at first the VEEV infection in cynomolgus macaques appeared to induce two disparate impacts upon EEG wave band power relative to baseline data: a small increase in delta EEG wave band during early infection prior and during the first febrile period, and a global decrease in all EEG wave band power during the second febrile period of VEEV. Only the latter result bore out statistical significance, but the implications of the first point remained pertinent to the data obtained from the trial of the anti-VEEV antibody. The global decrease of EEG wave band power relative to baseline values is perhaps indicative of the "generalized slowing" and other EEG dysrhythmias of the EEG described in human clinical cases of acute viral encephalitides, including Venezuelan equine encephalitis (303-305).

While the exact timing of intracranial pressure increases varied between individual macaques infected with VEEV, as well as the magnitude of ICP increase, the absolute percentage change from baseline ICP values across the group of VEEV-infected macaques experienced a statistically significant increase to over 50% of baseline values during the second febrile period of VEEV infection. As such, as in the EEEV-infected cohort of macaques, the utility of the increase in ICP probably remains limited with respect to predictive applications for disease detection but nonetheless remains an indicator of VEEV encephalitis. The relationship between increased ICP and fever onset in VEEV encephalitis in the macaque model remains characterized by a delayed onset of ICP increase that occurs subsequent to fever onset.

Investigation of the disposition of copies of VEEV viral genomes by q-RT-PCR as a surrogate measure of virus titer placed peak VEEV plasma viremia at 2 DPI, a feature corroborated by viral plaque assay. This timing places the onset of VEEV viremia during the putative lymphotropic phase of VEEV infection in the macaque and well before the global decrease of EEG power noted during the second febrile peak. The cross-sectional nature of CSF sampling occurred at 6 DPI, 26-35 DPI, and 48-52 DPI; this aspect of sampling demonstrated that vRNA, regardless of its association with competent virus, persists in the intrathecal space well after the resolution of clinically visible signs of disease as well as signs of disease detectable by EEG or ICP modalities. However, during the 6 DPI cross section, no detectable vRNA was assayed by q-RT-PCR in the

CSF, nullifying an association between the decrease in EEG power that occurred during the same time frame. Overall, plasma viremia appears to occur before increases in EEG and ICP occur, and while VEEV-infected macaque brain, lymph nodes, and CSF test positive for viral RNA, there appears to be none but a weak, if any, temporal relationship between the appearance of detectable changes in EEG/ICP and vRNA in CSF. More work is necessary to clarify this relationship.

## 7.4 Implications of Trial Data

The assessment of windowing periods for the study of EEEV- and VEEV-infected macaques determined an optimal windowing time of 1 days' worth of data, whether in the form of power spectral density plots or in the form of grand averages of temperatures or ICP.

The study of the effects of an anti-VEEV antibody administered via various routes in comparison to a control anti-DENV antibody and mock-infected macaques revealed a number of interesting findings. The experimental design in this work differed from the approach in previous studies with the same antibody in that anti-VEEV antibodies were administered on a prophylactic basis before aerosol challenge in this study, in contrast to the post-aerosol administration of the antibody after the first febrile period of VEEV. The totality of the results obtained from this study suggests that intravenous administration of the anti-VEEV antibody mitigates EEG changes, both increases and decreases in global EEG power, when the anti-VEEV antibodies are administered prophylactically via the intravenous route, either alone or in conjunction with intranasal administration of the same. While the combined route (IN+IV) of administration for such antibodies appears to have a slightly more beneficial effect with respect to the mitigation of signs

of VEEV disease detectable by EEG than the intravenous route of administration alone, but the results were not statistically significantly different from each other.

In contrast, the control group administered the anti-DENV antibodies seemed as if it did not experience a precipitous global drop in EEG wave band to the same degree that the cohort of VEEV-infected macaques that received no treatment detailed in the previous chapter. This appears somewhat problematic in two regards: i) despite the appearance of clinical signs of neurological disease (via observation by veterinary staff), which still manifested in all groups regardless of ab/normal EEG, the disease as characterized by EEG appeared somewhat milder in the control group administered the anti-DENV antibodies; and ii) both the control group and the group of VEEV-infected macaques treated with intranasal administration of anti-VEEV antibodies showed modest increases in *both* delta and alpha wave band power to a degree of statistical significance not seen in the untreated VEEV-infected macaques. The origins of this issue may stem from the possibility that the treatment of the macaques with antibody may have altered the electroencephalographic presentation of the disease, or that perhaps the anti-DENV antibodies were cross-reactive with VEEV and conferred some degree of neurological protection against VEEV, however unlikely.

Similarly, it appears that the impact of antibody treatment upon the circadian indices of the macaques was also negligible; with respect to the control group of macaques treated with anti-DENV antibodies, the fundamental frequencies of the circadian indices did not differ significantly from each other across all periods of disease. The same held true for differences between treatment groups; most groups appeared to show negligible changes between both each other and against the control antibody. Furthermore, it would appear that the actual periodicity of the circadian indices in this cohort of macaques was not affected; only the amplitude. Further study is warranted to reach firmer conclusions.

Finally, none of the macaques studied were implanted with intracranial pressure monitors; the assessment of ICP in the context of an experimental antibody trial would have conferred an additional dimension in which to gauge the onset and/or resolution of disease pursuant to the evaluation of the efficacy of the candidate countermeasure.

## 7.5 Conclusions

Electroencephalography is a direct electrophysiological measure of brain function that is highly conserved across macaques and humans. The continuous long-term monitoring in mockinfected animals allowed us to describe in great detail the normal range and rhythms of healthy macaque brain function. This data established a baseline against which to evaluate pathological brain function caused by infection with an encephalitic alphavirus.

Pre-infection, all frequency bands exhibited circadian variation. As may be expected, the slow-frequency wavebands delta and theta, exhibited higher magnitudes during the night and lower magnitudes during the day, while the high-frequency wavebands alpha and beta exhibit lower magnitudes at night and higher magnitudes during the day. Subtle differences in timing make themselves manifest; for instance, the delta magnitude peaks early during the night, whereas theta and alpha magnitude tend to peak after midnight. The beta magnitude also begins ramping up after midnight, with a sharp uptick after lights-on. The fundamental frequencies of the circadian indices, as modeled by Lomb-Scargle methodology, appear to undergo decreases in value concurrently with the onset of febrile illness.

