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Pharmacogenetics of Antiepileptic Drug Efficacy in Childhood Absence Epilepsy

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Abstract

Objective—Determine if common polymorphisms in CACNA1G, CACNA1H, CACNA1I, and ABCB1 are associated with differential short term seizure outcome in Childhood Absence Epilepsy (CAE).

Methods—446CAEchildren in a randomized double blind trial of ethosuximide, lamotrigine and valproate had short term seizure outcome determined. Associations between polymorphisms (minor allele frequency ≥15%)in four genes andseizure outcomes were assessed. *In vitro* electrophysiology on transfected CACNA1H channels determined impact of one variant on T-type calcium channel responsiveness to ethosuximide.

Results—80% (357/446) of subjects had informative short term seizure status (242 seizure free, 115 not seizure free). In ethosuximide subjects, two polymorphisms (CACNA1Hrs61734410/ P640L, CACNA1I rs3747178) appeared more commonly among not seizure free participants (p=0.011; Odds Ratio(OR):2.63; 95% Confidence limits(CL):1.25–5.56, p=0.026; OR:2.38; 95%CL:1.11–5.00). In lamotrigine subjects, one ABCB1 missense polymorphism (rs2032582/

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AUTHOR CONTRIBUTIONS

Study concept and design: T.A.G., K.H., V.O., M.K, P.O.C., A.C., D.D., D.G.H., S.S., and G. G. Data acquisition and analysis: T.A.G., K.H., V.O., M.K, L.J.M., P.O.C., A.C., D.D., D.G.H., S.S., and G. G.; Drafting manuscript and figures: T.A.G., K.H., L.J.M.

CONFLICTS OF INTEREST

Dr. Glauser, Dr. Holland, Ms. O'Brien, Dr. Keddache, Dr. Martin, Dr. Clark, Dr. Cnaan, Dr. Dlugos, Dr. Hirtz, Dr. Shinnar and Dr. Grabowskihave nothing to disclose.

S893A, p=0.015; OR:2.22; 95%CL:1.16–4.17) was more common in not seizure free participants, two CACNA1H polymorphisms (rs2753326, rs2753325) were more common in seizure free participants (p=0.038; OR:0.52; 95%CL:0.28–0.96). In valproate subjects, no common polymorphisms associated with seizure status. *In vitro* electrophysiological studies showed no effect of the P640L polymorphism on channel physiology in ethosuximide's absence. Ethosuximide's effect on rate of decay of $Ca_V3.2$was significantly less for P640L channel compared to wild type channel.

Interpretation—Four T-type calcium channel variants and one ABCB1 transporter variant were associated with differential drug response in CAE. The *in vivo*P640L variant's ethosuximide effect was confirmed by *in vitro* electrophysiological studies. This suggests genetic variation plays a role in differential CAE drug response.

INTRODUCTION

Childhood Absence Epilepsy (CAE), the most common pediatric epilepsy syndrome[1], is characterized by the classical triad of frequent absence seizures, normal cognition, and generalized 3Hz spike-wave discharges on EEG[2]. Until recently, CAE was considered a "benign" epilepsy syndrome with easily controlled seizures[3, 4]. An NIH sponsored double blind, randomized comparative trial of 446 children with newly diagnosed CAE found that the overall short term effectiveness of the three most commonly used antiepileptic medications was 47% with significant variability between the three meds (ethosuximide 53%, lamotrigine 29%, and valproic acid 58%)[5, 6].

The hallmark seizure in CAE, the absence seizure, results from disruptions in thalamocortical pathways specifically involving T-type calcium channels[7, 8]. Three genes (CACNA1G, CACNA1H, and CACNA1I) code for the T-type calcium channels involved in these disrupted thalamocortical pathways[8]. Antiepileptic medications effective against absence seizures have been proposed to exert their effect at these channels. All three drugs involved in the NIH CAE trial exert action at these channels[7, 9]. Secondly, drug response may be affected by the action of P-glycoprotein, a drug efflux transporter located in the intestine and at the membrane of brain capillary endothelial cells[10]. Evidence in animals and humans indicate that lamotrigine is a definite substrate[9–15] for P-glycoprotein, valproic acid is a possible substrate with conflicting evidence[9–11, 13–17], and ethosuximide is clearly not a substrate[18]. P-glycoprotein is coded for by the ABCB1 transporter gene[10]. Thus, variants in the three genes coding for T-type calcium channels and the ABCB1 transporter gene could impact drug responsiveness in Childhood Absence Epilepsy.

