Epilepsy & Behavior xxx (xxxx) xxx

Contents lists available at ScienceDirect

Epilepsy & Behavior

journal homepage: www.elsevier.com/locate/yebeh

Molecular tools for the characterization of seizure susceptibility in genetic rodent models of epilepsy

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ARTICLE INFO

Article history:
Received 26 August 2019
Revised 20 September 2019
Accepted 25 September 2019
Available online xxxx

Keywords:
DNA analysis
Gene expression studies
Immunodetection
Post-translational protein modifications
Proteomic

ABSTRACT

Epilepsy is a chronic neurological disorder characterized by abnormal neuronal activity that arises from imbalances between excitatory and inhibitory synapses, which are highly correlated to functional and structural changes in specific brain regions. The difference between the normal and the epileptic brain may harbor genetic alterations, gene expression changes, and/or protein alterations in the epileptogenic nucleus. It is becoming increasingly clear that such differences contribute to the development of distinct epilepsy phenotypes. The current major challenges in epilepsy research include understanding the disease progression and clarifying epilepsy classifications by searching for novel molecular biomarkers. Thus, the application of molecular techniques to carry out comprehensive studies at deoxyribonucleic acid, messenger ribonucleic acid, and protein levels is of utmost importance to elucidate molecular dysregulations in the epileptic brain. The present review focused on the great diversity of technical approaches available and new research methodology, which are already being used to study molecular alterations underlying epilepsy. We have grouped the different techniques according to each step in the flow of information from DNA to RNA to proteins, and illustrated with specific examples in animal models of epilepsy, some of which are our own. Separately and collectively, the genomic and proteomic techniques, each with its own strengths and limitations, provide valuable information on molecular mechanisms underlying seizure susceptibility and regulation of neuronal excitability. Determining the molecular differences between genetic rodent models of epilepsy and their wild-type counterparts might be a key in determining mechanisms of seizure susceptibility and epileptogenesis as well as the discovery and development of novel antiepileptic agents.

This article is part of the Special Issue "NEWroscience 2018"

Abbreviations: 2D-DIGE, 2D Fluorescence Difference Gel Electrophoresis; aCGH, array comparative genomic hybridization; *Acsm3*, coenzyme acyl-CoA synthetase; ATP5H, ATP synthase subunit d, mitochondrial protein; *C1q*, complement C1q chain; *Camkk2*, calcium/calmodulin-dependent protein kinase 2; CDKL5, cyclin-dependent kinase like 5; cDNA, complementary DNA; CGH, comparative genomic hybridization; ChIP-Seq, chromatin immunoprecipitation sequencing; CNVs, copy number variations; CSF, cerebrospinal fluid; *Egr1,2,3*, early growth response protein 1, 2 or 3; EWCE, expression-weighted cell-type enrichment; FISSEQ, fluorescent in situ RNA sequencing; *FN1*, encodes fibronectin; GABA(A)R, $GABA_A$ receptor; GAERS, Genetic Absence Epilepsy Rat from Strasbourg; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; GASH/Sal, genetic audiogenic seizure hamster from Salamanca; GFAP, glial fibrillary acidic protein; *Gpr126*, G-protein coupled receptor 126; *Gria2*, glutamate ionotropic receptor AMPA type subunit 2; *Grin2a*, glutamate ionotropic receptor NMDA type subunit 2A; H3/H4, histone H3/H4; HDAC, histone deacetylase; HIF-1, hypoxia inducible factor 1; HPLC, high-performance liquid chromatography; *Il10rb*, interleukin 10 receptor subunit beta; IC, inferior colliculus; iTRAQ, isobaric tags for a relative and absolute quantification; JAK/STAT signaling pathway, Janus kinases (JAK)/signal transducer and activator of transcription proteins (STATs); *KIF5A*, kinesin heavy chain isoform 5A; LCM, laser capture microdissection; MALDI, matrix-assisted laser desorption/ionization; MAPK, mitogen-activated protein kinase; MDH1, malate dehydrogenase; Methyl-Seq, massive methylation sequencing; miRNA, micro RNA; MS, mass spectrometry; MTLE, mesial temporal lobe epilepsy; MTLE-HS, mesial temporal lobe epilepsy with hippocampal sclerosis; mTOR, mammalian target of rapamycin; NEFL, neurofilament light polypeptide; NGS, next generation sequencing; PCDH19, protocadherin 19; PK, piruvate kinase; PNPO, pyridoxamine 5′-phosphate oxidase; PRDX2, peroxyredoxin-2; *Qdpr*, enzyme dihidopterina reductase quinoide; RNA-Seq, RNA-sequencing; RT-qPCR, quantitative real time PCR; *Rtel*, regulator of telomere elongation; SBL, in situ sequencing by ligation; SC, superior colliculus; scRNA, seq: single-cell RNA sequencing; SE, status epilepticus; seqFISH, sequential fluorescence in situ hybridization; SINEs, short interspersed nuclear elements; SLC25A22, solute carrier family 25, member 22; *Slc6a1*, solute carrier family 6, member 1; smFISH, single-molecule RNA fluorescence in situ hybridization; *Stat3*, signal transducer and activator of transcription 3; STXBP1, syntaxin-binding protein 1; *Timp-1*, tissue inhibitor of metalloproteinases 1; TIVA, the transcriptome in vivo analysis; TLE, temporal lobe epilepsy; TOF, time-of-flight; WAG/Rij, Wistar Albino Glaxo from Rijswijk; WAR, Wistar Audiogenic Rat; WES, whole exome sequencing; WGS, whole genome sequencing.