The utility of monitoring EEG was demonstrated by the differences seen between mockinfected and alphavirus-infected macaques; infection of the macaques with EEEV and VEEV in this cohort provided a tangible contrast in EEG to the mock-infected macaques. Infected macaques displayed a variety of EEG abnormalities during the post exposure period, namely differences in the delta, alpha, and/or beta band. In both EEEV and VEEV, a prominent suppression and loss of circadian rhythm in delta magnitude is seen during the second febrile period (2.5-8 DPI). Delta wave band power returned to baseline values in VEEV-infected macaques after two weeks post infection while EEEV-infected macaques with severe disease typically required euthanasia.

Measurements of ICP provide another dimension for the measurement of onset and resolution of the encephalitic disease state. The results show that we can successfully measure ICP in the macaque using a blood-pressure monitor. As a proof of concept, we demonstrated that the blood-pressure monitor can pick up physiological changes of ICP, such as cardiac ICP pulsations, and that these physiological signals did not degrade even after several weeks' worth of data collection. Most importantly, our results show the EEG/ICP implants can detect the expected increases of intracranial pressure during the acute phase of encephalitis. Increase of ICP was found to lag behind the onset of fever and instead emerge around the time of the peak of the fever. For VEEV, increase of ICP was tied to the second phase of the fever that is believed to reflect the infection of the brain.

The resultant data from this study of EEEV-infected macaques leads to the conclusion that delta wave band EEG increases contemporaneously with the febrile period in severe cases of EEEV. The appearance of abnormal EEG power in any wave band, in cases of known EEEV encephalitis in cynomolgus macaques, is correlated with the incidence of seizures. In EEEVinfected macaques, a trend of increase in intracranial pressure begins following the onset of fever and can increase to over 100% of baseline ICP values in severe EEEV encephalitis in macaques. Fundamental frequencies associated with the circadian index in EEEV decrease during the febrile period, and a putative link may exist between such decreases and downregulation in the PER3 gene. EEEV-infection of cynomolgus macaques causes increased expression of interferon-gamma associated genes both in the cerebellum and thalamus at 6 DPI. Contemporaneous upregulation of LIF and MMP-9 expression in the same tissues point to neuronal injury and blood brain barrier breakdown as part of the infection and/or host response to EEEV. Not all macaques develop febrile encephalitis in EEEV infection. This contrasts with approximately 100% of human cases recorded in the literature that exhibited fever as a sign of a disease (82). However, if the analogy of nonsevere in EEEV-infected macaques holds for humans, wherein no signs of disease exist despite proof by plaque reduction neutralization test confirmation of the occurrence of infection, a significant possibility arises that nonsevere human cases of EEEV may not seek health care due to mild or negligible signs/symptoms of disease.

Although gene expression of cytokines and genes associated with traumatic brain injury showed some changes during VEEV infection of macaques, the increases were not statistically significant. This information leads to the conclusion that cytokine gene expression is mildly upregulated in the context of limited neuronal damage at 6 DPI of VEEV infection. Though no differences occurred in the expression of genes of circadian rhythm in VEEV-infected macaques, EEG abnormalities in VEEV encephalitis in cynomolgus macaques include a global decrease in EEG activity across large swaths of the physiologically relevant frequency spectrum of the electroencephalogram, with a possible mild elevation of delta wave band EEG during the immediate post-infection period. Similarly, contemporaneous decreases in the fundamental frequency of circadian indices constructed for VEEV show a decrease in fundamental frequencies, or the suppression of circadian indices, during the second febrile period of VEEV that is presumably induced by a decrease in delta EEG wave band power, corroborated by literature on electroencephalography that suggests that delta activity varies according to a roughly defined "sleep need" (213). The data lead to the conclusion that intracranial pressure increase in VEEV occurs subsequent to the development of fever, after the second febrile peak, and experiences its maximal deflection of over 50% of baseline values during the second febrile period of VEEV in cynomolgus macaques. Plasma viremia appears to peak at 2 DPI following VEEV infection, while viral genomic RNA can be isolated from CSF at or after 28 DPI, well after signs of neurological disease detectable by EEG have subsided.

Finally, the conclusion of the trial of anti-VEEV antibody administered via several routes of administration in a cynomolgus macaque model of VEEV implied that anti-VEEV antibody, administered prophylactically to cynomolgus macaques by an intravenous or intranasal + intravenous route, could produce a modest mitigation of signs of neurological disease detectable by EEG.

The above conclusions lead also to a more overarching conclusion that the analysis of EEG spectra and the derived circadian indices may portend significant findings in higher-powered studies of acute viral encephalitic diseases in the future. The methods detailed in this work may prove generalizable to the investigation of other encephalitic disease states. The finding of accurately documented, raised intracranial pressure remains of particular interest in better characterizing encephalitic diseases of infectious origin. These findings in telemetered macaques hold the potential to track acute viral encephalitis disease courses for the testing and evaluation of vaccine candidates to mitigate or prevent human disease.

## 7.6 Future Work

Limitations of this work and the data presented here are myriad. The EEG/ICP implant used for this study permitted only one single bipolar EEG recording. Standard EEG caps in humans typically contain 32 to 64 channels that are spaced approximately evenly across the scalp. Such dense spatial sampling enables the use of source reconstruction techniques that can map certain EEG events to specific brain regions. In the current context, a larger number of electrodes might similarly enable the mapping of pathological changes to specific brain regions. Such analyses would be particularly informative in combination with post-mortem pathology in different brain regions.

EEG electrodes are easily affected by electric sources other than the brain, e.g., eye movements, blinking, and chewing (254, 306). The pre-processing steps were aimed at excluding epochs with obvious artifacts, but it was impossible to detect and exclude many more subtle artifacts. In future studies, artifact detection can be improved by using multi-electrode EEG arrays and using independent component analysis to identify artifacts based on their spatial distribution and temporal properties.

