2005

Post-Juvenile Brain Development Modulates Seizure Characteristics and Diazepam Efficacy in the Rat Pilocarpine-SE Model

William H. Holbert II
Virginia Commonwealth University

POST-JUVENILE BRAIN DEVELOPMENT MODULATES SEIZURE CHARACTERISTICS AND DIAZEPAM EFFICACY IN THE RAT PILOCARPINE-SE MODEL

A Thesis submitted in partial fulfillment of the requirements for the degree of Master of Science at Virginia Commonwealth University.

by

WILLIAM HAROLD HOLBERT II
Bachelor of Science
East Carolina University
1989

DIRECTOR: SEVERN B. CHURN, PHD
ASSOCIATE PROFESSOR, DEPARTMENTS OF NEUROLOGY, ANATOMY AND NEUROBIOLOGY, PHARMACOLOGY AND TOXICOLOGY, AND PHYSIOLOGY
DIRECTOR MOLECULAR NEUROSCIENCE RESEARCH FACILITY

Virginia Commonwealth University
Richmond, Virginia
July 2005

ACKNOWLEDGEMENTS

The author would like to thank several people for their help. First, I would to thank my parents, Pat and Harold, for their lifetime of support and instilling in me a strong work ethic. I especially would like to thank my sister for her endless love and devotion during this journey. I would like to thank the members of my lab for their help, insight, and suggestions: Dr. Travis Parsons, Ph.D., Mike Singleton M.S., Rajendar Kumar M.D. (Ph.D. student), Jonathan Kurz (M.D./Ph.D. student), James M. Bracey M.S., Pallavi Ramnarian (B.S.), Anh Lee Anderson (lab technician), and Matt L. Ryan (lab technician). I would like to thank the Department of Pharmacology & Toxicology for giving me the opportunity to complete this degree. I would also like to thank my committee members Dr. Susan Robinson Ph.D., and Dr. Steven Shapiro M.D., for their time and service during this project. Finally, I would like to thank my advisor Dr. Severn B. Churn, Ph.D. for his patience, training, and encouragement during this endeavor.

TABLE OF CONTENTS

Page

LIST OF TABLES

Page

LIST OF FIGURES

Page

LIST OF ABBREVIATIONS

AED	Anti-Epileptic Drug
Bl	Baseline
CSA	Composite spectral analysis
DF	Degrees of freedom
EC	Early continuous
EEG	Electroencephalogram
EPSP	Excitatory post synaptic potential
F	F test
FFT	Fast Fourier Transformation
F/S	Fast and Slow spiking
FSP	Fast Spiking w/Pausing
Hz	Hertz
I.P.	Intraperiotneal
GABA	Gamma amino butyric acid
$_A\alpha 2$	Alpha 2 subunit
LC	Late Continuous
P1	Postnatal day 1
P21	Postnatal day 21
P30	Postnatal day 30
P40	Postnatal day 40
P50	Postnatal day 50
P60	Postnatal day 60
P70	Postnatal day 70
P80	Postnatal day 80
P90	Postnatal day 90
qEEG	Quantitative electroencephalogram
SB	Seizure body
SE	Status epilepticus
SO	Seizure onset
ST	Seizure termination
μV	Micro volts
W/W	Wax/Wane

ABSTRACT

POST-JUVENILE BRAIN DEVELOPMENT MODULATES SEIZURE CHARACTERISTICS AND DIAZEPAM EFFICACY IN THE RAT PILOCARPINE-SE MODEL

By WILLIAM H. HOLBERT II, M.S.

A thesis submitted in partial fulfillment of the requirements for the degree of Master of Science at Virginia Commonwealth University.

Virginia Commonwealth University, 2005

Major Director: Severn B. Churn, Ph.D
Associate Professor, Departments of Neurology, Anatomy and Neurobiology, Pharmacology and Toxicology, and Physiology
Director, Molecular Neuroscience Research Facility

These studies were completed to examine how status epilpeticus seizure characteristics are modulated during post-juvenile brain development. This may determine if postnatal age in rats is a better identifier of stages of post-juvenile brain development. The first study fully detailed the acute discrete seizure phase of the rat pilocarpine-SE model. Results for this study showed that Racine behavioral severity score, spike

frequency, and seizure severity during the acute discrete seizure phase change in relation to post-juvenile brain developmental stages. The second study fully detailed early and late patterns of status epilepticus. Results for this study displayed modulation of time in pattern, spike frequency, and relative delta power for seizure pattern during post-juvenile ages. The third study displayed modulation of diazepam efficacy during post-juvenile ages. The data suggest characteristics in the acute discrete seizure phase, chronic SE phase, and therapeutic window of SE change in relation to age during post-juvenile brain development. This establishes that age is a better estimator of developmental stage than animal bodyweight.

INTRODUCTION

Epilepsy

Epilepsy is classified as a syndrome of recurrent partial or generalized seizures that are a result of abnormal electrical activity originating in cerebral neurons. This syndrome affects 1-2% of the United States population and 50 million people worldwide. The highest incidence has been shown to be in children five years old or younger [1] . Although some cases are genetic, the majority are caused by hypoxia, brain trauma, and the neurological emergency, status epilepticus [1].

Status Epilepticus

The classical definition of status epilepticus (SE) is continuous seizure activity lasting 30 minutes or longer without self termination [2-4]. A more recent definition is that SE can be identified as five minutes or more of continuous seizure activity [5]. Approximately 150,000 cases are reported each year with many resulting in morbidity and mortality [2, 5-8]. SE can result in high mortality in the elderly, intellectual dysfunction, and motor deficits in children [2, 4-8]. In addition, 20-40% of epilepsy cases are a direct result of an episode of status epilepticus [5]. The insult from SE can cause a predictable pattern of serious neuronal injury [5].

As SE progresses different seizure patterns have been identified [9-11]. One study, Treiman et al , organized an episode of SE into five predictable seizure patterns [9]. First came a period of acute self terminating discrete seizures; second was a pattern of merging seizures which cycled between periods of waxing and waning intensity [9]. The third stage was identified as a pattern of continuous ictal activity; the fourth was continuous ictal activity with electro-decrimental pauses (flat periods); and the fifth observed pattern a period of periodic lateralized epileptiform discharges [9]. Another study, Handforth et al 1995, further detailed an episode of SE into early and late stages of predictable seizure patterns [10, 11].

Many anti-epileptic drugs fail to terminate status epilepticus [5, 12-14]. In addition, of the drugs that do terminate SE many lose their therapeutic efficacy from the onset and during progression of SE [5, 12-14]. One compound that has been shown to work is the benzodiazepine *diazepam* [13, 15-17]. This compound has a high therapeutic index and rapidly crosses the blood brain barrier. However, *diazepam* can also lose therapeutic effectiveness as SE progresses [12-14].

Electroencephalogram (EEG)

One tool to measure brain activity during an SE event is the electroencephalogram or EEG [18]. The earliest publication on the use of the human EEG was completed in the 1920's by Hans Berger, a German neuro-psychiatrist [18]. Since the 1920's the EEG has been used to diagnose a variety of neural ailments, brain trauma, and the syndrome of epilepsy. The EEG functions through scalp recordings that measure the brain's electrical activity [18]. The electrical activity that is measured is summed excitatory and inhibitory post synaptic potentials [18]. In addition, rhythmic oscillations from the thalamus create

cortical neuron firing. Alterations of normal brain activity such as epilepsy often result in a slowing EEG [18]. This results in changed frequency activity in the EEG and may be represented as spikes or spike and wave complexes.

Pilocarpine-SE model

Many animal models are used to study status epilepticus [9-11, 19]. The accepted model of choice is the pilocarpine-SE model of limbic epilepsy [19, 20]. This model provides a good representation of the human condition and has a predictable timeline. First, animals are pretreated with methylscopolamine, a muscarinic antagonist that is excluded by the blood brain barrier, approximately twenty minutes prior to pilocarpine injection to reduce peripheral effects [21-24]. Once pilocarpine, a muscarinic agonist, is injected, the first ictal activity begins approximately 17.0±1.5 minutes later [21-25]. This starts the discrete seizure phase of SE that can last between 8.0-16.0 minutes [9-11, 21-25]. At the end of this phase, seizures merge without a clearly defined termination [9-11, 21-25]. This indicates the onset of the SE phase and the progression into predictable seizure patterns [9-11, 21-25]. One caveat exists with this model; there is high degree of variability in reported results of SE [20, 21].

Aging and Post-Juvenile Brain Development

Several problems exist when analyzing the incidence of SE in the general population. It has been shown that a higher incidence of SE exists in children and the elderly [1, 2, 5, 20, 21, 26-31]. In addition, of the SE cases reported, most are in children five years or younger, and the elderly experience a high degree of mortality when afflicted with SE [1, 2, 5, 20, 21, 26-31]. This has led to a major amount of research done on infant, juvenile, and geriatric animals [10, 11, 24, 29, 32-45]. Few studies exist

that fully characterize SE in post-juvenile aged animals. Furthermore, many laboratories that do study SE in adolescence or adulthood use weight as an estimator for developmental stage [9-11, 24, 41, 42, 45]. It has been shown that body weight does not parallel brain development in humans and rats [38, 46-50].

This study was comprised of three phases that investigated seizure characteristics, severity, and drug efficacy of SE during post-juvenile ages. The first part of this study was performed to detail seizure characteristics and severity of the acute discrete seizure phase of SE during post-juvenile brain development. Ages were represented by postnatal day (P) in 10 day increments. Seizures were induced using the model previously described and EEG data obtained. Animals were characterized in the acute discrete seizure phase for seizure termination profiles, composite spectral analysis, behavioral severity, spike frequency, time characteristics, and absolute and relative delta band power. The data suggest that discrete seizures in the acute discrete seizure phase change during post-juvenile brain developmental ages.

The second phase of this study examined the status epilepticus phase. Animals were characterized in the SE phase for time in seizure pattern, spike frequency in seizure pattern, and relative delta percentage in seizure pattern. In addition, correlations were established with characteristics of the first phase. The data suggested that severity was altered by age and not seizure pattern. In addition, age was a better estimator of post-juvenile developmental stage than animal body weight.

The third phase was a study conducted on post-juvenile animals concerning drug efficacy in the therapeutic window. Three postnatal ages (P30, P50, P90 days) were subjected to SE and injected with diazepam in time increments that allowed for the

discrete seizure phase and ensured injection during SE progression. The data for this study suggested that efficacy loss in the therapeutic window may be a result of age as well as progression of the disease.

The hypothesis for the overall study was that SE characteristics and diazepam efficacy are modulated during post-juvenile brain development. In addition, the postnatal age was a better representation of post-juvenile developmental stages than body weight. The data supported this hypothesis through several observations. Behavioral severity correlated directly with age of the animal. Seizure patterns did not alter in severity during SE progression; rather, severity in SE was a function of developmental age. A third observation was that SE induced loss of drug efficacy was related to age. Furthermore, many of the observed changes in seizure characteristics occurred in animal ages that were close in bodyweight. The following chapters that support our observation are companion papers (A, B), and a brief communication being sent for publication. The first paper, "Post-Juvenile Brain Development Modulates Seizure Characteristics in the Rat Pilocarpine Model: Discrete Seizure Phase", is being sent to *Epilepsia*. The second paper, "Post-Juvenile Brain Development Modulates Seizure Characteristics in the Rat Pilocarpine Model: Status Epilepticus Phase", is being sent to *Epilepsia*. The third paper; which is a brief communication, "Status Epilepticus-Induced Loss of Diazepam Efficacy is Age Dependent in the Rat Pilocarpine-SE Model", is being sent to *Epilepsy Research*. Each paper is formatted according to the guidelines established by each specific journal.

POST-JUVENILE BRAIN DEVELOPMENT MODULATES SEIZURE CHARACTERISTICS IN THE RAT PILOCARPINE MODEL: DISCRETE SEIZURE PHASE

ABSTRACT

Purpose: This study utilized the rat-pilocarpine model of status epilepticus to characterize ictal activity during the acute discrete seizure phase in post-juvenile ages.
Methods: Seizure activity was induced by pilocarpine injection (300 mg/kg) in rats ranging in age from P30 to P90. Behavioral and electrographic activities were monitored by video EEG. Behavioral observations were scored according to the scale of Racine. Electrographic activity was characterized for seizure profiles, time characteristics, and spike frequency measurements. Composite Spectral Analysis (CSA) and Quantitative Electroencephalogram analysis (qEEG) were performed using algorithmic calculations from Insight, Insight II software (Persyst, Prescott, AZ).
Results: Discrete seizures showed significant modulation of Racine scores, spike frequency, and power components. Additionally, seizure profiles transitioned from a predominate dreadnaught shape in P30-40 animals to a predominate cyclone shape in P90 animals. Animal ages P50-P90 showed a significant correlation between Racine scores and spike frequency
Conclusions: Seizure characteristics are age dependent in the acute discrete seizure phase of SE during post-juvenile brain development.

INTRODUCTION

Status epilepticus is a neurological emergency defined as continuous seizure activity for 30 minutes or longer without self termination [3, 4]. Approximately 150,000 cases of SE are reported each year in the United States, with many resulting in mortality [5, 6, 8, 51]. In addition, individuals that survive SE usually suffer long-term cognitive deficits and motor dysfunction [13]. Multiple studies have suggested that SE onset changes by developmental age [5, 7, 19, 21, 28, 52, 53]. Therefore, a systematic characterization of the acute discrete seizure phase is necessary to understand changes prior to status epilepticus onset.

Previous developmental investigations of SE have focused on P1-30 (juvenile) animals [34, 38, 39, 54]. During this age range, rats develop in several stages of brain growth [49, 50, 55]. However, it is widely accepted that rats continue to develop and finalize brain circuitry during post-juvenile (P30-80) ages until adulthood (P90) [40, 50, 56]. Similarly, the human brain finalizes neural circuitry development during post-juvenile ages [46, 47]. Although this age range has been investigated as a group [19, 21, 25, 57] , there has been no systematic characterization of the electrographic and behavioral seizure characteristics during post-juvenile brain development. Furthermore, studies utilizing the pilocarpine and other models of SE have reported a high degree of variability in multiple parameters including death rate and SE induction probability [19, 21]. In many investigations, body weight is used as an estimator for age [22-24, 58]; however, brain development does not parallel body growth or weight [46-50].

Therefore, characterization of the effect of developmental age on seizure characteristics is warranted.

This study examined seizure activity during the acute discrete seizure phase in the pilocarpine-SE model. Animal ages P30 through P90 were utilized to approximate the human brain's post-juvenile aging process. The results from this study identified multiple characteristics that changed during post-juvenile ages. Understanding how post-juvenile brain development affects seizure characteristics may provide information to decrease the variability for this model.

MATERIALS AND METHODS

Induction of Status Epilepticus

Male Wistar rats (P30-P90) were purchased from Harlan Laboratories (Indianapolis, IN, USA), housed with food and water provided *ad libutum*, with lighting on a 12 hour on/off cycle. All animal use procedures were in strict accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals and were approved by the Virginia Commonwealth University Institutional Animal Care and Use Committee.

Five days prior to SE induction, four surface electrodes were surgically implanted into the skull of male Wistar rats under ketamine (125 mg/kg i.p.)-xylazine (1 mg/kg) anesthesia. Two frontal electrodes (F1 and F2) were implanted over the frontal cortex (3.5 mm anterior to bregma; ±2.5 mm left or right of sagittal suture). Two posterior electrodes (P1 and P2) were implanted over parietal cortex and hippocampus (2.0 mm posterior to bregma, ±2.5 mm left or right of sagittal suture). Electrodes were secured with dental acrylic, and the animals were allowed to recover for at least five days. Four separate bipolar channels were recorded with a montage of F1-F2, F1-P1, F2-P2, and P1-P2. On the day of the experiment, animals were connected via headset to video-EEG machines (BMSI 5000, Nicolet), and baseline recordings were obtained. Using a sampling rate of 420 Hz per channel, a low pass frequency of 100 Hz, a high pass frequency of 2 Hz, and notch filter set at 60 Hz; animals were monitored for behavioral and EEG activities. To induce seizure activity, pilocarpine (300 mg/kg i.p.), a muscarinic agonist, was injected. Methylscopolamine, a muscarinic antagonist that does not cross the blood brain barrier, was administered i.p. (1 mg/kg) 30 minutes prior to pilocarpine

injection to reduce peripheral affects[21, 25, 57]. All materials were reagent grade and purchased from Sigma Chemical Company (St. Louis, MO, USA) unless otherwise stated.

Behavior observations

Fifty-six animals were used in this study of the acute discrete seizure phase of status epilepticus. In the discrete seizure phase, rats were assigned a score based on the scale of Racine [59]. Scores ranged from 0-5 according to movements and behavior. 0= normal behavior, wet dog shakes, arrest; 1= mouth and facial movements; 2= head nodding, mouth and facial movements; 3= forelimb clonus ; 4= rearing with forelimb clonus; 5= rearing and falling [59]. The maximum score for each animal was recorded and averaged for each age group. Postnatal-90-day old animals were defined as adults [21, 34, 60] and were used as a standard for comparison in the present study.

Visual and Electrographic analysis:

During each discrete seizure, spike frequency was determined using Insight II and averaged for each animal for the duration of the discrete seizure. Final results for all discrete seizures were then averaged for each postnatal age giving the overall average for the age group. Maximum and minimum spike frequencies for each discrete seizure per animal within each age group were also obtained. The number of discrete seizures was averaged per age group and an average duration for each discrete seizure was calculated. The total time in seizure activity was recorded for each discrete seizure phase along with average latency time to SE for each postnatal age.