This study's primary a-priori hypothesis was that common polymorphisms in either the three T type calcium channel genes coding for CAE drug targets (CACNA1G, CACNA1H, and CACNA1I) or the ABCB1 gene coding for a drug transporter would be associated with differential short term seizure free rates in the NIH CAE clinical trial cohort.

SUBJECTS AND METHODS

Subject population

Key inclusion criteria included a clinical diagnosis of CAE consistent with the International League Against Epilepsy's published criteria, an EEG demonstrating generalized 2.7–5 Hz spike waves with normal background and at least one burst lasting ≥ 3 seconds, and age 2.5–13 years at study entry. Key exclusion criteria included treatment for CAE with antiepileptic drugs for >7 days prior to study drug randomization and a history of major psychiatric disease or autism spectrum disorder. Full details of the eligibility criteria for this randomized double blind comparative effectiveness trial have been previously reported[5, 6].

The Institutional Review Boards of all 32 sites along with the clinical and data coordinating centers approved the study. Written parental informed consent and, when appropriate, child assent was obtained from all subjects. The trial was conducted under a Food and Drug Administration–approved IND (69,423) and was listed at http://clinicaltrials.gov/under ClinicalTrials.govidentifier: NCT00088452.

Study Design

The study was a double blind, parallel group, randomized, comparative effectiveness study with partial crossover to open label (at treatment failure only) with subsequent follow-up. This report describes only results from the initial double blind therapy portion. The details of the baseline and subsequent intervention evaluations up to and including the primary outcome at the week 16–20 visit have been previously reported in detail[5, 6]. In general, subjects continued receiving double blind study medication as long as they did not meet any treatment failure criteria.

The short term seizure status was classified as seizure free if a subject achieved i) seizure freedom on both EEG and by clinical history at the week 16–20 visit or ii) discontinued prior to the week 16–20 visit and both the EEG and clinical history at the discontinuation visit showed no seizures. Participants' short term seizure status was classified as not seizure free if i) their EEG showed electrographic seizures or their clinical history documented ongoing clinical absence seizures at the week 16–20 visit or ii) discontinued from the study prior to the week 16–20 visit due to report of worsening of absence seizures or development of a new type of seizure (focal or tonic clonic). Participants' short term seizure status was classified as uninformative if they either i) discontinued from the double blind study prior to the week 16–20 visit without an EEG at the discontinuation visit and the reason for discontinuation was either adverse events and/or patient/family desire to withdraw but not seizures or ii) discontinued from the double blind study prior to the week 16–20 visit with an EEG at the discontinuation visit showing ongoing absence seizure (or unclear if still having absence seizures) but no clinical report of either worsening of absence seizures or development of a new type of seizure[5, 6].

Sample collection and DNA extraction

Blood samples for genetic analysis were obtained at the first study visit after initiation of double blind study medication, shipped by overnight express mail and stored at room

temperature until DNA extraction. DNA was extracted using the 5-prime PerfectPure DNA Blood kit (formerly Gentra system's Puregene) and resuspended in 1.5mL of TE. After an overnight rehydration 1/3 of the DNA (500ul) was aliquoted, sheared by 10 passages through a 27 ½ gauge needle to reduce viscosity, quantitated using a Nanodrop spectrophotometer and diluted to a 25ng/ul working solution in 10mM Tris. The remainder of the DNA was stored at −20°C. DNA samples were aliquoted in deep 384 well plates on a Tecan Freedom Evo 200 robotic laboratory workstation according to a predefined layout containing 12 positive and 12 negative controls in different location for each plate.

Comparative PCR sequencing

The complete coding sequence of the four candidate genes (CACNA1G, CACNA1H, CACNA1I and ABCB1) was determined by comparative PCR sequencing. PCR primers were designed using the online tool ExonPrimer (http://ihg2.helmholtz-muenchen.de/ihg/ExonPrimer.html) to amplify at least 12 bp of each exon/intron boundary and each coding exon. Forward and reverse primers were tailed at their 5′ end with the sequence of the M13(−21) and M13-reverse universal primers respectively in order to obtain PCR products that can be sequenced directly with these universal primers. Primers and nucleotides were removed from PCR reactions prior to sequencing by enzymatic treatment with USB Corp's ExoSAP-IT, a mixture of Shrimp Alkaline Phosphatase and Exonuclease I. Both PCRs and Sequencing were conducted in 384 well format using a 384 well TecanTeMo as a replicating tool to preserve the original layout of the DNA plate with positive and negative controls at all steps of the data generation. After precipitation to remove the unincorporated nucleotides, the sequencing reactions were resolved on an Applied Biosystem 3730xl equipped with a 36cm capillary array.