The current study used a blood-pressure sensor to measure ICP. While the sensor clearly was able to measure physiological changes of ICP, it is plausible that a dedicated ICP pressure sensor that is designed to have maximal sensitivity in the range of ICP would have provided less noisy data. Furthermore, it is known that ICP can be affected by body posture. Thus, it is plausible that accounting for body posture using an automated posture detection mechanism could further improve the signal-to-noise of the ICP measurements.

One may wonder: why bother separating EEEV nonsevere disease from VEEV disease if what we are concerned about is disease phenotype? One cannot interchange findings between the two virus groups; the concept of "Severe disease = EEEV" and "Mild Disease = VEEV" remains a useful conceptual framework and approach from which to study pathology, but risks the overconclusion of distinctions between EEEV and VEEV if those distinctions do not in actuality exist. The results are only internally generalizable to each specific alphavirus.

Future work can also capitalize upon the ever-growing number of tools and talent that can facilitate the automated review of seizure activity from recorded closed-circuit video. An additional step forward would comprise linking the spectrographic analysis of seizures to flagged seizures from improved methods in computer vision. Advancements such as these would greatly augment the capacity of laboratory staff to monitor indirectly the status of the macaque from a remote location, allowing for increased utility regarding humane endpoints of such investigations.

Likewise, improvements to the methodology of circadian and/or sleep analysis of the macaques sickened by alphavirus encephalitis can occur on two fronts: i) the analysis of circadian indices by fundamental frequency or other algorithmic methods, and/or ii) the *in vitro* study of circadian rhythms by gene expression in tissues isolated during necropsy or in serial sacrifice studies of alphavirus infection in another model animal. This alternative might consist of, for example, the conduction of similar experiments in mice, wherein serial sacrifices may be performed more economically, or to perform *in vitro* exposures to alphavirus on synchronized neural networks to determine whether expression patterns of genes of circadian regulation are or are not evocative of a response to virus infection.

The following questions also beg to be addressed – what impacts do age or biological sex precipitate on the outcome of infection by alphavirus via the aerosol route? Presumably, intracranial pressure increases would occur regardless of age or sex, but the impact of age or sex cannot be ruled out from an impact on functions such as circadian patterns or more generally, the electroencephalogram. For example, the periodicity of hormonal changes may interfere or otherwise modify the outcome, as documented by EEG. These differences may be negligible, but only further study can bear them out.

#### **8.0 Implication to Public Health**

The public health significance of this project lies contained in the ever-present need to protect the public from epidemic or pandemic diseases of natural or artificial origin. At the time of this writing, no part of the globe has been left untouched by the seemingly unstoppable force of COVID-19. Indeed, through the 21<sup>st</sup> Century, several candidates for the Disease X enumerated by the WHO: the 2014 and 2016 Ebola outbreaks, the 2014 Zika outbreak, and especially the 2003 SARS outbreak have not only captured the human imagination as they have roiled through bodies and continents, but have made an indelible impact on the very foundations of society that shall reverberate through years to come (307-309). Arguably, the furious race to meet challenges posed by the present coronavirus pandemic has undoubtedly eclipsed the recent 2019 epidemic of EEEV in North America that represented a more than two-fold increase in annual cases (88).

The risks associated with equine encephalitis viruses to be deliberately spread as a biological weapon, in the face of ineffective or inadequate responses by world governments and organizations ill-prepared to contain large outbreaks of naturally occurring disease are significantly amplified in the event that no established treatments nor prophylactics exist to combat the spread of disease, as recent events have shown.

This project sought to optimize the utility of a telemetered macaque model of inhalational viral encephalitic disease caused by alphaviruses whose vaccine candidates cannot be evaluated in humans due to the lack of effective treatments and the high risk profile associated with infection. The findings from this inquiry can provide actionable information to investigators over the course of clinical trials to evaluate the efficacy of antiviral lead compounds or vaccines in altering post-exposure outcomes.

Again, the current global crisis wrought by an emerging infectious disease threat highlights the need for effective research programs, development of appropriate technologies and infrastructure, against the backdrop of more competent leadership and governance at the global, federal, state, local, and institutional levels. Complacency, apathy, ignorance, and decrepitude must no longer sit the throne. An uncompromising approach to providing the tools and materials needed to combat infectious disease in the rests in the investment of national and international resources into an effective biosafety and biosecurity program that addresses nascent threats and prepares for epidemics of the future such that society does not come to a grinding halt.

#### 8.1 Biodefense in the Twenty-First Century

Dual use research of concern (DURC) has weaved in and out of the US public consciousness throughout the twenty-first century ever since the 2001 anthrax bioterror attacks in the wake of the 9/11 terrorist attacks. Briefly, DURC describes research methods or findings that could suffer diversion or deliberate misapplication. As the decades preceding the Biological Weapons Convention (BWC) of 1972 saw decades' worth of research on the weaponization of VEEV, any subsequent research on VEEV runs the risk of diversion for nefarious purposes and potentially constitutes DURC.

Against the backdrop of the Cold War between the United States of America and the Soviet Union, both superpowers sponsored biological weapons programs intended to develop equine encephalitis viruses as a tactical weapon for unconventional warfare for the induction of nonlethal, incapacitating casualties *en masse* to delay a conventional, numerically superior, attacking force. The time and resources devoted to those *hors de combat* could in theory sufficiently alter the composition/disposition of enemy forces to stop the attack. The consideration of VEEV to fill such a role first received attention from the United States, due to the mildness of disease and low mortality, fast growth in culture, stability when aerosolized, propensity for infection by inhaled, infectious aerosol (coupled with a low inhaled dose to achieve said infection) (79, 105). A program was thus begun, with offensive and defensive arms, to create aerosolized VEEV-based biological weapon stockpiles and a human vaccine, respectively; numerous contributions to the study of aerobiology came as a result of this work. Likewise, the pathogenesis of alphaviruses received much attention as a result of this focus (79, 102, 152, 310). However, espionage during this period led the Soviet Union to adopt VEEV into its own biological weapons program (12).

