Early Seizure Blockade: Preventing Long-Term Epileptic Activity in Wag/Rij Rats

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A Thesis Submitted to the
Yale University School of Medicine
In Partial Fulfillment of the Requirements for the
Degree of Doctor of Medicine

by

April Robyn Levin

2007
EARLY SEIZURE BLOCKADE: PREVENTING LONG-TERM EPILEPTIC ACTIVITY IN WAG/RIJ RATS. April R. Levin1, Matthew L. Vestal1, Frederick Wang1, Kathryn Giblin1, Crystal Paul-Laughinghouse1, Tim Rice1, Anne M. Phadke1, Davender S. Khera1, Joshua P. Klein1, John Mission1, Chhitij Bashyal1, Joshua Motelow1, Hrachya Nersesyan1, R.K. Agarwal1, Dario J. Englot1, Ulrich Schridde1, Steven G. Waxman1, Hal Blumenfeld1,2,3. Department of 1Neurology, 2Neurobiology, and 3Neurosurgery, Yale University School of Medicine, New Haven, CT.

The purpose of this study was to determine how early seizure blockade with ethosuximide (ESX) would influence ion channel expression and long-term spike-wave discharge (SWD) activity in epileptic WAG/Rij rats. The goal was to elucidate the question “Do seizures beget seizures?” in a genetically prone model and if so, to attempt to interrupt this cycle by early intervention. In our first experiment, we used immunocytochemistry to determine the effect of ESX on cortical expression of ion channels in treated and untreated WAG/Rij rats and age-matched Wistar controls. This experiment revealed that treatment with ESX blocked the upregulation of Nav1.1 and Nav1.6 as well as the downregulation of HCN1 that is associated with epileptic activity in rats (p < .05). In a second experiment, WAG/Rij rats were divided into 3 groups: untreated (H2O), temporary early treatment (ESX 4 month), and continuous early treatment (ESX continuous), and SWD activity was measured by electroencephalogram (EEG) at various timepoints. This second experiment revealed that animals in the ESX 4 month group spent less percent time in SWD (0.242 ± .068 SEM) than animals in the H2O group (0.769 ± .060 SEM, p < .001), although they spent slightly more percent time in SWD than animals in the ESX continuous group (0.020 ± .065 SEM, p = .003). This effect was predominantly due to seizure number, and average seizure duration did not vary among the three groups. Additionally, power spectrum analysis revealed a significant correlation when the difference between power spectra for H2O and ESX 4 month rats was compared to the power spectrum of a seizure (Pearson correlation equals 0.955, 2-tailed significance < .000000001), suggesting quantitatively that seizures were reduced by temporary early treatment. This suggests that early prevention of SWD may reduce the burden of seizures later in life, and that possibilities for prevention of genetic absence epilepsy should be further investigated.
Acknowledgements

I would like to thank Dr. Hal Blumenfeld for his constant support and encouragement for this thesis project as well as many other endeavors during medical school. I would also like to thank the many members of the research team for this project for their thoughtful input and countless hours of hard work: Matthew Vestal, Frederick Wang, Kathryn Giblin, Crystal Paul-Laughinghouse, Tim Rice, Anuradha Phadke, Dr. Davender Khera, Dr. Joshua Klein, John Mission, Chhitij Bashyal, Joshua Motelow, Dr. Hrachya Nersesyan, Ravi Agarwal, Dario Englot, Dr. Ulrich Schridde, and Dr. Steven Waxman. Additionally, I am very grateful to Dr. Edward Novotny for his helpful suggestions in the writing of this thesis. Finally, I would like to thank my family for their undying love and support through this project and many others.

This work was supported by the generosity of the Office of Student Research at Yale University School of Medicine and NIH R01 NS049307.
Introduction

Background

Epilepsy constitutes an important medical issue. 120 in 100,000 people seek medical attention for newly recognized seizure disorders each year, and among this population there is tremendous variability in seizure types and manifestations, treatments and side effects, and associated comorbidities. Epilepsy is especially prevalent and often severe in children, with approximately 0.5-1% of children affected with the disorder [1].

An important issue in the treatment of children with epilepsy is not only how best to treat the immediate seizures, but how treatment and seizures will affect brain development and hence propensity for seizures later in life [2-4]. A number of in vitro and in vivo studies have been done in a variety of seizure models to investigate the question, “Do seizures beget seizures?” Many of these models involve the experimental induction of seizure activity, and they do suggest that either the induction of a seizure or the seizure itself may increase the likelihood of epileptic activity later in life [2, 5-7]. However, in order to determine whether seizure activity alone can increase the propensity for further seizures, it is necessary to use a model of spontaneously occurring epilepsy [8], and to determine whether prevention of early seizure activity can help to prevent the development of epilepsy, known as epileptogenesis.