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https://doi.org/10.1016/j.yebeh.2019.106594
1525-5050/

1. Introduction

Currently, epilepsy affects around 50 million people in the world [1], and its heterogeneous etiology includes structural alterations due to traumatic brain injury [2], genetic disorders [3], or idiopathic epilepsy with no certain cause [4]. Epileptogenesis is a dynamic and gradual process that involves changes in the brain after a precipitating injury or insult that results in the development of spontaneous recurrent seizure activity or epilepsy [5]. The complexity of this neurological disorder requires models for exploring different aspects of epilepsy [6]. Among the most used and well-characterized in vivo genetic models of epilepsy are those genetically predisposed animal species, in which seizures occur in response to high intensity acoustic stimulation, the so-called genetic audiogenic seizure models [7,8]. Genetic and protein alterations have been described as possible contributors to the development of epilepsy phenotypes in the audiogenic seizure models [9–11]. Thus, studies focused on molecular disruptions of the epileptogenic nucleus are of utmost interest, as they provide insights into the mechanisms involved in seizure susceptibility and regulation of neuronal excitability. Challenging these key assumptions may lead to discovery of molecular biomarkers, which can be used for the epilepsy diagnosis and prognosis as well as for assessing the efficacy of new antiepileptic treatments. In the present paper, we reviewed the molecular techniques used for the understanding of seizure susceptibility in genetic models of epilepsy, particularly in genetically epilepsy-prone rodents. We also compared the results obtained from the main models of epilepsy and showed some our own findings to highlight the strengths and weaknesses of different molecular approaches.

2. DNA studies

The need to identify a large set of genes involved in complex human diseases has led to an increased use of molecular techniques in biomedical research and clinical practice. The discovery of genes associated with epilepsy, excluding those involved in metabolic disorders and intellectual disability that may present high prevalence of epilepsy, has exponentially increased from 30 to 90 genes in the last decade [12]. However, most pathologic genetic variants were identified in monogenic epilepsy and represent a small subset of all epilepsy types [13]. All of these achievements have been possible because of the development and cost reduction of next generation sequencing (NGS)-based panel tests that allowed the advent of multigene panels, exome, and genome-scale sequencing. Additionally, neurophysiological and molecular studies of mutations underlying seizure susceptibility might also contribute to the successful development of new therapeutic targets and antiepileptic drugs. A large percentage of the mutations identified in epilepsy involves altered genes that encode calcium, potassium, sodium, and chloride ion channels, making up a family of channelopathies [14], neurotransmitter receptors (e.g., γ-aminobutyric acid (GABA) receptors) [15], several structural proteins [16,17], mammalian target of rapamycin (mTOR) pathway genes, modulation of synaptic vesicle docking and release (e.g., syntaxin-binding protein 1 [STXBP1]) [18], cell signaling (e.g., cyclin-dependent kinase like 5 [CDKL5]) [19], cell–cell adhesion (e.g., protocadherin 19 [PCDH19]) [20], transcription (e.g., the aristaless-related homeobox gene (ARX)) [21], DNA repair the bifunctional polynucleotide phosphatase/kinase gene (PNKP) [22], mitochondrial glutamate symporter (e.g., solute carrier family 25, member 22 [SLC25A22]) [23], and enzymes involved in metabolic pathways (e.g., pyridoxamine 5′-phosphate oxidase [PNPO]) [24].