The period of de-escalation in tensions between the United States and the Soviet Union known as *Détente* led to numerous arms limitations treaties signed between the two nations, and the BWC was among such pacts; although offensive weapons development was supposed to have stopped, research for defensive purposes for vaccine development remained legal. The subsequent decades and the end of the Cold War sharply reduced the risk of the deployment of offensive biological weapons in combat or conflict between nation-states (12). In fact, the possession of a stockpile of biological weapons has perhaps in modern times become more of a liability than an asset for nation-states, as the prerequisite of secrecy in the face of violating international arms agreements, not to mention international norms, coupled with the extant risks of espionage between nation-states have sharply diminished the deterrent value of offensive biological weapons (13, 150, 151, 311). Moreover, recent developments and scholarly work have shown that the labor and technical expertise required in securing, servicing, and modernizing an aging nuclear weapons stockpile raise unique risk profiles that demand equally unique and/or expensive methods of risk mitigation and management (312, 313). This extends by analogy to offensive biological weapons

stockpiles; the political and economic costs of the possession of offensive biological weapons stockpiles by a nation states do not make possession of such a stockpile, at this time, seem a particularly wise choice.

The geopolitical order of the early 21<sup>st</sup> Century was defined by a largely unipolar world of nation-states concentrating, with larger states such as the Russian Federation successor state to the Soviet Union and the People's Republic of China vying for regional hegemony and supremacy (314, 315). Increases in wealth inequality and cultural backlash have led to the breakdown of public trust in institutions and have brought about an ascendant populism in fractious democratic societies around the world while illiberal authoritarian governments chip away at the international status quo to establish a more isolationist, multipolar world that favors the security of their own regimes and spheres of influence (316-321). Through the latter half of the 20<sup>th</sup> Century and into the 21<sup>st</sup>, various groups with significant political, economic, and military means but without direct access to the levers of power of nation-states, defined in this work as non-state actors, have sought to weaken and subvert the contemporary international order or at least manipulate it to achieve their own social, political, or economic agendas against this chaotic backdrop.

Preludes to biological terrorism by non-state actors took the form of various incidents in the 20<sup>th</sup> Century, for example the Rajneeshee bioterror attack in the US state of Oregon in 1984 comprised a case in which a cult facilitated the sickening of hundreds of individuals with Salmonella in a clumsily-orchestrated attempt to influence the outcome of a local election (322, 323). Fortunately, no fatalities occurred from that incident. However, more brazen attacks in Japan between 1994 and 1995 involving numerous pathogens and toxins such as anthrax, botulinum, and chemical agents such as sarin, orchestrated by the doomsday cult Aum Shinrikyo (324-326). Although not technically biological terrorism, the 1995 sarin subway attacks on the Tokyo metro were by far the highest profile attacks in which 13 fatalities occurred and thousands were poisoned. Both examples thus far have highlighted the potential for non-state actors to indiscriminately attack civilians with little regard for the costs and consequences of their actions.

The 2001 Amerithrax attacks took place in the wake of the 9/11 terrorist attacks sickening 22 high-profile victims and killing 5 (105, 311). The principal difference between the 2001 Amerithrax attacks and the preceding attacks resides in the fact that an extremely plausible scenario exists wherein the perpetrator of the anthrax attacks remains at large. Despite a subsequent FBI investigation that identified a troubled researcher as the perpetrator of the attack, a review of both investigative practices as well as the evidence dredged up by the investigation have pointed to the possibility that other and/or more individuals were involved with the planning and perpetration of the anthrax attacks (327-329). The ability for the perpetrators of such an attack to i) accomplish the attack and ii) evade detection by law enforcement for a decade-long period pose a pernicious threat to national security from foreign or domestic enemies of the United States. The achievement of the long-term strategic goals of non-state actors through bioterrorist gambles, should they couple the technical expertise required for an attack like the Amerithrax attacks to a fanatical goal appears more plausible in such a light.

The Islamic State of Iraq and the Levant (ISIL/ISIS) represents a troubling recent example of a non-state actor that, through the organization of disaffected young men and women from locations around the globe, the leveraging of social media, capital, military materiel worked to acquire territory and construct a pseudo-legal bureaucratic structure to perpetuate itself and lend itself legitimacy as a nation-state under the guise of a resurrected Islamic caliphate (330-332). The prolonged inability of international cooperation to squelch this terrorist threat marks the emergence of such a powerful non-state actor that was alarmingly adept at asymmetric warfare such that the legitimate governments of fragile nation-states such as Syria or Iraq remained embattled with ISIS for more than half a decade (150). The length of the conflict was no doubt drawn out by the bickering of more powerful nation-states and regional blocs, each of which had its own vested interests in various outcomes of the conflict; what remains certain is that the instability of the prevailing conditions was conducive to the development and proliferation of, if not necessarily deployment, of biological weapons as deterrent or perhaps vengeance weapons by non-state actors.

Regardless, these developments of the early 21st Century underscore the need for an effective response to not only terrorism generally but also specifically against biological warfare threats. The stratified Categories of the Select Agent lists by the CDC reflect the ranking of various pathogens and toxins according to an assessment of their risk profiles, which includes the equine encephalitis viruses (7). As stated previously, the relatively low barrier of achieving technical and facilities resources capable of sustaining the manufacture, storage, and dispersal of equine encephalitis virus, as well as the difficulty of establishing robust defenses against attacks with such pathogens make nefarious diversion of equine encephalitis viruses an attractive candidate to people or groups seeking to undermine US national security (13, 150, 311).

All weapons of mass destruction, regardless of their nature: nuclear, chemical, or biological, objectively make the world a less secure place; however, the scholar would be remiss in shying away from questioning the *reason* for their existence. The entire pantheon of human history and internecine warfare within our species lends credence to the Hobbesian outlook that as long as large groups of people live within the confines of an anarchic political order, these groups shall, at the risk of their own extirpation, invest resources in the creation of weapons to defend themselves against rival groups for geopolitical dominance.