Spectral Analysis

During the acute discrete seizure phase, Composite Spectral Analysis (CSA) was created in four different sections for each discrete seizure: pre-ictal baseline section (Bl), a build up or seizure onset (SO), a seizure body or primary ictal event (SB), and seizure termination (ST). Visual inspection of the composite was made in four frequency bands: delta (0.5-3.99 Hz), theta (4.0-7.99 Hz), alpha (8.0-12.99 Hz), and beta 1 (13.0-20.99 Hz) [41, 42]. The major contributory frequencies from discrete seizures were analyzed between 2.5-50.0 μV. Frequency amplitude of 2.5 μV or lower was considered baseline; minor peaks were observed between 2.5 μV through 15.0 μV; and major peaks were observed at 15 μV or higher. One epoch of twelve seconds for each section per discrete seizure was used for quantitative EEG (qEEG) analysis and was completed in five frequency bands: delta (0.50-3.99 Hz), theta (4.0-7.99 Hz), alpha (8.0-12.99 Hz), beta-1 (13.0-20.99 Hz), beta-2 (21-44.0 Hz) [41, 42]. Absolute values were obtained for each band and relative delta contribution (% of total power) was derived as described [41, 42]. A one-twofold increase in absolute values from baseline defined seizure onset (SO) and a return to baseline defined seizure termination (ST) for absolute qEEG values. The software used for spectral analysis (Insight, Insight II) was purchased from Persyst Corporation (Prescott, AZ).

Statistical Analysis

Single parametric comparisons were made with paired Students t test. Multiple comparisons were tested with one-way analysis of variance (ANOVA) with Tukey post hoc analysis to reduce type-1 errors. Nonparametric comparisons were performed using Kruskal-Wallis test with Dunns multiple comparison, and correlations were determined

by Spearman's Rank correlation and linear regression analysis. All statistics were completed using Graph Pad Prism 4.0 for windows (Graph Pad Software, San Diego CA, USA, www.graphpad.com). Data are expressed as mean ± standard error of the mean.

RESULTS

Behavioral Characteristics:

There was a significant overall effect of postnatal age on Racine behavioral score ($p<0.001$ by Kruskal-Wallis, Kruskal-Wallis statistic=31.33, n=56 animals). After Dunns multiple comparisons between groups were completed, animals were compared against P90 animals' behavioral score. The P90 (adult) animal average maximum Racine behavioral score was 4.44±0.24 (Figure 2, n=9 animals). Dunns multiple comparison test showed that postnatal-30 and 40-day old animals (pubescent) had Racine behavioral scores similar to P90 animal behavioral scores ($p>0.05$, Figure 2, n=7 animals, P30-40, respectively). Unlike P90 or P30-40 animals, P50 (early adolescent) animals displayed a significantly reduced seizure severity when assessed by behavioral methods. Postnatal-50-day animals displayed the lowest Racine score of 2.10±0.27 out of all age groups. This was significantly lower than P90 animals ($p<0.001$, Kruskal-Wallis with Dunns multiple comparison test, n= 10 animals). In addition, the P50 animals were significantly lower than either P30 or P40 animals ($p<0.01$, $p<0.05$, Kruskal-Wallis, P30-40 respectively). Postnatal-60 and 70-day animals (mid-adolescent) displayed a similar reduced behavioral severity ($p<0.05$, Kruskal-Wallis with Dunns multiple comparison, n=6 animals, n=11 animals, P60-70 respectively). After P60-70 animals, behavioral seizure severity scores increased in P80 animals, however; these were not significantly lower when compared to P90 animals ($p>0.05$, Kruskal-Wallis, with Dunns multiple comparison).

To determine if developmental age had an association with behavioral severity, Racine scores were correlated with post-juvenile age. When all age groups were included, no significant correlation existed between post-juvenile age and behavioral severity ($r=0.10$, $p>0.05$, Spearman's rank). However, when the P30-40 age group was removed a significant correlation was observed between P50 ages through P90 ($r=1.00$, $p<0.01$, Spearman's rank). The data suggest that late stages of brain development (P50-80) affect seizure severity when characterized by behavioral measures.

Visual and Frequency Analysis of EEG

Time characteristics were observed to quantify if the average time frame for seizure characteristics changed with age in the pilocarpine-SE model. The duration of the discrete seizure phase for P90 animals averaged 14.33 ± 3.00 min (Table 1) with approximately 3 discrete seizures per period. Postnatal-90-day animals averaged 50.36± 4.73 sec per seizure (Table 1, ~149 sec/phase). This seizure duration of 50.36± 4.73 sec and 3 discrete seizures per period resulted in P90 animals spending approximately 22.58±3.97% of the 14.33± 3.00 min discrete seizure phase in ictal activity (Figure 4).

There was a significant age dependent effect on the duration of discrete seizures for all animals ($F=4.25$, $df=54$, $p<0.01$, by one way ANOVA, $n=56$ animals). After Tukey post hoc analysis, however; no significant difference exists when all separate age groups are compared to P90 animals ($p>0.05$, one way ANOVA). The average latency period to SE from seizure onset for all animals did not have a significant age dependent effect ($F=1.29$, $df=55$, $p>0.05$, by one way ANOVA, $n=56$ animals). Conversely, there was a significant overall effect of postnatal age on percent time in ictal activity during post-juvenile ages ($F=2.29$, $df=55$, $p<0.05$, by one way ANOVA, $n=56$ animals). For

individual age groups after Tukey post hoc analysis, postnatal-50-day animals spent a significantly greater percent of time in ictal activity, 49.98±10.47%, than P90 animals (Figure 4, $p<0.05$, one-way ANOVA with Tukey post hoc analysis). This peak resulted from a trend of increasing time spent in ictal activity that originated in P30 animals and continued in P40 animals (Figure 4). This was not significant when compared to P90 animals ($p>0.05$, P30-40, one way ANOVA, n=7 animals respectively). A decline of time in ictal activity was observed in P60 animals and remained lower than P50 animal time in ictal activity for P70-80 animals (Figure 4). The data suggest that younger animals (P30-50) spend more overall time in seizures than older animals (P60-80, Figure 4).

To determine if seizure severity is age modulated, spike frequency was recorded for each discrete seizure in all age groups. One way analysis of variance showed a significant effect of postnatal age on spike frequency ($F=10.49$, $df=54$, $p<0.001$, by one way ANOVA, n=56 animals). After Tukey post hoc analysis spike frequencies were compared against P90 animals. The average spike frequency for P90 animal discrete seizures was 7.43±0.32 Hz, which was the highest average in any of the animal age groups studied (Table 1). Postnatal-30, 40, and 50-day animals (n=7, 7, 10 animals, respectively) displayed the lowest average spike frequency per discrete seizure studied and were significantly lower than P90 animal spike frequency values (Figure 3, P30: $p<0.001$; P40-50 $p<0.01$, one-way ANOVA with Tukey post hoc analysis). This began a progression of increasing values until P90 levels were reached (Figure 3). Spike frequency values for P50 animals provided a transition point through postnatal ages averaging 5.83±0.42 Hz per discrete seizure, which was significantly higher than P30

animals (Figure 3, $p<0.01$, one-way ANOVA with Tukey post hoc analysis, n=10 animals) but these values were significantly lower than those of P90 animals ($p<0.01$, one-way ANOVA with Tukey post hoc analysis).

To test if an age dependent correlation for severity exists, linear regression analysis was performed between spike frequency averages for each age and postnatal age. Results showed a significant correlation exists between spike frequency and post natal age ($r^2=0.90$, $p<0.001$). The data suggest that as animals' age during post-juvenile brain development, seizure severity increases.

Spectral Analysis of Seizure Severity

Significant slowing of the EEG was observed as an increase in amplitude in the lower frequency bands (Figure 1C). Major frequencies were identified in the delta (0.50-3.99 Hz), theta (4.00-7.99 Hz), and alpha band (8.00-12.99 Hz). It appeared that P90 animals had an increased delta contribution when compared to the other age groups examined.

To quantify the contribution of each frequency band to total ictal activity, qEEG analysis was performed using Insight II software. Five frequency bands were measured for absolute and relative power as described (see Materials and Methods). Increases over the average baseline qEEG values were used as a standard for analysis. Overt ictal activity was associated with a significant, five-tenfold increase over baseline values in the SB section for absolute delta power in all animals (all P30-90, $p<0.01$, paired Students t test, n=56 animals). The average absolute delta power for the seizure body section for all animals was $18.33\pm1.33\ \mu V^2$ (st.dev 6.29) and $3.08\pm0.30\ \mu V^2$ (st. dev 1.80) for baseline values.

Relative delta contribution has been utilized in previous studies to compare ictal severity [41, 42]. To further quantify age dependent modulation of seizure severity, increases in the seizure body section over baseline relative delta values was calculated for all ages and age was found to have significant effect on relative delta power increase ($F=7.76$, $df=48$, $p<0.001$, by one-way ANOVA, $n=56$ animals). After Tukey post hoc analysis delta increases for each animal group were compared to the P90 animal average of 18.47±2.38% (increased delta contribution, $n=9$ animals). Although postnatal-30-day animals did display a similar increase in relative delta values of 17.08±1.0% ($n=7$ animals), postnatal-40-day animals began a significant decline in relative delta power increase in relation to P90 animals (7.16±2.05%, $p<0.05$, one-way ANOVA with Tukey post hoc analysis, n=7animals). Postnatal-50-day animals continued this decline in relative delta values with the lowest percentage increase of 1.96±2.85% ($n=10$ animals) out of all age groups. This small increase was the greatest difference in relative delta percentage increases in this study when compared to P90 animal increases ($p<0.001$, one-way ANOVA with Tukey post hoc analysis). Furthermore, some calculated percentages were lower than baseline values. A sharp return to higher relative delta increases, that were similar to P90 animal values, was observed for P60 animals (16.29±1.50%, Figure 5, $p>0.05$, One way ANOVA). This observation of similar values (~16-18%) remained for the P70-80 age group relative delta increase (Figure 5).

To determine the relationship between relative delta contribution over baseline values and behavior severity, values from each age group were compared. While a similar age-dependent change in both of these parameters was observed, a significant correlation did not exist between these two variables ($r=0.71$, $p>0.05$, Spearman's rank).

Seizure severity most likely does not explain the observed age dependent changes in behavioral severity.

DISCUSSION

The pilocarpine-SE model was used to characterize events related to the discrete seizure phase of status epilepticus through post-juvenile developmental ages (P30-P80). This model was chosen due to its approximation of the human condition and the ability to study developmental influence on seizure characteristics. The data demonstrated that seizure profiles, Racine behavior scores, spike frequency recordings, and spectral analysis changed in relation to post-juvenile developmental ages (P30-P90). The results demonstrated that developmental age modulates specific seizure characteristics. Since many laboratories report animal weight ranges and not specific postnatal ages, the present study may provide information to reduce inter-study variability with this model.

Previous investigations have shown seizure sensitivity is age modulated in immature animals [2, 19, 21, 36, 40, 61]; however, no systematic characterization of the discrete seizure phase has been conducted. In our study, P30-40 aged animals displayed significant pathology when measured by behavioral standards. These observations were confounded, however; when measuring spike frequency, percent time in ictal activity, and seizure profiles. Within the P30-40 age group there exist multiple possibilities that may explain these disparate observations. This age group may be heavily influenced by pubescent hormones, which may modulate seizure severity and activity [38, 50, 62-64]. In addition, animals in this age group experience a final rapid growth stage of dendritic connections [50]. Following this stage the brain systematically culls dendritic density and interneuron number until P90 levels are reached [56, 65, 66]. Thus, this stage may represent a critical stage of increased seizure sensitivity. An earlier study using perforant path stimulation displayed an increase in seizure development with similar aged animals

(P35) [67, 68]. However, despite the increased sensitivity, the immature P30-40 brain displayed decreased spike frequency. This suggests that the P30-40 (peri-pubescent) brain may not have the completed neural circuitry to reduce seizure severity when compared to P90 (adult) animals.

Out of the many characteristics studied, P50 (adolescence) was the earliest stage to clearly define late stage developmental influence on seizure characteristics. Large differences in behavior, electrographic, and power characteristics between P50 and P90 animals were observed. The data suggest that as animals' age during P50-80 development, seizure severity increases. To complement these observations, a linear regression between Racine scores and discrete seizure spike frequency in ages corresponding with post-juvenile brain development was calculated. When compared through all postnatal a significant correlation did not exist (r^2=0.03, p=0.70, linear regression, n=56 animals). However, when this study separated away the P30-40 age group a significant correlation was observed between P50 through P90 animal group (P50-90) with regard to Racine scores and spike frequency values (r^2=0.93, p<0.01, linear regression, n=42 animals). These correlations further suggest that seizure characteristics are developmentally modulated through the age range P50-80: ages corresponding to late stage brain development.

Previous experiments using the kindling model related brain status and seizure severity during ictal activity to scores (1-5) for certain behavior [69]. These experiments revealed that inducing seizures through the amygdala produced scores of 1-3 and a limbic status of ictal activity (less severe) [69]. Scores of 4-5 exhibited an extra-limbic progression to a generalized full brain status (more severe)[69]. Given that a Racine

score can determine discrete seizure severity, a score of 3 is the critical value in this ranking system and needs to be examined. Age modulation was observed between P80 and P90 animals. Postnatal-80-day animals averaged 3.3 as a maximum score with four animals having a maximum score of 3 and two animals having a maximum score of 4. Postnatal-90-day animals had one animal with a maximum score of 3 and eight animals above 4. These observations suggest that at the P80 age animals transitioned between the limbic (less severe) and extra-limbic generalized (more severe) seizure status [69]. Conversely, the majority of P90 animals attained the generalized seizure. These two age ranges averaged a 4-5% change in body weight (P80:364±8.96 g vs P90:383.39±2.95 g) yet displayed disparate characteristics for severity and localization of seizures. The data demonstrate significant modulation of seizure severity during late adolescent animal aging. The modulation may explain apparently disparate findings and high variability across multiple laboratories using this model.

It is well known that the EEG provides a summation of the brain's electrical activity [18]. Electrodes will receive most signals generated by excitatory postsynaptic potentials (EPSP) and some action potentials from cortical neurons [18]. It is speculated that the thalamus is the originator of EEG rhythmic oscillations that activate cortical neuron firing, which are represented as a slowing EEG [18]. This study found a progression through postnatal ages of increasing spike activity. Out of this progression, significant differences between P30-P40, P50, and P90 animals were observed allowing for the identification of discrete seizure characteristics which change during post-juvenile ages.

There were five principal frequency bands used for power analysis in our investigation as described (see Materials and Methods). The delta band (0.50-3.99 Hz) power has been utilized as an indicator of neuropathology; representing brain lesions, cerebral damage, edema, and white matter destruction in animal experiments [70, 71]. Results from this study demonstrated that relative delta power did change during developmental ages (P40, P50). When tested for significance, the relative delta component for P50 animals was the lowest value out of all postnatal age groups. Thus, like behavioral observations, P50 animals expressed less severe seizure activity when measured by delta power contribution. In addition, the subsequent increase in delta power suggests that developmental mechanisms occur in the late stages of brain development. These mechanisms may participate in the increased seizure severity observed in this model.

Previous investigations have translated human years to animal ages through measurements of brain development between humans, primates, and rats in terms of anatomical growth [56, 72]. One such study established timelines suggesting early postnatal ages for rats were similar to the human pre-natal brain [72]. Other studies have suggested that postnatal 21 (P21) rats are analogous to five year old humans [56]. Our study utilized the pilocarpine-SE model to study post-juvenile brain development and anthropomorphically translate animal ages. We estimate that P30-40 animals were analogous to a 11-14 year old human due to the hormonal surges preceding and during this age range [63, 64]. Several studies have also defined this age range as pubescent [73-75]. This study estimated P50 as a 15-16 year old human due to the continuing transitional point in the results through out this study and the previous definition of

adolescence in P50 animals [62, 63, 73]. Postnatal-60-day animals were considered a 17-18 year old human due to results which were similar to both P50 and P60 animals. Postnatal-70 and 80-day animals were estimated to be analogous to a 19-24 year human due to their results closely approximating P90 rats. Postnatal-90-day rats were considered fully developed and resembling a 25-year-old human. The age ranges identified are a best-guess anthropomorphic estimate of late stage brain development.

This study investigated seizure characteristics in the discrete seizure phase in the pilocarpine-SE model and how they change during post-juvenile brain development. Many characteristics during the acute discrete seizure phase displayed developmental modulation such as seizure profiles, Racine scores, power analysis, and spike frequency. These results establish that age may explain the developmental variability in characteristics for this model. Future studies will determine what characteristics change during the chronic SE phase of the pilocarpine –SE model and if the variability reported in this model are age-dependent.

Figure 1: Post-juvenile Brain Development Modulates Seizure Characteristics in the Discrete Seizure Phase Preceding SE.

Typical seizure profiles (left) and frequency distributions (right) observed in postnatal 30-day-old (A), postnatal 50-day-old (B) and postnatal 90-day-old rats (C). Age did not affect the total number of seizures observed or the duration of seizure activity. Seizure length averaged between 30 and 90 seconds and was characterized by a ten to fifteen-fold increase in average absolute delta band power (0.50-3.99 Hz). Seizure (SO) activity started with a gradual slowing of the EEG that progressed to high amplitude, increased slowing of the seizure body (SB). Postnatal-90-day animals (C) displayed slowing in the seizure body section (SB). Postnatal-30 and 40-day animals (A) had a similar slowing profile when compared to P90 animals. Postnatal-50-day animals (B) displayed reduced signal amplitude when compared to P30 and P50 animals. Seizure termination (ST) for all animals either ended abruptly (B, C) or faded to baseline (A) (termination). In addition, P30-40 animals had a majority of seizure terminations that faded to baseline resulting in a dreadnaught appearance. Postnatal-50-day animals through P90 animals displayed abruptly ending seizure terminations which gave a cyclone shape seizure appearance.