Despite these significant advances, much less is known about the altered genes in animal models of epilepsy, with the exception of those related to monogenic epilepsies. Animal models of epilepsy arose from artificial selection of seizure-susceptible strains over many generations that resulted in high predisposition to epilepsy, as is the case of audiogenic epilepsy models. Examples of rodent audiogenic seizure models are the Genetic Absence Epilepsy Rat from Strasbourg (GAERS; [25]), the Wistar Albino Glaxo from Rijswijk (WAG/Rij; [26]), the Wistar Audiogenic Rat (WAR; [27]), and the genetic audiogenic seizure hamster from Salamanca (GASH/Sal; [7]). Activation of auditory pathways are required for the onset and progression of seizures in all audiogenic seizure models, and many studies pointed out the inferior colliculus (IC), a critical integration center in the auditory midbrain pathway, as the epileptogenic nucleus [6]. However, the genetic alterations underlying epileptogenesis in the IC are not fully known. With the increase in availability of genome-editing techniques that assess the relationship between mutation genotype and phenotype, nonrodent species like the zebrafish are also used to optimize and expedite genetic testing as well as development of antiepileptic drugs [28].

The multiple molecular approaches and the many options for linkage of different analysis may detect a number of mutations and DNA modifications involved in epilepsy. Fig. 1 depicts the analytic tools used in genome-wide studies of epilepsy as well as a brief description of their corresponding outcomes, showing possible genetic alterations. It should be noted that no single genetic testing has all the requisite capacity to contend with the complexities of all genetic mechanisms, and in some cases, the genetic variants identified through the variety of approaches are not fully understood [29].

2.1. Comparative genomic hybridization (CGH)

The comparative genomic hybridization (CGH) and, lately, arrays of CGH (aCGH) are the simplest approaches to study DNA variations, particularly if used species with known genome such as rat and mouse. The aCGH method allows reliable detection of DNA sites in multiple genome loci by comparing the relative amounts of DNA from two genomes, the control and the sample to be tested. Both samples are labeled with different fluorochromes that bind with DNA fragments of known sequences or "probes", fixed to a slide or glass holder (Fig. 1A). The color of the fluorescence at each point of the aCGH informs about the relative amount of each DNA and allows inferring the presence of gains or losses in specific regions of the genome [30]. The aCGH method is used to evaluate targeted regions throughout the chromosomes for copy number variations (CNVs). There are commercial rodent microarrays that contain 720,000 probes/oligonucleotides of 50–75 mer (average size) and an average distance of 3537 pb. Those with less than 10 consecutive oligonucleotides are considered as an aberration. In the GASH/Sal model, we used commercial arrays of mice and found genomic alterations at the level of 17qE5. The gain and loss detected in this chromosome region could be indicative of CVNs, involving the neurexin I gene; however, no association with epilepsy was found (unpublished data). Mullen et al. [31] demonstrated that CNVs are overrepresented in patients with genetic generalized epilepsy, pointing out CNVs as an important risk factor. Taking into account the large pattern of genetic and phenotypic heterogeneity in epileptic syndromes, the applicability of CGH microarrays to evaluate the CVNs becomes controversial [32,33]. Olson et al. [34] suggested assessing CVNs through conventional chromosomal microarray to shed light upon the idiopathic epilepsy, but the greatest impediment for CNVs detection might be the limit size of 10 kb. In this regard, more recent studies argue in favor of using other techniques such as the whole exome sequencing (WES, see below) that is able to identify small CNVs less than 10 kB in size [35].

2.2. Whole genome sequencing (WGS)

The feasibility of implementing gene sequencing methods has made tremendous contributions to science advancement in general, and especially in epilepsy research. Although sequencing technologies, some years ago, were relatively expensive and time consuming, nowadays, a rapid development is taking place. Thus, novel sequencing platforms and supporting technologies involved in processes such as *targeting* and data analysis have emerged and are used routinely to reduce costs and determine the complete genome sequences of different species.

