Article

Differential Gene Expression Profile Induced by Valproic Acid (VPA) in Pediatric Epileptic Patients

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Abstract: Epilepsy is a neuronal disease that affects up to 70 million people worldwide. The development of effective therapies to combat childhood epilepsy requires early biomarkers. Here, we performed a whole-genome microarray analysis in blood cells to identify genes differentially expressed between epileptic and epileptic valproic acid (VPA)-treated children versus normal children to obtain information about the gene expression to help us to understand genetic aspects of this disease. We found that the most significant differentially expressed genes were related to the transcriptional factor cAMP-response element binding protein (CREB) that is overexpressed in children with epilepsy compared with normal children, and 6 and 12 months of VPA treatment reversed several of these changes. Interestingly, leukocyte-associated immunoglobulin-like receptor 1 (LAIR1), a type I transmembrane glycoprotein that binds collagen proteins and contains CREB binding sites, was one of the more up-regulated genes in epileptic children compared with normal children, and 6 and 12 months of VPA treatment reversed several of these changes. CREB up-regulates genes related to epilepsy; here, we suggest that LAIR1 could activate CREB, and together, they trigger epilepsy. After VPA treatment, LAIR1 repressed genes by disrupting the functional LAIR1–CREB complex, resulting in successful treatment. A functional microarray analysis offers new information that could open novel avenues of research in biomarker discovery, which may be useful for the early identification of children with a predisposition to epilepsy.

Keywords: epilepsy; valproic acid; microarrays; transcriptome analysis
1. Introduction

According to the International League Against Epilepsy (ILAE), epilepsy is defined as a disease of the brain characterized by the occurrence of two unprovoked seizures more than 24 h apart, one unprovoked seizure and a probability of further seizures similar to the general recurrence risk (at least 60%) after two unprovoked seizures occurring over the next 10 years, or the diagnosis of an epilepsy syndrome [1]. This condition affects many people; in a recent study, it was reported that 70 million people have epilepsy worldwide, and approximately 90% of them are in developing regions, with a major prevalence in rural zones [2]. In addition, an estimated incidence has been reported of just over 100 patients per 1,000,000 inhabitants, with a prevalence of 3.7% in the population group aged 18–64 years, 1.6% in urban areas and 2.1–4.1% in rural children [3,4]. This condition has many risk factors, including family history, congenital, infections, trauma, neuronal diseases and malformations [2]. Epilepsy is characterized by many molecular mechanisms related to the immune system, such as deregulation of synaptic transmission, brain plasticity, apoptosis, neuroinflammation, oxidative stress and other functional alterations in the neuronal and neurovascular unit [5–9], resulting in gene expression alterations [10]. Antiepileptic drugs (AEDs) are used for epilepsy control, and valproic acid (VPA) is one of the more commonly used AEDs [11]. Several studies have demonstrated that AEDs (including VPA) may induce injury or show a neuroprotective effect in experimental models and humans [8,9,12].

Valproic acid is a branched short-chain fatty acid and is a widely used drug for the treatment of epilepsy, migraines and bipolar disorders [13]. In the treatment of epilepsy, VPA has several biochemical and molecular mechanisms: (a) it enhances inhibitory GABAergic activity and inhibits glutamatergic transmission [14,15]; (b) it modulates sodium and potassium channels [16,17]; (c) it modulates antioxidant defense and the production of oxidant metabolites [8]; (d) it modulates the activity and expression of protein kinases [18]; (e) it modulates neurogenesis, neuronal differentiation and neuronal survival [19,20]; and (f) it exerts effects on gene expression regulation by acting on transcription factors (mainly by regulating phosphorylation) and by acting as a histone deacetylase inhibitor [21,22].

We hypothesized that administration of VPA as an AED might alter the expression of some relevant genes related to neuroprotection and neurotoxicity pathways in patients with epilepsy. Here, we analyzed, for the first time, the gene expression profile of epileptic children as well as the molecular mechanism of VPA after different periods of administration in these patients by performing genome-wide array analysis to obtain novel transcript-based biomarkers that are predictive of epilepsy.