Sequence analysis and data management

Variations in the sequence files were analyzed using SoftGenetics' Mutation Surveyor software which compares the shape of the electropherogram with a reference electropherogram visually inspected for the absence of variation from the consensus sequence in each amplicon. A web based sequence management system was created to organize the sequence files and the single nucleotide polymorphism calling files output from Mutation surveyor. The system kept track of which regions of the genes had been covered with reliable sequence data, what variations were observed and output which combinations of samples and amplicons were missing so they could be repeated.

Mutagenesis, Cell Culture, and Transfection

To investigate the physiological impact of the CACNA1H rs61734410 mutation (P640L) in relation to ethosuximide's effects, HEK-293 cells were transiently transfected with constructs containing the human CACNA1H and green fluorescent protein (GFP) channel, $Ca_V3.2$ (wild type) or a construct encoding $Ca_V3.2$ with the rs61734410 mutation (P640L) and GFP. Cells were grown in Dulbecco modified Eagle's medium supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 50 units/ml penicillin, and 50 μg/ml streptomycin in a humidified 5% CO_2 atmosphere at 37ºC. Expression of $Ca_V3.2$ was achieved using transient transfection with the reagent Lipofectamine2000 (Life Technologies; 5 μg total plasmid DNA was transfected). Mutagenesis was applied by using QuikChange II XL Site-

Directed Mutagenesis Kit (Agilent Technologiess). The wild-type CACNA1H construct was provided by Dr. Edward Perez-Reyes.

Electrophysiology

The methods used for these studies were based on those of Gomora and colleagues[19]. Calcium channel currents were recorded at room temperature 48–72 h after transfection. For electrophysiological recordings the intracellular solution contained 135 mMCsCl, 10 mM EGTA, 4 mM Mg-ATP, 0.3 mM Na_3GTP, and 10 mM HEPES, pH adjusted to 7.3 with CsOH; osmolality: 305 and the extracellular solution contained 5 mM $CaCl_2$, 130 mMtetraethylammonium (TEA) chloride, 2 mM 4-aminopyridine, 1 mM $MgCl_2$, 10 mM D-Glucose and 10 mM HEPES, pH adjusted to 7.4 with TEA-OH; osmolality: 312. Ethosuximide was freshly dissolved in external solution.

Whole cell currents were recorded using the ruptured patch method using an Axopatch 200B amplifier, Digidata 1200 A/D converter, and pCLAMP 10.1 software (Axon Instruments)[20]. Patch pipettes were fabricated from TW-150F-6 glass capillaries (WPI) with a P-97 multistage Flaming-Brown micropipette puller (Sutter Instruments). Once filled with internal solution the pipette resistance was typically 5 to 6 MΩ. Series resistance was compensated 90%, with 70% prediction. Leak currents were subtracted by using an online P/4 procedure. All whole-cell currents were low-pass Bessel filtered at 5 kHz and digitized at 50 kHz. Cell capacitance ranged from ~25–50 pF.

The recording chamber was a RC-26 (Warner Instrument), which has a volume of 0.17 ml. Each test solution was perfused at 3 to 6 ml/min for 1–3 minutes before testing at a specific concentration. For concentration-response experiments currents were measured by stepping the membrane potential from a resting potential of −110 mV to −30 mV for 300 mS. Cells were washed with extracellular solution between each concentration and the degree of inhibition was calculated using the average responses to ethosuximide measured before and after exposure to various concentrations ethosuximide concentrations. Up to 3 different concentrations were used in each cell using progressively higher concentrations. Some run-down of current was observed. Therefore, stability of currents was monitored in all experiments, and effects of ethosuximide on peak currents were analyzed only when the run-down rate was less than 1%/min. This limited the ability to measure effects of ethosuximide on the peak current at concentrations below < 3 mM.

Statistical Analysis

This study's primary outcome was the short term seizure status for subjects in each of the three study medication arms. All analyses were prespecified in the original protocol. Baseline characteristics were compared by means of either an exact chi-square test for categorical variables or a t-test for continuous variables.

Hardy Weinberg Equilibrium (HWE) test was performed for identified variants. Variants that exhibited marked deviations from HWE ($p \leq 0.0001$) were excluded from further analyses. To test for the association between seizure free status and the genetic variants, logistic regression was used coding the genetic variants as additive (the number of minor alleles

present) including race and age as covariates. Analyses were performed separately by medication.