Please cite this article as: J.R. Bosque, R. Gómez-Nieto, S. Hormigo, et al., Molecular tools for the characterization of seizure susceptibility in genetic rodent models of epilepsy, Epilepsy & Behavior, https://doi.org/10.1016/j.yebeh.2019.106594

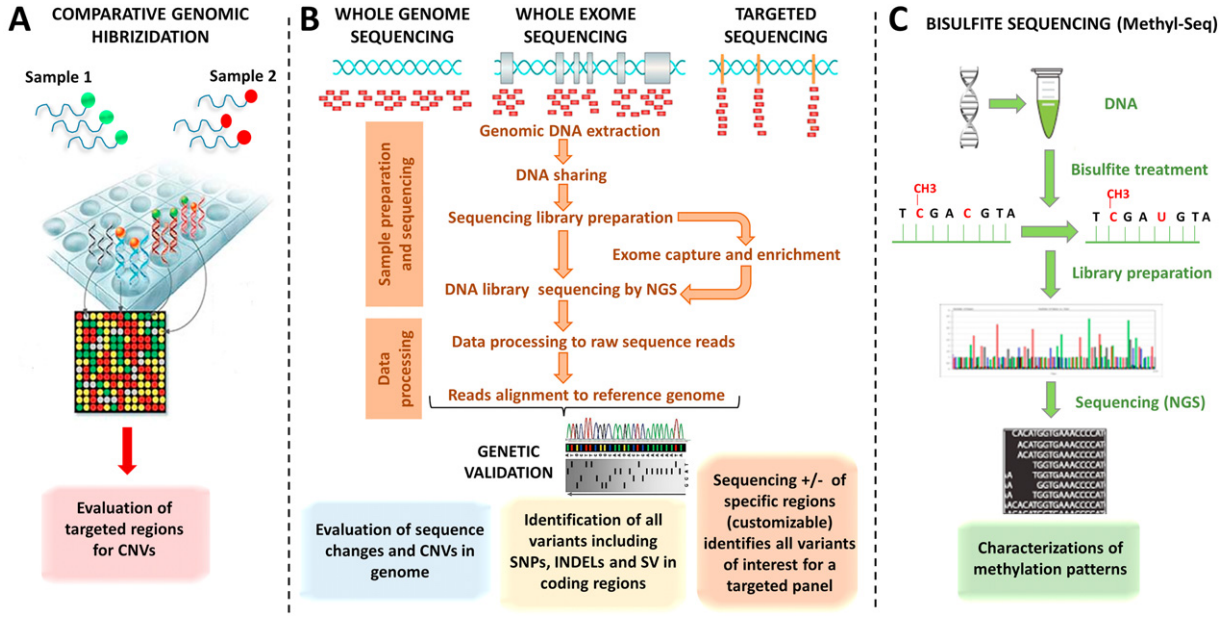

Fig. 1. Overview of techniques used for determining DNA alterations. A. Diagram of the microarray-based comparative genomic hybridization processes. Samples are labeled with fluorescent dyes and applied to the microarray. Complementary nucleotide sequences are bound to the microarray wells and the microarray scanner measures the fluorescent signals to generate plots. B. Workflow of genome, exome, or panel gene sequencing. The genomic DNA is cut into short fragments to generate of DNA library by PCR. Using NGS, the library is sequenced, and the corresponding data are analyzed through bioinformatics analysis. Finally, SANGER sequencing is necessary to confirm the mutations. C. Workflow of histone modifications analysis by Methyl-Seq. Genomic DNA is bisulfite-treated and nonmethylated cytosine residues are converted to uracils, then library is performed and sequenced in NGS platform, and are further analyzed using bioinformatics tools.

When whole genome sequencing (WGS) is applied to animal models of epilepsy, the identification of gene variants associated with epilepsy is possible by comparing differences between the WGS on the wild type and the epileptic strain (Fig. 1B).

As an example, the complete genome sequence of the GAERS was performed bringing promising results [36]. The GAERS model shows a genetic generalized epilepsy that is analogue to the human absence epilepsy as verified by electroencephalic, pharmacological, and behavioral results, and like the human condition, this model appears to have a complex genetic architecture. The GAERS genome study identified 1.12 million single nucleotide variants, 296.5 K short insertion–deletions, and 354 putative copy number variants that result in complete or partial loss/duplication of 41 genes. Among the variants with high quality criteria, 25 variants gain a stop codon, 56 have putative essential splice sites, and 56 were predicted to result in a frameshift mutation. The information obtained from the GAERS genome study was too extensive and varied that the analysis is still in progress, and it is difficult to make a correlation with genetic modifications responsible for seizure susceptibility [36]. The WGS approach can also be applied to identify susceptible genes in human, which may allow high rates of molecular diagnosis in patients with epilepsy and increase the identification of epilepsy genes [37,38].