Figure 1

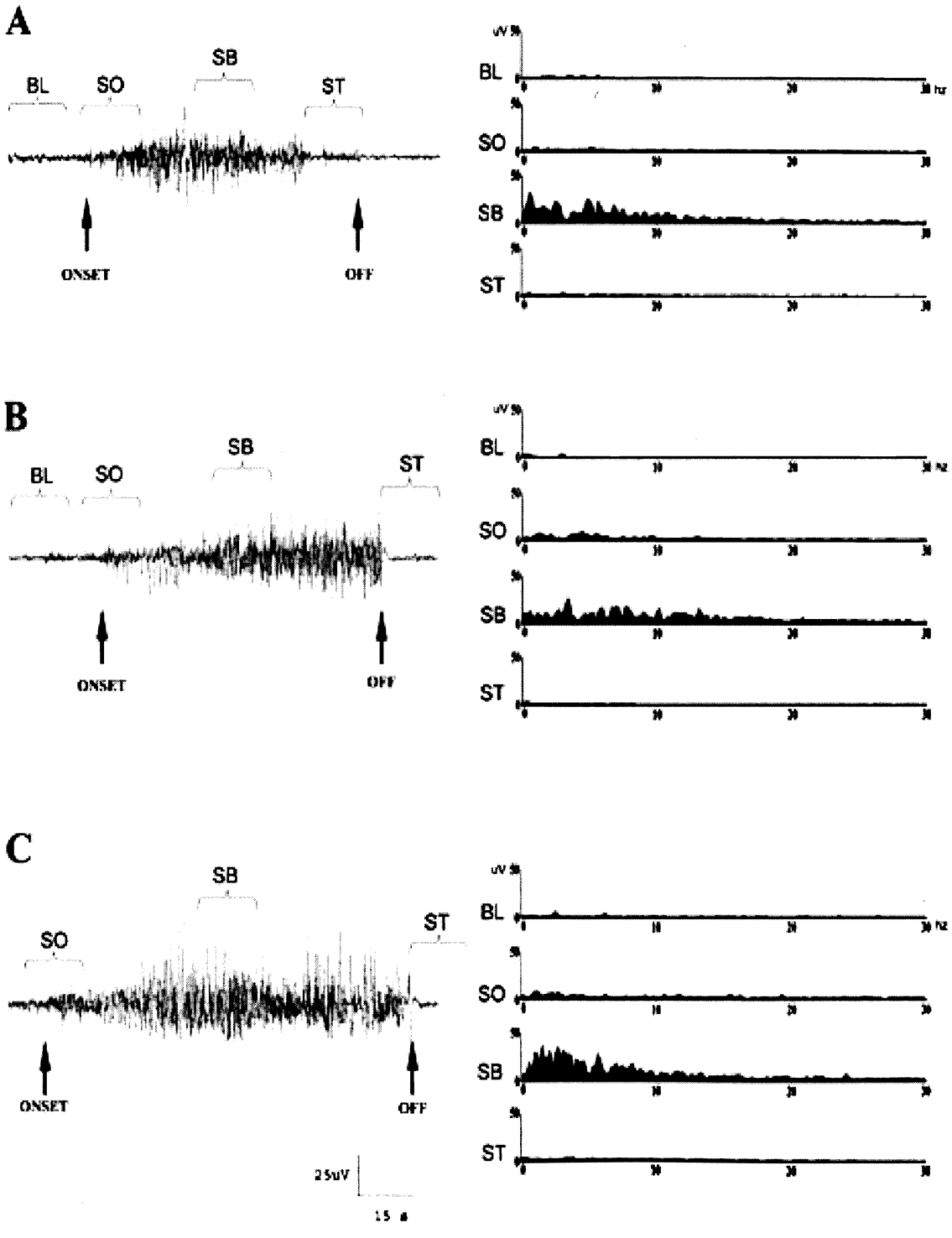

A
BL
SO
SB
ST
ONSET
OFF
B
BL
SO
SB
ST
ONSET
OFF
C
SO
SB
ST
ONSET
OFF
25uV
BL
SO
SB
ST

Table 1: Time and Frequency characteristics. Data are expressed as mean ± standard error of the mean.

	N	Max freq per seizure (Hz)	Min freq per seizure (Hz)	Avg freq per seizure (Hz)	Average Discrete Seizure Period (minutes)	Avg Seizure Duration (sec)
P30	7	4.79±0.58	3.35±0.22	3.96±0.35	12.0±1.76	52.40±9.17
P40	7	5.98±0.51	4.74±0.26	5.41±0.34	11.33±1.75	79.91±9.17
P50	10	6.59±0.31	4.99±0.49	5.83±0.42	8.88±2.00	57.93±8.47
P60	6	7.17±0.35	5.21±0.46	6.27±0.23	16.41±1.75	69.10±10.14
P70	11	7.29±0.33	5.87±0.16	6.55±0.23	8.38±1.74	33.54±2.19
P80	6	7.11±0.27	5.93±0.20	6.51±0.21	9.68±2.00	51.44±8.52
P90	9	8.32±0.48	6.73±0.28	7.43±0.32	14.33±3.00	50.36±4.73

Table 2: Absolute power values for discrete seizures (SB) in each post natal age. Data are expressed as mean±standard error of the mean.

	Delta μV^2	Theta μV^2	Alpha μV^2	Beta 1 μV^2	Beta 2 μV^2
P30	22.39±1.91	9.86±0.86	5.69±0.45	3.32±0.23	1.60±0.10
P40	14.20±1.16	8.99±0.79	5.49±0.50	3.45±0.34	1.73±0.18
P50	14.70±1.78	10.26±1.55	8.11±1.43	5.14±1.14	2.98±1.06
P60	17.41±1.62	7.70±0.50	4.23±0.27	2.98±0.22	1.50±0.61
P70	19.60±3.11	10.32±1.64	7.50±1.21	4.71±1.07	2.17±0.92
P80	20.68±3.30	9.69±0.76	5.64±0.56	3.50±0.36	1.88±0.18
P90	20.80±3.16	8.10±0.84	4.85±0.51	3.05±0.31	1.56±0.19

Figure 2: Behavioral Observations Reveal Age Dependent Seizure Severity

Animals were observed for behavior during discrete seizures and assigned a score based on the scale of Racine (see Materials and Methods) [69]. Scores of 1-3 distinguished a less severe limbic seizure and scores of 4-5 a more severe generalized seizure [69]. Late stage developmental modulation of seizure severity was observed in P50 animals having the lowest average score and significantly lower than P90 animals (2.10 ± 0.27, $p<0.001$, Kruskal-Wallis with Dunns multiple comparison). Postnatal-60-day animals were also significantly lower than P90 animal behavioral severity scores ($p<0.01$, Kruskal-Wallis with Dunns multiple comparison). * denotes statistical significance. ***=$p<0.001$, **=$p<0.01$,*= $p<0.05$ when compared to P90 animals.

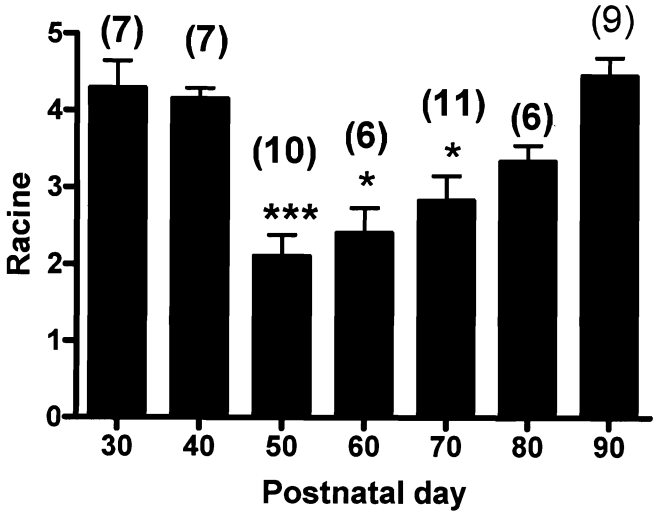


Figure 2: Racine behavioral severity scores for each postnatal age. Calculated means are expressed has columns and standard error of the mean +1 are expressed as error bars.

Figure 3: Spike Frequency Recordings Display Significant Differences during Post-Juvenile Brain Development.

Spike frequency was recorded using Insight software (Persyt, Prescott, AZ), and all seizures were averaged for each animal. Age was found to have a significant effect on data for all age groups ($F=10.49$, $df=54$, $p<0.001$, by one way ANOVA, $n=56$ animals). After Tukey post hoc analysis postnatal-90-day animals displayed significantly higher frequency recordings than P30-40 and P50 animals (P30: $p<0.001$; $p<40$-P50: $p<0.01$, one-way ANOVA with Tukey post hoc analysis). Additionally, spike frequency values for P50 animals were significantly higher than P30 animals ($p<0.01$ P50 vs P30, one-way ANOVA with Tukey post hoc analysis).* denotes statistical significance. ***=$p<0.001$, **=$p<0.01$ when compared to P90 animals.

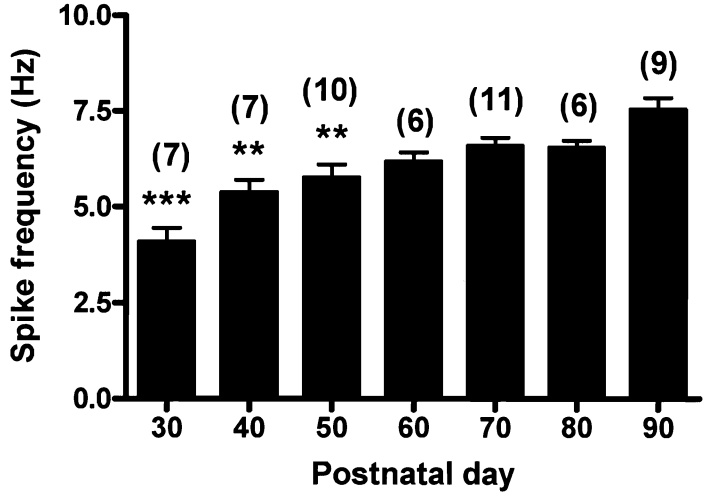


Figure 3: Spike frequency averages for discrete seizures in each postnatal age. Calculated means are expressed as columns and standard error of the mean +1 are expressed as error bars.

Figure 4: Postnatal-50-day Animals Spend Significantly More Time in Ictal Activity than P90 Animals.

For time characteristics, animals averaged 3.0 discrete seizures, with 30-90 seconds per seizure, and 11.50±1.50 minutes for the discrete seizure phase in this study (Table 1, n=56 animals). Percent time in ictal activity for P50 animals was significantly higher than P90 animal time in ictal activity during the discrete seizure period (49.98±10.47%, $p<0.05$, one-way ANOVA with Tukey post hoc analysis). This was the only age group significantly higher when compared to P90 animals in time characteristics. However; age did have an overall effect on percent time in ictal activity ($F=2.29$, $df=55$, $p<0.05$, by one way ANOVA, n=56 animals). * denotes statistical significance. *= $p<0.05$ when compared to P90 animals.

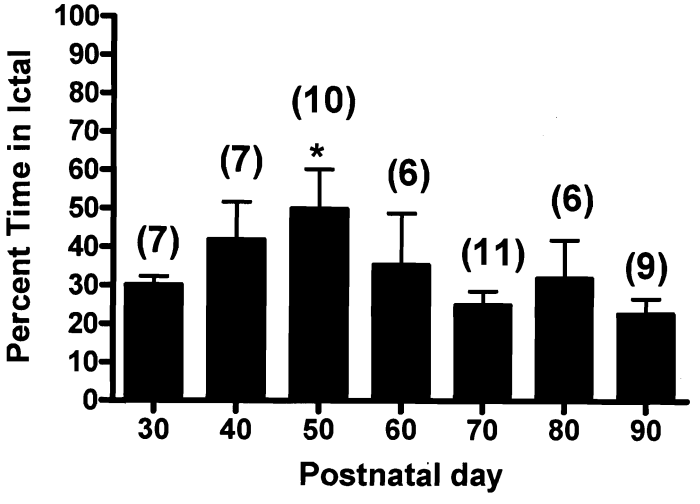


Figure 4: Time spent in seizure (ictal) activity for the duration of the discrete seizure period. Calculated mean are expressed as columns and standard error of the mean +1 are expressed as error bars.

Figure 5: Post-Juvenile Brain Development Modulates Relative Delta Power: Power estimates were completed in five bands: delta (0.50-3.99 Hz), theta (4.0-7.99 Hz), alpha (8.0-12.99 Hz), beta 1 (13-20.99 Hz), and beta 2 (21-44 Hz) [41, 42]. Relative delta power (% total power) increases over baseline displayed significant changes through post-juvenile brain development. Age was found to have a statistically significant effect on relative delta increase ($F=7.76$, $df=48$, $p<0.001$, by one-way ANOVA, n=56 animals). Observations after Tukey post hoc analysis showed postnatal-30-day animals with relative delta percentage increases close to P90 animal percentage increases (17.08±1.01%). The postnatal-40 day animal's relative delta power increase was significantly lower than postnatal-day-90 animal (7.16±2.04%, $p<0.05$, one-way ANOVA with Tukey post hoc analysis). Postnatal-50-day animals followed this with the lowest relative delta percent increase in the age groups studied (1.96±2.85%). This observation was significantly lower than postnatal-30, 60, and 90-day animals (P30, P90: $p<0.001$; P60: $p<0.01$, one-way ANOVA with Tukey post hoc analysis). Postnatal-60-day animals returned relative delta percentage increases to P90 levels where they remained for P70-80 animals. * denotes statistical significance. *** =$p<0.001$, **=$p<0.01$,*=$p<0.05$ when compared to P90 animals.

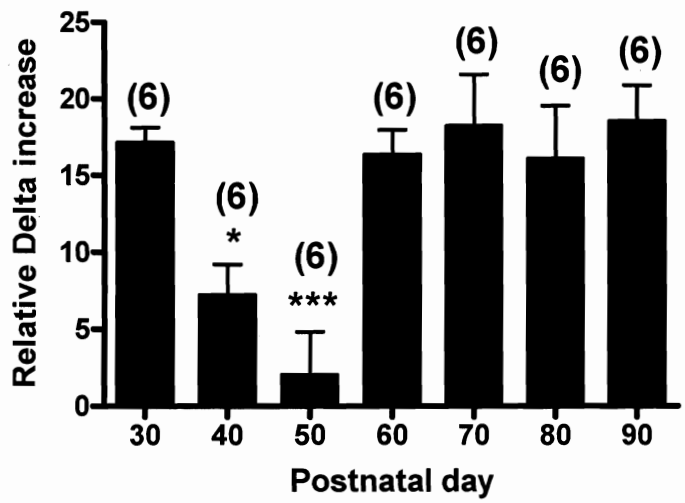


Figure 5: Relative delta percentages for discrete seizures in each postnatal age. Calculated means are expressed as columns and standard error of the mean +1 are expressed as error bars.

POST-JUVENILE BRAIN DEVELOPMENT MODULATES SEIZURE CHARACTERISTICS IN THE RAT PILOCARPINE MODEL: STATUS EPILEPTICUS PHASE

ABSTRACT

Purpose: This investigation was conducted to identify seizure characteristics and severity of status epilepticus (SE) changes during post-juvenile brain development

Methods: The rat pilocarpine model was used to fully characterize seizure patterns of status epilepticus (SE). SE was induced by pilocarpine (300 mg/kg) in rats ranging in age from P30 (pubescent) and P90 (adult). Behavioral and electrographic activities were monitored by video EEG (BMSI 5000, Nicolet). Electrographic activity was characterized for early and late predictable patterns of SE: Early; merging seizures with waxing and waning (W/W), Fast and Slow spiking (F/S); Late; Early Continuous Spiking (EC), Continuous Fast Spiking w/pauses (FSP), and Late Continuous spiking (LC). Quantitative Electroencephalogram analysis (qEEG) was completed using algorithmic calculations from Insight, Insight II software (Persyst, Prescott, AZ).

Results: Modulation of SE characteristics during post-juvenile brain development was observed for SE onset probability, time in seizure patterns, and spike frequency in seizure patterns. Relative delta values for P50 animal early and late SE patterns were significantly lower than P90 animal early and late SE seizure patterns. Significant correlations were established between onset probability and discrete seizure phase characteristics.

Conclusions: Early and late SE characteristics are age dependent during post-juvenile brain development. Animal age is a better estimator for developmental stage than body weight.

INTRODUCTION

Status epilepticus (SE) is a serious, life threatening, neurological emergency defined as continuous seizure activity lasting 30 minutes or longer [3, 4]. The annual occurrence of SE is approximately 150,000 cases per year in the United States with young children and the elderly experiencing the greatest percentage of incidence [2, 5-8]. Many of these occurrences will result in mortality [2, 5-8]. In addition, survivors suffer secondary effects including intellectual deficits, motor dysfunction, and the development of epilepsy [2, 5, 8, 13]. Therefore, development of animal models of SE that closely represent the human condition are necessary to determine the molecular mechanisms that underlie this devastating disease.