This returns to the significant public health imperative that drives the continued study and research of equine encephalitis viruses; the production of weapons based on equine encephalitis virus as a pathogen that causes fulminant, incapacitating neurological disease is within the reach of well-funded non-state actors (150, 333) Public health researchers as well as clinicians are beholden to explore new methods of detecting and treating disease not only for the sake of biosecurity and the safeguarding of public health, but also for the maintenance of national security and the liberal democratic world order from the encroachment of illiberal authoritarianism and the nefarious intentions of terrorist groups. To date, no licensed vaccines nor treatments exist to prevent or directly counteract equine encephalitis infection, leaving the population vulnerable. The decentralized, disseminated nature of infectious disease transmission knows no boundaries or national borders, and in the case of an outbreak, natural or man-made, unpreparedness shall be society's collective downfall.

#### 8.2 Alphavirus Research and Medical Countermeasures

The role that vaccine and therapeutic development plays in the grand scheme of preparedness and deterrence against biological warfare attacks remains an incredibly important one. Research into alphaviruses and medical countermeasures against them comprises one of the highest-order priorities of biotherapeutic development as outlined by US government agencies (334). Although transmission of these viruses in nature typically occurs through mosquito vectors as the intermediary between avian or equine amplification hosts and humans, the propensity for aerosolized virus to cause human disease was discovered through laboratory accidents subsequent to the initial discovery of EEEV and VEEV in the 1930s. The largest obstacle lying in the path of

progress to the development of vaccines or therapeutics against equine encephalitic viruses remains the demonstration of efficacy in human-similar animal models. A premier hurdle in the evaluation of disease in animal models rests in the practice of tracking encephalitic EEV disease by fever and/or in-person veterinary assessments of animal neurological disease, which in the absence of reportable symptoms in animal models, remains too nonspecific or subjective, respectively, to provide a diagnosis of encephalitis. Radiofrequency telemetry, or radiotelemetry modalities such as EEG or ICP thereby offer a putative solution to this unmet need by providing objective, quantitative data for the detection of neurological disease or infection of the central nervous system.

The augmentation of this telemetered cynomolgus macaque model to produce encephalitis holds utility towards the development of improved vaccines against aerosol-induced disease. The macaques that exhibited courses of EEEV or VEEV disease exhibited deviations from the typical downward baseline frequency spectra in either or both EEG and ICP modalities. This work provides evidence that changes in morphology of the frequency spectra can be better characterized such that a divergence from the baseline spectra can be treated as a pathognomonic differentiator of sick and well states. Likewise, the methods detailed in this work may prove generalizable to the investigation of other encephalitic disease states, especially those of infectious, viral origin. These findings in telemetered macaques hold the potential to track acute viral encephalitis disease courses for the testing and evaluation of vaccine candidates to mitigate or prevent human disease.

## **Appendix A Glossary and Abbreviations**

## **Appendix A.1 Glossary – Definitions**

Ad Libitum – As necessary or as needed.

Aerosol – A spray or fine mist, a substance or particulate suspended in air.

Alphavirus – A genus of positive sense single-stranded RNA viruses.

**Encephalitis** – Inflammation or infection of the brain.

**Fourier Transformation** – A mathematical operation that isolates frequency components of a signal in the time domain.

**Select Agent** – A pathogen or biological toxin registered by the United States federal government due to the potential to pose severe threat(s) to public health and safety.

**Transcranial Doppler** – A specialized form of ultrasonography that can resolve images of vascularized brain parenchyma.

Ventriculostomy – Opening of the ventricles of the brain, often for therapeutic purposes.

**Zeitgeber** – Literally: "time-giver;" a stimulus that can entrain or help to entrain a circadian rhythm.

**Zero-Drift** – Drift away from the offset calibration of a digital device.

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## **Appendix A.2 Abbreviations**

- BWC 1972 Biological Weapons Convention
- C84 Cell culture derived experimental vaccine against VEEV.
- **CNS** Central nervous system.
- **CPP** Cerebral perfusion pressure
- CRY1 Cryptochrome circadian regulator 1
- **CRY2** Cryptochrome circadian regulator 2
- CSF Cerebrospinal fluid
- **DENV** Dengue virus
- DURC Dual use research of concern
- **EEE** Eastern equine encephalitis
- **EEG** Electroencephalography
- EEV Equine encephalitis virus (EEVs; plural)
- **EEEV** Eastern equine encephalitis virus
- GFAP Glial fibrillary acid protein
- HDPE High-density polyethylene
- **HPA** Hypothalamic-pituitary axis
- **ICP** Intracranial pressure
- IFN Interferon
- IL Interleukin
- **IND** Investigational new drug
- **IP-10** Interferon gamma inducible protein (aka CXCL-10)
- LIF Leukemia inhibitory factor

- MAP Mean arterial pressure
- MCM Medical countermeasures
- MCP-1 Monocyte chemoattractant protein 1, aka CCL2
- MMP-9 Matrix metalloproteinase 9
- mRNA messenger RNA
- NTC Negative temperature coefficient
- **PAPR** Powered air purifying respirator
- PE6 Experimental, formalin-inactivated vaccine against EEEV infection
- **PER3** Period 3 gene
- **PVT** Paraventricular nucleus
- q-RT-PCR quantitative real time polymerase chain reaction
- **SCN** Suprachiasmatic nucleus
- TC-83 Experimental live, attenuated vaccine against VEEV infection
- **Th1** Type 1 Helper T (CD4<sup>+</sup>) Cell
- **Th2** Type 2 Helper T (CD4<sup>+</sup>) Cell
- USAMRIID United States Army Medical Research Institute of Infectious Diseases
- **VEE** Venezuelan equine encephalitis
- **VEEV** Venezuelan equine encephalitis virus
- **vRNA** viral genomic RNA

## Appendix B Pertinent MATLAB Code for Analyses

The MATLAB code highlighted in this section was modified a script generously provided by Dr.

Teichert; this code utilizes the EEGLab MATLAB plugin to transcribe European Data Format

(.edf) recordings to a MATLAB-interpretable format.