2. Material and Methods

2.1. Patients

All samples were collected from the Emergency Services, Military Hospital of Specialties of Women and Neonatology, Secretary; of National Defense (Secretaría de la Defensa Nacional, SEDENA) in Mexico City, Mexico. Two different groups of patients with available electronic health records were included in these experiments: (a) a group of 13 children (5 healthy children and 8 epileptic patients) were used for the microarray experiments, and (b) a confirmation group of 49 children (17 healthy children and 32 epileptic patients that followed 12 months of uninterrupted VPA monotherapy, including the patients in the microarray experiment) were used for the validation of some specific genes identified in the microarray analysis via real time-PCR (RT-PCR).

This study was approved by the Bioethics in Research Committee of the Military Hospital of Specialties of Women and Neonatology, SEDENA (Registration number 35, approval date 18 December 2015). The committee’s human experimentation guidelines were followed, and written informed consent was obtained from each patient.

The sample collection was conducted from December 2015 to March 2018 and each sample was considered according to the inclusion, exclusion and elimination criteria. The inclusion criteria
were as follows: peripheral blood samples from newly admitted pediatric patients at the Military Hospital of Specialties of Women and Neonatology and the Central Military Hospital, SEDENA from 2016 to 2018; pediatric patients diagnosed with epilepsy or an epileptic syndrome; untreated pediatric patients for the first sampling and then 12 months of uninterrupted VPA treatment as the only AED (monotherapy). The exclusion criteria were as follows: pediatric patients with chronic diseases such as hematological, cardiac, hepatic, renal or thyroid disorders; pediatric patients with obesity; pediatric patients presenting infectious diseases or who had performed excessive physical exercise before sampling; pediatric patients taking drugs that interfere or alter the antioxidant or inflammatory state; pediatric patients taking an AED prior to the start of the study; and pediatric patients with a convulsive crisis with refractoriness of AED treatment. The elimination criteria were as follows: those cases of pediatric patients whose initial diagnosis of epilepsy was modified to another disease; pediatric patients who did not follow the therapeutic regimen during the study; and samples with low quality RNA. All recruited epileptic patients followed the inclusion, exclusion and elimination criteria, and none of the healthy children developed diseases or had a family history of epilepsy during the sampling.

2.2. Blood Sampling and RNA Extraction

For the study with epileptic children, a 5 mL sample of peripheral venous blood was taken at the time of initial diagnosis (drug-free stage) and after VPA monotherapy (samples after 6 and 12 months of AED administration). A single blood sample was taken from the healthy children. The samples were collected in Vacutainer tubes containing EDTA (Becton, Dickinson and Company, Franklin Lakes, NJ, USA), and leucocytes were isolated. The isolation of RNA from the leucocytes was performed using Trizol reagent (TRI Reagent® Solution, RNA/DNA/Protein Isolation Reagent, Invitrogen-Ambion, ThermoFisher Scientific, Waltham, MA, USA). Total cellular RNA was extracted according to the manufacturer’s protocol. RNA integrity, quality and quantification were assessed using QIAxcel and QIAxpert equipment (Qiagen, Germantown, MD, USA). All extracted RNA was preserved with nuclease inhibitor solution and stored at −80 °C.