Animal Models

A number of animal models of spontaneously occurring epilepsy are available, including Wistar Albino Glaxo from Rijswijk (WAG/Rij) rats [9-12], Genetic Absence Epilepsy Rats from Strasbourg (GAERS) [7, 12, 13], and epileptic beagles [7]. Both rat
models were bred for spontaneous absence seizures from the Wistar rat strain, but the underlying genes for the epilepsy in these rats are not known [14-16]. Numerous strains of mice also exhibit spike-wave discharges (SWD) characteristic of absence epilepsy, and the question of whether seizures beget seizures has been studied in some of these mouse models. For example, previous studies in *tottering* mice have shown network burst abnormalities which are more pronounced during development than at adulthood, suggesting that these abnormalities do not represent a secondary consequence of repeated seizures [17]. However, this experiment studied paroxysmal depolarizing shifts in response to elevated extracellular potassium in vitro as opposed to spontaneous seizure activity in vivo, and did not examine the effects of seizure blockade. Therefore, this experiment suggested a mechanism for increased seizure susceptibility during development but did not rule out the possibility that seizure activity could beget further seizures by other mechanisms. Along these lines, short-term seizure blockade with ESX after seizure onset in *stargazer* mice was not found to alter intermediate early gene protein (IEGP) expression associated with mossy fiber sprouting in induced seizure models [18]; however, this study also showed that IEGPs are not an obligatory step in mossy fiber sprouting in absence seizures, and the ESX treatment examined here was short-term and was not given prior to seizure onset. Additionally, in “tottering,” “stargazer,” and numerous other strains of mice exhibiting SWD characteristic of absence epilepsy, including spontaneously mutated “lethargic,” “ducksy,” “mocha 2j,” “slow-wave epilepsy,” “coloboma,” “leaner,” “rolling Nagoya,” and “rocker” and *HCN2* knock-out mice, SWD are associated with severe motor abnormalities typically associated only with less common forms of absence epilepsy in humans [15, 19-21]. More recently, the
genetic basis of spike-wave seizures was also characterized in C3H/He mice, which do not exhibit any brain or motor abnormalities [15]. In a study of GAERS rats, levetiracetam treatment beginning one week prior to seizure onset did not prevent expression of SWD during adulthood; however, again in this study ESX treatment was short-term and was stopped before adulthood [22].

Among the models of spontaneous absence epilepsy, WAG/Rij rats are considered to have “face validity” as a model for human absence epilepsy. This is based on the presence of SWD concurrent with decreased responsiveness, preferential occurrence of SWD during transitional states between sleep and waking, modulation of SWD by physical and mental activity, effect of antiabsence drugs (except lamotrigine), and inheritability, all of which are seen in human absence epilepsy as well [11]. A major difference between this model and human absence epilepsy is that absence seizures in WAG/Rij rats appear after puberty (around age 2-3 months) and worsen throughout life, whereas in humans the seizures generally occur before puberty and then disappear or convert to more severe forms of epilepsy [11, 21, 23-26]. Of note, GAERS rats develop SWD earlier, after approximately 30 days of life [21]. Another major difference is that SWD occur at a frequency of 7-10 Hz in the rat and 3-4 Hz in the human [10, 23]. Notably, the frequency of SWD in mouse models of absence epilepsy is also higher (5-7 Hz) than in humans [21].

Two types of SWD are known to occur in WAG/Rij rats. The first type is seen during both sleep and wakefulness, lasts at least 1 second, shows a train of sharp spikes and slow waves directed upwards with a frequency of 7.5 to 9.5 Hz and an amplitude of at least twice the background, occurs at an average of 18 discharges per hour depending
on the age of the rat, and is accompanied by behavioral concomitants reminiscent of human absence seizures. The second type occurs almost exclusively during wakefulness, lasts at least 0.5 seconds, shows opposite polarity of the spikes and another position to the waves compared to type I, has a frequency ranging from 7.6 to 8.3 Hz and an amplitude of at least twice the background, occurs at an average of 14 discharges per hour, and is not accompanied by behavioral correlates [9].

The exact underlying genes controlling SWD in WAG/Rij rats remain unknown. Genetic control of SWD was recently mapped to chromosome 5 for type I SWD and chromosome 9 for type II SWD [14]. One dominant gene does appear to determine the occurrence of type 1 SWD in WAG/Rij rats, but multiple other background genes likely manipulate the number and duration of the SWD [27].

SWD in rats, as in humans, occur most often during passive wakefulness or light slow-wave sleep, less often during active wakefulness, and rarely during slow wave and rapid eye movement (REM) sleep [11, 23, 28, 29]. As in humans, slow-wave sleep in rats is characterized by large-amplitude, low-frequency, irregular delta waves, whereas waking is associated with high-frequency, low-amplitude beta waves. Interestingly, both rats and humans can be aroused during SWD. Rats have been shown to be more likely to be aroused by a conditioned stimulus than an unconditioned stimulus, suggesting that information continues to flow through the brain even during SWD [10].

**Pharmacology**

A number of pharmacological agents have been used to treat absence seizures. Ethosuximide (ESX), valproate, and trimethadione all act as antiabsence drugs in both
humans and rats, whereas anticonvulsant drugs such as carbamazepine and
diphenylhydantoin have no effect or even worsen absence seizure activity [11, 30].
Currently, ESX is considered a first choice anti-absence drug [31].

The exact mechanism of ESX in blocking SWD is controversial. Although
blockade of thalamic T-type Ca\(^{2+}\) channels is often cited as the most likely mechanism
[32], a reduction of nonactivating Na\(^+\) current and Ca\(^{2+}\)-activated K\(^+\) current in thalamic
and cortical neurons is also likely to contribute to the therapeutic action of ESX [33].