# **Appendix B.1 Data Processing Code**

function [ output\_args ] = prep\_raw15( args ) % ultimate goal: create a set sampled at 1/15 minutes that includes: % delta, theta, alpha, beta and gamma power % activity % EEG envelope FFTWinSec = 4; % 4 second long FFT windows binDurMin = 15: % 15 minutes bin width targetSR = 1/(binDurMin\*60); % average in bins of 15 minutes % EEGLab structures created during the process % EEG : channel 1: EEG trace at 500 Hz 50 Hz low-pass filtered % channel 2: chewing artefact detection channel. high-pass, rect, low-pass % channel 3: up-sampled activity monitor % % FFT15 : Spectra averaged in bins of 15 minutes % MA15 : High-frequency envelope and activity in bins of 15 minute %% load the data [header, recorddata] = edfread( [args.rawDir 'M' args.animal '-' args.extn '\_EEG\' args.file] ); %% identify all EEG channels eegChanInd = strcmp(header.label, 'EEG') | strcmp(header.label, 'ECG');

NEEG = sum(eegChanInd);

```
EEGchan = find(eegChanInd);
minEEG = 1000*header.physicalMin(EEGchan)*.99;
actChanInd = strcmp(header.label, 'Activity');
        = sum(actChanInd);
NACT
ACTchan = find(actChanInd);
tmpChanInd = strcmp(header.label, 'Temp');
         = sum(tmpChanInd);
NTMP
TMPchan = find(tmpChanInd);
          = strsplit(header.starttime, '.');
tmp
                = 3600 * str2num(tmp{1}) + 60 * str2num(tmp{2}) +
args.onsetTime
str2num(tmp(187));
for chnd = 1: NEEG
 args.picDir = ['~\Dropbox\amy\plots\' args.exp '_' args.animal '\' ]; %'_ch'
num2str(chnd) '\'];
 if ~exist(args.picDir,'dir')
   mkdir(args.picDir);
 end
 cd( args.picDir )
 args.fileName1 = [args.date, '_sleepindex_' num2str(chnd)];
 args.fileName2 = [ args.date, '_spectrumByActivity_' num2str(chnd)];
 %% put EEG data into a EEGlab structure, and set dropped data to 0; sampled at 500 Hz
 EEG
              = struct():
 taxis
              = (0:(size(recorddata,2)-1)) / header.frequency(EEGchan(chnd));
 tst
             = \max(\text{find}(\text{abs}(\text{recorddata}(\text{EEGchan}(\text{chnd}),:)) > 1e-6));
                 = recorddata(EEGchan(chnd),1:tst);
 rawEEG
              = taxis(1:max(tst));
 taxis
 EEG.txS
                = taxis + args.onsetTime;
 EEG.txH
                = EEG.txS/3600;
 EEG.txD
                = EEG.txH/24;
 EEG.data
                 = 1000*recorddata(EEGchan(chnd),1:tst);% + 10*sin(2*pi*taxis*10);
 dropEEGBin
                   = EEG.data < minEEG;
 dropEEGInd
                   = find(dropEEGBin);
 EEG.data(drop EEGInd) = 0;
```

= 500;EEG.srate EEG.trials = 1: EEG.event  $= {}:$ = size(EEG.data,2); EEG.pnts EEG.nchan = NEEG: % highpass filter to isolate epochs with strong muscle artifacts locutoff = 50: filtorder = 128; %[tmp, com, b] = pop\_firws(EEG, 'fcutoff', locutoff, 'forder', filtorder, 'ftype', 'highpass'); tmp.data = abs(tmp.data);[MUA, com, b] = pop firws(tmp, 'fcutoff', 20, 'forder', filtorder, 'ftype', 'lowpass'); % ======== low-pass filter the data hicutoff = 50: locutoff = 1/50;filtorder = 128: %%[EEG, [locutoff com, b] = pop\_firws(EEG, 'fcutoff', hicutoff], 'forder',filtorder,'ftype','bandpass'); [EEG, com, b] = pop\_firws(EEG, 'fcutoff', hicutoff, 'forder', filtorder, 'ftype', 'lowpass'); % EEG.data(2,:) = MUA.data;EEG.nchan = NEEG + 1; %% put activty data into a EEGlab structure sampled at 10 HZ ACT = struct(); = (0:(size(recorddata,2)-1)) / header.frequency(ACTchan(chnd)); taxis = find(recorddata(ACTchan(chnd),:) > 0); tst %rawACT = recorddata(ACTchan(chnd),1:max(tst)); ACT.data = recorddata(ACTchan(chnd),1:max(tst));% + 10\*sin(2\*pi\*taxis\*10); taxis = taxis(1:max(tst)); ACT.txS = taxis + args.onsetTime; ACT.times = ACT.txS; ACT.txH = ACT.txS/3600; ACT.txD = ACT.txH/24; mnACT = mean(ACT.data); ACT.data = ACT.data;% - mnACT;

```
ACT.srate= header.frequency(ACTchan(chnd));ACT.trials= 1;ACT.event= {};ACT.pnts= size(ACT.data,2);ACT.nchan= NACT;
```

upACT

resample(ACT.data,header.frequency(EEGchan(chnd)),header.frequency(ACTchan(chnd))
);