2.3. Microarrays

Total RNA samples from five control children and eight epileptic children were analyzed using a microarray according to the manufacturer’s protocol (Two-Color Microarray-Based Gene Expression Analysis/Low Input Quick Amp Labeling; Agilent Technologies, Santa Clara, CA, USA). Briefly, 75 ng of total RNA was converted to cDNA, followed by in vitro transcription and incorporation of Cyanine 3-CTP into the nascent complementary RNA (cRNA), followed by fragmentation and hybridization to Agilent SurePrint Human GE 8 × 60 K Microarrays (Agilent Technologies) for 17 h at 65 °C. The quality control parameters used for cRNA labeling and hybridization were specified by the manufacturers. The microarrays were scanned using a NimbleGen microarray scanner (Roche, Basel, Switzerland), and the signal intensities in the TIFF images were calculated using Feature Extraction software (FE, version 12.0; Agilent Technologies). The microarray data were analyzed, and the associated biological pathways were determined using GeneSpring GX 13.0 software (Agilent Technologies). Differentially expressed genes were selected with a fold change >2.0 and \( p < 0.05 \). The Benjamini–Hochberg algorithm was used to compute false discovery rates [23]. The classification of the identified pathways was based on the Kyoto Encyclopedia of Genes and Genomes pathway database (KEGG Pathway Maps). The gene ontology analysis for down- and up-regulated genes was submitted to the bioinformatics and functional annotation tool of the Database for Annotation, Visualization and Integrated Discovery (DAVID Bioinformatics Resources) v. 6.8 of the NIAID (National Institute of Allergy and Infectious Disease), NIH (National Institutes of Health).
2.4. Gene Expression Validation

To confirm the microarray analysis results, RT-PCR validation was performed for the most representative dysregulated genes from the total sample population (which included 17 healthy control children and 32 children with epilepsy). Reverse transcription was performed using a One-Step qRT-PCR KAPA SYBR FAST® Kit (Kapa Biosystems, Sigma-Aldrich, St. Louis, MO, USA) according to the manufacturer’s protocol, with a Rotor-Gene Q (Qiagen) and specific gene primers (provided upon request). To determine the relative gene expression levels, the number of cycles in which the fluorescence intensity increases above the baseline fluorescence of the sample (CPs) of endogenous candidates genes (glyceraldehyde-3-phosphate dehydrogenase (GAPDH), β-2-microglobulin (B2M) and actin-β (BACT)) and each of the analyzed genes were exported from Rotor-Gene Q v.2.3.1 software (Qiagen) to calculate the efficiencies using the REST© statistical model [24,25], and the data were plotted by constructing a linear regression comparing the logarithmic concentration (total RNA) against the CP. To correlate the candidate endogenous genes and determine the more stably expressed genes, BestKeeper software was used by exporting the CP values from the Rotor-Gene Q software using the Excel tool to show the melting temperature (Tm) characteristics of each amplified product. The expression levels of the housekeeping genes were analyzed using the BestKeeper statistical model, which analyzed the CP values via Pearson correlation [26].

2.5. Statistical Analysis

All statistical analyses were performed using GraphPad Prism version 6.0 software (La Jolla, CA, USA) and XLSTAT for Excel 2018 (Addinsoft, NY, USA). The data are expressed as the mean ± standard deviation (SD). The Kolmogorov–Smirnov normality test was performed based on the null hypothesis that the data was normally distributed. Data from the absolute quantification of all the samples were normalized with housekeeping genes and were analyzed using the Student’s t-test. Differences between groups were tested using analysis of variance (ANOVA) with Bonferroni post hoc analysis.

3. Results

3.1. Characteristics of the Patients Included in the Microarray Study

The random sample for the microarray study consisted of 13 children, distributed with respect to weight and sex. Overall, 13 patients participated, with five children in the healthy group and eight epileptic children in the drug-free and VPA monotherapy group, with a range of ages (months of age, mean ± SD) between 36.6 ± 13, and 58.3 ± 55.2 for healthy and epileptic children, respectively. Of the healthy children, 40% were female and 60% were male, and of the epileptic children, 25% were female and 75% were male. The characteristics of the patients with epilepsy used in the microarray study are presented in Table 1. In terms of family history of epilepsy, 3 (37.5%) had relatives with epilepsy, 3 (37.5%) had a perinatal history, 2 (25%) presented with other diseases, 6 (75%) showed generalized epilepsy with idiopathic etiology, 6 (75%) presented abnormal findings in the image studies; the mean serum level of VPA (µg/mL) was 28.9 ± 16.9 and the mean weight-based divided dose of VPA (mg/kg) was 24.04 ± 9.34.
Table 1. Clinicopathological characterization of the patients included in the microarray study. VPA: Valproic Acid.