While several animal models are used to investigate SE, the pilocarpine model provides a good representation of the human condition with similar pathology and seizure patterns [10, 11, 19]. One caveat exists concerning this animal model; a significant variability has been reported in results with respect to death rate and percent response in post-juvenile ages (P30-90) [19, 21, 58]. During this period of development, many laboratories approximate adolescence or adulthood in animals using body weight ranges even though there is a lack of synchrony between the brain and body weight during the maturation process in animals [46, 47, 49, 55, 76, 77]. Brain development occurs in spurts during juvenile ages with adolescence finalizing circuitry development [46, 47, 49, 55, 56, 76, 77]. Thus, accurate characterization of SE using postnatal age is warranted.

This study examined developmental modulation of SE characteristics in the rat pilocarpine model through post-juvenile and adult ages (P30-80, P90). The data demonstrated age modulation for the probability of SE onset, time in specific seizure

pattern, and spike frequency within seizure patterns. Significant differences were found in relative delta percentage contribution in the F/S, EC, and FSP seizure patterns within developmental stages during post-juvenile aging. The data suggest that age is a more accurate description for developmental stage than body weight, and SE characteristics were age modulated during post-juvenile brain development. The results may provide a model to elucidate the molecular and cellular events surrounding age dependence of SE onset and to reduce the variability in this model.

MATERIALS AND METHODS

Induction of Status Epilepticus

Male Wistar rats (P30-P90) were purchased from Harlan Laboratories (Indianapolis, IN, USA), housed with food and water provided *ad libitum* with lighting on a 12 hour on/off cycle. All animal use procedures were in strict accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals and were approved by the Virginia Commonwealth University Institutional Animal Care and Use Committee. The software used for analysis (Insight, Insight II) was purchased from Persyst Corporation (Prescott, AZ).

Five days prior to SE induction four surface electrodes were implanted into the skull of male Wistar rats under ketamine (125 mg/kg i.p.)-xylazine (1 mg/kg) anesthesia. Two frontal electrodes (F1 and F2) were implanted over the frontal cortex (3.5 mm anterior to bregma, ±2.5mm left or right of sagittal suture). Two posterior leads (P1 and P2) were implanted over parietal cortex and hippocampus (2.0 mm posterior to bregma, ±2.5mm left or right of sagittal suture). Electrodes were secured with dental acrylic and animals were allowed to recover for at least five days. Four separate bipolar channels were recorded with a montage of F1-F2, F1-P1, F2-P2, and P1-P2. Electrodes for each animal were connected via headset to video-EEG machines (BMSI 5000, Nicolet), and baseline recordings obtained. EEG data were obtained using a sampling rate of 420 Hz per channel; low pass frequency set at 100 Hz, high pass frequency set at 2 Hz, and the notch filter set at 60 Hz. To induce seizure activity, pilocarpine (300 mg/kg i.p. Sigma, St. Louis, Mo), a muscarinic agonist, was injected. Methylscopolamine, a muscarinic antagonist that does not cross the blood brain barrier, was administered i.p. (1 mg/kg) 30

minutes prior to pilocarpine injection to reduce peripheral affects [21, 25, 57]. Animals were monitored for electrographic activity throughout the procedure. Status epilepticus was broken down into two stages: early; with merging seizures that wax and wane (W/W, Figure 6B), and Fast/Slow spiking (F/S, Figure 6C); late, with early continuous fast spiking (EC, Figure 6D), continuous fast spiking with electro-decrimental pauses (flat periods) lasting 0.10-0.50 seconds (FSP, Figure 6E) [11, 12]. The late continuous fast spiking pattern (LC, Figure 6F) was not observed in all age groups and was not graphed [10]. Animals were measured for time in pattern, frequency per pattern, absolute, and relative power (% total power) [41, 42] in the delta band (0.50-3.99 Hz) in each pattern. All materials were reagent grade and purchased from Sigma Chemical Company (St. Louis, MO, USA) unless otherwise stated.

Electrographic analysis

Beginning with the wax and wane seizure pattern, animals were monitored throughout the procedure for time and spike frequency in pattern using Insight software (Persyst Corporation) and EEG machines (BMSI 5000, Nicolet). Time recordings were calculated in total seconds and converted to percentiles for each pattern through all postnatal ages. Spike frequency was recorded in each pattern using 6-18 second epochs. During each pattern spike frequency was quantified by measuring an average for each channel for the duration of the epoch. Final results for all patterns were then averaged for each postnatal age.

Quantitative EEG analysis (qEEG) was completed using Insight II software (Persyst Corporation, Prescott, AZ) and was recorded in the five patterns using 6-18 second epochs (Figure 1). One to twenty-five measurements were used to record each

pattern. Quantitative EEG (qEEG) analysis (absolute and delta) was measured and recorded for all four patterns in the delta frequency band (0.5-3.99 Hz) [42].

Statistical Analysis

Multiple comparisons were completed using one-way analysis of variance (ANOVA) with Tukey post hoc analysis to reduce type-1 errors. Correlations were determined by Spearman's Rank correlation and linear regression analysis. Fisher's exact test was used to determine age dependence and completed through Vassar College: http//:faculty.vassar.edu/lowry/webtext.html [78]. One-way analysis of variance (ANOVA), Spearman's rank, and linear regression were completed using Graph Pad Prism 4.0 for windows (Graph Pad Software, San Diego, CA USA, www.graphpad.com).

RESULTS

SE Onset Probability and Age Correlations:

One hundred thirty-three animals through seven postnatal ages were used in this study. Animals for each age group were observed for 60 minutes of SE, a standard duration for investigations of biochemical and pharmacological changes during SE [22-25, 45]. The induction of status epilepticus (SE) was defined as the onset of ictal activity without a clearly defined termination (Figure 6B). This activity initiated approximately 11.0±1.5 minutes after the first discrete seizure in all animal age groups studied.

Onset probability was calculated in all age groups studied with P90 animals having the highest probability of SE onset (P90: 96.0%, n=24 animals, Figure 7). Postnatal-30 and 40-day animals had a similar probability of SE onset (P30: 89.0%, n=19 animals; P40: 77.0%, n=9 animals). However, P50 and P60 animals had the lowest percent of SE onset out of all animals studied (P50: 56.0%, n=30 animals; P60:72.0% P60, n=11 animals). Induction of SE for P70-80 animals was also lower when compared to P90 animals (P70: 86.0%, n=21 animals; P80: 84.0%, n=19 animals).

Specific seizure characteristics during the acute discrete seizure phase were examined to determine their predictive value for SE probability. Behavioral severity of discrete seizures had a positive correlation with SE onset probability (Figure 2 and 7, $r= 0.96$, $p<0.01$ Spearman's rank). However, other measures were confounded by the high SE onset probability observed in P30-40 animals. Postnatal-30 and 40-day animals had a higher onset probability than older animals (mid-late adolescence, P60-80) which confounded age dependent calculations for onset probability through all postnatal ages (P30-90) (Figure 7, P=0.17, P30-P90, Fishers exact, N=133 animals). In addition, when

onset probability and discrete seizure spike frequency averages for all postnatal ages (P30-P90) were plotted, no significant age correlation existed (r^2=0.009, p=0.74, linear regression, P30-P90).

Previous studies have suggested that P30-40 animals could have confounding factors [36-38, 50, 63, 73] that may interfere with age correlations in P30-40 animals. In addition, several laboratories utilize P60 to P90 animals for biochemical analysis [21, 36, 64, 79]. Therefore, the late developmental ages (P50 to P90) were characterized to determine any predictive characteristics within this age group. When P30-40 animals were omitted as a developmental stage, developmental age became a significant predictor for SE onset ($p<0.05$, P50-P80, Figure 7, Fishers exact, n= 81 animals). This suggests that a significant modulation of SE onset exists during late stage brain development.

Post-Juvenile Brain Development Affects Early Patterns of SE:

To determine how brain development affects SE severity, animals were grouped into 10 day incremental age groups (P30-90) and characterized for early and late SE seizure patterns previously described [10, 11]. Postnatal-90-day old rats have been defined through previous investigations as adults [21, 34, 60], and were used as a comparison for this study in each SE pattern with post-juvenile ages as described (see Materials and Methods).

The wax/wane (W/W) pattern was identified by the merging of discrete seizures without a clearly defined self termination. Developmental age did not significantly affect the duration of the W/W seizure pattern for all age groups (P30-90) studied (F=0.94, df=46, p=0.47, by one-way ANOVA, Figure 8 A, n=47 animals). In P90 animals, seizure severity was also not affected by age. After Tukey post hoc analysis animals were

compared to P90 animal spike frequency averages. The average spike frequency for the P90 W/W seizure pattern was 6.11±0.48 Hz (n=8 animals). The wax period averaged 6.64±0.46 Hz and the wane period averaged 5.21±0.47 Hz for P90 animals (Figure 9 A). The W/W pattern and individual wax or wane period spike frequency were similar to P90 animal W/W spike frequency in all age groups studied ($p>0.05$, P30-P80; Figure 9 A).

The end of the W/W seizure pattern was characterized by a noticeable progression into an alternating fast and slow (F/S) spiking pattern. The F/S pattern for P90 animals persisted for 16.13 ± 4.97% of total time of observed SE (Figure 8 B, n=8 animals). This pattern averaged two fast and slow cycles for P90 animals of which the majority was spent in the fast spiking period (14.1±3.24% fast, 4.12 ±1.73% slow).

Age did have a significant effect with regard to time in pattern ($F=5.71$, $df=46$, $p<0.001$, by one way ANOVA, n=47 animals). Analyzing after Tukey post hoc analysis and then comparing against P90 animals, postnatal-30-day animals spent a significantly greater time in fast/slow spiking pattern when compared to P90 animals (53.41±5.57%, $p<0.01$, by one-way ANOVA with Tukey post hoc analysis, n=7 animals). The remaining age groups spent similar time as P90 animals in the F/S pattern with the same average number of cycles ($p>0.05$, Figure 8 B). Like the preceding wax/wane pattern, fast and slow periods cycled between high and low spike frequencies for P90 animals (8.73±0.33 Hz, fast; 6.84±0.33 Hz, slow, n=8 animals). However; after Tukey post hoc analysis P30-80 animal age groups were similar to P90 animals ($p>0.05$, Figure 9 B). The data suggest that time and spike frequency characteristics in the early stages of SE (W/W, F/S) [11] minimally changed during ages corresponding to post-juvenile brain

development. However, the percent time an animal showed in the early seizure patterns decreased with increasing age.

Post-Juvenile Brain Development Modulates Late Stages of SE:

The later patterns of SE [10] (EC,FSP) constituted the majority of time spent during the observed time in SE for all animals. Early Continuous (EC, Figure 6 D) was identified as a pattern absent of F/S spiking revolutions. This pattern had a noticeable increase in amplitude from the F/S pattern (Figure 6 E) and age did have a significant effect on this pattern ($F=4.77$, $df=47$, $p<0.001$, by one way ANOVA, $n=48$ animals). Postnatal-90-day animals spent 16.0±4.52% of observed SE time in the EC pattern ($n=8$ animals). Similar to P90 animals, postnatal-30-day animals spent 24.40±7.36% of observed time in SE in the EC pattern ($p>0.05$, Figure 8 C, $n=7$ animals). However, P40 animals spent a significantly greater time in EC than P90 animals (42.00±4.38%, $p<0.05$, one-way ANOVA with Tukey post hoc analysis, $n=7$ animals). Postnatal-50-day animals were like P90 animals with 35.60±8.10 % ($n=8$ animals) of observed SE time in EC and started a decreasing trend which continued in P60 and P70-80 animals (Figure 8 B). Postnatal-30 and 40-day animals spent more of the observed time in the late stage SE pattern of EC when compared to P50-P80 and P90 animals.

The EC pattern had the highest spike frequency recorded for P90 animals during the observed time in SE (9.07±0.54 Hz). Postnatal-30 and 40-day animal spike frequency in the EC pattern was not significantly different than P90 animal spike frequency; however it did begin a trend of increasing spike frequencies (Figure 9 C). Spike frequency averaged 9.02±0.93 HZ for P50 animals in the EC pattern and remained high in late developing animals P60-80 and P90 animals (Figure 9 C). For P50-80 and

P90 animals, spike frequency in the EC pattern was consistently 2-5 Hz higher than P30-40 animals in the same pattern.

The fast spiking w/pauses pattern (FSP, Figure 6 E) was characterized as a pattern of high amplitude and frequency with intermittent electro-decrimental pauses (flat periods) lasting 0.10-0.50 seconds. For all age groups, there were significant differences between groups in the FSP pattern in relation to time in pattern ($F=7.87$, $df=47$, $p<0.001$, by one way ANOVA, n=48 animals). Tukey post hoc analysis was used to compare groups to P90 animal percentages. Postnatal-90-day animals spent the majority of their time during the observed period of SE in the FSP pattern (57.34±5.09%, n=9 animals). Postnatal-30-day animals spent a significantly lower time of 17.38±4.49 in this pattern when compared to P90 animals ($p<0.01$, one way ANOVA, n=7 animals). In addition, postnatal-50-day animals spent 23.60±8.55% of observed SE time in the FSP pattern (Figure 8 D, n=8 animals). This was significantly lower than P90 animal time in the FSP pattern ($p<0.01$, one-way ANOVA, n=8 animals). However; P50 animals started a trend of increasing time spent in the FSP during late stage brain development. Postnatal-60-day animals continued this trend of increasing time in the FSP pattern were it remained near to P70-80 animals (43.83±5.18%, n=6 animals).

Age was found to have a significant effect on spike frequency in the FSP pattern ($F=50.03$, $df=48$, $p<0.001$, by one way ANOVA, n=49 animals). Using Tukey post hoc analysis, animals were compared to P90 animal values. Spike frequency for P90 animals averaged 8.89±0.31 Hz for the FSP seizure pattern (Figure 9 D, n=8 animals). Postnatal-30 and 40-day animals did measure significantly lower spike frequencies in the FSP pattern when compared to P90 animals (Figure 9 D, P30: $p<0.001$; P40: $p<0.01$, one-way

ANOVA with Tukey post hoc analysis, n=7 animals respectively). Postnatal-50-day animals recorded a significantly higher spike frequency when compared to P30-40 animals (Figure 9 D, P30: $p<0.001$; P40: $p<0.01$, one-way ANOVA with Tukey post hoc analysis, n=4 animals). Spike frequency for this pattern remained at a high level in P60-80 animals until P90 values were reached (Figure 9 D). The data suggest that late SE seizure pattern time and frequency characteristics change in relation to post-juvenile brain development.

Spectral Analysis:

To more thoroughly characterize seizure severity in SE over specific ages, spectral analysis of each seizure pattern was performed. Relative (% total power) delta power contribution was determined as described (see Materials and Methods). The delta band (0.5.00-3.99 Hz) [41, 42] was selected due to its relation to severity and indication of neural pathology [70, 71]. Postnatal-90-day animals displayed cycles of revolving intensity through the early patterns of observed status epilepticus. Wax periods had higher relative delta percentage values when compared to wane periods (44.09±1.00% wax vs 43.80±2.8% wane, n= 4 animals). Fast and slow periods followed a similar pattern of revolving intensity for P90 animals (45.57±1.66% fast vs 42.48±2.39% slow, n=4 animals). This resulted in an average relative delta power of 43.95±1.38% for the W/W pattern and 43.27±1.85% for the F/S pattern. In the latter patterns of SE, relative delta percentage for P90 animals were similar to the early patterns of observed SE (EC; 42.94±2.16%: FSP; 41.70±2.01, n=4 animals). The results show seizure severity is not related to type or progression of seizure patterns of SE.

To further assess severity in each pattern increases over baseline delta values were measured for each age group and compared against P90 animal values. Postnatal-30 through 80-day animal age groups did not differentiate from P90 animals in the observed W/W pattern of SE for relative delta increases ($F=0.75$, $df=78$, $p>0.05$, by one way ANOVA, Figure 10 A, n=79 animals). Conversely, two age groups had an increase over delta baseline values in the F/S pattern (P50:9.93±1.89%, $p<0.01$; P80:11.39±1.99 $p<0.05$, one way ANOVA with Tukey post hoc analysis). The data suggest that age had a minimal impact on relative delta increases per pattern in the early stages of SE.

For the late stages of SE, EC and FSP patterns, age had a significant effect for both patterns concerning relative delta increases (EC: $F=14.92$, $df=65$; FSP: $F=15.85$, $df=70$, $p<0.001$, by one way ANOVA, n=66 animals, n=71 animals, respectively). Using Tukey post hoc analysis, animals were then compared between groups. Postnatal-30-day animals did have significantly higher relative percentages than P50 animals in those patterns (Figure 10 C, D; $p<0.001$ EC and FSP, one-way ANOVA, n=14 animals, n=8 animals, respectively). Postnatal-40-day animals were significantly higher than P50 in the EC pattern ($p<0.001$, one way ANOVA, n=14 animals). These percentages for P50-80 animal age groups remained lower than both P30-40 and P90 animal relative delta in the EC and FSP patterns (Figure 10 B-D). Postnatal-50-day animals had significantly lower relative delta percentage increases than P90 animals for both EC and FSP patterns (Figure 10 C, D; $p<0.001$, one-way ANOVA with Tukey post hoc analysis, n=8 animals, n=11 animals). Postnatal-60-day animals continued with significantly lower percentages for the FSP patterns (Figure 10 D: $p<0.001$ respectively, one-way ANOVA with Tukey post hoc analysis, n=11 animals). In addition, P80 animals were significantly lower than

P90 animal relative delta increases in both the EC and FSP patterns (Figure 10 C, D: $p<0.001$, one-way ANOVA with Tukey post hoc analysis, n=7 animals, n=9 animals). The data suggest that seizure severity changes during post-juvenile brain development in the late stages of status epilepticus.

