Transcriptome Analysis of the Hippocampus in Novel Rat Model of Febrile Seizures

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Abstract

Febrile seizures (FS) are the most common type of convulsive events in infants and young children, but the precise underlying genetic mechanism remains to be explored. To investigate the underlying pathogenic factors in FS and subsequent epilepsy, alterations in gene expression between the two new strains of rats (hyperthermia-prone [HP] vs hyperthermia-resistant [HR]), were investigated by using the Whole Rat Genome Oligo Microarray. This process identified 1,140 differentially expressed genes (DEGs; 602 upregulated and 538 downregulated), which were analyzed to determine significant Gene Ontology (GO) categories, signaling pathways and gene networks. Based on the GO analyses, the modified genes are closely related to various FS pathogenesis factors, including immune and inflammatory responses and ion transport. Certain DEGs identified have not been previously examined in relation to FS pathogenesis. Among these genes is dipeptidyl peptidase 4 (DPP4), a gene closely linked to interleukin 6 (IL-6), which played a key role in the gene network analysis. Furthermore, sitagluptin, a DPP4 inhibitor significantly decreased epileptic discharge in rats, observed via electroencephalogram, suggesting an important role for DPP4 in FS. The effectiveness of sitagluptin in reducing seizure activity may occur through a mechanism that stabilizes cellular Ca2+ homeostasis. In addition, DPP4 expression may be regulated by DNA methylation. The hippocampal gene expression profiles in novel rat models of FS provides a large database of candidate genes and pathways, which will be useful for researchers interested in disorders of neuronal excitability.

Introduction

Febrile seizures (FS) are the most common seizure disorders in children, occurring in 2–5% of children before age 5 years [1,2]. Retrospective studies with epilepsy showed that 10–15% of these patients had previous FS [3,4]. In prospective follow-up studies of large cohorts of children with FS, febrile seizures occur in 2–7%, which is up to ten times the prevalence in the general population [5]. FS are complex and heterogeneous, and genetic factors contribute significantly to the etiology of FS [6]. This is supported by the findings in twin and family studies which demonstrated an important genetic component in the etiology of FS [7,8]. Eleven genetic loci (FEB1-11) have been mapped in the Online Mendelian Inheritance in Man (OMIM) database (http://www.omim.org/). In these genetic loci, some susceptibility genes (Scn1a, Scn9a, IL-1β, IL-10, Gpr98 and Cap6) have been confirmed[6,9–12], but not in other six other genetic loci FEB1, 2, 5, 7, 9 and 10. The precise underlying pathogenic factor is still unclear which has hampered many aspects of the study of FS.

Genetic animal models, particularly those with controlled genetic and epigenetic backgrounds, are useful tools for investigating the pathogenesis of a disorder without interferences from many confounding factors [13,14]. Hyperthermia influences neuronal protein expression and cell properties by manipulating gene transcription and epigenetic modification[15–17]. Prolonged experimental FS have been found to enhance hippocampal network excitability and promote epilepsy, which may result from broad modifications of the gene-expression programs of numerous molecules that govern neuronal excitability and network response [18,19]. We previously developed two new strains of FS rats by using an established model of hyperthermia-induced seizures combined with a selective breeding process, which were named Hyperthermia-Prone (HP, lower seizure-threshold) and Hyperthermia-Resistant (HR, higher seizure-threshold). The two strains of rats were housed under standard laboratory conditions and hyperthermia-induced seizures were produced using hot water bath immersion. The most sensitive rats from the HP group and the most resistant rats from the HR group were used as breeders to create the next generation. This process resulted in a inheritable
significant difference in seizure sensitivity and subsequent epilepsy between the two groups of rats [20]. As a result of the simple external interference in the modeling process, these two strains of rats provide a novel tool to study the underlying pathogenesis induced by hyperthermia in FS.