| Patient | Age at the Beginning of Treatment (Months) | Family History of Epilepsy | Perinatal History | Other Diseases | Type of Convulsion | Etiology of Epilepsy | Findings in the Image Studies | Mean Daily Convulsions before Treatment | Mean Daily Convulsions after Treatment | VPA Dose (mg/kg of Body Weight) |
|---------|-------------------------------------------|-----------------------------|-------------------|----------------|-------------------|----------------------|-------------------------------|--------------------------------------|--------------------------------------|-------------------------------------|
| 1       | 12                                        | None                        | Prematurity       | None           | Generalized       | Idiopathic           | Left cortical atrophy         | 4                                    | 0                                    | 30                                  |
| 2       | 144                                       | Mother diagnosed with epilepsy | Threatened abortion | None           | Generalized       | Idiopathic           | None                          | 1                                    | 0                                    | 48                                  |
| 3       | 84                                        | Aunt diagnosed with epilepsy | None              | None           | Generalized       | Idiopathic           | Abnormal paroxysm            | 6                                    | 0                                    | 25                                  |
| 4       | 132                                       | No                          | None              | Asthma         | Generalized       | Idiopathic           | Irritative cortical activity  | 1                                    | 0                                    | 10                                  |
| 5       | 48                                        | Brother diagnosed with epilepsy | None              | None           | Generalized       | Idiopathic           | Ventricular asymmetry        | 18                                   | 0                                    | 20                                  |
| 6       | 12                                        | None                        | Prematurity       | Bronchopulmonary dysplasia | Generalized       | Symptomatic           | Epileptiform activity in the left parietal region | 1                                    | 1                                    | 20                                  |
| 7       | 10                                        | None                        | None              | None           | Generalized       | Idiopathic           | Ventricular asymmetry with increase in left occipital volume | 5                                    | 0                                    | 25                                  |
| 8       | 24                                        | None                        | None              | None           | Generalized       | Symptomatic           | None                          | 3                                    | 1                                    | 38.9                                |
3.2. Gene Expression Profiling in Peripheral Blood Cells

Of the 50,378 probes tested in our microarray analysis, 21,056 probes remained after background correction and were used for further analysis. In total, 978 unique genes were found to be significantly differentially expressed between normal and epileptic children before treatment. There were 341 unidentified genes in a microarray data set and these were not considered in our analysis. From the remaining 637 known coding genes, 451 were down-regulated, and 186 were up-regulated in epileptic children compared to normal children (Table S1, Supplementary Materials). The results from the array concerning the 637 genes differentially expressed between both conditions are depicted in a heat map (see Figure 1).

3.3. Functional Analysis

To study the functions of the differentially expressed genes, DAVID Bioinformatics Resources were applied to known gene ontology pathways. The 637 known genes with significantly different expression levels between epileptic patients before treatment and normal children were classified into several biological processes according to their function. The biological processes showing the highest number of differentially expressed genes were related to transcription/translation machinery (n = 28), Poly(A) RNA binding (n = 51), a cytokine-mediated signaling pathway (n = 11), the immune system (n = 19), cytokine-cytokine receptor interactions (n = 14), DNA binding (n = 67), protein binding (n = 254), and positive regulation of smooth muscle cell proliferation (n = 5), among other functions.

The functional annotation clustering of genes differentially expressed are shown in Table 2. Notably, most of the genes involved in these pathways were up-regulated, and they had not previously been associated with epileptic children.