DISCUSSION

The pilocarpine model was used to characterize SE progression during post-juvenile developmental ages (P30-80) and adulthood (P90) in rats. Electrographic analysis examined post-juvenile age dependent modulation of SE characteristics progressing through early and late stage SE patterns as previously described (see Materials and Methods) [10, 11]. Significant differences were observed in multiple SE characteristics as animals progressed from pubescence (P30) to adulthood (P90). These changes include spike frequency, time in seizure pattern, and severity observations in post-juvenile (P30-90) animals. Significant correlations existed between SE induction and Racine scores. The data demonstrate that seizure patterns and severity modulate during post-juvenile brain development in the pilocarpine-SE model. These findings show that accurate estimation of brain development is important to reduce inter-study variability in animal models of status epilepticus.

Typically, animal body weight has been used to determine developmental age. However; weight change does not accurately approximate brain maturation from infancy to adulthood in animals [46, 47, 49, 55]. There is a lack of steady synchronous growth between the brain and body weight during the maturation process [46, 47, 49, 55]. The rat brain develops in stages of rapid growth until P30-35 with many neurotransmitter systems becoming functional [19, 38, 55, 80]. After this initial period of rapid growth stages, it has been shown that the rat brain continues to change through postnatal ages P30-P80 [38, 56, 65]. Specifically, there is a steady culling of neuron number, loss of synaptic density, and finalizing of neurotransmitter circuitry lasting until adulthood [56, 66]. Furthermore, between the P30-40 age range animals experience a rapid growth

stage, hormonal influences on GABAergic tone, and continued development of neurotransmitter systems [36, 38, 62-64]. The lack of synchrony was evident in this study when analyzing results and weight of the animals studied. Postnatal-30-day animal body weight was the lowest out of all animals studied at 117.05±2.60 grams. From this age until P50 (279.43±6.13 grams) animals grew approximately 160 grams over a twenty day period. During this period many results were similar to P90 animals for both P30-40 animal ages yet animals experienced >100% change in bodyweight. Furthermore, these animal ages were an approximate 64-70% change in bodyweight when compared to P90 animals. However; during the next 40 day growth period (P50-90) animals gained roughly 90-100 grams of bodyweight. This was less than half body weight of a P50 animal and approximately 20-25% change in bodyweight when compared to P90 animals. During these ages many parameters were significantly different than P90 animals which suggest that body weight should not identify development.

Similar to animals, humans continue to develop and finalize circuitry development during adolescence [76]. The size and weight of the human brain remains stable after growth spurts during 0-6 years of age until the age of 20, yet many internal changes occur during this maturation process [76]. The human adolescent brain increases myelination and white matter volume in a linear pattern [76]. The basal ganglia, which are involved in cognitive processing, have been shown to lose volume in the caudate nucleus during adolescence [76]. Neural connections are active during adolescent years along the left arcuate fasiculus that connects speech processing areas Wernicke's (reception) and Broca's (production) [76]. Furthermore, the density of pyramidal cell axons with $GABA_A\alpha 2$ subunit, have been shown to decline in the monkey prefrontal

cortex from infancy through adolescence [76]. The $GABA_A\alpha2$ subunit has a higher affinity for GABA, higher efficacy, and fast activation times which may increase GABAergic tone [76]. It has also been shown that during late developmental ages the incidence of SE decreases in adolescence while the occurrence of schizophrenia and bipolar disorder increase [77]. In addition, humans enter and complete reasoning stages during adolescence leading to adulthood [46, 47, 55]. Therefore, postnatal age is a better estimator for developmental stages than animal bodyweight.

Previous studies have established that onset of SE is age dependent in both the human condition and the rat pilocarpine model [19, 21]. What have not been characterized are the possible predictors or causes of this relationship. The previous paper determined that post-juvenile age has a positive correlation with behavioral seizure severity within the acute discrete seizure phase. The data for this study demonstrated an age dependence in P50-80 animals and P90 animals with onset probability ($p<0.05$, $n=105$ animals, Fishers exact). This probability correlated directly with Racine behavioral seizure severity scores in the acute discrete seizure phase. This suggests behavioral severity in the acute discrete seizure phase may be a predictor for SE during late developmental stages.

There have been separate studies Treiman et al and Handforth et al that have identified patterns in SE [9-11]. The present investigation chose the 1995 characterization due to the fact it was done primarily with animals. The 1990 characterization was based mostly on human electroencephalograms. Furthermore, the observation that W/W and F/S periods had unique, revolving characteristics led to the full characterization of each period in these patterns through all ages. Spike frequencies for

certain patterns averaged close to values established in Handforth et al [11, 12]. In addition, the late continuous pattern for this study had significantly lower delta values when compared to earlier pattern values. This differentiated the EC pattern from the FSP pattern. Therefore, the detailed breakdown from Handforth et al [11, 12] was a better vehicle to describe post-juvenile SE modulation.

The 60 minute time band was chosen due to its relevance to previous investigations quantifying this time epoch for biochemical and pharmacological changes [21-25]. Although SE was fully characterized, animals were not observed for the development of epileptogenesis, which has been shown to be variable in relation to the frequency of seizures and their severity. Future studies could investigate SE severity and pathology with the magnitude of seizures, existing correlations, and development of epileptogenesis after the initial insult of SE.

This study completed a detailed characterization of the SE phase provided by the pilocarpine-SE model of status epilepticus. The data demonstrated significant modulation during late stage brain development concerning time in pattern, spike frequency, and seizure severity. Further studies will focus on SE induced loss of drug efficacy, epileptogenesis, and whether the therapeutic window of SE is age dependent.

Figure 6: Typical Seizure Progression During Status Epilepticus

Postnatal-90-day animals progress through specific seizure patterns as SE duration increases. (6A); a discrete seizure with displaying a gradual increased amplitude developing with overt ictal activity followed by an abrupt seizure termination (cyclone shape,). The discrete seizure phase typically begins 17.5 ± 1.5 minutes after pilocarpine injection. Approximately 11.50±1.5 minutes after the first ictal activity discrete seizures merge without a clearly defined terminus, W/W, (6B) defining the onset of SE. This pattern lasted 5.50±0.39% of the observed time in SE for the average animal. The end of W/W was indicated by a noticeable progression into cycles of fast and slow spiking (6C). The F/S pattern generally averaged 24.10±5.54% of the observed time in SE. The next pattern, early continuous (6D) was defined as high amplitude fast spiking with occasional electro-decrimental pauses (flat periods) absent slow spiking revolutions. This pattern lasted 21.22±5.05% for the average animal during the observed time in SE. Fast spiking w/pauses (6E) was characterized with periods of consistent electro-decrimental pausing separated with periods of fast spiking. This pattern persisted for the greatest amount of observed time in SE for the average animal (42.41±7.21%). This was followed by a diminution of amplitude and intensity: the late continuous pattern (6F) lasting 6.85±2.58% of observed time in SE.

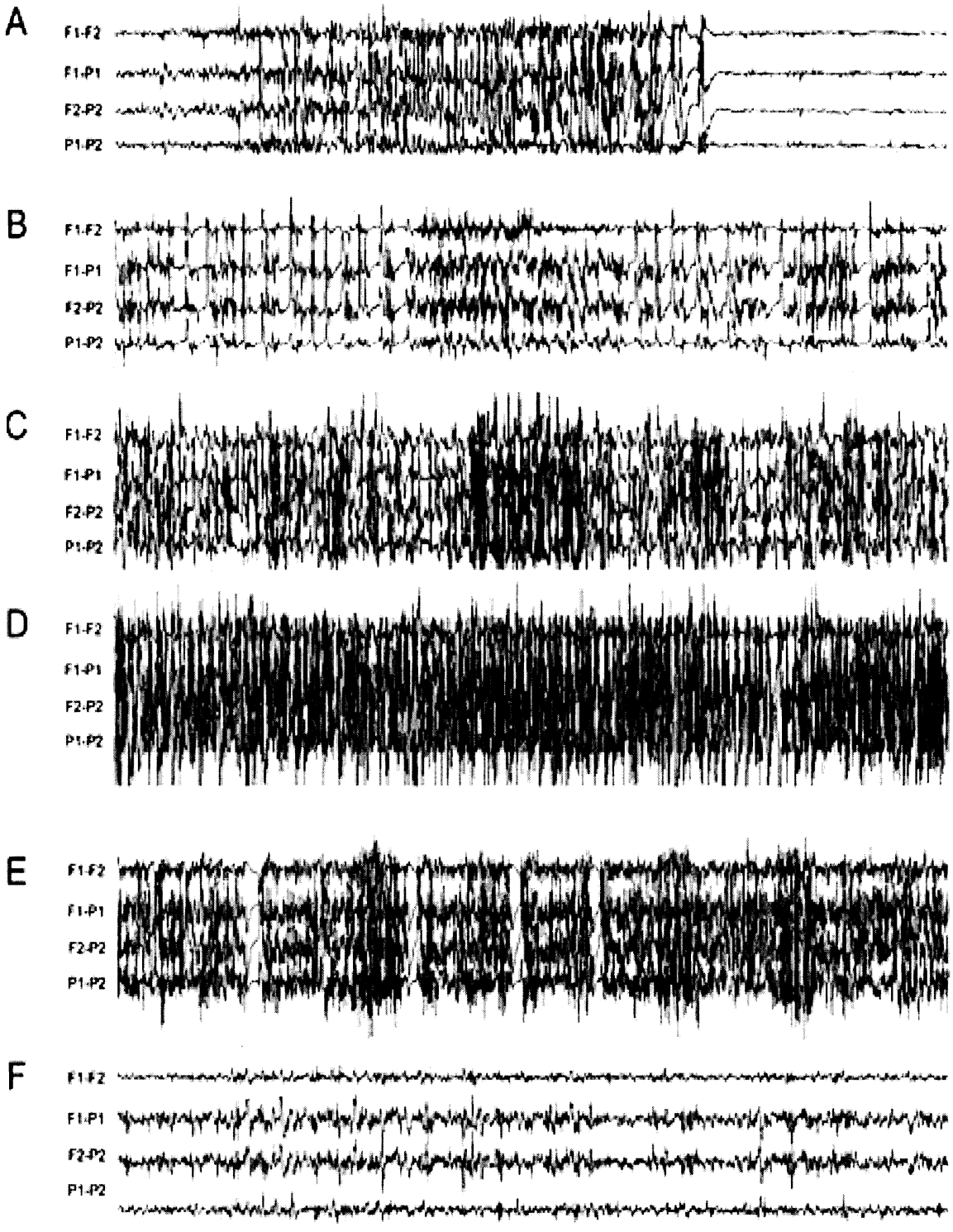


Figure 6: Seizure patterns during the progression of status epilepticus.

Figure 7: Developmental Age Modulates Specific Seizure Characteristics During Status Epilepticus.

Specific characteristics during the acute discrete seizure phase during ages corresponding to post-juvenile brain development may predict SE onset probability: a percent of animals that develop SE displayed a noticeable reduction at P50 (56.0%) and P60 ages (72.0%). This is contrasted by a higher percent for P30-40 and P90 animals obtaining SE (90.0% P30, 89.0% P40, 96.0% P90). A significant correlation exists with Racine scores and onset probability ($r=0.85$, $p<0.05$, Spearman's rank) suggesting severity may be linked to SE onset. Previous epidemiology data have shown that a higher incidence of SE in children and the elderly exists when compared to P50-60 ages [5, 7]. (Statistical significance when compared against P90 animals is denoted by *. *=$p<0.05$, **=$p<0.01$, ***=$p<0.001$ when compared to P90 animals.)

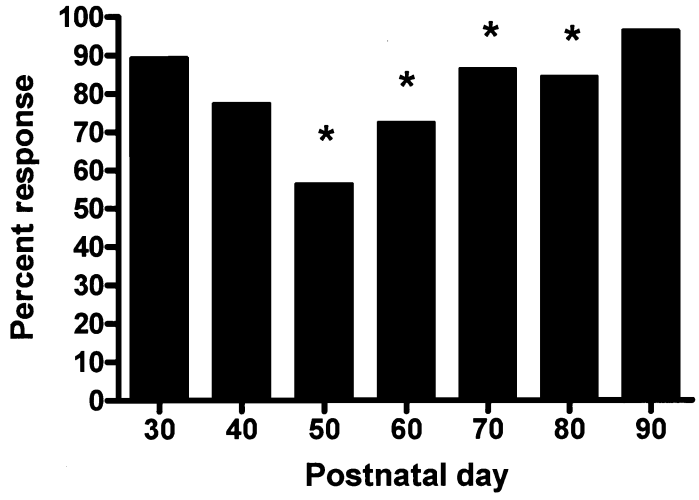
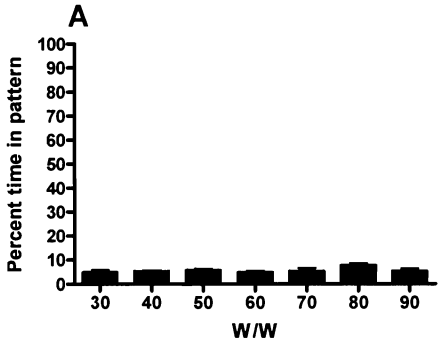
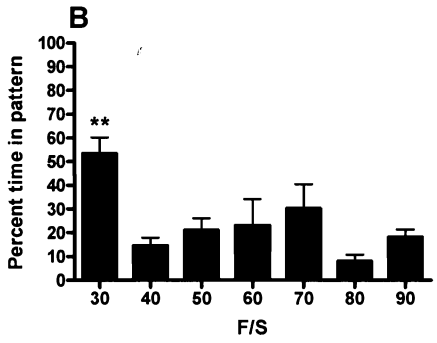


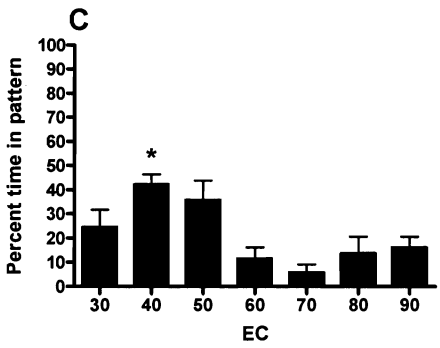
Figure 7. Onset probability for each postnatal age group. Columns express final calculated percent of onset probability.

Figure 8: Developmental Age Modulated Progress into Late Seizure Patterns During Status Epilepticus Progression.

Animals were characterized for time in the early (W/W, F/S) and late (EC, FSP,) patterns of SE previously described [10, 11] in all postnatal ages. The W/W and F/S were broken down into single component patterns and number of cycles. For P90 animals there were two wax and two wane cycles per pattern which combined for 5.31±1.63% of observed SE time. Although P90 animals did average two slow periods per F/S pattern, the majority of this pattern was spent in fast spiking (14.10±3.24%, 2 periods, Figure 8 B). The last two patterns, early continuous and fast spiking w/pauses, constituted the majority of observed time in SE. Postnatal-90-day animals spent 16.00±4.52% in EC (8 C) and 57.34±5.09% of time in fast spiking w/pauses (8 D). The data demonstrates that younger animals spent more time in EC while P50-80 animals spent more time in FSP. (* was used to denote statistical significance. *= $p<0.05$, **= $p<0.01$ when compared to P90 animals)


A
Percent time in pattern
100
90
80
70
60
50
40
30
20
10
0
30 40 50 60 70 80 90
W/W


B
Percent time in pattern
**
100
90
80
70
60
50
40
30
20
10
0
30 40 50 60 70 80 90
F/S


C
Percent time in pattern
*
100
90
80
70
60
50
40
30
20
10
0
30 40 50 60 70 80 90
EC

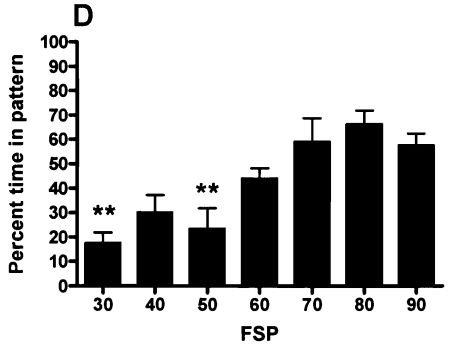

D
Percent time in pattern
**
**
100
90
80
70
60
50
40
30
20
10
0
30 40 50 60 70 80 90
FSP

Figure 9: Spike Frequency Modulates for Seizure Pattern and Postnatal Age

Spike frequency was quantified for each pattern using 6-12 second epochs of time. Spike frequency showed a noticeable increase in the early stages of SE. Spike frequency increased 1.0-2.0 Hz from W/W pattern to the F/S spiking pattern in all animal ranges studied (W/W: 5.91±0.24 Hz ; F/S: 7.20±0.25 Hz). Spike frequency increased 1-2 Hz again and remained at a high level through EC and FSP patterns (EC: 9.04±0.60 Hz; FSP: 8.32±0.48 Hz). Postnatal-30 and 40-day animals were differentiated from P90 animals by significant lower recordings in spike frequency values (P30: $p<0.001$ 6.26 ±0.50 Hz; FSP, P40: $p<0.01$, 6.80±0.65 FSP, one-way ANOVA with Tukey post hoc analysis). Spike frequency was also different than P90 animals in the fast and slow patterns (P40, fast: $p<0.001$, 8.07±0.56 Hz; P40 slow $p<0.05$, 4.52±0.15 Hz, one-way ANOVA with Tukey post hoc analysis). On average these age ranges were lower than P50-P90 age ranges. (* was used to denote statistical significance when compared against P90 animals. *= $p<0.05$, **= $p<0.01$, ***= $p<0.001$ when compared to p90 animals)