For the primary analyses, it was pre-specified that only polymorphisms with a minor allele frequency ≥ 15% were included. This was chosen to maximize power for clinically relevant effect sizes and common enough to have the potential to change clinical practice. It was the study investigators' consensus opinion that this frequency would be common enough to change their clinical practice and lead to ordering genetic testing if a positive result was identified. Exploratory analyses were performed for polymorphisms with minor allele frequencies ≥ 5% but < 15%.

Analyses were carried out using JMP Genomics 6.1 (SAS Institute, Cary NC). As multiple variants and subgroups were considered, there was an increased risk of family wise error. However, given the unique sample design enabling us to evaluate the genetic association with response to medication in a clinical trial, correction for multiple testing or replication of the findings would not have been possible while retaining reasonable power. Rather, a p-value of ≤ 0.05 was considered to indicate statistical significance. However, to ensure findings were not false positives, we sought to support these findings with independent evidence, such as biologic studies, when possible. Associations which reach statistical significance without secondary support should be interpreted with caution.

The clinical trial's study cohort sample size of 446 was originally designed to detect a 20% difference in the clinical trial's primary outcome (freedom from failure rate – a measure of effectiveness rather than only efficacy) among the three medications at the week 16–20 visit with 80% power at a two-sided P value of 0.017 and one interim analysis. This study's overall and each study medication arm's sample size was determined by the original clinical trial[5, 6]. An independent data and safety monitoring board appointed by the National Institutes of Healthmonitored the trial.

Electrophysiological validation studies used parameters for current-voltage relationships, voltage dependence of activation and steady state inactivation detailed in the figure legends. Current-voltage and steady-state channel availability curves were fit with Boltzmann functions to determine the voltage for half-maximal channel activation or inactivation ($V_{1/2}$) and slope factor (k). Concentration–response relationships were fit with the Hill equation with the IC_{50} representing the concentration that produced half-maximal inhibition. Clampfit 10.2, Origin 8.1, and Excel 2010 software were used for the analyses. Values are reported as the mean ± S.E.M. Student's *t*-tests were used to compare differences between the channel variants and paired Student's *t*-tests were used when comparing the effects of ethosuximide within a channel variant. For electrophysiological testing, the Bonferroni correction was used to correct for multiple comparisons.

RESULTS

Patient population

Overall 446 children enrolled in the efficacy/effectiveness trial[5, 6] of which three did not have a DNA sample for analysis. A total of 242 children were classified as seizure free, 115

were classified as not seizure free, and 86 children had uninformative seizure status(Table 1). Thus, overall 80% (357/446) of the original cohort were included for pharmacogenetic analysis. There were no significant differences in demographics between either the three pharmacogenetic drug groups, between those included in the overall pharmacogenetic analysis versus those excluded (Table 1), and those seizure free in each treatment group versus those not seizure free (data not shown).

Polymorphism frequency

Sequencing identified 472 variants in the four target genes (ABCB1, CACNA1G, CACNA1H, and CACNA1I). Of these, 37 had frequencies of ≥ 5% or greater. Three of these variants exhibited marked deviations from Hardy Weinberg Equilibrium($p \leq 0.0001$) and thus were excluded from further analyses. All remaining 34 variants had missing rates less than 10% of the sample.

Overall, 22of these polymorphisms had minor allele frequency ≥ 15% in the overall PG cohort of 357 subjects. These polymorphisms were distributed across all four genes (ABCB1 n=4, CACNA1G n=3, CACNA1H n=10, and CACNA1I n=5). Fourteen polymorphisms were synonymous variants, six were missense variants with single cases of 3′-UTR variant and combined splice region variant/intron variant (Supplementary Table 1). Three of the missense variants were in the CACNA1H gene (rs1054645/R2071H, rs4984636/V664A, and rs61734410/P640L), two were in the CACNA1I gene (rs136853/I1005V, rs2294369/G1747R) and one was in the ABCB1 gene (rs2032582/A893S).

There were an additional 12 polymorphisms identified with minor allele frequency ≥ 5% but less than 15% (data not shown), mainly in CACNA1H (n=7) with some in ABCB1 (n=3) and single cases in each of the CACNA1G and CACNA1I genes.

Polymorphisms and seizure status

In the ethosuximide cohort analysis, the minor allele of two polymorphisms(CACNA1H rs61734410/P640L and CACNA1I rs3747178)were associated with increased rates of not seizure free outcomes. The missense variant rs61734410 (P640L) appeared more commonly in the not seizure free cohort compared to the seizure free cohort (73.9% versus 42.5%, OR = 2.63 (1.25 – 5.56), p=0.011). The CACNA1I polymorphism rs3747178 was also more common in the not seizure free cohort (60.9% vs 47.4%, OR 2.38 (1.11 – 5.00), p = 0.026, Table 2).