**Absence seizures: Classification**

Seizures are generally classified as focal (involving only part of the brain) or
generalized (involving the whole brain) [26, 34]. Absences are a type of generalized
seizure, and typical absences demonstrate clinical impairment of consciousness and 3-
4Hz SWD on EEG [34]. They are most commonly associated with childhood absence
epilepsy (CAE), which peaks at age 6-7 [26] and remits by adolescence in 70% of
patients [21]. Other idiopathic generalized epilepsies associated with absence include
juvenile absence epilepsy (JAE), in which onset peaks at puberty. Absences tend to be
less severe than in CAE, but the disorder is associated with infrequent myoclonic jerks
and generalized tonic-clonic seizures. Additionally, juvenile myoclonic epilepsy (JME)
demonstrates the same seizure types and age of onset as JAE, but the myoclonic jerks are
more severe than the absences in this syndrome. Cryptogenic or symptomatic epilepsies
involving absence have been identified as well, including myoclonic absence epilepsy,
Lennox-Gastaut syndrome, and epilepsy with continuous spike-waves during slow sleep
[26].
Mutations of several genes, including those encoding for various GABA, glutamate, acetylcholine, sodium, potassium, and calcium channels have been associated with some cases of familial CAE, JAE, and JME [21, 35]. Additionally, it has been suggested that mouse models of absence and ataxia may be representative of combined absence and ataxia syndromes in humans [21]. Although 16-45% of patients with CAE report of positive family history, penetrance is incomplete, with concordances of 75-80% among monozygotic twins and 33% among first-degree relatives [21]. The majority of human absence epilepsies are therefore likely due to complex genetic inheritance factors, and several studies are currently underway to investigate the genotype phenotype correlations in absence and other human epilepsies [35, 36].

Absence seizures: Generation and maintenance

Although absence seizures are considered to be generalized, it is known that specific cortico-thalamic networks play an important role in the generation and maintenance of these seizures, while other regions are relatively spared [11, 37, 38]. Although debate continues about the region of seizure onset in humans, onset in WAG/Rij rats was recently localized to the facial region of the somatosensory cortex. However, after the first 500 msec of a seizure in WAG/Rij rats, cortical and thalamic sites interact bi-directionally, with the direction of coupling varying throughout the seizure [38, 39]. Similarly in GAERS rats, microinfusion of ESX into the peri-oral region of the primary somatosensory cortex (S1po) produces an immediate cessation of seizure activity, whereas infusion of ESX into the thalamus produces only a modest and delayed reduction in SWD [40].
Multiple ion channels are likely to be involved in the initiation and maintenance of absence seizures [41, 42]. Studies in collaboration with our lab showed that the expression of messenger ribonucleic acid (mRNA) and protein for the sodium channels Nav1.1 and Nav1.6 is upregulated in the WAG/Rij epileptic cortex compared to non-epileptic controls [24], and seizure activity has been shown to induce changes in sodium channels in non-absence seizures as well [43-48]. Similarly, alterations in the cation channel HCN1 and its depolarizing current I_h have been associated with absence seizures in WAG/Rij rats [49, 50], GAERS rats [51], and stargazer mice [52].

Such results beg the question of whether the changes in channel level are a cause or an effect of seizure activity. To answer this question, we first conducted an experiment in which ESX was used to block spontaneous seizure activity in WAG/Rij rats, and the effects on channel expression were measured. If blocking seizures could block alteration in channel levels, and if alteration in channel levels is associated with alterations in neuronal bursting, the question follows whether early seizure blockade might break a self-reinforcing cycle in which seizures alter channel levels, resulting in increased seizure susceptibility. In our second experiment, therefore, we studied the effects of early temporary ESX treatment on the frequency and duration of later seizures.
Hypothesis and Specific Aims

The goal of our experiments was to investigate the broad question, “Do seizures beget seizures?” Given that Nav1.1 and Nav1.6 are known to be upregulated in WAG/Rij cortex [24] and HCN1 is known to be downregulated in this region [49, 50], we hypothesized that treatment with ESX would block these changes in ion expression. As a corollary, we hypothesized that treatment with ESX during the usual period of seizure onset in these rats would decrease SWD even once the ESX was withdrawn.

To investigate these hypotheses, our first aim was to determine total seizure time in treated and untreated WAG/Rij and Wistar rats to quantify the effect of ESX on blockade of SWD activity. We then planned to determine the expression of Nav1.1, Nav1.6, and HCN1 in the cortices of these rats. Finally, we aimed to determine the effect of early temporary treatment with ESX by using electroencephalography (EEG) to quantify seizure frequency and duration in treated, untreated, and temporarily treated WAG/Rij rats, and to use power spectrum analyses to determine quantitatively how these treatment regimens would alter EEG activity during seizures and at baseline.
Methods

Animals

The WAG/Rij rat strain originated from the Radiobiological Institute TNO in Rijswijk, and a colony is maintained at Yale University. Non-epileptic female Wistar rats were obtained from Charles River Laboratories in Wilmington, MA. These rats were screened by EEG and were not included in the study if significant SWD activity was noted. Animals were all female, were housed in groups of two or three and were kept on a 12-hour light/dark cycle with unlimited access to food and water, in accordance with NIH guidelines for the care and use of laboratory animals. Animal protocols were all approved by the Yale University Institutional Animal Care and Use Committee.

In the treated groups, ESX was administered orally at a dose of 300 mg/kg/d by adding it to the drinking water beginning at the time of weaning (p21). Based on pilot measurements, we determined that the rats drink approximately 120 cc/kg/d, and drink slightly more (~200 cc/kg/d) in the first 1.5 months. Therefore, ESX 300mg/kg/d was given using 250mg/5ml syrup (Pharmaceutical Associates, Inc. Greenville, SC) by adding 3cc of syrup per 100cc H₂O for p21 through p45, and 5cc per 100cc H₂O for p45 onward. Pilot experiments showed that lower doses of ESX (100 - 200 mg/kg/d) did not consistently block SWD, whereas higher doses (400 mg/kg/d) caused significant lethargy and gait ataxia. The dose of 300 mg/kg/d was effective in completely blocking SWD, and was also well tolerated without any side effects of lethargy, ataxia, or reduced food intake. Water bottles were coated on the outside with black paint since the medication is light-sensitive, and the medication in the bottles was replaced at least weekly to ensure that therapeutic doses were continuously available.
In experiment #1, a total of 31 animals were used. Epileptic WAG/Rij rats (n=15) were compared to age-matched Wistar controls (n=16), and each group was subdivided into those treated with ESX (n=8 Wistar, n=8 WAG/Rij) and those not treated (n=8 Wistar, n=7 WAG/Rij). The treated group received ESX beginning at weaning (p21) (Fig. 1A).