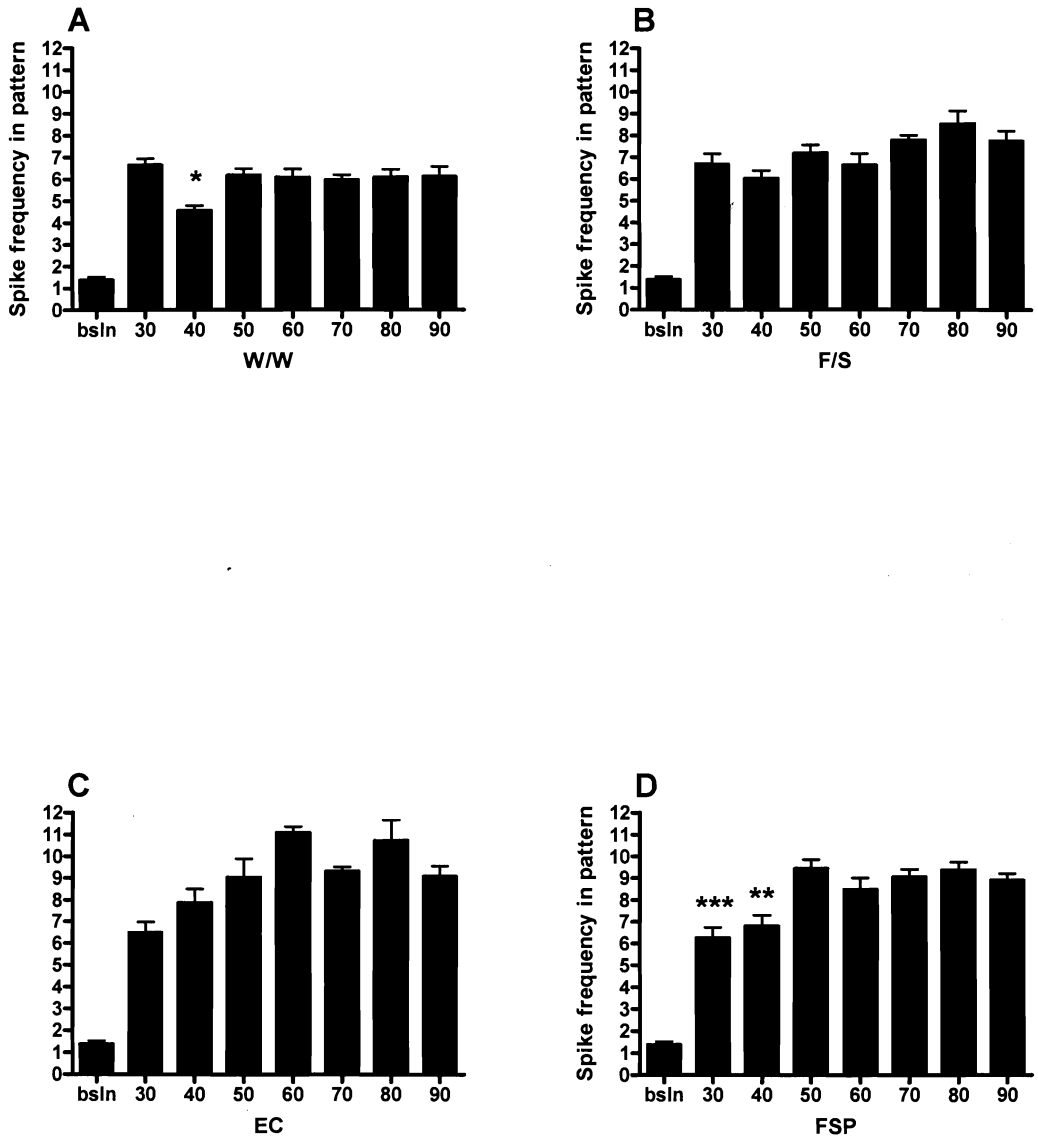


Figure 9: Spike frequency in seizure pattern for each postnatal age. Calculated means are expressed as columns and standard error of the mean +1 are expressed as error bars.

Figure 10: P50 Animals Show Age Dependent Modulation of Seizure Severity in qEEG Analysis

The delta frequency range (1.0-3.99 Hz) has been shown to measure brain pathology and severity during seizures [70, 71]. Relative percentage (% of total power) values for this frequency range suggest that severity is dependent on age, not pattern. Delta qEEG analysis displayed lower values in P50-80 day animals when compared to P90 and P30-40 animals for different seizure patterns. During each pattern, there was a noticeable dip for P50 ages and significant differences with P90 animal values (FSP and EC; $p<0.001$ P50, P80: $p<0.01$, P60, one-way ANOVA with Tukey post hoc analysis). Postnatal-30 and 40-day animals had significantly higher relative delta values when compared to P50-80 animals which differentiated this age group ($p<0.001$, one-way ANOVA with Tukey post hoc analysis). * was used to denote statistical significance when compared against P90 animals. *= $p<0.05$, ***= $p<0.001$ when compared to P90 animals)

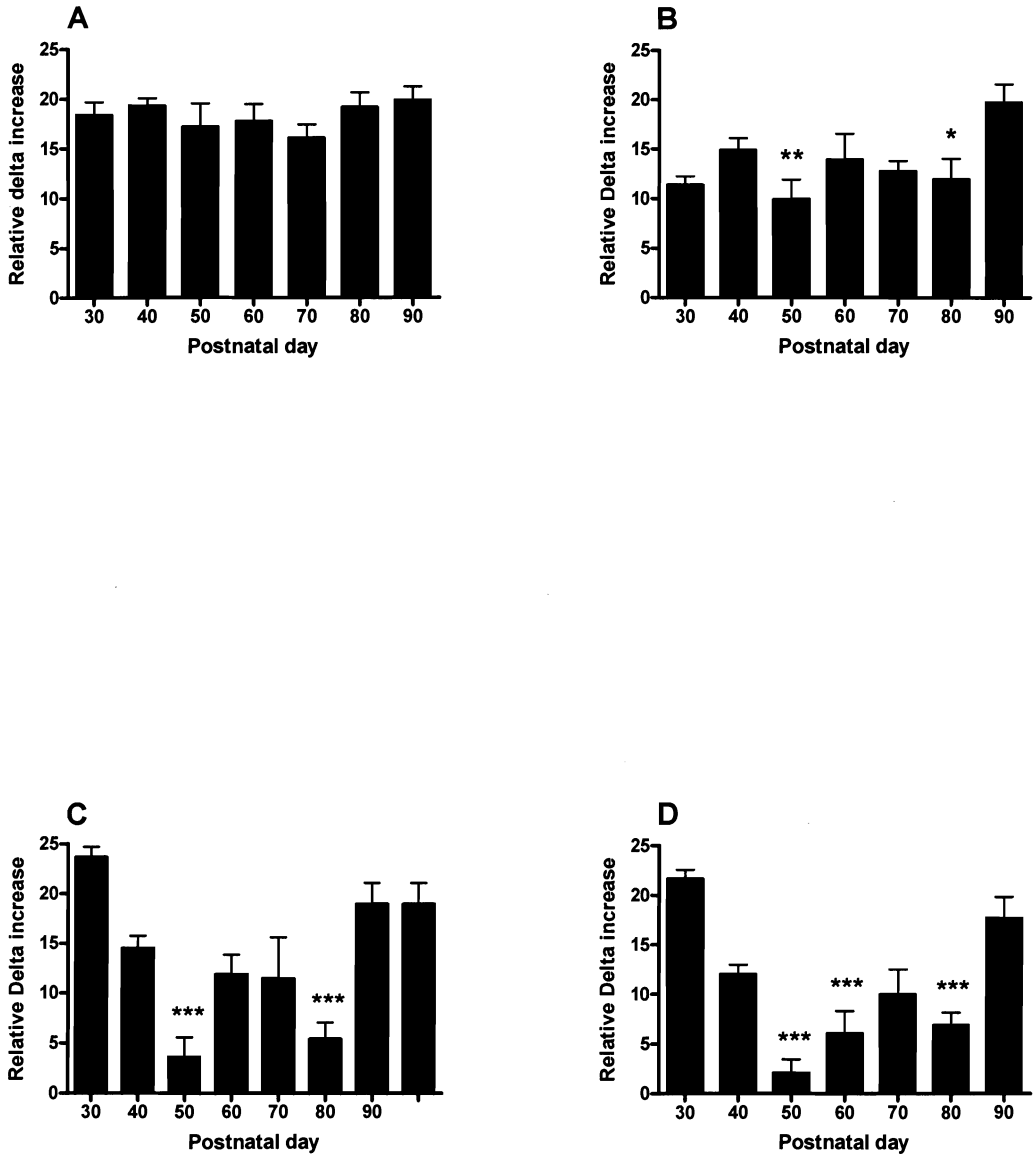


Figure 10: Relative Delta percentage for seizure pattern during SE. Calculated means are expressed as columns and standard error of the mean +1 are expressed as error bars.

STATUS EPILEPTICUS-INDUCED LOSS OF DIAZEPAM EFFICACY IS AGE DEPENDENT IN THE RAT PILOCARPINE-SE MODEL

ABSTRACT

Purpose: This study was completed to characterize drug efficacy in the initial therapeutic window of SE during post-juvenile ages.
Methods: SE was induced by pilocarpine (300 mg/kg) in Wistar rats aged P30, P50, and P90. Diazepam was administered at 10, 15, 20 minutes after the onset of the first discrete seizure (P1DS). Electrographic activities were monitored by video EEG (BMSI 5000, Nicolet) and spike frequency obtained using Insight software.
Results: Postnatal-90-day animals showed no significant spike frequency reduction at 10 minute P1DS (paired Students t test, $p>0.05$) and displayed significant spike frequency increases at 15 and 20 minute P1DS (paired Student's t-test, $p<0.05$). P50 animals displayed a significant response to *diazepam* at 10 minute P1DS (paired Student's t-test, $p<0.05$) and a small reduction in frequency at 15 and 20 P1DS. Pubescent animals had a similar response as P50 animals at 10 minute P1DS.
Conclusion: These results suggest that there is an age dependent change of drug efficacy during SE.

INTRODUCTION

Status Epilepticus (SE) is a neurological emergency of continuous or intermittent seizure activity that has been described and identified since 600-700 B.C. [4, 5, 13]. Approximately 150,000 cases of SE are reported each year with many cases resulting in mortality [4, 5, 13] . The highest incidence of SE occurs in children less than three years old and may precede the development of epilepsy in later life [1, 4, 5]. Many of the anti-epileptic drugs in use fail to terminate seizure activity, and of the drugs that are effective, most lose their therapeutic efficacy as SE progresses [5, 12-14, 68, 81]. Thus, an examination of the age effects of SE using a common anti-epileptic drug is warranted.

Previous studies have suggested SE characteristics are age-dependent in the pilocarpine model of limbic epilepsy [19, 21]. What these studies failed to investigate is SE-induced loss of drug efficacy using a common benzodiazepine during post-juvenile ages: P30 (pubescent), P50 (adolescent), and P90 (adult). These ages have been shown to correspond to post-juvenile and adult humans, an age group understudied in relation to SE characteristics [21, 62, 73].

To combat SE, many anti-epileptic drugs are used in combination or singly to treat this life threatening condition. Specifically, the benzodiazepines (diazepam, clonazepam, clorazepate, lorazepam, midazolam) are a frontline treatment with *diazepam* a drug of choice [13]. Through its rapid crossing of the blood brain barrier and allosteric modulation of the GABA receptor *diazepam* has been shown to stop SE. This compound should be administered immediately due to a correlation with SE duration and loss of drug efficacy [14, 68]; however, few studies have characterized SE induced loss of

diazepam efficacy as age dependent. Therefore, a detailed study concerning the loss of drug efficacy and it relation to age was undertaken.

MATERIALS AND METHODS

Induction of Status Epilepticus

Male Wistar rats were purchased from Harlan Laboratories (Indianapolis, IN, USA), housed with food and water *ad libitum*, with lighting on a 12 hour on/off cycle. The software used for analysis (Insight, Insight II) was purchased from Persyst Corporation (Prescott, AZ). All animal use procedures were in strict accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals and were approved by the Virginia Commonwealth University Institutional Animal Care and Use committee.

Five days prior to the experiment, four surface electrodes were surgically implanted into the skull of Male Wistar rats under ketamine (125 mg/kg i.p.)-xylazine (1 mg/kg) anesthesia. Two frontal electrodes were implanted over the frontal cortex (3.5 mm anterior to Bregma, ±2.5 mm left or right of sagittal suture). Two posterior leads were implanted over parietal cortex and hippocampus (2.0 mm posterior to bregma, ±2.5 mm left or right of sagittal suture). Electrodes were secured with dental acrylic and animals were allowed to recover for at least five days. Four separate bipolar channels were recorded with a montage of F1-F2, F1-P1, F2-P2, and P1-P2. On the day of the experiment animals were connected via headset to EEG machines (BMSI 5000, Nicolet), and baseline recordings were obtained. To induce seizure activity, pilocarpine (300 mg/kg i.p. Sigma, St. Louis, Mo), a muscarinic agonist, was injected. Methylscopolamine, a muscarinic antagonist that does not cross the blood brain barrier, was administered i.p. (1 mg/kg) 30 minutes prior to pilocarpine injection to reduce peripheral affects [21, 25, 57]. All materials and *diazepam* were reagent grade and

purchased from Sigma Chemical Company (St. Louis, MO, USA) unless otherwise stated. Animals were monitored for behavior and electrographic activity throughout the procedure using video and EEG recordings.

Drug Treatment

Diazepam was administered i.p. (4 mg/kg) 10, 15, and 20 minutes post onset of the initial discrete seizure (P1DS). Twenty-five spike frequency recordings were completed in twelve second epochs beginning five minutes prior to drug injection. After drug injection, 15 minutes were allowed for absorption and distribution of the drug [82], and then frequency recordings were repeated. Efficacy was determined measuring the posttreatment spike frequency values then comparing them to pretreatment values.

Electrographic and Statistical Analysis:

For EEG analysis, the notch filter was set at 60 Hz, the sampling rate set at 420 /sec per channel, the low pass frequency set at 100 Hz, and high pass set at 2 Hz. Preinjection and postinjection comparisons were made with paired Student's t-test using Graph Pad–Prism 4.0 for windows (Graph Pad software, San Diego, CA USA, www.graphpad.com). Data are expressed as mean ± standard error of the mean.

RESULTS

P90 Animal Diazepam Efficacy

Seventy-two animals in three postnatal age groups (30, 50, 90) were used to characterize diazepam efficacy in the initial stages of status epilepticus. Animals typically began the acute discrete seizure phase 17.0±1.5 minutes after pilocarpine injection. From the initial onset of ictal activity the acute discrete seizure phase averaged 11.5±1.5 minute's time in phase, ranging between 9.50-14 minutes, and averaged 3 discrete seizures per phase in all age groups studied. Thus, the ten-minute treatment mark was selected to ensure that diazepam was injected near onset of SE by accounting for the discrete seizure period. Using this as a standard injection point P50 and P90 animal posttreatment spike frequencies were measured in the EC seizure pattern (~9.00 Hz); P30 posttreatment measurements were completed in the fast/slow spiking pattern (~8.00 Hz).

Seven of the eight P90 animals displayed reductions in spike frequency posttreatment for the ten minute treatment mark (n=8 animals). The pretreatment recorded spike frequency for P90 animals was 4.39±0.75 Hz and the posttreatment spike frequency was 3.38±1.01 Hz. This resulted in a 23.0% frequency reduction in posttreatment values which was not significantly lower than pretreatment values (Figure 11A). However; this represented an approximate 5.50-6.00 Hz reduction for the EC seizure pattern.

To ensure that efficacy was measured during the duration of SE, the 15 minute treatment mark was chosen to approximate five minutes of elapsed SE. Out of the eight animals that progressed to SE, one P90 animal displayed posttreatment spike frequency

reductions. The pretreatment spike frequency was 5.27±0.71 Hz and posttreatment spike frequency was 6.67±0.83 Hz for P90 animals. This was a significantly higher spike frequency posttreatment value when compared to pretreatment values in P90 animals (25% increase; $p<0.05$, paired Student's t-test, Figure 11A). Even though posttreatment values did increase in spike frequency, there was average reduction of two Hz in spike frequency for the EC seizure pattern in P90 animals.

To observe the loss of therapeutic efficacy as SE progresses, the 20 minute post first discrete seizure treatment mark was selected. Two P90 animals showed spike frequency reductions at the twenty minute treatment mark out of eight P90 animals that progressed to SE. The pretreatment average spike frequency was 6.87±0.40 Hz and posttreatment average spike frequency 7.82±0.71 Hz for P90 animals (Figure 11A). This resulted in significantly higher posttreatment spike frequency average in P90 animals (14% increase, $p<0.05$, paired Student's t-test). This was only a 1 Hz reduction in the average EC spike frequency. The data suggest that diazepam efficacy is lost very early in the progression of SE in P90 animals.