To identify genetic factors that contribute to the FS, especially in hippocampus, a gene microarray screen was performed to compare the hippocampus of the HP and HR strains. Furthermore, using a range of data mining and information annotation approaches, we were able to identify many candidate genes that were significantly differentially expressed between the two groups. These identified candidate genes might shed some light in elucidating the pathogenic factors in FS and the subsequent development of epilepsy.

**Materials and Methods**

**Ethics Statement**

All protocols complied with the recommendations in the Guide for the Care and Use of the Animal Biosafety Level 3 (ABSL-3) Laboratory and were approved by the Animal Ethics Committee of the Wuhan University (Permit Number: SCXK 2008-0004). All surgery was performed under chloral hydrate anesthesia, and all efforts were made to minimize suffering.

**Seizures Induction and Brain Sampling**

Sprague-Dawley rats were obtained from the ABSL-3 Laboratory. Unless noted otherwise, 21 days old HP and HR rats at the beginning of the experiments were used. Rats were housed individually on a 12-h light-dark cycle with ad libitum access to food and water. Seizure induction and selective breeding procedure of HP and HR rat strains were carried out as previously described [20]. Hyperthermia-induced seizures were produced using hot water bath. The animals were placed in a temperature-controlled water bath and were immediately removed from the water when seizures were induced.

After the finish of the whole seizure-induction process, rats were anesthetized with 10% chloral hydrate (3 ml/kg). The brains were quickly removed and placed in ice-cold, and the hippocampi were dissected and placed into liquid nitrogen, and transferred into a −80°C low-temperature refrigerator for storage and use.

**RNA Extraction and Microarray Hybridization**

Three rats were selected randomly from HP group and HR group, respectively. Whose hippocampus were collected under chloral hydrate anesthesia, and all efforts were made to minimize suffering. Following hybridization, the microarray slides were checked for background evenness.

**Differentially Expressed Genes (DEGs), Gene Ontology (GO) and Pathway Analysis**

**Linear Models for Microarray Data** and empirical Bayes methods were used to further investigation of the DEGs between the two groups. DEGs were considered significant if both the P value <0.05 and the fold change (FC)>1.5.

The GO annotations of the DEGs were downloaded from the GO project [http://www.geneontology.org] and NCBI [http://www.ncbi.nlm.nih.gov]. The “elim Fisher” algorithm described by Alexa et al was used for the GO enrichment test [21]. GO categories with a P value <0.01 were reported.

The pathway analysis was obtained from the Kyoto Encyclopedia of Genes and Genomes (KEGG) database [http://www.genome.jp/kegg]. A Fisher exact test was used to find significant enrichment for pathways. Pathway categories with a P value <0.05 were reported.

**Gene Network Analysis**

Functional association networks were constructed using the GeneSpring GX software (version 11.0, Agilent Technologies, USA). The annotation data presented in this analysis were obtained from NCBI's Gene Expression Omnibus (GSE23165).

All DEGs were selected to construct a co-expression network. The network edges were specified to feature Pearson correlation coefficients above 0.99 to ensure strong gene co-expression relationships. Degree description of the number of single gene that regulate other genes. Diffdegree describe the difference in value between the HP and HR groups. A k-core of a gene co-expression network stands for the ability of co-expression.

**Quantitative Real-time PCR (qRT-PCR) Analysis**

To confirm the microarray results, ten representative genes were confirmed by qRT-PCR, as described [22]. Six rats (HP n=3 and HR n=3) were selected randomly from each group. After hippocampus isolation and RNA extraction, briefly cDNA was prepared using the RevertAid™ First Strand cDNA Synthesis Kit (ThermoFisher, USA). By employing the Real Master mix (SYBR Green) Kit (Tiangen, China) and a LightCycler (Roche Diagnostics, Germany), qRT-PCR was performed following the manufacturer’s protocols. Single PCR products were further verified by melting curve analysis. Note that rat β-actin was always amplified in parallel with the representative genes. The relative expression ratio was determined by the formula 2^(-ΔΔct) method.

