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Preliminary Behavioral, Biochemical and Neuropathological Characterization of a New Epilepsy Mutant (Poster)

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Preliminary Behavioral, Biochemical and Neuropathological Characterization of a New Epilepsy Mouse Mutant

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ABSTRACT

Background: Studies in epilepsy have focused primarily upon younger patient populations, with fewer papers published on adult-onset forms of the disease. We are studying a genetic mouse model representative of adult-onset epilepsy. The goal of the larger project is to identify the gene(s) that is mutated in this unique mouse strain. Three hypotheses were tested: i) behaviorally, a proxy for seizure phenotype can be determined earlier in the life cycle, ii) biochemically, a microdeletion found before the promoter region of the ROBO gene in chromosome 16 is affecting its expression in seizure mice, and iii) there will be pathological changes in seizure mice brains.
Methods: A prospective cohort of mice from the CCOR15155 inbred strain from which the seizure mutation(s) arose, and first generation mice created by mating CCOR15155 seizure mice with FVB/NJ inbred strains were studied. Seizure threshold was measured using electroshock testing. Western blotting studied the ROBO1 protein. Nissl and Timm staining assessed the morphology of major neuroanatomical structures.
Results: Measurement of electroshock seizure threshold in a series of F1 mice between 6-8 months of age revealed a single cluster of responses with no evidence for a bimodal distribution. Nissl and Timm staining did not reveal gross brain structural abnormalities. Western blot analysis was unsuccessful in testing the hypothesis as described.
Conclusions: Our work provides insight into several biological characteristics of this new genetic mouse model of adult-onset epilepsy and forms a foundation upon which subsequent studies can build to ultimately identify the underlying gene mutation(s).

INTRODUCTION

Epilepsy is a common heterogeneous neurological disease characterized by spontaneously recurrent seizures and a number of associated behavioral co-morbidities.[1] Numerous anti-epilepsy drugs and other alternative therapies are available to treat patients with epilepsy; however, up to 30% of patients do not obtain satisfactory clinical benefit emphasizing the need for better understanding of pathophysiology.[2] Genetic studies using both animal models and patient DNA have been fruitful in providing clues to underlying pathogenic mechanisms in epilepsy. Genetic mouse models in particular have been integral in helping to shape the understanding of epilepsy as an "ion channelopathy" of the brain and complement human studies in which ion channel gene mutations are documented as pathogenic for epilepsy.[3,4,5] The three classic seizure mutant strains recognized for their important insight into human epilepsy pathogenesis are stargazer, tottering and lethargic.[6,7,8]
The age at which seizures first appear in humans with epilepsy is highly variable and dependent on many factors, especially epilepsy subtype. Overall, a large fraction of cases begin early in life, and this includes the vast majority of known genetic causes of epilepsy. However, it is becoming more clear that risk for developing epilepsy follows a U-shaped curve and begins to increase after the age of 55, and that up to half of these cases are idiopathic in nature.[9,10,11] The genetic factors that underlie the late onset of common, idiopathic forms of generalized epilepsy are mostly unknown and represent a critical gap in our knowledge with regard to understanding and treating epilepsy in the largest growing segment of the population.[13] The rare model studied in this project represents a viable means to begin to fill this gap.

AGE OF SEIZURE ONSET

Females		Males	
Mouse #	First seizure (mo)	Mouse #	First seizure (mo)
96	18	54	7
160	12	97	5
161	12	191	6
		267	8
		367	5
		397	10
		413	8
		501	6
		502	7
Mean ± SD	14.0 + 3.5*	Mean ± SD	6.9 + 1.6

Table 1. Seizure onset in female and male mice. This gender difference is statistically significant (p=0.036; exact 2-sided Wilcoxon rank sum test).

DISTRIBUTION OF SEIZURES IN F2 MICE

	Females		Males	
	Seizure	No seizure	Seizure	No seizure
F1	4	11	5	10
F2	7	32	20	41

Table 2. Distribution of seizures among F1 and F2 intercross mice. Mice were gently handled and systematically observed for 20 minutes each week starting at the time of weaning (~1 month). Expression of behavioral seizures was recorded by a trained observer. The age of onset for female F2 mice was 6.5 ± 2.4 months; for males it was 6.9 ± 3.3 months. Age of onset in F2 generation female mice is notably lower than in parental OR15155 female mice. The ratio of seizure to non-seizure F2 females (1:4.5) is not significantly different than the ratio for F2 males (1:2) (P = 0.1, Fisher's Exact t-test).