For experiment #2, a total of 37 animals were used. In the first group, called “H2O” (n=13), rats received no ESX at any point. In the second group, called “ESX 4 months” (n=11), rats had ESX added to their water bottles at weaning (p21) and continued for 4 months, at which point ESX was discontinued and rats resumed receiving plain water. In the third group, called “Continuous” (n=13), ESX was given from weaning but not discontinued (Fig. 1B).
Figure 1.  (A) Experiment I: Effects of early ESX on cortical ion channel expression. Rats were divided into 4 groups: Wistar nonepileptic rats receiving no treatment (NE control H2O), Wistar nonepileptic rats receiving treatment (NE control ESX), WAG/Rij rats receiving no treatment (WAG/Rij H2O), and WAG/Rij rats receiving treatment (WAG/Rij ESX). Rats in the ESX groups began receiving ESX at weaning (p21). At age 5 months, 6-hour EEG was recorded for each rat, followed by sacrifice for immunostaining.  (B) Experiment II: Effects of early ESX on seizure development. WAG/Rij rats were divided into 3 groups: H2O, ESX 4 months, and ESX continuous. Rats in the H2O group received no ESX at any point. Rats in the ESX 4 months group began receiving ESX at weaning (p21) and continued for 4 months until a baseline recording was completed (-1d), at which point they resumed receiving plain water for the remainder of EEG recordings (1, 14, 30, 60, and 90 days following ESX withdrawal). Rats in the ESX continuous group began receiving ESX at weaning (p21), and continued throughout the duration of all EEG recordings. White bars = Rat receiving plain H2O; Black bars = Rat receiving ESX 300mg/kg/d.

EEG Recordings

Electrodes were implanted at age 5 months for experiments #1 and #2. For implantation of electrodes, animals were deeply anesthetized with intramuscular ketamine (100mg/kg), xylazine (5.2mg/kg) and acepromazine (1.0mg/kg) and placed in a stereotactic frame (David Kopf Instruments, Tujunga, CA). Level of anesthesia was
measured by respiration, heart rate, glabrous skin perfusion, and response to foot pinch. Small burr holes were made in the skull without disturbing the dura and tripolar electrodes (Plastics One, Roanoke, VA) were secured to the skull using 1.60-mm stainless steel screws (Plastics One). EEG recording electrodes were placed at AP +2.0, ML +2.0 mm and AP -6.0, ML +2.0mm. A ground electrode was placed in the midline over the cerebellum. Dental acrylic (Henry Schein Inc, Indianapolis, IN; Lang Jet Denture Repair Acrylic) was used to fix the electrode unit in place.

Continuous EEG data was recorded from awake-behaving rats between 10:00 AM and 4:00 PM (light cycle). For experiment #1, following a 1-week recovery period after electrode implantation, EEG was recorded for 2 hours/day over a consecutive 3-day period for each animal, for a total of 6 hours of recording time per animal. For experiment #2, at least 3 hours of baseline EEG was recorded at the following time intervals in the ESX 4 months group and age-matched controls in the H2O and ESX continuous groups: 1 day prior to stopping ESX; and then 1 day, 14 days, 30 days, 60 days, and 90 days after stopping ESX.

EEG signals were recorded via commutator (Plastics One, Inc.) using a Grass CP 511 amplifier (Grass-Telefactor, Astro Med, West Warwick, RI), with band pass filter settings of 1 to 300 Hz. Signals were digitized at a sampling rate of 1 kHz with a National Instruments USB-6008 A/D converter and LabView 7.1 software (National Instruments Ltd.), and analyzed using Spike2 software (Cambridge Electronic Design, Cambridge, UK).

Start and end times of all SWD were marked by visual inspection of EEG recordings in both experiments. Inspection was done by a total of five trained observers,
blinded to the experimental group and time point of each analysis. Criteria for marking SWD included trains of rhythmic sharp spikes and slow waves with a frequency of 7-8 Hz, lasting at least 1 second, with a peak-to-peak amplitude at least twice the background. In experiment #1, for each experimental group, percent time spent in SWD was then calculated as (sum of SWD interval durations/total recording time).

For experiment #1, where the goal of EEG recordings was simply to determine whether or not ethosuximide was effectively blocking SWD (i.e. a binary decision), we included all EEG data from a given file, as long as most of the file was free from artifact and from slow wave sleep (which obscures SWD). For experiment #2, where precise and quantitative measurement of SWD duration and frequency was critical, all data files were subdivided by marking segments of “usable” (awake, artifact-free) and “unusable” (artifact or slow wave sleep) data. More specifically, unusable data was defined as that in which slow-wave sleep (1-3 Hz, large amplitude waves or spindles) or signal-obscuring artifact occurred for more than 75% of a 40-second period. Since blocks of unusable data were required to last at least 40 seconds, this meant that any interruptions of the unusable data (i.e., segments of usable data) had to occur within the 40 second block and not on either end. Usable data was defined as lack of artifact or large amplitude irregular slow activity on EEG occurring for >50% of a 40-second block, with blocks of unusable data allowed to be on the ends. Blocks of usable data were therefore required to last >20 seconds. Any SWD occurring during unusable time were recorded but were not included in further analyses. For each experimental group at each time point, data was analyzed for percent of usable time spent in SWD, average number of seizures per hour of usable time, and average seizure duration.
Additionally, for each experimental group at each time point, aggregate power spectra were run for all seizures using Spike2, with programming done by Cambridge Electronic Design (Cambridge, England). Aggregate power spectra were also run for usable time, and any data files noted by the reader to have more than very little artifact were not included in the power spectral analyses.