**Surgery and Electroencephalogram (EEG) Recording**

SD rats (21 days old, n=12) were surgically implanted with an injection guide cannula and recording electrodes using stereotactic guidance under deep chloral hydrate anesthesia, as previously described [20]. Two electrodes were implanted bilaterally into the dorsal hippocampus (from the posterior fontanelle: nose bar 0; anteroposterior 2.5 mm, lateral 2.5 mm and 2.5 mm below dura mater). At the same time, a guide cannula was positioned unilaterally below the dura mater to enable the intracerebroventricular (icv) injection of drugs (from the bregma: nose bar 0;anteroposterior 1.5 mm, lateral 1.5 mm and 3.5 mm below the dura mater). EEGs recordings were obtained in freely moving rats 1 week after surgery. The baseline EEG for each rat was stable and
reproducible after the drug injection or before exposure to hyperthermia. A first recording period (0–5 min) was used to assess the normal baseline before drug injection. Subsequently, the icv injection of drugs (n = 6) or vehicle (n = 6) was performed using a microsyringe and rats were allowed to rest for 20 min after injection. Another recording period (25–30 min) was used to assess the baseline before exposure to hyperthermia, and then EEG activity was measured continuously throughout the entire process including 4 min of exposure to warm water at a temperature of 44°C and an observation period. EEG recording were stopped after hyperthermia exposure and monitored until we observed a 10 min EEG trace that was similar to baseline. All EEG power data were recorded and analyzed using the RM6240 physiological signal acquisition and processing system (Chengdu instrument, China).

Cell Culture and Intracellular Ca\(^{2+}\) Detection

The rat glioma C6 cell line (ATCC, USA) was grown in Dulbecco’s modified eagle medium (GIBCOQ, USA) supplemented with 15% heat-inactivated fetal calf serum, 50 U/ml penicillin and 50 μg/ml streptomycin. The culture dishes were kept at 37°C in a humidified atmosphere (5% CO\(_2\)).

The fluorescence ratio of the Ca\(^{2+}\) indicator dye Fura-3-acetoxymethyl (AM) ester (Beyotime, China) was used to quantify Ca\(^{2+}\) concentrations in the C6 cells. The cells were incubated for 60 min in the presence of 2 μM Fura-3 AM, washed twice with Krebs-Hepes buffer and allowed to incubate with sitagliptin (100 μM) or glutamate (60 μM) for 20 min before imaging. The [Ca\(^{2+}\)] values are presented in terms of relative fluorescence intensity, and the fluorescence ratio (RFU) of [Ca\(^{2+}\)] was determined using the VICTOR ×5 Multilabel Plate Reader (PerkinElmer, USA). Fluo-3 was excited at a wavelength of 488 nm and fluorescence was measured at a wavelength of 515 nm. Values for each group represent the total [Ca\(^{2+}\)] of 3.5×10\(^4\) C6 cells.

Western Blot

After decapitation, the freshly isolated hippocampal tissues were collected and protein content was determined by BCA Protein Quantitation Kit (Thermofisher, USA). The protein amount loaded per lane was 10 μg. After separation, the proteins were transferred to nitrocellulose paper and unspecific protein binding sites were blocked with 5% skim milk. Tublin was used as loading control for DPPI. The blots were incubated overnight with the primary antibodies, antibodies against DPPI and Tublin were purchased from Abcam (1:2000). Followed 2 h incubation with horseradish peroxidase-conjugated secondary antibody purchased from Proteinlab (1:10,000). Immunoreactivity was visualized using the ECL detection system (Thermofisher, USA). Data were analyzed by Quantity one software.

Bisulfite Sequencing

After decapitation, genomic DNA was extract from freshly isolated hippocampal tissues using DNA extraction kit (Yuansinghao Bio, China) according to the manufacturer’s instructions and then modified with sodium bisulfite using Epit ect Bisulfite kit (Qiagen, Germany). DNA was then PCR amplified using the dippeptidyl peptidase 4 (DPPI) Cpg island 1 (−229/+94) primer pair forward: 5’-AGGGA GGAAT AGTTA AATT TTAATG TGTA-3’ and reverse: 5’-AAAA AATCTA CGAAA ACACC ACCGAC-3’ or Cpg island 2 (+221/+325) primer pair forward: 5’-TGAGG GTAA TATA GGTTG TAATA-3’ and reverse: 5’-AAACCA CTAAA TTATTA ACGC AAAAG-3’. The PCR products were subcloned into pMD18-T simple vector (Takara, Japan) and sequencing was accomplished by Sangon Company (Shanghai, China).