In the lamotrigine cohort analysis, one ABCB1 polymorphism (rs2032582, p=0.015) appeared more commonly in the not seizure free cohort compared to the seizure free cohort (Table 2). In contrast, two CACNA1H polymorphisms (rs2753326 and rs2753325) were significantly more common in the seizure free cohort. The frequency of the other polymorphisms with minor allele frequency ≥ 15% was not significantly different between lamotrigine seizure status groups.

In the valproic acid cohort analysis, no polymorphisms with minor allele frequency ≥ 15% were significantly different between seizure status groups.

In an exploratory analysis, no differences were seen in the frequency of the 12 polymorphisms with minor allele frequency ≥ 5% but less than 15% between seizure status groups in the ethosuximide or lamotrigine subgroups. One polymorphism of CACNA1H (rs2235634) in the valproic acid cohort appeared more commonly in the not seizure free cohort compared to the seizure free cohort (50.0% versus 18.6%, p =0.0008, Table 2).

Biophysical and biopharmacologic effects of the rs61734410 (P640L) polymorphism

In the absence of ethosuximide there were no significant differences in voltage dependence activation or inactivation between the wild type and P640L channels (Figure 1A and 1B; Supplementary Table 2) and those values were similar to reported by others[21]. The rate of inactivation (⊤) was faster in the P640L variant than the wild-type channel at the lowest membrane potential studied but not at other membrane potentials (Fig 1C).

The peak current was reduced by ethosuximide in a concentration-dependent manner both in the wild type and the P640L variant. There was no significant difference in the IC_{50} between the variants (57.5 ± 3.3 mM for wild type; 59.8 ± 2.8 mM for P640L) but in concentrations below the IC_{50} there was a tendency toward greater inhibition in $Ca_V3.2$ in the wild-type compared with theP640L variant. However these differences were not statistically significant when corrected for multiple comparisons and the concentrations studied were above those that are therapeutically relevant[19]

In contrast to the effects on peak currents, pronounced differences in the effect of ethosuximide between wild type and P640L were seen on the rate of decay of the $Ca_V3.2$ (Fig 1C) even at low ethosuximide concentrations. Ethosuximide-induced acceleration in the rate of decay (quantified by reduction in the rate of inactivation) of $Ca_V3.2$ was seen at even at the lowest concentration of ethosuximide studied (1 mM) in the wild-type channel but was not seen in the P640L variant until 10 mM (Fig 1D). There was a tendency toward greater ethosuximide-induced shifts in voltage dependence of activation and inactivation for P640L compared to wild type however this only approached statistical significance for voltage dependence of activation and only at high ethosuximide concentrations (Supplementary Table 2).

DISCUSSION

Identifying genetic predictors of antiepileptic drug response has been challenging due to both clinical and methodological obstacles[9, 22]. Prior epilepsy efficacy/effectiveness pharmacogenetic studies involved heterogeneous patient populations with variable medication histories and a variety of antiepileptic medications[22, 23]. Additionally, retrospective study designs increase the difficulty of accurately and reliably phenotyping efficacy and effectiveness. These methodologic issues resulted in either negative or conflicting positive efficacy and effectiveness findings with lack of confirmation in subsequent validation cohorts[24, 25]. As such, the greatest advances in epilepsy pharmacogenetics have occurred in studies involving severe cutaneous reactions because the target phenotype is reliable and verifiable, the medication responsible is clearer and the epilepsy duration, specific previous drug trials, and medication dosage are less impactful than the genetics[26].

This study's design addresses many of these previous studies' limitations. This study's patient population consisted of a homogenous recognizable common epilepsy syndrome (verified both at the site and by central reviewers) and all subjects were treatment naïve at study entry. The prospective design with randomization, double blinding, and titrations with pre-specified dosing adjustments based on clinical response minimized treatment bias between and within treatment arms. The pre-specified definition of seizure freedom based on objective, verifiable and reliable criteria was assessed both at the site and the coordinating center (with conflicts decided by a third reviewer)[5, 6].

Although double blind treatment failures were randomized to second monotherapy and subject to the same titration and assessments, this second therapy cohort was not considered a potential validation cohort due to differences in prior drug exposure and response. Insteadan *in vitro* electrophysiologic approach to variant confirmation was conducted for this study.