For experiment #2, all statistical analyses for percent time in SWD, seizure number, and seizure duration were run in SPSS (v. 14, SPSS Inc., Chicago, Il, USA) using one-way ANOVA or MANOVA for repeated measures with Wilks’ Lambda multivariate analysis, followed by Games-Howell posthoc analyses if appropriate. The between factor for all analyses was group (H2O, ESX 4 months, ESX continuous), and the within factor was time (-1d, 1d, 14d, 30d, 60d, 90d). A p < .05 was regarded as significant.

For analysis of power spectra, differences were assessed in SPSS by Pearson correlation. A significance (2-tailed) value < .01 was regarded as significant.

**Perfusion**

When recordings were complete, rats were deeply anesthetized with ketamine/xylazine (80/5 mg/kg) given intraperitoneally, using a #26 needle. The skin was then incised and the heart exposed. For animals treated with ESX until perfusion, 3ml of blood was drawn from the inferior vena cava at this time, transferred into a K2 EDTA (3.6mg) BD Vacutainer, and centrifuged for 10-12 minutes at a speed of 1000x. Supernatant was then transferred to a CryoTube vial, placed on dry ice, and sent to National Medical Services (Willow Grove, PA) for measurement of ESX levels.
To perfuse, the inferior vena cava was incised to allow blood to escape, and an 18.5 gauge needle attached to a perfusion pump with 0.01 M phosphate buffer solution (PBS) was then inserted into the left ventricle and clamped against the heart muscle. Perfusion with PBS was continued at 8ml/min until liquid in the tubing became clear (5 minutes). Perfusion was then switched to 4% cold buffered Paraformaldehyde (PFA) and continued until muscles were stiff (10-15 minutes). Rats were then decapitated, skin was removed with a scalpel and skull was removed with a bone crusher. Exposed brain was postfixed and cryoprotected in 30% sucrose solution.

**Immunocytochemistry**

For experiment #1, coronal sections (10μm) of the cerebral hemispheres were cut at AP +0.0, ML +6.0mm using the pial surface and deep white matter border as anatomical landmarks to obtain layers II-III of barrel somatosensory cortex, previously shown to have altered levels of Nav1.1 and Nav1.6 mRNA and protein in epileptic WAG/Rij compared to nonepileptic Wistar rats [24]. Bilateral sections were obtained, and data from both hemispheres was combined. The sections were mounted onto slides and incubated in blocking solution (5% normal goat serum and 1% bovine serum albumin in phosphate buffer solution (PBS)) with 0.1% Triton X-100 and 0.02% sodium azide at room temperature for 30 minutes. Serially consecutive slices with identical preparations were then incubated with subtype-specific antibodies to sodium channel α-subunit Nav1.1 (residues 465-481, 1:100 dilution, Alomone, Jerusalem), sodium channel α-subunit Nav1.6 (residues 1042-1061, 1:100, Alomone, Jerusalem) and HCN1 (Chemicon rabbit anti-rat, 1:100, Temecula, CA) overnight at 4°C. Slides were then washed in PBS,
incubated with biotinylated goat anti-rabbit serum (1:1000, Sigma) in blocking solution for 3h, washed again in PBS, incubated in avidin-horse radish peroxidase (1:1000, Sigma) in blocking solution for 3h, washed again in PBS, and incubated with goat anti-rabbit IgG-Cy3 (1:2000, Amersham, New Jersey). Immunofluorescence signal was detected using fluorescein illumination (emission wavelength 516-565 nm).

A Nikon Eclipse TE300 light microscope was used for sample observation, and quantitative microdensitometry of immunostaining signals was obtained using IPLab v3.0 Image Processing software (Scanalytics, Fairfax, VA). Signal intensities were determined by outlining individual cortical neurons, and IPLab integrated densitometry functions were used to calculate mean signal intensities. Results from identical regions and layers of cortex in WAG/Rij and Wistar rats, both treated (ESX) and untreated (H2O), were processed in parallel. Immunopositivity was quantified by averaging multiple counts within a defined area (1.9 x 10^4 μm^2) within layers II-III of barrel somatosensory cortex. Only neurons with distinct borders whose nuclei fell within the plane of section were analyzed. Approximately 50 neurons were analyzed for each antibody (Nav1.1, Nav1.6, and HCN1) per animal. Analysis of neurons from left versus right somatosensory cortex showed no significant difference in level of expression within each group of rats, so these data were combined.