METHODS

Overall. A cohort of mice was bred to try to identify the pattern of inheritance of the spontaneous seizure trait as well as genetically map the putative mutation(s). Electroconvulsive shock was used to measure the seizure threshold of each mouse. Brains were analyzed with Nissl stain for gross pathology and Timm stain to assess nerve terminal sprouting in the hippocampus.
Mice. This project is based on the Collaborative Cross (CC) strain OR15155. A single mouse from this strain (male #54) was originally identified as having spontaneous seizures and we were able to breed him to generate the family that is shown in Figure 1. In order to begin mapping the chromosome location of the gene(s) underlying the seizure phenotype, males #54 and #97 were mated with female mice from another inbred strain FVB/NJ. First filial (F1) generation mice were cross-bred to generate F2 mice. Mice were studied for seizure phenotype by daily observation
Genotyping. Tail DNA from a subset of mice was used for SNP genotyping on the GigaMUGA platform, a 143,000-probe array based on the Illumina Infinium II technology. We have genotyped 11 mice from the Figure 1 pedigree. Six genotyped mice are known seizure mice: #367, #54, #97, #161, #191 and #267; and five genotyped mice have not been observed to exhibit spontaneous seizures #369, #15, #52, #107 and #215.
Western Blot Analysis. We developed an assay to assess the levels of ROBO protein in the brain of control mice. Blots were analyzed with NIH ImageJ which is developed specifically for densitometric analysis of images.
Electroshock Testing. Maximal electroshock seizure threshold (MEST) was conducted with F1 mice and parental OR15155 mice to test for the hypothesis that MEST will be lower in mice more likely to develop spontaneous seizures later on in life. Shocks were delivered via ear-clip electrodes. The current level at which mice first exhibit a generalized seizure is taken as the generalized electroshock seizure threshold (GEST). A maximal seizure is defined by bilateral tonic extension of hind limbs. The current level at which mice exhibit tonic hind limb extension is taken as the maximal electroshock seizure threshold (MEST).
Histopathology. Goals are to cut seizure mouse brains using the cryomicrotome to stain them using a Nissl stain (cresyl violet) and Timm stain to analyze them using high-powered microscopy.

PEDIGREE STRUCTURE

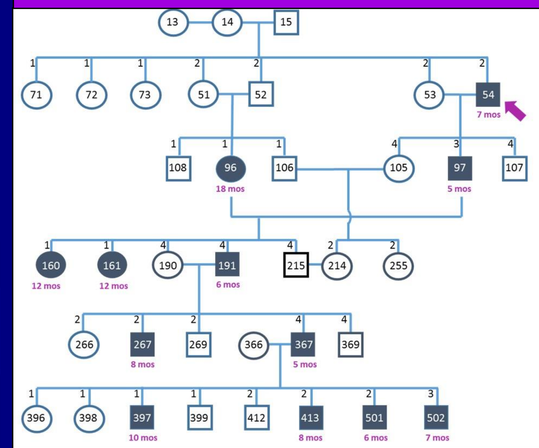

Figure 1. Pedigree diagram of the CCOR15155 seizure family. Circles represent females, squares represent males. Numbers inside shapes identify each mouse. Progenitors (females 13, 14, and male 15) were obtained from UNC. The "index case" is male 54 who first exhibited spontaneous generalized seizures at 7 months of age. The small numbers near the top of each vertical line indicate the litter number.

BEHAVIORAL SEIZURE PHENOTYPE

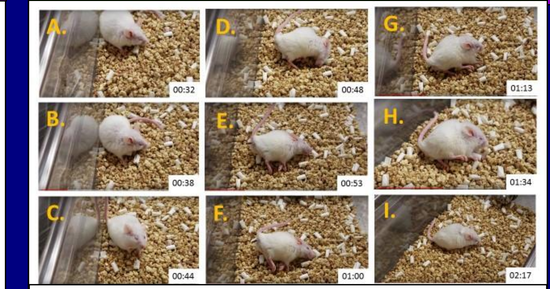

Figure 2. Time line of a representative spontaneous behavioral seizure expressed by male 191 (October 2017). The seizure begins with the mouse becoming immobile and exhibiting a facial grimace (A). Subtle head bobbing is observed several seconds later (B). This is followed by unilateral forepaw clonus (C) which becomes bilateral and then generalizes, with the mouse losing postural control and exhibiting dorsoflexion (D), as well as bilateral hind limb clonus (E and F). The mouse retains an abnormal posture in the immediate postical phase (G), and this persists for some time (H) before some locomotor activity is observed (I). It typically takes 5-10 minutes following a seizure before mice exhibit normal home cage behavior.