Table 2. Functional annotation clustering of genes differentially expressed in epileptic children.

| Pathway                                           | n   | p-Value        | Fold Enrichment | Benjamini False Discovery Rate |
|---------------------------------------------------|-----|----------------|-----------------|-------------------------------|
| Translation                                       | 28  | 6.12 × 10⁻10   | 4.2236          | 2.18 × 10⁻7                   |
| Poly(A) RNA binding                               | 51  | 3.12 × 10⁻4    | 1.6854          | 0.0866                        |
| Coiled-coil                                       | 104 | 0.0036         | 1.3327          | 0.1019                        |
| Cytokine-mediated signaling pathway               | 11  | 0.0023         | 3.2046          | 0.2925                        |
| Alternative splicing                              | 304 | 0.0032         | 1.1171          | 0.1449                        |
| Receptor binding                                  | 20  | 0.0032         | 2.1113          | 0.3725                        |
| Cytokine-cytokine receptor interaction            | 14  | 0.0075         | 2.2894          | 0.1655                        |
| Immune response                                   | 19  | 0.0288         | 1.7223          | 0.9340                        |
| DNA-binding                                       | 67  | 0.0317         | 1.2715          | 0.6044                        |
| Jak-STAT signaling pathway                        | 9   | 0.0372         | 2.3437          | 0.4948                        |
| Regulation of immune response                     | 10  | 0.0446         | 2.1440          | 0.9055                        |
| Protein binding                                   | 254 | 0.0494         | 1.0774          | 0.9599                        |
| Phosphoprotein                                    | 230 | 0.0640         | 1.0852          | 0.7827                        |
| Positive regulation of smooth muscle cell proliferation | 5   | 0.0716         | 3.1803          | 0.9618                        |
| Non-syndromic deafness                            | 6   | 0.0730         | 2.6831          | 0.806                         |
| Metal-binding                                     | 107 | 0.0844         | 1.1437          | 0.799                         |
| Zinc ion binding                                  | 40  | 0.0919         | 1.2751          | 0.9309                        |
| Adaptive immune response                          | 8   | 0.0938         | 2.0629          | 0.9861                        |

Additionally, we applied bioinformatics analyses to detect any enrichment of transcriptional factors. We found a cluster of 349 of the 637 genes with significant expression (54.8%) with CRE motifs (TGACGTCA), whose transcription is controlled by the cAMP-response element binding protein (CREB) transcription factor in the transcriptome of epileptic children before treatment (in comparison with healthy children). Of these 349 CREB genes, 106 were up-regulated (30.4%), and 243 were down-regulated (69.6%) (Table S2, Supplementary Materials). The results for the ten genes related to CREB that displayed the greatest changes are shown in Table 3.
Figure 1. Gene expression changes triggered by epileptic disease. Microarray heat map shown genes that were differentially expressed and unchanged during epileptic episodes without treatment. A color code scale was used to show gene expression differences in logarithmic fold change units between the groups (red represents lower expression; green represents higher expression).

Table 3. Most representative deregulated cAMP-response element-genes (CRE-genes) in pediatric patients with epilepsy in comparison with healthy children.

| Official Symbol | Official Full Name | Fold Change |
|-----------------|-------------------|-------------|
| RCHY1           | Ring finger and CHY zinc finger domain containing 1 | 193.7847 |
| CCL13           | C-C motif chemokine ligand 13 | 133.6095 |
| LAIR1           | Leukocyte associated immunoglobulin-like receptor 1 | 80.0391 |
| RPSAP58         | Ribosomal protein SA pseudogene 58 | 62.9540 |
| IL6R            | Interleukin 6 receptor | 60.4950 |
| CCDC14          | Coiled-coil domain containing 14 | 49.6876 |
| C21orf131       | Long intergenic non-protein coding RNA 320 | 42.6516 |
| AEPAP1L2        | Actin filament associated protein 1-like 2 | 36.8321 |
| TRIM24          | Tripartite motif containing 24 | 14.5041 |
| CSMD1           | CUB and Sushi multiple domains 1 | 13.5815 |
Real time-PCR was used to validate the expression of selected genes related to the CREB transcription factor: RCHY1, CCL13, LAIR1 (leukocyte-associated immunoglobulin-like receptor 1), TRIM24, CCR2, MYO6, BMS1 and DSP. The results showed that RCHY1, CCL13, LAIR1 and TRIM24 were significantly up-regulated genes and CCR2, MYO6, BMS1 and DSP were significantly down-regulated genes in pediatric patients with epilepsy (see Figures 2 and 3).