For experiment #1 (immunocytochemistry), differences were assessed by one-way ANOVA, followed by Bonferroni posthoc analyses if appropriate. A p < .05 was regarded as significant.
Student Contribution

For experiment #1, the writer assisted in performing EEG recordings and maintaining consistent delivery of ESX to experimental animals, analyzed EEG data, and prepared figures. For experiment #2, the writer analyzed 58% of the EEG data, managed and organized analyses completed by other observers, ran all power spectra, completed all statistical analyses, and prepared all figures.
Results

Experiment 1

Examples of typical recordings from WAG/Rij rats treated with ESX and untreated are shown in Fig. 2A. In the animals we studied, ESX treatment in WAG/Rij rats decreased the percent time spent in SWD to the level of nonepileptic (NE) Wistar controls (Fig 2C). Mean percent times spent in SWD were 0.004 ± .002 SEM for NE control ESX, 0.095 ± .032 for NE control H2O, 0.100 ± .035 for WAG/Rij ESX, and 0.771 ± .142 for WAG/Rij H2O. ANOVA with post-hoc Bonferroni analyses showed significant differences between WAG/Rij H2O and all other groups (p < .001). No other between-group differences were significant. Notably, NE controls and WAG/Rij rats treated with ESX all spent a small amount of time in SWD, and treatment with ESX in Wistar controls did not significantly alter the time spent in SWD. ESX levels drawn from blood prior to perfusion showed an average of 87.7 ± 9.8 SEM µg/ml for all treated animals (Data not shown).
Figure 2 Effect of ESX on SWD activity. (A) Example of SWD activity in untreated (H2O) WAG/Rij rats (bottom trace) with an example of typical SWD at higher gain (top trace). (B) Example of SWD in WAG/Rij rats treated with ESX. (C) Mean percent time spent in SWD for each experimental group. Data is plotted as mean ± S.E., * = p < .05, ANOVA with post-hoc Bonferroni adjustment.
Immunocytochemical experiments using subtype-specific antibodies for Nav1.1, Nav1.6, and HCN1 have previously shown that Nav1.1 and Nav1.6 are up-regulated in layer II-IV cortical neurons of barrel somatosensory cortex of WAG/Rij rats compared to Wistar rats (Klein 2004) [24], and that HCN1 is downregulated in this region [49, 50]. Fig. 3A-L show staining of the barrel somatosensory cortex with antibodies for HCN1 (Fig. 3A-D), Nav1.1 (Fig. 3E-H), and Nav1.6 (Fig. 3I-L). These results demonstrate that in WAG/Rij rats treated with ESX, the levels of these proteins return to that in treated and untreated Wistar rats. Quantification of these data (Fig. 3M) demonstrates that there is a significant increase in Nav1.1 and Nav1.6 protein and a significant decrease in HCN1 in barrel somatosensory cortex of untreated WAG/Rij rats (p < .001, ANOVA with post-hoc Bonferroni adjustment). There is no significant difference in the levels of these proteins between Wistar rats and treated WAG/Rij rats. Mean optical intensity for each group is listed as follows (Table 1):

<table>
<thead>
<tr>
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<th>Mean</th>
<th>Std. Error</th>
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<td>NE control ESX</td>
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<tr>
<td>NE control ESX</td>
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</tr>
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Table 1. Quantification of mean optical intensity for each channel antibody (Nav1.1, Nav1.6, and HCN1) for nonepileptic Wistar control rats receiving no treatment (NE control H2O), nonepileptic Wistar control rats receiving ESX (NE control ESX), WAG/Rij rats receiving no treatment (WAG/Rij H2O), and WAG/Rij rats receiving ESX (WAG/Rij ESX).
Figure 3. Effect of ESX on ion channel expression.  (A-L) Immunostaining of layers II-III of barrel somatosensory cortex in each experimental group for HCN1 (A-D), Nav1.1 (E-H), and Nav1.6 (I-L). Clockwise from top left for each ion channel: NE control H2O, NE control ESX, WAG/Rij ESX, WAG/Rij H2O. (M) Quantification of ion channel protein expression in barrel somatosensory cortex by optic densitometry. WAG/Rij H2O rats (n=7) show a significant increase in Nav1.1 (white bars) and Nav1.6 (gray bars), and a significant decrease in HCN1 (black bars) protein levels compared to that in Wistar NE control H2O (n=8) and NE control ESX (n=8) rats. WAG/Rij rats treated with ESX (n=8) show protein levels of Nav1.1, Nav1.6, and HCN1 that is not significantly different from that in NE Wistar rats. Data is plotted as mean ± SEM, * = p < .05, ANOVA with post-hoc Bonferroni analyses.

Experiment 2

Figure 4 shows the effect of early ESX treatment on the percent time spent in SWD, as well as the number and duration of seizures. Once treatment was discontinued, we observed a marked group effect for percent time in SWD over the course of 90 days (F_{2,31} = 38.197, p < .001). The Games-Howell posthoc test revealed that rats in the ESX 4 month group spent significantly less percent time in SWD (0.242 ± .068 SEM) compared to untreated rats (0.769 ± .060 SEM, p < .001), although they do spend more
percent time in SWD than rats in the ESX continuous treatment group (0.020 ± .065 SEM, \( p = .003 \)).

Repeated-measurements MANOVA revealed a time effect (\( F_{5,24} = 5.335, \ p = .002 \)), indicating that percent time in SWD was altered depending on the timepoint of the EEG recording. This was expected based on previous observations [24]. Additionally, the repeated-measurements MANOVA revealed a time by group effect (\( F_{10,48} = 4.240, \ p < .001 \)), indicating that the pattern of change over time in percent time in SWD differed between treatment groups.

Percent time spent in SWD is the product of number of seizures and average seizure duration per unit of time. Notably, for seizure number there is a group effect (\( F_{2,31} = 42.818, \ p < .001 \)), a time effect (\( F_{5,24} = 6.128, \ p = .001 \)), and a time by group effect (\( F_{10,48} = 4.56, \ p < .001 \)); number of seizures per hour is 4.172 ± .978 SEM for ESX 4 months, compared to 11.819 ± .858 SEM for H\(_2\)O (\( p < .001 \)), and .393 ± .932 SEM for ESX continuous (\( p = .003 \)). For seizure duration, there is no significant group effect, time effect, or time by group effect.