Overall, four common T-type calcium channel variants (one missense variant, three synonymous variants) and one transporter missense variant were associated with differential drug response. The presence of the CACNA1H missense variant (rs61734410/P640L) was more often associated with lack of response to ethosuximide (73.9% in non-responders versus 42.5% in responders, p=0.011). The global minor allele (T) frequency of this C/T variant is 0.44 [27] compared to 0.29 in this trial's overall population (n=318) and 0.29 in the ethosuximide cohort. Although other CACNA1H variants have been associated with susceptibility to CAE, this variant is considered benign and not disease causing[28]. The variant was mentioned in one study as occurring in both CAE patients and controls[29] indicating this variant is unlikely to contribute to the risk of CAE.

The study's electrophysiology results on the CACNA1H variant (rs61734410/P640L) confirm the genetic findings. There were no significant clinically relevant differences in biophysical properties between the wild type T-type calcium channel and the P640L variant of the T-type calcium channel. This reinforces the conclusion that this variant is not disease causing. However, the biopharmacologic properties of the channels were different between the wild type and the P640L variant. Both channel forms exhibited physiologic response to ethosuximide. Compared to the wild type, the P640L channel demonstrated a minor effect on peak currents (less inhibition than the variant) but a pronounced effect on rate of decay (i.e. ethosuximide increased the rate inactivation in the wild type but not in variant channel). Combined, these findings indicate the variance channel has altered responsiveness to ethosuximide compared to the wild type.

The CACNA1I synonymous variant (rs3747178) was also more often associated with lack of response to ethosuximide (60.9% in non-responders versus 47.4% in responders, p=0.026). The global minor allele (T) frequency of this C/T variant is 0.33[27] compared to 0.28 in this trial's overall population (n=353) and 0.29 in the ethosuximide cohort. This CACNA1I variant is considered benign and not pathogenic for CAE[30]. There are no prior reports of any pharmacogenetic clinical impact of this specific polymorphism. Because this is a synonymous variant, we were unable to confirm our results in biologic studies, so this finding will need to be validated in future studies.

The presence of the minor allele (T) in the ABCB1 missense variant (rs2032582, T2677G, S893A) was more often associated with lack of response to lamotrigine (76.4% versus 46.7%, p=0.0149). Its global minor allele frequency is reported as 0.33[27] compared to 0.38 in the lamotrigine cohort. This variant is considered benign and not pathogenic[28]. However, multiple studies have examined whether this variant impacts overall antiepileptic drug responsiveness in mixed seizure populations taking a variety of medications in varied ethnic backgrounds[14, 31–34]. No consistent group differences were identified in the genotype, allele, haplotype, or diplotype frequencies of this variant for these analyses[14, 25, 31–34]. In contrast this study examined only initial monotherapy in a homogenous population of children with pre-specified, objective, and prospectively determined drug response criteria. Given our lack of biologic validation, this finding requires further validation.

Two synonymous CACNA1H variants, located essentially next to each other, (rs2753326 and rs2753325) were associated with greater seizure freedom in the lamotrigine group. Both have global minor allele frequency reported as 0.29[27] compared to 0.36 in the lamotrigine cohort. Their clinical impact is unknown.

Although no common polymorphisms were associated with differential valproic acid seizure response, one less common CACNA1H polymorphism (rs2235634) appeared more frequently in the not seizure free cohort compared to the seizure free cohort (50% versus 18.6%, p =0.0008). Its global minor allele frequency is reported as 0.13[27] compared to 0.12 in the valproic acid cohort. Its clinical impact is unknown.

There was not a statistically significant association between two other frequently reported ABCB1 polymorphisms (rs1045642, C3435T and rs1128503, C1236T)[35] and differential drug response for any of the three study medications. No CACNA1G variants were associated with differential drug response for any of the three study medications.

This study used a candidate gene approach rather than whole exome or whole genome sequencing. The sample size needed for an appropriately sized whole exome or whole genome analysis (in the thousands) would not have been possible in the context of a randomized comparative trial where each treatment arm enrolls approximately 150 subjects. We hypothesized that a randomized double blind clinical trial could enhance the pharmacogenetic signals through rigorous prospective phenotyping and homogenous subgroups.