**Table 3. Cont.**

| Official Symbol | Official Full Name | Fold Change |
|-----------------|-------------------|-------------|
| CCR2            | C-C motif chemokine receptor 2 | -190.5848  |
| ZSWIM7          | Zinc finger SWIM-type containing 7 | -167.3231  |
| MYO6            | Myosin VI         | -148.6852   |
| SNAPC2          | Small nuclear RNA activating complex polypeptide 2 | -118.0506  |
| BMS1            | Ribosome biogenesis factor | -108.2960  |
| TBX22           | T-box 22          | -78.6303    |
| MKLN1-AS        | MKLN1 antisense RNA | -69.2440   |
| EFTUD2          | Elongation factor Tu GTP binding domain containing 2 | -64.2970   |
| IDH3B           | Isocitrate dehydrogenase 3 (NAD(+)) beta | -61.9831   |
| DSP             | Desmoplakin       | -54.6393    |

**Figure 2.** Levels of normalized mRNA of the up-regulated genes RCHY1, CCL13, LAIR1 and TRIM24 in pediatric patients with epilepsy. The gene levels were normalized to the level of the housekeeping gene B2M and are expressed per 20 ng of total RNA. The values represent the means with standard deviations (SD) of the expression levels of each gene. We found significant differences between all genes. * \( p < 0.05 \) vs. the gene expression of CCL13, LAIR1, and TRIM24; ** \( p < 0.05 \) vs. the gene expression of LAIR1 and TRIM24; and *** \( p < 0.05 \) vs. the gene expression of TRIM24.
we found that six months of monotherapy were unable to change the expression of 40 genes, while were up-regulated by epilepsy in epileptic children before treatment. The expression of 14 of them genes, while after 12 months of monotherapy, only 14 remained unchanged. Among the genes that were highly increased after six months of VPA treatment, whereas the expression level of 40 genes decreased by 90.5% and 96% during 6 and 12 months of VPA treatment, respectively, compared with the drug-free stage. Between 6 and 12 months of VPA treatment, the gene expression of both CCR2 and DSP increased by more than 100%.

Figure 3. Levels of normalized mRNA of the down-regulated genes CCR2, MYO6, BMS1 and DSP in pediatric patients with epilepsy. The gene levels were normalized to the level of the housekeeping gene B2M and are expressed per 20 ng of total RNA. The values represent the means with standard deviations (SD) of the expression levels of each gene. We found significant differences between all genes. * p < 0.05 vs. the gene expression of MYO6, BMS1, and DSP; ** p < 0.05 vs. the gene expression of BMS1 and DSP; and *** p < 0.05 vs. the gene expression of DSP.