ESX levels drawn from blood prior to perfusion of ESX continuous animals showed an average of 52.3 ± 4.0 SEM µg/ml (Data not shown).
Figure 4. Effect of early ESX treatment on seizure activity. Percent time in SWD (A), number of seizures per hour (B), and mean seizure duration (C) is shown based on analysis of EEG prior to ESX withdrawal (-1 day) as well as 1, 14, 30, 60, and 90 days after ESX withdrawal in ESX 4 month rats (▲) and age-matched H₂O (■) and ESX continuous (X) groups. Data is plotted as mean ± SEM. Repeated-measurements MANOVA with Wilks’ Lambda multivariate analysis, followed by Games-Howell posthoc analyses when appropriate revealed significant group, time, and time by group effects for percent time in SWD as well as number of seizures per hour (p < .05), but no significant group, time, or time by group effects for duration of SWD.
Total power spectrum analyses were also run for SWD and usable time for each experimental group pooled over timepoints 1 day through 90 days (Fig. 5A-F). Difference in power between groups for each frequency band was then calculated and Pearson correlation analysis was performed to compare each resulting curve to the seizure curve in H₂O rats. For the difference between H₂O and ESX 4 month rats (Fig. 5G), Pearson correlation equals 0.955, 2-tailed significance < .000000001, suggesting highly significant correlation. For the difference between H₂O and ESX continuous rats (data not shown), Pearson correlation equals 0.795, 2-tailed significance < .0001, also suggesting significant correlation. For the difference between ESX 4 month and ESX continuous rats (Fig. 5H), Pearson correlation equals -0.259, 2-tailed significance = .300, suggesting no significant correlation.
Figure 5. Power spectra. Total power spectra for seizures (A-C) and all usable time (D-F) by group: H2O (top row), ESX 4 month (middle row), and ESX continuous (bottom row). Data is plotted as mean ± SEM. (G) Difference between all usable power spectrum for H2O and ESX 4 month rats. Pearson correlation analysis of this curve with the seizure curve shown in (A) equals 0.955, 2-tailed significance < .00000001. (H) Difference between all usable power spectrum for ESX 4 month and ESX continuous rats. Pearson correlation analysis of this curve with the seizure curve shown in (A) was not significant. For (G) and (H), correlation was considered significant at the .01 level (2-tailed).
Discussion

This study demonstrates that early seizure blockade prevents the abnormal expression of ion channels Nav1.1, Nav1.6, and HCN1 in WAG/Rij rat cortex and decreases seizure activity even after antiabsence medication is discontinued. The combination of these data suggest that seizures may beget further seizures by altering levels of ion channels, and by producing other cellular and molecular changes, which then lead to increased membrane excitability and hence the propensity for seizure activity.

A number of questions result from this discovery. Although the alteration of ion channels after treatment with ESX suggests that this may be the mechanism by which long-term seizure activity is altered, we have not yet shown that ion channel levels remain altered after ESX is stopped, or that ion channel levels are directly related to seizure frequency or duration. Further experiments to elucidate this question could involve early measurement of cortical ion channel expression in rats that were sacrificed after the 90-day timepoint, and correlation of these levels with seizure activity prior to sacrifice.

Our data show that treatment with ESX alters ion channel levels specifically in the cortex, and that early temporary ESX alters long-term seizure frequency but not duration. Previous experiments have shown that seizures are initiated in WAG/Rij cortex, but thalamocortical networks are necessary for the maintenance of seizure activity [38]. Taken in combination, this information suggests the possibility that alterations in cortical ion channels may contribute to seizure initiation and hence frequency. It would be interesting, therefore, to measure ion channel expression in the thalamus of our rats;
one might hypothesize that since seizure duration was not altered in our rats, ion channel
central increases in HCN1 which persist through adulthood in WAG/Rij
rats [49, 50, 52], epileptogenesis is associated with thalamic increases in HCN1 which
are later followed by adaptive changes in Ih which serve to terminate synchronous
network activity in GAERS rats [51, 53].

Along these lines, the specific action of the antiabsence drug should be noted.
Blockade of thalamic T-type Ca^{2+} channels is often cited as the most likely mechanism of
ESX activity [32], although a reduction of nonactivating Na^{+} current and Ca^{2+}-activated
K^{+} current in cortical and thalamic neurons likely contributes as well [33]. It would
therefore be relevant to study the effects of other antiabsence drugs on ion channels and
long-term seizure activity. For example, expanding on our hypotheses regarding cortical
effects on seizure frequency and corticothalamic effects on seizure duration, one could
consider that medication acting predominantly on the thalamus would only limit seizure
activity and channel alterations, whereas perhaps a medication with stronger cortical
effects would be more likely to fully prevent seizure onset and hence channel alterations.

Nav1.1, Nav1.6 and HCN1 are likely not the only channels involved in seizure
generation and propagation. For example, Ca^{2+} channel mutations likely underlies the
expression of absence in mice [21, 54] and has been implicated for some forms of human
absence epilepsy [21]. Presynaptic Ca_{v}2.1 channels are upregulated in the rostral
reticular thalamic nucleus of 6-month-old WAG/Rij rats when compared to
presymptomatic (3-month-old) WAG/Rij rats and age-matched non-epileptic ACI rats
[55]. The TASK4 K^{+} channel has also shown positive linkage to human absence
epilepsy, and may contribute to absence epilepsy in GAERS rats as well [56]. Additionally, the metabotropic glutamate mGlu2/3 receptor has been shown to be upregulated in the somatosensory cortex, ventrobasal thalamic nuclei, and hippocampus of 6-month-old WAG/Rij rats compared to nonepileptic ACI and 2-month-old pre-epileptic WAG/Rij rats [57]. GABA_B receptors have been implicated in determining neocortical excitability in WAG/Rij rats as well: mRNA levels for GABA_B(1) subunits are diminished, these GABA_B(1) subunits fail to localize to the distal dendrites of pyramidal cells, and paired-pulse depression is decreased in epileptic WAG/Rij neocortex compared to age-matched non-epileptic controls [58]. It would therefore be beneficial to study the effects of early temporary antiabsence treatment on some of these channels as well, to determine the role of these channels in begetting increased seizure activity. Specifically, channels exhibiting the strongest correlation with long-term seizure activity could be directly targeted for preventative treatment.