=

```
tmx = max( size(EEG.data,2),size(upACT,2) );
EEG.data(3,1:tmx) = upACT(1:tmx);
EEG.nchan = 3;
```

```
tmp = strsplit(header.starttime, '.');
startSec = str2num(tmp(187));
```

```
% find the first full 4 second period
skipSec = mod(FFTWinSec - mod(startSec,FFTWinSec), FFTWinSec);
startInd = skipSec * EEG.srate;
binSec
        = FFTWinSec:
                                   % length of each fft bin in seconds
        = floor( (size(EEG.txS,2) - startInd)/(binSec*EEG.srate)); % number of bins
Nbin
%Nseconds = floor(EEG.pnts/EEG.srate)-binSec-1;
        = EEG.srate * binSec;
                                      % number of FFT bins
NFrq
freqs
        = (0:1:(NFrq-1))/binSec;
valFrqInd = find(freqs<40);
NvalFrg = length(valFrgInd);
valfreqs = freqs(valFrqInd);
fftdat = zeros(NvalFrq,Nbin);
MAmax = zeros(3,Nbin);
        = (args.onsetTime + startSec + FFTWinSec*(0:(Nbin-1)));
fftaxS
fftaxH = fftaxS/3600;
```

```
fftaxD = fftaxH/24;
```

```
fftme = mod(fftaxD,24);
```

```
taper = ([1:200 200*ones(1,NFrq-400) 200:-1:1]/200)-1;
```

```
taper = (1+\cos(pi^*taper))/2;
```

```
for (i = 1:Nbin)
```

```
thisStartInd = startInd + binSec*(i-1)*EEG.srate;
thisInd = thisStartInd + (1:NFrq);
```

```
= fft(taper .* EEG.data(1, thisInd));
   thisfft
   fftdat(:,i) = thisfft(valFrqInd);
   MAmax(1,i) = max(EEG.data(2,thisInd));
   MAmax(2,i) = max(upACT(1,thisInd));
   MAmax(3,i) = max(dropEEGBin(1,thisInd));
 end
 powfft = abs(fftdat);\%.^2;
 mnpow = mean(powfft,1);
 % split day-time spectra in four groups based on activity monitor
 nightTimeIndx = find( (fftme<0.25 \mid fftme > 0.75));
 dayTimeIndx = find( fftme>0.25 & fftme<0.75);
 brks
          = quantile(MAmax(2,dayTimeIndx), [0 1/4 2/4 3/4 1]);
 %if length(dayTimeIndx)>40 && length(nightTimeIndx)>40
 % figure
 % plot(sort(MAmax(2,dayTimeIndx)), (1:length(dayTimeIndx))/length(dayTimeIndx),
'r', 'Linewidth',3)
 % hold on
                                                 MAmax(2,nightTimeIndx)
                                 plot(sort(
                                                                                ).
 %
(1:length(nightTimeIndx))/length(nightTimeIndx),'k', 'Linewidth',3)
 %end
 if length(dayTimeIndx)>40
   figure
   plot(log10(valfreqs), log10(mean(abs(fftdat(:,dayTimeIndx)),2)), 'r', 'Linewidth',3);
   hold on
   for (qx = 1:4)
     qxind = fftme>0.25 & fftme<0.75 & MAmax(2,:)> brks(qx) & MAmax(2,:)<
brks(qx+1);
     plot(log10(valfreqs),
                              log10(mean(abs(fftdat(:,qxind)),2
                                                                   )),'Linewidth',1,
Color',[qx/4,.5,1-qx/4]);
     hold on
   end
 end
 if length(nightTimeIndx)>40
   plot(log10(valfreqs), log10(mean(abs(fftdat(:,nightTimeIndx)),2)), 'k', 'Linewidth',3);
   hold on
    for (qx = 1:4)
```

```
qxind = (fftme < 0.25 | fftme > 0.75) \& MAmax(2,:) > brks(qx) \& MAmax(2,:) <
brks(qx+1);
     plot(log10(valfreqs),
                              log10(mean(abs(fftdat(:,qxind)),2
                                                                  )),'Linewidth',1,
'Color',[qx/4,.5,1-qx/4], 'Linestyle','--' );
     hold on
    end
 end
 saveas(gcf,args.fileName2, 'pdf');
 close all
 downsample to 1 / 15 minutes
 % find the first 15 minute bin
 tmp
         = strsplit(header.starttime, '.');
 startHr = str2num(tmp{1});
 startMin = str2num(tmp{2});
 startSec = str2num(tmp(187));
 startMinBin = startMin - mod(startMin,binDurMin);
 startBinS = 3600 * \text{startHr} + 60 * \text{startMinBin} + 0;
 startBinM = startBinS/60;
 startBinH = startBinM/60;
 % number of bins to be created
 N15bin = ceil( FFTWinSec * (max(EEG.txS)-startBinS)/3600);
           = zeros(NvalFrq,N15bin);
 fftdat15
           = zeros(5,N15bin);
 ma15dat
 Nval15
          = zeros(1,N15bin);
 valFFTaxSM = MAmax(1,:) < .1;
 valFFTaxSA = MAmax(2,:) < 1000000; %126000;
 valFFTaxSD = MAmax(3,:) < .5;
 for bx = 1:N15bin
   % range of times in seconds that contribute to this 15 minute bin
   trngS = startBinS + [bx-1, bx] * binDurMin*60;
   % indeces of all spectra in the 15 minute bin
   thisFFTaxSbin = find(fftaxS>trngS(1) & fftaxS<=trngS(2));
   ma15dat(1,bx) = mean(MAmax(1,thisFFTaxSbin),2);
   ma15dat(2,bx) = mean(MAmax(2,thisFFTaxSbin),2);
   %thisFFTaxSbin = find(TMP.txS>trngS(1) & TMP.txS<=trngS(2));
   %ma15dat(3,bx) = mean(TMP.data(1,thisFFTaxSbin),2);
```

% indeces of all valid spectra in the 15 minute bin

```
thisFFTaxSbin = find(fftaxS>trngS(1) & fftaxS<=trngS(2) & valFFTaxSM & valFFTaxSA
& valFFTaxSD);
   Nval15(bx) = length(thisFFTaxSbin);
   if (length(thisFFTaxSbin)>20)
     fftdat15(:,bx) = mean( powfft(:,thisFFTaxSbin),2);
                      = mean( MAmax(1,thisFFTaxSbin),2);
     ma15dat(3,bx)
     ma15dat(4,bx)
                      = mean( MAmax(2,thisFFTaxSbin),2);
   end
 end
 FFT15
            = struct();
 FFT15.srate = 60/binDurMin;
 FFT15.data
            = fftdat15;
 FFT15.setname = 'FFT15';
 FFT15.icawinv = []:
 FFT15.icaweights = [];
 FFT15.icasphere = [];
 FFT15.icaact = [];
 FFT15.chanlocs = [];
 FFT15.trials = [];
 FFT15.nbchan = size(FFT15.data,1);
             = size(FFT15.data,2);
 FFT15.pnts
 FFT15.filename = [args.date, '_fft15_ch_' num2str(chnd)];
 FFT15.xmin
               = 0:
               = FFT15.pnts/FFT15.srate;
 FFT15.xmax
               = (0:(FFT15.pnts-1))/FFT15.srate + startBinH;
 FFT15.times
 pop_saveset(FFT15, 'filename', FFT15.filename, 'filepath',args.picDir );
 MA15
             = FFT15:
 MA15.filename = [args.date, '_ma15_ch_' num2str(chnd)];
 MA15.data
             = ma15dat;
 MA15.nbchan = size(MA15.data,1);
             = size(MA15.data,2);
 MA15.pnts
 pop_saveset(MA15, 'filename', MA15.filename, 'filepath',args.picDir );
end
```

```
output_args = -1;
end
```

## Appendix C Supplementary Figures and Tables

Post-aerosol challenge, macaques were monitored for signs of clinical encephalitis. Checks occurred at least twice daily, with additional checks triggered if any individual macaque in the holding room manifested signs of disease. The checklist included excreta output and food/water intake, as mentioned previously in this chapter.