P50 Animal Diazepam Efficacy

Seven out of eight P50 animals observed progressing into SE showed reductions in spike frequency posttreatment. The pretreatment spike frequency average was 4.98±0.87 Hz and the posttreatment spike frequency was 1.80±1.24 Hz, for P50 animals (Figure 11B, n=8 animals). This resulted in a significantly lower percentage in posttreatment values as compared to pretreatment values (64%; $p<0.05$, paired Student's t-test). Furthermore, diazepam injection resulted in an approximate 7 Hz reduction for the average spike frequency for the EC seizure pattern in P50 animals.

Five P50 animals had reduced spike frequencies in posttreatment recordings for the 15-minute treatment mark (Figure 11B, n=8 animals). The pretreatment average spike frequency value for P50 animals was 8.60±0.46 Hz. This was similar to the posttreatment spike frequency average of 8.43±0.28 Hz for the 15 minute treatment mark and this resulted in a statistically insignificant 2.0% drop in frequency posttreatment for P50 animals (Figure 1 B). Similar to P90 animals, posttreatment spike frequency measurements were close to average spike frequencies for the EC seizure pattern in P50 animals.

Four P50 animals had spike frequency reductions out of the eight animals studied that progressed to SE. Postnatal-50-day animals had a slight frequency reduction of .01% in posttreatment values which was not significantly different than pretreatment values (Pre; 8.39±0.44 Hz: Post; 8.32±0.27 Hz, Figure 11B). This was similar to the 15-minute treatment mark. The data suggest diazepam does work in P50 animals at the onset of SE.

Pubescent Animal Diazepam Efficacy

All P30 animals displayed a reduction in posttreatment spike frequencies and had the greatest efficacy for diazepam out of all animals studied (Figure 11C). Pretreatment spike frequency averaged 5.72±0.82 Hz and posttreatment values were reduced to a baseline average of 0.57±0.10 Hz (Figure 11C, n=8 animals). This resulted in a significantly lower posttreatment spike frequency (90.0%) and a drop of approximately 8.00 Hz in the fast/slow pattern ($p<0.01$, paired Student's t-test, n=8 animals).

Pubescent animals had the best response to diazepam at the 15 treatment mark out of all animals studied with six animals responding to diazepam. Postnatal-30-day animals averaged 7.62±0.76 Hz for the pretreatment value and 6.53±1.7 Hz posttreatment

spike frequency average. Although these observations were similar ($p>0.05$, paired Students t test, n=8 animals), it still resulted in a 14% drop in spike frequency for the 15 minute treatment mark (Figure 1 C).

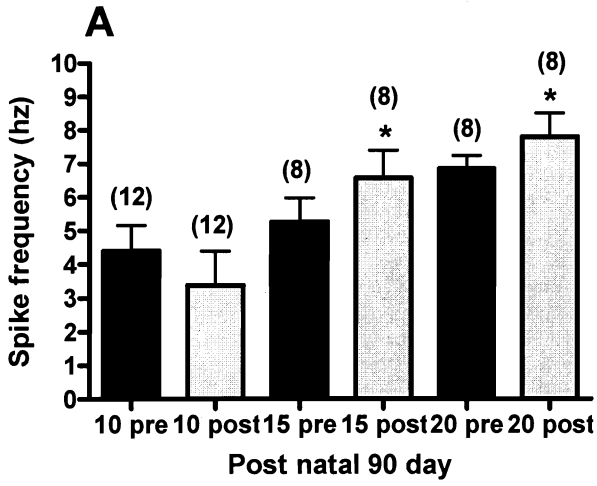
Similar to the 15 treatment mark, P30 animals displayed reduction in spike frequency at the 20 minute treatment mark. The pretreatment average spike frequency was 6.57%±1.06 Hz and posttreatment spike frequency average of 5.91±1.37 Hz in pubescent animals (Figure 11C). These measurements were similar to each other, yet there was a 10% reduction in posttreatment spike frequency values and an approximate three Hz reduction in the average spike frequency for the fast/slow pattern. The data suggest that P30 animals displayed the greatest response to diazepam at the onset of SE. The efficacy of this drug continued as SE progressed. However, the efficacy was not as great when compared to the initial stages of SE. The data further suggest that during post-juvenile ages, diazepam efficacy is altered during the initial stages of SE.

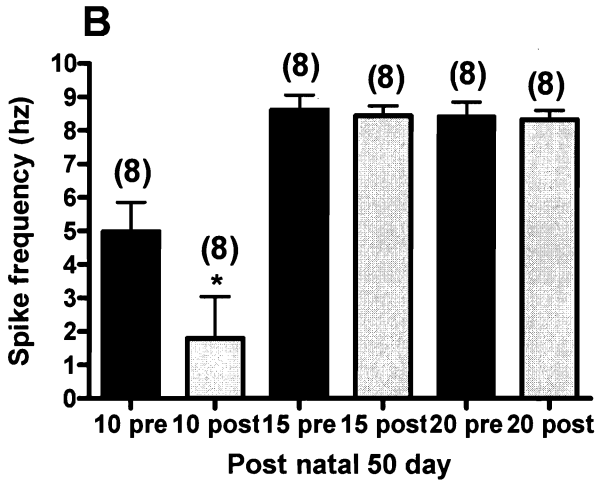
DISCUSSION

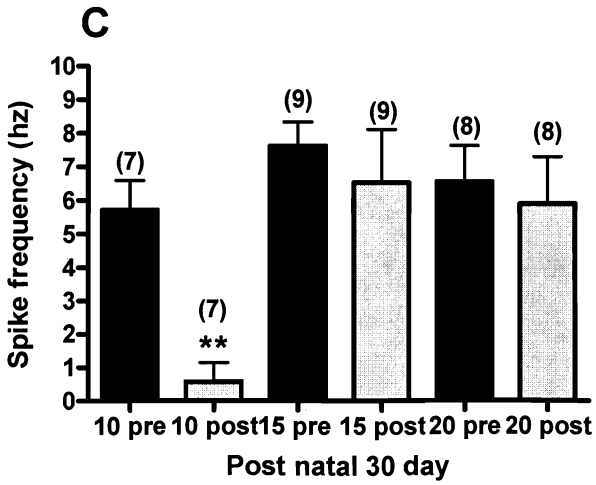
In summary, pubescent and P50 animals had a significant response to diazepam at 10 min P1DS. Additionally, at 15 minute P1DS both age groups showed, although not significant, slight responses to the compound. Even though P90 animals displayed a similar response to diazepam at 10 minute P1DS, the responses at 15 and 20 P1DS showed significant increases in spike frequency. The data suggest an age related response to diazepam during the therapeutic window of SE. Younger, immature animals had a significant response to *diazepam* early in SE and a diminished response as SE progressed. Postnatal-90-day animals had a slight response to *diazepam* early in the window and no response as SE progresses.

This study used the pilocarpine model of limbic epilepsy to characterize efficacy in the therapeutic window of SE. The data demonstrated that younger animals had a better response to the drug and significantly modulated efficacy in comparison to P90 animals. Future studies will encase all postnatal ages and may include other compounds to investigate this observation.

Figure 11: Status epilepticus was induced by pilocarpine, and the typical progression into seizures was observed. From the first ictal activity in the acute discrete seizure phase animals were injected with diazepam at 10, 15, and 20 minutes. In order to get a pretreatment average, frequency was measured for five minutes prior to that injection mark. After treatment, 15 minutes were allowed for drug distribution, and then another five minutes was measured. Efficacy was determined by analyzing pre and posttreatment frequencies. Postnatal-30 and 40-day animals had the greatest response to diazepam. The ten-minute mark for P30-40 and P50 animals was significantly lower in posttreatment values (P30: $p<0.01$; P50: $p<0.05$, paired Students t test). At the fifteen-minute mark, P30-40 and P90 animals did respond to diazepam. Postnatal-90-day animals had a significant increase in frequency posttreatment ($p<0.05$, paired Students t test). This significant increase in frequency for P90 animals was observed again at the 20 minute mark ($p<0.05$, paired Students t test). Postnatal-30 and 40-day and P50 animals did have a slight reduction in frequency at the twenty minute treatment mark. **=$p<0.01$, *=$p<0.05$ when compared to P90 animals.


A
Spike frequency (hz)
10
9
8
7
6
5
4
3
2
1
0
(12)
(12)
(8)
(8)
*
(8)
(8)
*
10 pre
10 post
15 pre
15 post
20 pre
20 post
Post natal 90 day


B
Spike frequency (hz)
10
9
8
7
6
5
4
3
2
1
0
(8)
(8)
*
(8)
(8)
(8)
(8)
10 pre
10 post
15 pre
15 post
20 pre
20 post
Post natal 50 day


C
Spike frequency (hz)
10
9
8
7
6
5
4
3
2
1
0
(7)
(7)
**
(9)
(9)
(8)
(8)
10 pre
10 post
15 pre
15 post
20 pre
20 post
Post natal 30 day

DISCUSSION

The rat pilocarpine-SE model was utilized to detail seizure characteristics of status epilepticus for 60 minutes, a standard time frame for observing status epliepticus [21-25]. This model was further utilized to examine diazepam efficacy in the therapeutic window of SE. The first study showed that seizure characteristics in the acute discrete seizure phase changed at certain developmental stages. Racine severity scores, seizure termination profiles, composite spectral analysis, and spike frequency were significantly modulated as animals continued to develop in post-juvenile ages (P30-80). The second paper examined seizure characteristics in the chronic status epilepticus phase and established correlations with the acute discrete seizure phase. Time and frequency characteristics established differences in seizure pattern between early and late SE. Spectral analysis defined severity as a function of age, not seizure pattern. Correlations were observed between SE onset and behavioral severity. The third study displayed SE-induced loss of diazepam efficacy may be related to post-juvenile developmental stage and seizure duration. Posttreatment spike frequencies for post-juvenile and P50 animals were significantly less at certain time points when compared to P90 animals. The data demonstrated that seizure characteristics of SE modulate during post-juvenile ages and that drug efficacy loss could be related to age. These findings suggest that the variability

in results reported in this model is age dependent and establishes postnatal age as an identifier of post-juvenile developmental stage.

The incidence of SE has been shown to be an event experienced mostly in juveniles and the elderly [7, 30, 31]. Studies have reported that adolescents and adults have a much lower occurrence of this disease [7, 30, 31]. To investigate this observation many animal models are utilized to replicate the human condition [10-12, 19, 20, 45, 70, 83-91]. The accepted model of choice has been the pilocarpine-SE model. Many of the seizure characteristics can mimic the predictable seizure patterns observed in the human condition [9]. This model has shown age dependence in juvenile and adult animals to the seizure inducing agent pilocarpine [19, 21, 25]. The reason for this may be found in the development of the cholinergic system and the cholinergic activity of pilocarpine [19, 58, 92]. Cavalheiro et al demonstrated that rats injected with pilocarpine at 7-12 postnatal days were not as susceptible to seizures as P15 to P21 rats [19]. This was suggested to be due to the final development of the cholinergic system in rats during postnatal ages P15-P21 [19]. Another study by Hamani et al , found that after creating bilateral anterior thalamic lesions and administering pilocarpine, animals did not produce seizures [58]. This may be due to the loss of thalamic stimulus and the cholinergic projection from the non-striatal telencephalon [93]. The thalamus has been shown to be the originator of EEG activity [18]. In addition, structures involved in cholinergic activity, such as the thalamus, amygdala, hippocampus, and subsantia nigra, may go through post-juvenile development that may decrease the susceptibility to pilocarpine. These structures have been shown to have widespread damage from pilocarpine induced seizures [92]. Although age dependence to pilocarpine in this model has been shown;

separate studies have reported a large variability in results for this model [19, 21, 57, 58]. This may be a direct consequence of using animal weight as a classifier of development in animal post-juvenile ages. Across many laboratories, there is high degree of variance between the definition of adolescence or adulthood using body weight of the animal.

Human and animal brains develop in a manner that does not parallel body growth rate [46-49, 55]. The human brain develops in growth spurts that are preceded by increases in cerebral blood flow [46-48, 55]. Many of the growth stages occur before the age of six years; from this age brain weight remains relatively stable until the age of 20 years [46-48]. During this aging process, human reasoning develops in stages of cognitive development identified by the Swiss psychologist Jean Piaget [46-48, 55]. Furthermore, adolescence is a time of many internal changes unrelated to brain size and growth [76, 77]. In adolescence, the brain increases myelination, volume of white matter, and many neural connections become active [76, 77]. Many of the reported incidents of schizophrenia and bipolar disorder occur during adolescence [77]. The observation of final brain development after juvenile ages is also observed in rats [38, 40, 49, 56, 65]. The rat brain does finalize circuitry during this time and changes in GABA receptor subunits are observed [38, 40, 49, 56, 65]. Thus, a lack of synchrony exists between body weight and the developing brain.

A common observation throughout this study was that results for P30-40 animals confounded expectations. This age group had many characteristics that were either similar or dissimilar to P90 animal results. Racine behavioral severity scores were similar to P90 animal scores. Increases in relative delta percentages over baseline for discrete seizures in this age group averaged 17.08%, which was similar to P90 animal

percentages. EEG slowing profiles for the delta component were similar to P90 animals. In addition, when this age group was included, a correlation existed with SE onset probability. Spike frequencies for P30-40 animals however, were significantly lower than P90s. When P30-40 animals were included in certain correlation tests with all animals, poor correlations were observed. When omitted, a positive correlation could then be established. In addition, postnatal-30-day animals had the greatest efficacy response to diazepam in this study. Conversely, this age range had a large difference in body weight when compared to P50 and P90 animals. Postnatal-30 and 40-day animals averaged 138.98±2.59 grams, which was 64% lower than P90 animal average weight of 383.40±2.82 grams. These findings suggest that this is a separate age range.

Postnatal-50 through 80 day animals in this study provided consistently lower values in seizure characteristics when compared to P90 animals. Racine scores for this age range remained less severe when compared to P90 animals. Spike frequency recording for this age range started significantly lower than P90 measurements then progressively increased to P90 levels. Composite spectral analysis for P50 animals displayed less EEG slowing in the delta frequency range when compared to P90 animals. Relative delta percentages for P50 discrete seizures were significantly lower than P90 animal percentages. In the chronic SE seizure phase, severity was related to animal age in P50-80 animals as opposed to specific seizure pattern. Furthermore, when these ages were analyzed for correlations between each phase, positive correlations existed in most parameters tested. These correlations establish that seizure characteristics in this model change in relation to postnatal age. The average weight of a P50 animal was approximately 279.92±6.13 grams, which was a 25% difference (90-100 gms) when

compared to P90 animals. As animals aged from adolescence this percentage progressively decreased. While there was a large difference in SE seizure characteristics between P50 and P90 animals, specifically, P50 animals were the lowest value observed throughout this study in the multiple parameters examined. This was not seen in P30-40 animals. Postnatal-30 and 40-day animals had many characteristics that were the same as P90 animals; however this age group had a much larger difference in body weight when compared to P90 animals. An average P50 animal had major differences with P90 animals in seizure characteristics examined, yet was close in body weight to P90 animals. This observation held true for P60 animals as well. Postnatal-70 and 80-day animals were approximately 4-5% (P70-80:~360±5 grams vs P90: 383±2.85 grams) different in body weight when compared to P90 animals. Racine scores, relative delta percentages for seizure pattern, and spike frequency were different for these animal ages when compared to P90 animals. This suggests that body weight does not affect seizure characteristics and cannot explain the variability in reported results for the pilocarpine-SE model.

One problem exists with the treatment of SE in humans. During the initial seizure patterns of SE, anti-epileptic drugs lose their therapeutic value as the condition progresses [12, 68]. The underlying mechanisms for this are unknown. The data demonstrated that diazepam efficacy may be related to age of the patient and not a function of time. This finding may have gone unnoticed in previous studies due to the use of body weight to identify developmental stage.

This project was made up of three separate studies that examined the neurological emergency, status epilepticus. The first study examined the acute discrete seizure phase

and found that many of the seizure characteristics were related to postnatal age. The second study examined the chronic SE phase and determined that severity was related to age and not pattern. The third study showed that therapeutic efficacy of an anti-epileptic drug may be related to age. These findings support the hypothesis that seizure characteristics of SE are age dependent during post-juvenile ages. Furthermore, age should be used to classify developmental stage. Future studies may expand the examination of the initial therapeutic time frame of SE and seizure severity as they relate to developmental stage.