Statistics

Unless noted otherwise, statistical significance of differences between groups was assessed using Students t tests. Throughout the text, summary data were presented as mean ± SEM.

Results

Sample Clustering

Hierarchical sample classification was applied to the expression matrix, each data set generally separated the HP samples (n = 3) from the HR samples (n = 3) (Fig. 1A). All six samples were categorized into two main distinct clusters, corresponding to our expected groups. It was identified that 1140 genes (|FC|<0.05, FC> 1.5) were significantly different in the HP compared to the HR groups (Fig. 1B, Table S1), of which 602 or 538 genes were upregulated or downregulated, respectively. Notably, some known susceptibility genes of FS (sodium channel, voltage-gated, type IX, alpha subunit [SN9A] and interleukin-1 beta [IL-1β]) were also identified in the DEGs.

To confirm the results obtained from the microarray, 10 selected DEGs (5 upregulated and 5 downregulated) were determined using qRT-PCR, based on their involvement in different functional groups and/or pathways. Our results suggested that majority of the investigated genes had congruent results between the microarray and qRT-PCR assays (Fig. 1C–D). Primers for the 10 genes were summarized in Table S2.

GO and Pathway Analysis

The significant GO categories were designated as those with a P value <0.01. The GO categories for genes that significantly differentially expressed between the HP and HR groups were associated with positive regulation of neurotransmitter secretion, negative regulation of synaptic transmission, and negative regulation of acute inflammatory response to antigenic stimulus (Fig. 2A–B). A portion of these GO terms were associated with inflammation (e.g. interferon-gamma [IFN-γ] and interleukin-6 [IL-6]). All significant GO terms of DEGs and related data were summarized in Table S3.

The significant KEGG pathways were designated as those with P value <0.05. The significant pathways of upregulated genes mainly included the steroid hormone biosynthesis pathway and PPAR signaling pathway (Fig. 2C). The significant pathways of downregulated genes mainly included neuroactive ligand-receptor interaction pathway and drug metabolism-cytochrome P450 pathway (Fig. 2D). The DEGs involved in significant pathways were listed in Table S4.

Gene Network Analysis

Genes do not function in isolation, but rather as interacting partners in complex molecular networks that control biological processes. To further study the relationships between DEGs, GeneSpring software were used to construct gene networks for direct and/or indirect contact among these genes, with integrative mining defining a molecular network depicting the generation of FS. In the current study, it was detected that IL-1β and IL-6 were in important positions in the constructed network map; whereas other DEGs in the network were directly or indirectly associated with the IL-1β or IL-6 (Fig. 3). Thus these data further suggests that immune response may be involved in the molecular network during the development of FS.
Figure 1. Hierarchical clustering analysis and qRT-PCR verification. (A) Hierarchical clustering analysis of all experimental samples. The reliable probes Horizontal stripes represent genes while columns show experimental samples (HP n = 3 and HR n = 3). Relationships were represented by the tree lines at the right side of the heat map (short line, close relation; long line, distant relation). (B) Heat map of the differentially expressed genes (DEGs) between the HP and HR groups. 1140 genes (P<0.05, FC>1.5) were identified from the hierarchical cluster, including 602 upregulated genes and 538 downregulated genes. Gene expression was shown in the heatmap as up-regulated (red color), down-regulated (green color) and no change (black color). (C) Hierarchical clustering analysis of 10 DEGs (P<0.05, FC>2). The genes (left) and probes (right) were shown in the heatmap. Gene expression profile was shown in the heatmap as up-regulated (red color), down-regulated (green color) and no change (black color). (D) The verification results of qRT-PCR. Adcy8, Cytl1, Pcdhb6, Rgs1, Dpp4, were up-regulated, Bcl3, Kcnma1, Tnnt1, Mfsd10, Plk3 were down-regulated in the HP group compared with the HR group. Fold change was calculated based on the mean intensity value from 3 rats by using the comparative Ct method and normalized to the housekeeping gene β-actin, *P<0.05, ns: no significant difference. doi:10.1371/journal.pone.0095237.g001
To identify the gene(s) play pivotal roles in the HP and HR groups, we also constructed a gene co-expression network that predicts the relationship among the DEGs. In this network, we applied the notion of k-core value to predict gene function similarity. Thirty-eight DEGs with higher degrees occupied more central positions within a large-scale gene network than other DEGs (Table S5), including ion channels (\textit{Kcnma1, Scn5a, Kcnq4}), precursor proteins (\textit{Avp, Hcrt}). It was speculated that these genes were crucial for the pathogenesis of FS.