## **Appendix C.1 Macaque Health Status Scoring**

Clinical scores consisted of the summation of three individual scores: temperature, activity, and neurological status. All scores were assigned on ordinal score rankings. Activity and neurological scores were assigned based off observations of such signs of disease with seizures or a comatose macaque ranked 5 or 6 on the neurological scoring scale.

Once the scores were over a cumulative threshold of 14, or the neurological score ranked over 5 (comatose macaque), the humane study endpoint was reached and the macaque euthanized (procedure detailed in Appendix C.2). These scores were formulated based off previous works and are described therein (17, 18, 95, 138, 168). A separate scoring key was used for EEEV- versus VEEV-infected macaques due to the observation of disparate clinical signs of neurological disease in each disease course. Once infected, NHPs were observed twice daily by either the RBL-DLAR staff, PI's staff, or both. Clinical signs were recorded at least once daily using the scoring categories/parameters described below until one week had passed with no clinical symptoms.
Score	Neurological Score	Activity Score	Temperature Score
1	N7 1		
1	Normal;	Normal: responds to observer	Normal: baseline to
		contact and body language	heading average
		interactions	temperature
2	Occasional loss of balance	Less active: responds to	Mild Fever: 1.6 to 3.0
-	or muscle control (ex:	observer approaching the cage:	degrees above
	stumbling unsteadiness):	body language interactions less	baseline average
	drooling	frequent or intense	temperature
			·····F · · · · · · ·
3	Occasional nystagmus (eye	Sluggish: only responds when	Moderate Fever: 3.1
	oscillation/twitching), head	prodded or when observer	to 4.0 degrees above
	pressing, tremors	rattles the cage, maintains	baseline average
		hunched posture with back to	temperature
		observer; limited eye contact	
		and body language	
		interactions; glassy eyes,	
		grimace or sad facial	
4	I and a flatter as	expression	C
4	Loss of balance,	Upright but inactive; does not	Severe Fever: greater
	tremore: occasional	cage ignores all stimuli	above baseline
	seizures	cage, ignores an sumun	temperature
	seizures		temperature
			OR
			Mild Hypothermia:
			0.1 to 2 degrees
			below baseline
			temperature
5	Frequent seizures	Recumbent/moribund, and	Moderate
		unresponsive (prompt	Hypothermia: 2.1 to 5
		euthanasia)	degrees below
			baseline average
	~		temperature
6	Comatose (prompt	N/A	Severe Hypothermia:
	euthanasia).		greater than 5 degrees
			below baseline
			average temperature

## Appendix Table 1. EEEV – Infected Macaque Scoring Key

Total score  $\geq 10$  - Increase observations to once every eight hours.

Animals that reach a cumulative score of 14 will be promptly euthanized

Score	Neurological Score	Activity Score	Temperature Score
1	Normal	Normal: responds to observer room entry, frequent eye contact and body language interactions	Normal: baseline to 1.5 degrees above baseline average temperature
2	Mild photophobia, muscle stiffness, jaw soreness, occasional loss of balance and/or nystagmus, infrequent swatting at head	Less active: responds to observer approaching the cage; body language interactions less frequent or intense	Mild Fever: 1.6 to 3.0 degrees above baseline average temperature
3	Head pressing, frequent loss of balance, tremors, moderate photophobia, or frequently swatting at head.	Sluggish: only responds when prodded or when observer rattles the cage, maintains hunched posture with back to observer or stays in back of the cage; limited eye contact and body language interactions; glassy eyes, grimace	Moderate Fever: 3.1 to 4.0 degrees above baseline average temperature
4	Seizure activity	Upright but inactive; does not respond to observer rattling the cage, ignores all stimuli	Severe Fever: greater than 4.0 degrees above baseline temperature OR Mild Hypothermia: 0.1 to 2 degrees below baseline temperature
5	Complex loss of balance and/or comatose	Does not respond to observer rattling the cage, ignores all stimuli, recumbent/moribund (prompt euthanasia)	Moderate Hypothermia: 2.1 to 5 degrees below baseline average temperature
6	N/A	N/A	Severe Hypothermia: greater than 5 degrees below baseline average temperature

## Appendix Table 2. VEEV - Infected Macaque Scoring Key

Total score  $\geq 10$  - Increase observations to once every eight hours.

Animals that reach a cumulative score of 14 will be promptly euthanized.

## Appendix C.2 Macaque Euthanasia Procedure

Euthanasia was carried out by anesthetization with 10 mg/kg ketamine, with final samples of blood (and CSF, if applicable) taken, followed by intravenous sodium nitroprusside diluted in heparin solution to facilitate vascular vasodilation, and a sublethal intravenous dose of 15 mg/kg of sodium pentobarbital. When the macaque was thus anesthetized, complete exsanguination via transcardial perfusion with 12L of sterile 0.9% saline solution. Confirmation of death was achieved by the observed cessation of cardiac function, stoppage of physical respiration (excluding agonal breathing), and by testing for corneal areflexia.

Animals will be promptly euthanized if any of the following are met:

1. Animal is found are either comatose or moribund.

2. Animal reaches a score of 6 the temperature scale, or a score of 5 on either the neurological or the activity scale.

Animals that reach a cumulative score of 14 will be promptly euthanized.

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