LITERATURE CITED

Literature Cited

1. Patel, M., *Mitochondrial Dysfunction and Oxidative Stress: Cause and Consequence of Epileptic Seizures.* Free Radical Biology & Medicine, 2004: p. 1951-1962.
2. Patel, M. and Q.Y. LI, *Age dependence of seizure-induced oxidative stress.* Neuroscience, 2003. **118**: p. 431-437.
3. Fountain, N.B., *Status epilepticus: risk factors and complications.* Epilepsia, 2000. **41 Suppl 2**: p. S23-30.
4. Holmes, G.L. and J.J. Riviello, Jr., *Midazolam and pentobarbital for Refractory status epilepticus.* Pediatr Neurol, 1999. **20**(4): p. 259-64.
5. Lowenstein, D.H., *Status Epilepticus: An Overview of the Clinical Problem.* Epilepsia, 1999. **40**(Suppl 1): p. S3-S8.
6. Lothman, E., *The biochemical basis and pathophysiology of status epilepticus.* Neurology, 1990. **40**(5 Suppl 2): p. 13-23.
7. DeLorenzo, R.J., et al., *A prospective, population-based epidemiologic study of status epilepticus in Richmond, Virginia.* Neurology, 1996. **46**(4): p. 1029-35.
8. Waterhouse, E.J., et al., *Synergistic effect of status epilepticus and ischemic brain injury on mortality.* Epilepsy Res, 1998. **29**(3): p. 175-83.
9. Treiman, D.M., N.Y. Walton, and C. Kendrick, *A progressive sequence of electroencephalographic changes during generalized convulsive status epilepticus.* Epilepsy Res, 1990. **5**(1): p. 49-60.
10. Handforth, A. and D.M. Treiman, *Functional mapping of the late stages of status epilepticus in the lithium-pilocarpine model in rat: a 14C-2-deoxyglucose study.* Neuroscience, 1995. **64**(4): p. 1075-89.
11. Handforth, A. and D.M. Treiman, *Functional mapping of the early stages of status epilepticus: a 14C-2-deoxyglucose study in the lithium-pilocarpine model in rat.* Neuroscience, 1995. **64**(4): p. 1057-73.
12. Jones, D.M., et al., *Characterization of pharmacoresistance to benzodiazepines in the rat Li-pilocarpine model of status epilepticus.* Epilepsy Res, 2002. **50**(3): p. 301-12.
13. Goodkin, H.P., X. Liu, and G.L. Holmes, *Diazepam terminates brief but not prolonged seizures in young, naive rats.* Epilepsia, 2003. **44**(8): p. 1109-12.
14. Kapur, J. and R.L. Macdonald, *Rapid seizure-induced reduction of benzodiazepine and Zn2+ sensitivity of hippocampal dentate granule cell GABAA receptors.* J Neurosci, 1997. **17**(19): p. 7532-40.
15. Treiman, D.M., *Therapy of status epilepticus in adults and children.* Curr Opin Neurol, 2001. **14**(2): p. 203-10.
16. Treiman, D.M., et al., *A comparison of four treatments for generalized convulsive status epilepticus. Veterans Affairs Status Epilepticus Cooperative Study Group.* N Engl J Med, 1998. **339**(12): p. 792-8.
17. DeLorenzo, R.J., *Status epilepticus: concepts in diagnosis and treatment.* Semin Neurol, 1990. **10**(4): p. 396-405.
18. Thakor, N.V. and S. Tong, *Advances in Quantitative Electroencephalogram Analysis Methods.* Annual Review of Biomedical Engineering, 2004. **6**: p. 453-95.

19. Cavalheiro, E.A., et al., *The susceptibility of rats to pilocarpine-induced seizures is age-dependent.* Brain Res, 1987. **465**(1-2): p. 43-58.
20. Cavalheiro, E.A., *The pilocarpine model of epilepsy.* Ital J Neurol Sci, 1995. **16**(1-2): p. 33-7.
21. Singleton, M.W., et al., *Age dependence of pilocarpine-induced status epilepticus and inhibition of CaM kinase II activity in the rat.* Developmental Brain Research, 2005. **156**(1): p. 67-77.
22. Parsons, J.T., et al., *Pilocarpine-induced status epilepticus causes N-methyl-D-aspartate receptor-dependent inhibition of microsomal Mg(2+)/Ca(2+) ATPase-mediated Ca(2+) uptake.* J Neurochem, 2000. **75**(3): p. 1209-18.
23. Kurz, J.E., et al., *A significant increase in both basal and maximal calcineurin activity in the rat pilocarpine model of status epilepticus.* J Neurochem, 2001. **78**(2): p. 304-15.
24. Churn, S.B., L.D. Kochan, and R.J. DeLorenzo, *Chronic inhibition of Ca(2+)/calmodulin kinase II activity in the pilocarpine model of epilepsy.* Brain Res, 2000. **875**(1-2): p. 66-77.
25. Holbert II, W.H., et al., *Late Stages of Neuronal Development Modulate Status Epilepticus Characteristics.* Society for Neurosciences, 2004. **31**.
26. Lowenstein, D.H. and B.K. Alldredge, *Status epilepticus at an urban public hospital in the 1980s.* Neurology, 1993. **43**(3 Pt 1): p. 483-8.
27. DeLorenzo, R.J., et al., *Epidemiology of status epilepticus.* J Clin Neurophysiol, 1995. **12**(4): p. 316-25.
28. DeLorenzo, R.J., et al., *Status epilepticus in children, adults, and the elderly.* Epilepsia, 1992. **33 Suppl 4**: p. S15-25.
29. Wasterlain, C.G., *Recurrent seizures in the developing brain are harmful.* Epilepsia, 1997. **38**(6): p. 728-34.
30. Waterhouse, E.J. and R.J. DeLorenzo, *Status epilepticus in older patients: epidemiology and treatment options.* Drugs Aging, 2001. **18**(2): p. 133-42.
31. Waterhouse, E.J., et al., *Prospective population-based study of intermittent and continuous convulsive status epilepticus in Richmond, Virginia.* Epilepsia, 1999. **40**(6): p. 752-8.
32. Sankar, R., D. Shin, and C.G. Wasterlain, *Development of temporal lobe epilepsy in 21-day-old rats.* Epilepsia, 2003. **44**(6): p. 872; author reply 872-3.
33. Sankar, R., et al., *Granule cell neurogenesis after status epilepticus in the immature rat brain.* Epilepsia, 2000. **41 Suppl 6**: p. S53-6.
34. Sayin, U., T.P. Sutula, and C.E. Stafstrom, *Seizures in the Developing Brain Cause Adverse Long-term Effects on Spatial Learning and Anxiety.* Epilepsia, 2004. **45**(12): p. 1539-1548.
35. Wasterlain, C.G. and B.E. Dwyer, *Brain metabolism during prolonged seizures in neonates.* Adv Neurol, 1983. **34**: p. 241-60.
36. Wasterlain, C.G., et al., *Seizure-induced neuronal death in the immature brain.* Prog Brain Res, 2002. **135**: p. 335-53.
37. Wasterlain, C.G. and Y. Shirasaka, *Seizures, brain damage and brain development.* Brain Dev, 1994. **16**(4): p. 279-95.
38. Park, M., et al., *Postnatal development of the dopaminergic neurons in the rat mesencephalon.* Brain & Development, 2000. **22**: p. S38-S44.

39. Haugvicova, R., H. Kubova, and P. Mares, *Qualitative changes of anticonvulsant action of felbamate during development in rats.* Brain & Development, 1998. **20**: p. 222-226.
40. Haut, S.R., J. Veliskova, and S.L. Moshe, *Susceptibilty of immature and adult brains to seizure effects.* The Lancet (Neurology), 2004. **3**: p. 608-17.
41. Holschneider, D.P., et al., *Effects of cholinergic deafferentation and NGF on brain electrical coherence.* Brain Res Bull, 1998. **45**(5): p. 531-41.
42. Holschneider, D.P., et al., *Changes in cortical EEG and cholinergic function in response to NGF in rats with nucleus basalis lesions.* Brain Res, 1997. **765**(2): p. 228-37.
43. Handforth, A. and D.M. Treiman, *A new, non-pharmacologic model of convulsive status epilepticus induced by electrical stimulation: behavioral/electroencephalographic observations and response to phenytoin and phenobarbital.* Epilepsy Res, 1994. **19**(1): p. 15-25.
44. DeLorenzo, R.J., et al., *Comparison of status epilepticus with prolonged seizure episodes lasting from 10 to 29 minutes.* Epilepsia, 1999. **40**(2): p. 164-9.
45. Churn, S.B., Franks, P.D., Thiessen, M., *Efficacy of Topiramate in Otherwise Refractory Status Epilepticus in the Rat.* Epilepsia, 2004. **Submitted for Publication**.
46. Epstein, H.T., *Phrenoblysis: special brain and mind growth periods. I. Human brain and skull development.* Dev Psychobiol, 1974. **7**(3): p. 207-16.
47. Epstein, H.T., *Phrenoblysis: special brain and mind growth periods. II. Human mental development.* Dev Psychobiol, 1974. **7**(3): p. 217-24.
48. Epstein, H.T., *Stages in human brain development.* Brain Res, 1986. **395**(1): p. 114-9.
49. Gottlieb, A., I. Keydar, and H.T. Epstein, *Rodent brain growth stages: an analytical review.* Biology of the Neonate, 1977. **32**(3-4): p. 166-76.
50. Pan, H.S., et al., *Deficits in the Brain Growth of Rats Induced by Methyl Mercury Treatment during the Brain Growth Spurt.* Journal of Health Science, 2005. **51**(1): p. 41-47.
51. Fountain, N.B. and E.W. Lothman, *Pathophysiology of status epilepticus.* J Clin Neurophysiol, 1995. **12**(4): p. 326-42.
52. Maytal, J. and S. Shinnar, *Febrile status epilepticus.* Pediatrics, 1990. **86**(4): p. 611-6.
53. Maytal, J., et al., *Low morbidity and mortality of status epilepticus in children.* Pediatrics, 1989. **83**(3): p. 323-31.
54. Sankar, R., D.H. Shin, and C.G. Wasterlain, *GABA metabolism during status epilepticus in the developing rat brain.* Brain Res Dev Brain Res, 1997. **98**(1): p. 60-4.
55. Epstein, H.T., *Stages of increased cerebral blood flow accompany stages of rapid brain growth.* Brain & Development, 1999. **21**: p. 535-539.
56. Avishai-Eliner, S., et al., *Stressed-out, or in (utero)?* TRENDS in Neurosciences, 2002. **25**(10): p. 518-524.
57. Singleton, M.W., et al., *Modulation of CaM Kinase II Is Conicident with Induction of Status Epilepticus in the Rat Pilocarpine Model.* Epilepsia, 2005. **46**(9): p. 1-12.

58. Hamani, C., et al., *Bilateral Anterior Thalamic Nucleus Lesions and High-Frequency Stimulation are Protective Against Pilocarpine-induced Seizures and Status Epilepticus.* Neurosurgery, 2004. **54**(1): p. 191-197.
59. Racine, R.J., *Modification of seizure activity by electrical stimulation. II. Motor seizure.* Electroencephalogr Clin Neurophysiol, 1972. **32**(3): p. 281-94.
60. Stafstrom, C.E. and D.M. Sasaki-Adams, *NMDA-induced seizures in developing rats cause long-term learning impairment and increased seizure susceptibity.* Epilepsy Research, 2003. **53**(1-2): p. 129-137.
61. Turski, W., et al., *Age dependency of the susceptibility of rats to aminooxyacetic acid seizures.* Brain Res Dev Brain Res, 1992. **67**(2): p. 137-44.
62. White, A.M., *Substance use and adolescent brain development.* Youth Studies Australia, 2003. **22**: p. 39-45.
63. Little, P.J., et al., *Differential effects of ethanol in adolescent and adult rats.* Alcohol Clin Exp Res, 1996. **20**(8): p. 1346-51.
64. Luster, M.I., J.H. Dean, and D.R. Germolec, *Consensus Workshop on Methods to Evaluate Developmental Immunotoxicity.* Environmental Health Perspectives, 2003. **111**(4): p. 579-583.
65. Sharpe, N.A. and J.M. Tepper, *Postnatal development of excititatory synaptic input to the rat neostriatum: an electron microscopic study.* Neuroscience, 1998. **84**(4): p. 1163-75.
66. Markus, E.J. and T.L. Petit, *Neocortical synaptogenesis, aging, and behavior: lifespan development in the motor-sensory system of the rat.* Experimental Neurology, 1987. **96**(2): p. 262-78.
67. Mazarati, A.M., et al., *Self-sustaining status epilepticus after brief electrical stimulation of the perforant path.* Brain Res, 1998. **801**(1-2): p. 251-3.
68. Mazarati, A.M., et al., *Time-dependent decrease in the effectiveness of antiepileptic drugs during the course of self-sustaining status epilepticus.* Brain Res, 1998. **814**(1-2): p. 179-85.
69. Coulter, D.A., D.C. McIntyre, and W. Loscher, *Animal Models of Limbic Epilepsies: What can They Tell Us?* Brain Pathology, 2002. **12**: p. 24-256.
70. Carpentier, P., et al., *Delta Activity as an Early Indicator for Soman-Induced Brain Damage: A Review.* Neurotoxicology, 2001. **22**: p. 299-315.
71. McDonough Jr, J.H., et al., *Protection Aqainst Nerve Agent-Induced Neuropathology, But Not Cardiac Pathology, is associated with the Anticonvulsant Action of Drug Treatment.* Neurotoxicology, 1995. **15**(1): p. 123-132.
72. Clancy, B., R.B. Darlington, and B.L. Finlay, *Translating Developmental Time Across Mammalian Species.* Neuroscience, 2001. **105**(1): p. 7-17.
73. Spear, L., *Modeling adolescent development and alcohol use in animals.* Alcohol Res Health, 2000. **24**(2): p. 115-23.
74. Moshe, S.L. and B.J. Albala, *Maturational changes in postictal refractoriness and seizure susceptibility in developing rats.* Ann Neurol, 1983. **13**(5): p. 552-7.
75. Moshe, S.L., et al., *Maturation and segregation of brain networks that modify seizures.* Brain Res, 1994. **665**(1): p. 141-6.
76. Giedd, J.N., *Structural Magnetic Resonance Imaging of the Adolescent Brain.* Annals New York Academy of Sciences, 2004. **1021**: p. 77-85.

77. Lewis, D.A., et al., *Postnatal Development of prefrontal Inhibitory Circuits and the Pathophysiology of Cognitive Dysfunction in Schizophrenia.* Annals New York Academy of Sciences, 2004. **1021**: p. 64-76.
78. Lowry, R., *Concepts and Application of Inferential Statistics:Chapter 8a;Fishers exact*. 1 ed, ed. R. Lowry. 1999, Poughkeepsie, NY: Web.
79. Sankar, R., et al., *Epileptogenesis after status epilepticus reflects age- and model-dependent plasticity.* Ann Neurol, 2000. **48**(4): p. 580-9.
80. Veliskova, J., et al., *Seizures in the developing brain.* Epilepsia, 2004. **45**(8): p. 6-12.
81. Walton, N.Y. and D.M. Treiman, *Response of status epilepticus induced by lithium and pilocarpine to treatment with diazepam.* Exp Neurol, 1988. **101**(2): p. 267-75.
82. Garattini, S., E. Mussini, and L.O. Randall, *The Benzodiazepines*. 1 ed. 1973, New York: Raven Press. 685.
83. Berzaghi, M.P., et al., *Effect of amygdaloid kindled seizures during pregnancy on neonatal brain biogenic amines.* Braz J Med Biol Res, 1990. **23**(9): p. 827-30.
84. Bortolotto, Z.A., et al., *Effects of 2-chloroadenosine on amygdaloid and hippocampal kindled seizures.* Arch Int Pharmacodyn Ther, 1985. **277**(2): p. 313-20.
85. Cavalheiro, E.A., et al., *Intracortical and intrahippocampal injections of kainic acid in developing rats: an electrographic study.* Electroencephalogr Clin Neurophysiol, 1983. **56**(5): p. 480-6.
86. Clifford, D.B., et al., *Effect of anticonvulsant drugs on kainic acid-induced epileptiform activity.* Exp Neurol, 1982. **76**(1): p. 156-67.
87. Erakovic, V., et al., *Lithium plus pilocarpine induced status epilepticus--biochemical changes.* Neurosci Res, 2000. **36**(2): p. 157-66.
88. Ferraz, A.C., et al., *Ricinine-elicited seizures. A novel chemical model of convulsive seizures.* Pharmacol Biochem Behav, 2000. **65**(4): p. 577-83.
89. Lothman, E.W. and R.C. Collins, *Kainic acid induced limbic seizures: metabolic, behavioral, electroencephalographic and neuropathological correlates.* Brain Res, 1981. **218**(1-2): p. 299-318.
90. Lothman, E.W., R.C. Collins, and J.A. Ferrendelli, *Kainic acid-induced limbic seizures: electrophysiologic studies.* Neurology, 1981. **31**(7): p. 806-12.
91. Racine, R.J., *Modification of seizure activity by electrical stimulation. I. After-discharge threshold.* Electroencephalogr Clin Neurophysiol, 1972. **32**(3): p. 269-79.
92. Turski, L., et al., *Review: cholinergic mechanisms and epileptogenesis. The seizures induced by pilocarpine: a novel experimental model of intractable epilepsy.* Synapse, 1989. **3**(2): p. 154-71.
93. Cooper, J.R., F.E. Bloom, and R.H. Roth, *The Biochemical Basis of Neuropharmacology*. Oxford University Press, 2003. **Eighth Edition**.

VITA

William Harold Holbert II (Bill) was born on July 24, 1965 at the United States Naval hospital in Portsmouth, Virginia. He graduated from Bayside High School in Virginia Beach, Virginia, in 1983. After this he attended East Carolina University in Greenville, North Carolina and graduated with a Bachelor of Science in 1989. During his time at East Carolina he enlisted in the United States Marines Corps (reserves). Following graduation from East Carolina University he entered private industry until 1994 where he entered Old Dominion University to complete post bachelor work. In 2001 he began graduate work at Virginia Commonwealth University in the biochemistry certificate program. He transferred to Pharmacology and Toxicology in 2003 where he will graduate with a MS degree. During his time at Virginia Commonwealth University he worked for the Department of Neurology. In this period Bill sent three papers for publication. The first paper is entitled, "Post-juvenile Brain Development Modulates Seizure Characteristics in the Rat Pilocarpine Model: Discrete Seizure Phase". The second paper, "Post-juvenile Brain Development Modulates Seizure Characteristics in the Rat Pilocarpine Model: Status Epilepticus Phase". The third paper which is a brief communication is entitled, "Status Epilepticus-Induced Loss of Diazepam Efficacy is Age Dependent in the Rat Pilocarpine-SE Model". He will also be second author on several other publications.