The impact of genetic variants in drug metabolizing enzymes will be presented in a separate paper as part of a complete pharmacokinetic pharmacodynamic population modeling analysis. However, we have already published that for each study medication, there were no significant differences in the steady-state trough serum concentrations between seizure-free and not seizure subjects at the week 16–20 visit[5]. This implies that any impact of an ABCB1 variant occurred at the brain rather than by affecting absorption or bioavailability in the intestine. The relationship between common polymorphisms in genes coding for receptor, transporter and drug metabolizing enzymes and treatment emergent adverse events from the CAE trial will be presented in a separate report.

The study's pre-specified approach examined polymorphisms with minor allele frequency ≥15% in four genes. Given this a priori restriction, we did not perform further corrections for multiple comparisons in the pharmacogenetic discovery phase in order to not miss a signal or finding. Because of this limitation, it is imperative to obtain validation of our findings to have confidence that these results were not simply false positives. Having identified a T-type calcium channel polymorphism associated with ethosuximide response, we did do multiple corrections for the confirmatory electrophysiology to minimize Type 1 errors. This biologic support provides important validation that CACNA1H P640L is unlikely to be a false positive. There are two limitations to our confirmatory electrophysiology studies. First, the studies were conducted at room temperature. Subsequent attempts to perform the electrophysiological confirmation at physiological temperatures were not successful due to excessive run down of the currents that occurred. Secondly, only one expression system (transiently transfected HEK-293 cells) was used to confirm the clinical trial findings.

The CACNA1H variant validation was performed *in vitro* rather than *in vivo* since the second therapy segment of the trial would not really represent a replication cohort because the subset of subjects whose initial therapy failed may be genetically different than the overall initial treatment group.

These results indicate that four common T-type calcium channel variants and one common ABCB1 transporter variant were associated with differential drug response in children with newly diagnosed CAE. The *in vitro* electrophysiologic studies of the P640L variant's effect on ethosuximide response are consistent with the clinical trial's *in vivo* findings. These pharmacogenetic biomarkers of CAE drug response can now be integrated with previously identified non-heritable biomarkers of CAE drug response (e.g. duration of shortest pretreatment EEG seizure[36]) to create predictive decision support models that can form the foundation for precision medicine approaches to CAE therapy. The prospects are bright for integrated genetic/non-heritable precision therapies for children with CAE.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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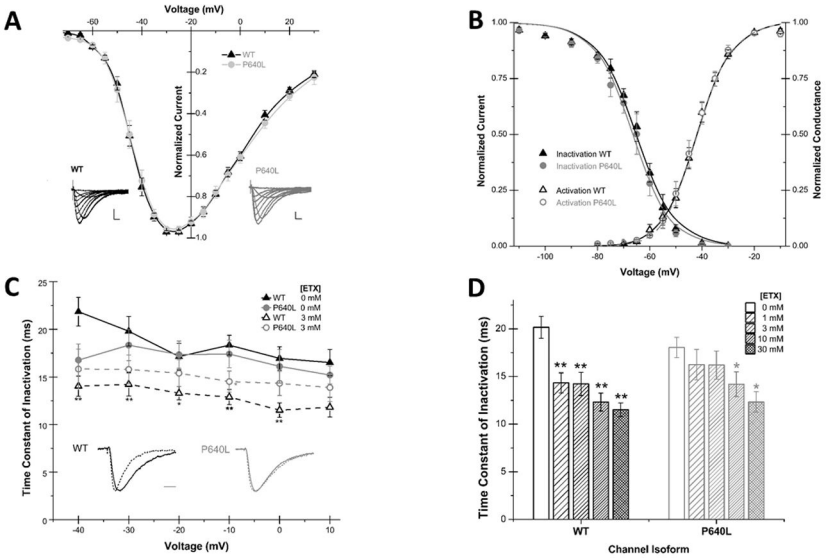

Figure 1. Biophysical and Pharmacological Properties of wild type (WT) $Ca_V3.2$ and rs61734410 (P640L) variant of $Ca_V3.2$

A. Current-voltage relationships of whole cells currents are similar between cells transiently transfected with WT (black) or P640L (gray). Currents were activated by 5–10 mV voltage steps to between −80 to +40 mV for 150 ms from a holding potential of −110 mV. Currents were normalized to the maximal current attained from each cell. The insets are representative families of whole cell currents from WT (black) and P640L (gray) obtained by stepping from a holding potential of −110 mV to various test potentials. Scale bars for the insets are 200 pA and 10 ms

B. Voltage dependence of activation (open symbols, dotted lines) and steady-state inactivation (closed symbols, solid lines) for WT (black) and P640L (gray) are the same. The voltage dependence of activation was estimated by measuring the calcium current during a test potential from a holding current of −110 mV using the voltage steps in A. The current at each membrane potential was divided by the driving force and normalized to the maximal conductance. The voltage dependence of inactivation was obtained from 2-second steps to various voltages (ranging from −130 to +30 mV in 5–10 mV increments) followed by a 90 ms test pulse to −30 mV. The lines were fitted using the Boltzmann equation.