SEIZURE THRESHOLD IN F1 MICE

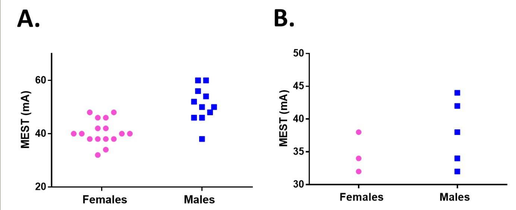

Figure 3. Maximal electroshock seizure threshold (MEST) in F1 (A) and parental (B) OR15155 mice. We tested the hypothesis that the putative seizure mutation(s) lowers MEST, and that this effect can be detected prior to the onset of spontaneous seizures. MEST was measured using a ramp-up method (daily shock via earclip electrodes and step-wise current increase) when mice were 4-6 months of age, at a time when none had been observed to have spontaneous seizures. Both females (N=16) and males (N=11) were studied. A statistically significant gender difference was documented ($P < 0.01$ Student t-test). Several outliers were detected (A). Measurement of MEST in several older parental OR15155 mice (12-14 months of age) revealed a lower range of values compared to the younger F1 mice (B) for both genders. The female with the lowest MEST and the 2 males with the lowest MEST are known to express spontaneous seizures.

Western Blot Analysis

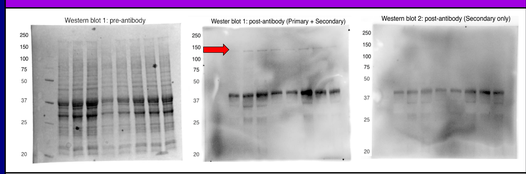

Figure 4. Western blot assay to measure ROBO1 in brain frontal cortex from naïve FVB/NJ mice. As seen in Figure 4, Molecular weights are in kD. On left, Western blot 1 shows eight lanes with equal amounts of loaded mouse brain tissue. Center, Western blot is shown with the addition of both primary ROBO1 antibody and secondary antibody after the usage of blocking agent. A band is prominent at ~150kD at the red arrow. On right, Western blot 2 depicts the use of secondary antibody without primary antibody. Notice the disappearance of what we presume is ROBO1 at ~150kD on the left.

GENOTYPING RESULTS

Table 3A: Chromosome 16

Chr 16			Seizure Mice						Non-seizure Mice					
Marker	Start pos	End pos	#54	#97	#161	#191	#267	#367	#15	#52	#107	#215	#369
JAX00424398	72224700	72224945	C	C	C	C	C	C	C	C	C	C	C
UNC27091880	72231017	72231262	A	A	A	A	A	A	A	A	A	A	A
JAX00424419	72298631	72298876	N	A	A	N	A	N	A	A	A	A	A
UNC27093387	72307443	72307688	N	A	A	N	A	N	A	A	A	A	A
UNC27093975	72344349	72344594	N	G	G	N	G	N	G	G	G	G	G
UNCJPD006434	72390734	72390979	N	C	C	N	C	N	C	C	C	C	C
UNC27095048	72414153	72414398	N	C	C	N	C	N	C	C	C	C	C
JAX00424449	72446634	72446879	N	C	C	N	C	N	C	C	C	C	C
UNC27096197	72475719	72475964	N	C	C	N	C	N	C	C	C	C	C
UNCHS043101	72552109	72552354	N	C	C	N	C	N	C	C	C	C	C
UNC27098619	72611972	72612217	T	T	T	T	T	T	T	T	T	T	T
UNCJPD008732	72615447	72615692	G	G	G	G	G	G	G	G	G	G	G