3.4. Association Studies of Gene Expression in Epileptic Patients Undergoing Valproic Acid Monotherapy

To investigate the effect of VPA monotherapy on the change in the expression of several mRNAs in epileptic patients, we selected the CREB genes to measure again after 6 and 12 months of VPA monotherapy. As shown in Figure 4, 106 genes of the 637 genes with significant expression (16.6%) were up-regulated by epilepsy in epileptic children before treatment. The expression of 14 of them returned to healthy levels after six months of VPA treatment, whereas the expression level of 40 genes began to decrease after six months and continued decreasing after 12 months of treatment with VPA. Consistent with the progression of the VPA treatment time and the improvement in epileptic episodes at 12 months, the gene expression levels of 92 of the 106 transcripts (86.8%) were changed. Interestingly, we found that six months of monotherapy were unable to change the expression of 40 genes, while after 12 months of monotherapy, only 14 remained unchanged. Among the genes that were highly expressed in the epileptic group, we found RCHY1, CCL13, LAIR-1, RPSAP58, IL6R, CCDC14, AFAPI12, TRIM24, CSMD1, ZNF704, CDK5RAP3, FRAS1, Ska1, CY5T1, IL5, LHFP and CPB1. Interestingly, VPA treatment reduced their expression, suggesting that these genes promote epilepsy, since this condition was also diminished as the treatment period progressed. Specifically, RCHY1, CCL13, LAIR-1, RPSAP58 and TRIM24 were down-regulated after six months of VPA treatment. In the validation experiment, we observed that the expression levels of TRIM24 and LAIR1 were significantly decreased after six months of VPA treatment, whereas the expression levels of CCR2 and DSP were significantly increased after six months of VPA treatment compared with the drug-free stage (see Figure 5). TRIM24 gene expression decreased by 90.5% and 96% during 6 and 12 months of monotherapy, respectively, and LAIR1 gene expression decreased by 66.1% and 95% during 6 and 12 months of monotherapy, respectively, compared with the drug-free stage. Between 6 and 12 months of VPA treatment, the gene expression of TRIM24 and LAIR1 decreased by 57.8% and 85.4%, respectively. CCR2 gene expression increased by more than 100% between 6 and 12 months of monotherapy, and DSP gene expression increased by more than 100% during the same time period compared with the drug-free stage. Between 6 and 12 months of VPA treatment, the gene expression of both CCR2 and DSP increased by more than 100%.
In addition to the genes that increased during epilepsy and without treatment, we found that the expression of 243 genes of the 637 genes with significant expression in epileptic children before treatment (38.1%) was decreased. Of these 243 genes, after treatment with VPA for 6 months, 13 genes were unchanged, 92 were expressed at healthy levels, the expression of 102 decreased more than expected, and 36 had increased expression levels. After 12 months of VPA treatment, only 10 of the 243 (4%) genes did not have changed expression levels (see Figure 4 and Table S3, Supplementary Materials). These results suggest that many of the benefits of VPA treatment of epilepsy may be attributed to the effect on the transcription of several genes.
4. Discussion

In the present study, we explored the transcriptome of leucocytes during epilepsy in pediatric patients before and after different lengths of treatment with VPA monotherapy. Microarray studies in pediatric patients with epilepsy indicated that short and long-term (6 and 12 months) exposure to the well-established therapeutic drug VPA induces altered mRNA levels of a large number of genes, and importantly, reverting most of the genes altered by the disease to a healthy level.

Potential mediators for the positive regulation of epileptic gene transcription include CREB [27], which is a well-known master regulator of metabolic pathways. CREB is an attractive candidate because it was recently identified as a key regulator of a pathway that involves LAIR-1 [28] (Figure 6).

Potential mediators for the positive regulation of epileptic gene transcription include CREB [27], which is a well-known master regulator of metabolic pathways. CREB is an attractive candidate because it was recently identified as a key regulator of a pathway that involves LAIR-1 [28] (Figure 6).
(NF-L), regulating its ubiquitination. If it is deregulated, it triggers neurodegeneration [35]. Given its related function, TRIM24 might play a similar role in the development of epilepsy. As with LAIR-1, we demonstrated that VPA treatment also dramatically down-regulates its expression throughout the entire 12 months of VPA treatment.

The CUB structural domain (for complement C1r/C1s, Uegf and Bmp1) and sushi multiple domains 1 gene, CSMD1, which is highly expressed in epithelial tissues and the central nervous system, plays a role as an important regulator of complement initiation and inflammation. Patients who carry a deletion of this gene show language delay, learning difficulties and epilepsy [36]. Spindle and kinetochore associated complex subunit 1 (SKA1), has been suggested as being involved in human brain development [37]; however, its participation in epilepsy has not been studied.

Our data showed that changes in the expression of the above mentioned genes were diminished after VPA treatment. The gene C21orf131 is known to be highly expressed in the human brain, but its specific functions are not clear, nor is it known whether it has lower expression in patients with epilepsy. This study is the first report showing neurodevelopment problems related to the C21orf131 gene. We suggest that it is necessary to investigate the role of this gene to understand the development of human epilepsy.

In addition to the increased transcription of CRE-regulated promoters during epilepsy in pediatric patients and the decrease in their transcription through VPA monotherapy treatment, we also observed a set of genes containing CRE motifs with decreased expression in epileptic patients. After VPA treatment, this group of genes showed a tendency to increase their expression levels.