Hyperthermia Induced Seizures could be Reduced by the DPP4 Inhibitor Sitagliptin

DPP4 was chosen to explore the role of DEGs in FS pathogenesis. To study this, icv injection of sitagliptin was applied during EEG power analysis (Fig. 4A). Rats were exposed to hyperthermia at a core temperature of 44°C for 4 min after sitagliptin (2 mg/kg, n = 6) or vehicle (normal saline) was injected icv. The EEG results revealed that sitagliptin decreased both the frequency and the amplitude of epileptiform spikes significantly compared to that of the vehicle treated group. The average EEG power spectrum in the whole frequency band (0–40 Hz) was significantly lower ($P < 0.001$, independent $t$-test) in the sitagliptin treated group compared to the vehicle treated group (Fig. 4B–D). These data suggest that DPP4 may plays a critical role in seizure generation, which can be attenuated by a DPP4 inhibitor.

The rat C6 glioma cell line was used to explore the function of sitagliptin \textit{in vitro}. We hypothesized that the method by which sitagliptin protects neurons from hyperexcitability is by stabilizing cellular Ca$^{2+}$ homeostasis. Intracellular Ca$^{2+}$ (Fig. 4E) revealed that although sitagliptin caused a rapid increasing [Ca$^{2+}$]i (2169±632.07 vs 2761±213.3, $P < 0.01$), it significantly decreased glutamate induced [Ca$^{2+}$]i increases (3167±50.44 vs 2475±52.14, $P < 0.001$). These data suggest that the neuroprotective role of sitagliptin may occur via the stabilization of cellular Ca$^{2+}$ homeostasis.
Different DNA Methylation Rate of DPP4 between HP and HR Rats

It was observed that the DPP4 mRNA expression was significantly greater (FC = 2.5367, \(P < 0.05\)) in the HP group compared to the HR group (Fig. 1D), which is consistent with the protein productions of these two groups. Compared to HR rats, the hippocampus of HP rats showed a significant elevation (0.5961 ± 0.0296 vs 0.3606 ± 0.0500, \(P < 0.05\)) in the expression of DPP4 (Fig. 5A–B). Meanwhile, immunohistochemical results from the hippocampus of HP rats revealed higher DPP4 expression than that observed in HR rats (Fig. S1). Subsequently, whether DNA methylation mediate the different expression levels of DPP4 between HP and HR rats was explored. Two CpG islands were analyzed from the DNA sequence of DPP4: CpG island 1 (−229/+94) and CpG island 2 (+221/+325). CpG island 1 contains 31 CG sites, 24 of which belong to the promoter and 7 of which belong to exon 1. All 7 CG sites of CpG island 2 belong to intron 1 (Fig. 5C). Bisulfite sequencing revealed a lower DNA methylation rate in HP rats than in HR rats in both CpG island 1 (0.9090 ± 0.5248 vs 2.828 ± 0.2672, \(P < 0.05\)) and CpG island 2 (7.667 ± 0.8819 vs 17.33 ± 0.8819, \(P < 0.01\)) of DPP4 (Fig. 5D–E).
Discussion

Gene expression profile analysis was used in this study to identify candidate susceptibility genes and expression patterns for FS in rat models. According to the results of the Hierarchical Cluster Analysis, all experimental samples were divided into two different clusters based on expression similarity, and we can conclude that these genetic results are consist with with the animals behavior (HP vs HR) [20], which provides a solid foundation for microarray analysis. Using a range of data mining and information annotation approaches, we were able to identify a number of transcriptome features that may shed light on the pathogenesis of FS.