Table 3B: Chromosome 5

Chr 5			Seizure Mice						Non-seizure Mice					
Marker	Start pos	End pos	#54	#97	#161	#191	#267	#367	#15	#52	#107	#215	#369
UNCJPD008627	4130472	4130472	G	G	G	G	G	G	G	G	G	G	G
JAX00572406	4193739	4193739	A	A	A	A	A	A	A	A	A	A	A
UNC8549503	4246073	4246073	H	H	H	H	A	H	C	C	C	H	C
JAX00572431	4265029	4265029	H	H	H	H	G	H	A	A	A	H	A
UNCHS013437	4279109	4279109	H	H	H	H	A	H	G	G	G	H	G
UNCHS013438	4282460	4282460	H	H	H	H	A	H	G	G	G	H	G
UNCHS013439	4326862	4326862	H	H	H	H	G	H	A	A	A	H	A
UNCHS013440	4330346	4330346	H	H	H	H	T	H	C	C	C	H	C
UNC8551116	4356749	4356749	H	H	H	H	G	H	A	A	A	H	A
CEAJAX00126552	4364099	4364099	C	C	C	C	C	C	C	C	C	C	C
UNCJPD008200	4385413	4385413	C	C	C	C	C	C	C	C	C	C	C
UNC8551996	4428504	4428504	G	G	G	G	G	G	G	G	G	G	G

Table 3. Chr. 16 (2A) and 5 (2B) SNP markers in seizure and non-seizure mice. Inspection of the genome data set revealed that most loci from the 8 CC progenitor strains are fixed in a homozygous state. Sporadic loci across the genome are heterozygous; however, there are several large unfixed segments including the proximal region of chr. 5 (0-45Mb). We also detected a ~350 Kb region on chr. 16 that appears to be deleted in 3 of 6 seizure mice, but not in any of the non-seizure mice. The deletion is 42 kb proximal to the promoter of the roundabout guidance receptor 1 gene (ROBO1). It is denoted in the table above as "N" for a continuous set of markers distal to base pair (bp) position 72231262 and proximal to bp position 72611972, suggesting that the region between these markers is deleted. The chr. 5 data show a region of heterozygosity in the seizure mice which is fixed in all but one non-seizure mouse.

Histopathology

Seizure mouse (#97, 191, 267) and non-seizure mouse (#52, 191, 215) brains were successfully fixed in paraformaldehyde and cut in 10 micrometer slices using the cryomicrotome. The olfactory bulb and the cerebellum were removed before taking slides from every mouse except #267, whose cerebellum was left intact. Eighty slides were obtained from each animal. Twenty-five percent of each brain was stained using the Nissl stain (using cresyl violet) on every fourth slide. Every eighth slide was stained using Timm Stain. Results of representative Nissl and Timm stains for seizure and non-seizure mouse brains can be seen in figures 5-7.

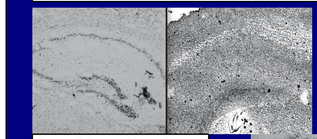

Figure 5 Above. Mouse 52 (non-seizure mouse) hippocampus with Nissl stain on left and Timm stain on right. Slide #17 was used for the Nissl stain and slide #18 was used for the Timm stain.

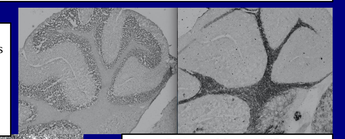

Figure 6 Below. Mouse #97 (seizure mouse) hippocampus with Nissl stain on left and Timm Stain on right. Slide #37 was used for the Nissl stain and slide #34 was used for the Timm Stain.

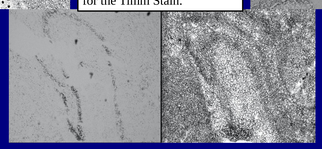

Figure 7 Above. Mouse #267 (seizure mouse) cerebellum with Nissl stain on left and Timm Stain stain on right. Slide #7 was used for the Nissl and #8 was used for the Timm Stain.

SUMMARY AND CONCLUSIONS

Our laboratory has discovered a unique adult-onset mouse model of genetic epilepsy. It is unique because previous mouse models of epilepsy begin in the neonatal or juvenile period. We plan to use this model to map and identify the causative gene mutation(s). Results presented in this Capstone project provide a preliminary characterization of the seizure phenotype, a basic histological analysis of the mouse brains, and the genetic mode of inheritance as well as information on genome-wide analysis of single nucleotide polymorphisms that may help to shed light on structural genetic alterations in these seizure mice. Future directions of this work should take into consideration genetic mapping and a larger number of histological subjects for analysis. Overall, our work provides initial insight into several biological characteristics of this new genetic mouse model of adult-onset epilepsy and forms a foundation upon which subsequent studies can build to ultimately identify the underlying gene mutation(s).

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