C. Low concentration of ESM accelerates the decay of $Ca_V3.2$ in wild type (black, n=9) but not P640L (gray, n=9). Voltage dependence of the time constant of inactivation in the absence of (solid symbols, solid lines) and presence of (open symbols, dashed lines) 3 mM ethosuximide demonstrating that ethosuximide increased the rate inactivation in the WT but not in P640L. Inactivation rates (⊤) were determined by fitting the current-voltage traces with two exponentials; one for activation and the other for inactivation. The insets represent representative current traces at −30 mV normalized to the peak current. The dotted lines represent the currents in the presence of 3 mM ethosuximide and the solid lines are currents without ESM. Scale bar is 5 ms.

D. Dose-Dependent acceleration of $Ca_V3.2$ decay occurs at lower concentrations in WT (left) than P640L (right). The time constant of inactivation at −30 mV at various concentrations of ESM. The inactivation time constant was determined as in C. * $p<0.05$; ** $p<0.01$ compared to responses without ESM (paired Student's t-test with Bonferroni

correction for multiple comparisons); (for WT n=6 at 1 mM; n=10 at 3 mM; n=9 at 10 mM; n=8 at 30 mM; for P640L n=5 at 1 mM; n=9 at 3 mM; n=8 at 10 mM; n=8 at 30 mM)

Table 1

Pharmacogenetic (PG) study cohorts' demographics

	Ethosuximide (n=118)	Lamotrigine (n=117)	Valproic Acid (n=122)	Overall PG study cohort (n=357)	Excluded (N=89)
Age	7.37 ± 2.12	7.78 ± 2.35	7.69 ± 2.17	7.61 ± 2.21	7.85 ± 2.08
Gender (Male)	48 (41%)	45 (39%)	60 (51%)	153 (43%)	39 (44%)
Hispanic	28 (24%)	27 (23%)	27 (22%)	82 (23%)	18 (20%)
Race					
Black	24 (20%)	20 (17%)	23 (19%)	67 (19%)	19 (21%)
White	83 (70%)	93 (80%)	89 (73%)	265 (74%)	66 (74%)
Other	11 (9%)	4 (3%)	10 (8%)	25(7%)	4 (4%)
Seizure Status					
Seizure Free	95	45	102	242	
Not seizure free	23	72	20	115	
Uninformative	36	27	23		86
No DNA	0	2	1		3

There were no statistically significant differences in age (p=0.36), gender (p=0.87), ethnicity (p=0.58) or race (p=0.63) between the overall PG study cohort (n=357) and the excluded subjects (n=89).

Table 2

Significant Associations between Common Polymorphisms and Short Term Seizure Outcome by Antiepileptic Medication

Cohort	RS number (Gene)	Number of Genotyped Individuals	Not seizure free N (%) with minor allele	Seizure Free N (%) with minor allele	Logistic Regression		
					WaldChiSq	p-value	Odds Ratio (CL) for NSF
Ethosuximide (n=118, NSF=23, SF=95)	rs61734410 (CACNA1H)	110	17 (73.9%)	37 (42.5%)	6.50	0.011	2.63 (1.25–5.56)
	rs3747178 (CACNA1I)	118	14 (60.9%)	45 (47.4%)	4.97	0.026	2.38 (1.11–5.00)
Lamotrigine (n=117, NSF=72, SF=45)	rs2032582 (ABCB1)	117	55 (76.4%)	21 (46.7%)	5.93	0.015	2.22 (1.16–4.17)
	rs2753326 (CACNA1H)	117	41 (56.9%)	30 (66.7%)	4.32	0.038	0.52 (0.28–0.96)
	rs2753325 (CACNA1H)	117	41 (56.9%)	30 (66.7%)	4.32	0.038	0.52 (0.28–0.96)
Valproic Acid (n=122, NSF=20, SF=102)	rs2235634 ** (CACNA1H)	122	10 (50.0%)	19 (18.6%)	11.27	0.0008	7.69 (2.38–25.00)

NSF = not seizure free at week 16–20 visit, SF = seizure free at week 16–20 visit, CL = 95% confidence limits

** = Note this variant was found during the exploratory analysis of polymorphisms with minor allele frequencies between ≥ 5% and < 15%.

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