Regarding mRNA with decreased expression levels during epileptic episodes in children, most had reverted levels of expression after 6 or 12 months of treatment with VPA; those genes were CCR2, ZSWIM7, MYO6, SNAPC2, BMS1, TBX22, MKLN1-AS, EFTUD2, IDH3B, and DSP.

Elevated levels of the chemokine C-C motif ligand 2 receptor CCR2 have been reported in experimental seizures, and the neuronal localization of CCR2 has been mapped [38,39]. However, its participation in relieving episodes of seizures due to VPA treatment has not been widely evaluated, and we demonstrated that after 6 months of monotherapy with VPA, the large reduction in CCR2 transcript levels was ameliorated as the treatment progressed, thus possibly contributing to the reduction in seizures. MYO6, an unconventional myosin, has been previously associated with deafness [40]. BMS1 is a ribosome biogenesis factor already reported to be involved in epilepsy. Its reversion to control levels due to VPA and its participation in the development of convulsive episodes has not been studied.

The decrease of the ZSWIM7, MYO6, SNAPC2, BMS1, TBX22, MKLN1-AS, EFTUD2, IDH3B, and DSP transcripts had not been previously associated with epileptic episodes in pediatric patients, and even less is known about their re-establishment by monotherapeutic treatment with VPA. We confirmed that the gene expression of DSP, similar to CCR2, increased dramatically throughout the 12 months of VPA monotherapy. This generates new horizons in the study of signaling pathways for the treatment and prevention of epileptogenesis. Diminished expression of this group of genes could be mediated by ICER, an inducible cAMP early repressor, which is part of the CRE binding protein family that shares the ability to also bind to the highly conserved DNA 8-mer (TGACGTCA) sequence, a CRE motif [41,42]. In laboratory models, following kainic acid-induced or electroconvulsive seizures, there is an increase in the level of ICER [42], which could explain the blocking of the expression of a specific group of genes that contain the CRE motif and could consequently explain why treatment with VPA eliminates this blockage and re-establishes the expression of most genes. It is relevant to mention that these alterations in the expression of genes are from patients with generalized epilepsy with principally idiopathic etiology. Other studies will be focused on determining the effects of VPA in other kinds of seizures with other originating causes in epileptic children.
5. Conclusions

Functional microarray analysis suggests a possible therapeutic gene bank that may be a useful approach for early identification of children with predispositions to epilepsy. Moreover, our results showed that VPA differentially regulates the gene expression profile in epileptic children during the 6 and 12 months of treatment, principally in one group of genes related with the CREB transcription factor, suggesting the LAIR1-CREB axis as a possible action mechanism of this AED in generalized epilepsy.

Supplementary Materials: The following are available online at http://www.mdpi.com/2073-4425/9/7/328/s1, Table S1: Complete list of differentially expressed genes between normal and epileptic children, identified by microarray analysis, Table S2: List of differentially expressed genes between normal and epileptic children with significantly enriched CRE motif, Table S3: List of differentially expressed genes with CRE motif in epileptic children and the effect of VPA administration. Orange: up-regulated, Purple: down-regulated.

Author Contributions: N.C.-R. and D.O.-C. conceptualized the study, conducted the formal analysis, supervised the project completion and drafted the original manuscript. N.C.-R., D.O.-C., E.M.-A. and I.I.-M. conducted the studies and visualization of data. E.F.-S., F.B., I.I.-M., P.R.-M., E.C.-V., M.G.C.-E. and C.K.A.-S. conducted the methodology, performed data curation and the validation experiments. R.S.-P. recruited the patients, collected the biological samples and revised the clinical-pathological information. L.C.-A. revised the clinic-pathological information. E.F.-S., F.B., D.O.-C., I.I.-M., E.M.-A., L.C.-A. and N.C.-R. analyzed the information. N.C.-R. and E.F.-S. conducted the formal analyses, administered the project and secured the funding. N.C.-R. realized investigation and writing-review and editing. All authors revised the manuscript.

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