We identified some important genes whose GO and signal pathways may be associated with the pathogenic factors of FS (Fig. 2A–B). Upregulated genes were associated with the cellular response to IFN-γ and IL-6. In addition, downregulated genes were associated with the negative regulation of T cell proliferation and immune system processes, which may help clarify the role of immunity and inflammation in FS. An increasing amount of evidence supports the involvement of immune and inflammatory processes in the etiopathogenesis of seizures [23,24]. Various

Figure 4. Anticonvulsant effects of sitagliptin in rats exposure to hyperthermia. (A) Schematic diagram of experimental protocol. Data were binned in 60 min intervals. (B) The representative EEG tracings from rats in vehicle group (n = 6). Hyperthermia-induced seizure were shown in details in the expanded lower trace during seizure induction period (left panel) or observation period (right panel). (C) The representative EEG tracings from rats in sitagliptin group (n = 6). Hyperthermia-induced seizure were shown in details in the expanded lower trace during seizure induction period (left panel) or observation period (right panel). (D) Power spectral analysis of EEG between the two groups. Differences between the vehicle (black line) and sitagliptin groups (blue line) were statistically significant (P<0.001). The vertical axis was the average EEG power spectral (dB), and the horizontal axis was the frequency (HZ). (E) C6 cells in control group were treated with 5 μl PBS; sitagliptin group were treated with 100 μM sitagliptin; glutamate group were treated with 60 μM glutamate; sitagliptin+glutamate group were treated with both 100 μM sitagliptin and 60 μM glutamate. **P<0.01, ***P<0.001.

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Experimental observations provide evidence for an active role of brain-derived inflammatory molecules and the activation of related cell signaling pathways (e.g., the IL-1 receptor/Toll-like receptor pathway, NF-κB pathway and MAPK pathway) in seizure pathogenesis [25]. According to our KEGG pathway analysis, the steroid hormone biosynthesis pathway was the most significant pathways of upregulated genes (Fig. 2C), which concurs with research reporting that neurosteroids are synthesized within the brain and rapidly modulate neuronal excitability [26,27]. However, an association between steroid hormones and the immune response in the brain has also been detected [28]. Because the inflammatory response could have serious detrimental effects in the brain, local steroid generation negatively regulates NF-κB dependent signaling pathways, and therefore serves as an important element for the control of inflammation in the central nervous system [28].

Using advanced molecular network techniques, we were able to propose a molecular network (Fig. 3). In this network, IL-1β and
**Supporting Information**

Figure S1 Immunohistochemistry detection of DPP4 in hippocampal samples. The representative DPP4 expression of HP rats (n = 3) in hippocampal (A), CA3 (C), and dentate gyrus (E), and representative DPP4 expression of HR rats (n = 3) in hippocampal (B), CA3 (D), and dentate gyrus (F). (TIF)
Table S1  The Differential Expressed Genes between the HP group and the HR group. (XLS)

Table S2  Sequences of Primers for qRT-PCR. (XLS)

Table S3  Significant GO analysis between the HP group and the HR group. (XLS)

Table S4  Significant Pathways analysis between the HP group and the HR group. (XLS)

Table S5  38 genes identified by gene co-expression network. (XLS)

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Author Contributions
Conceived and designed the experiments: ZW YF WL XH. Performed the experiments: ZW YF JX LJ DH SH JY BP. Analyzed the data: ZW YF JX. Wrote the paper: ZW YF XH.

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