In addition to ion channels, other mechanisms that may be important for epileptogenesis should be studied as well. Recurrent seizures induced by various mechanisms during early development in rodents have been shown to result in aberrant mossy fiber sprouting, reduced neurogenesis, and morphological changes in dendritic spines [3]. Additionally, it has been hypothesized that interictal spikes result in post-injury epileptogenesis [59]. Experiments involving the prevention of these changes, either by seizure blockade, treatment of interictal spikes, or other mechanisms, will be helpful in determining which processes lead most strongly to epileptogenesis and therefore which processes should be targeted and blocked for the purpose of seizure prevention.
The power spectrum analysis of our data raises interesting questions as well. The difference between the power spectrum for usable data for H2O rats and that of ESX 4 month rats correlates closely with the power spectrum of seizures in an untreated H2O rat. This correlation provides an additional objective measure of the increased seizure activity in H2O rats compared to ESX 4 month rats, since it does not require identification of individual seizures by either humans or by computer. This data also suggests that quantification of power spectrum data may eventually be useful in determining the adequacy of preventative seizure treatments.

Interestingly, the comparison of usable data for ESX 4 month rats compared to ESX continuous rats showed a peak at 2-3 Hz with a second, smaller peak at 9-10 Hz, which did not correlate significantly with the seizure power spectra. One possible explanation for this is that ESX treatment at various timepoints may prevent synchronous activity in a variety of frequency bands, including but not limited to that of seizures.

The generalizability of our absence seizure data to other types of seizures is another important issue. It has been suggested that absence epilepsy is related to a predominance of inhibitory activity, whereas generalized convulsive and focal seizures are characterized by an excess of excitatory activity [12, 60]. This may explain why anticonvulsant drugs such as carbamazepine and diphenylhydantoin have no effect or even worsen absence seizure activity [11, 30]. As described in the introduction, many seizures-beget-seizures models involve experimental induction of convulsive seizures or status epilepticus, whereas our model involves prevention of spontaneous non-convulsive seizures. It is possible that the excessive inhibitory activity in absence seizures actually
counters the tendency towards other types of excitatory brain activity. For example, studies show that WAG/Rij rats are more resistant to amygdala kindling than Wistar rats [60], a kindling-resistant subpopulation of WAG/Rij rats shows significantly longer SWD durations compared with kindled WAG/Rij rats [60], and that GAERS rats are fully resistant to kindling [13, 61]. Further studies would therefore be necessary to determine the effects of early absence seizure blockade on later susceptibility to other seizure types.

Along these lines, an interesting angle for our study comes from data suggesting that seizures are sensitive to environmental manipulations. In one study, neonatal handling and maternal deprivation led to a decrease in absence seizures later in life as well as an increase in HCN1 protein and Ih [50]. On the other hand, rats exposed to neonatal isolation in addition to status epilepticus induced by lithium-pilocarpine at P10 had prolonged seizure duration and increased seizure susceptibility compared to non-isolated rats with induced status epilepticus [62]. These thought-provoking studies highlight the necessary balance between excitation and inhibition for normal development of the nervous system as well as the far-reaching effects of early environmental stimulation versus neglect on brain function. Additionally, these studies suggest that the developing nervous system may be more sensitive to the effect of seizures, which may alter normal developmental expression patterns of ion channels and other molecules that regulate excitation and inhibition [2, 3].

Clinically, it is especially important to recognize the magnitude of the effect of both pharmacologic and environmental changes on nervous system development. Our study may indicate that early treatment, perhaps even prior to the onset of clinical seizure activity for known genetic cases of absence epilepsy, would be beneficial in reducing
later epileptic activity. However, it is important to note that whereas rats have increasing seizures with age [24], 70% of human absence epilepsy spontaneously remits around adolescence; the remaining 30% persists into adulthood and often coexists with other types of seizure activity [21]. The variability in the clinical outcome is likely related to the underlying molecular basis and type of absence epilepsy [21, 35, 36]. Whereas brain maturity occurs at P25-P35 in rodents (4-14 days after the start of ESX in our experiments), brain maturity occurs post-puberty in humans [4]. Further experiments involving earlier AED treatment in rodents might therefore help further elucidate the time course of alterations in ion channels and epileptogenicity. Additionally, the effects of very early ESX treatment in humans is not known, although ESX exposure in utero has been associated with neonatal hemorrhage due to a deficiency of vitamin K dependent clotting factors [63]. Further investigation of these effects would therefore be necessary prior to recommending more aggressive early treatment for genetic absence epilepsy in humans. Additionally, alternative agents should be explored where the effect may be related to the molecular basis of the absence epilepsy in patients.

Overall, these data provide exciting new insights into the world of seizure development. In the future, such information will likely prove useful in guiding the timing and specificity of treatment and perhaps even the prevention of childhood epilepsy.
References