2.3. Whole exome sequencing (WES)

The WES, a genomic technique for sequencing the entire protein-coding region of genes in a genome, may be an excellent option when other methods fail (Fig. 1B). Allen et al. [39] used WES to investigate mutations in epileptic encephalopathies and found 329 de novo mutations; some of them have a clear statistical evidence of association with epileptic encephalopathy, such as *GABRB3* (that encodes the subunits beta 3 of the GABA type A receptors) and *ALG13* (that encodes the ALG13 UDP-N-Acetylglucosaminyltransferase Subunit).

The WES, despite its high potential, is not commonly used in experimental models of epilepsy. However, it was seemingly a good choice in the first genetic studies of the GASH/Sal model, as the genome used as a reference was incomplete. Once the DNA is isolated and fragmented, it is necessary to create a genomic library. To do this, the oligonucleotides are amplified via polymerase chain reaction (PCR), then transcribed *in vitro* in the presence of biotinylated uridine triphosphate (UTP) to generate single-stranded RNA "bait." Genomic DNA is sheared, ligated to Illumina sequencing adapters, and selected for lengths between 200 and 350 bp. A quality control of the sequences is carried out with the FASTQC program [40], assembling those that pass the checking with the SPAdes program [41]. As a result, the genome can be compared with the sequences of the reference genome, identifying point mutations, structural variations, and genomic reorganizations by using the GATK, the Lumpy, and the MAUVE software, respectively [42–44]. After a bioinformatics analysis, the variants with higher or lower impact can be obtained [45]. Although the WES data analysis is a difficult task, variant catalogs such as ClinVar [132] and Human Gene Mutation Database (HGMD) [133] are suitable for guiding variant classifications among known disease genes, which facilitate the interpretation of a novel missense variant. Despite this, the final variant interpretation can be a highly subjective process, contributing to the variability reported in variant classification. For this reason, the American College of Medical Genetics and Genomics (ACMG) has drastically reduced variability in the classifications by systematizing the variant interpretation process.

The WES has also some limitations, for example, it is not able to detect alterations in noncoding regions or methylation abnormalities, and hence, the use of other techniques is required [46,47].

Several studies performed different techniques in a complementary way to identify deleterious genetic variants in epilepsy and to detect the responsible genes for a given type of epilepsy. For example, a combination of aCGH, targeted sequencing, and WES successfully allowed the identification of de novo mutations that affected the maturation and development of neural networks [48].

The WES results must be validated, ensuring that the mutations are found in all tested animals. To do so, primers near the mutated regions are designed to subsequently amplify those regions and perform the SANGER sequencing (Fig. 1C).

The large number of gene variants detected in the GASH/Sal using WES analysis allowed us to identify high impact mutations in genes

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that might be related to epilepsy (unpublished data). A first step to reduce such a big number of gene variants is to filter out based on the mutation characteristics. Once the variants of interest have been identified, the next step will be to assess the implication of each gene variant in the seizure susceptibility.

2.4. DNA modifications

The study of DNA modifications includes DNA methylation, specific chromatin changes such as histone modifications, and noncoding RNAs among other components that are tightly interconnected. All these DNA modifications form integral part of the epigenetic machinery that regulates the gene expression [49,50]. Alterations in DNA methylation of specific genomic regions may contribute to the development of complex disorders (e.g., Prader–Willi and Angelman syndromes), as they could prompt cells to gain or loss biological functions of genes typically expressed only from the maternal or the paternal copy [51,52].

Recent studies show that epilepsy and epileptogenesis are associated with changes in each of the factors that affect the epigenome [134]. Aberrant DNA methylation signatures link general pathomechanisms of epileptogenesis and epilepsy to epileptic brain tissue in experimental animal models and humans. The repressive capacity of cytosine DNA methylation is mediated by recruitment of silencing complexes, in which methyl-CpG binding domain proteins aid in the mediation of gene silencing. There are standard procedures used to study those modifications and patterns of methylations based on the bisulfite conversion of genomic DNA, methylation-sensitive restriction enzyme digestions, and the immunoprecipitation of methylated DNA using methyl CpG-specific antibodies [53]. The procedure consists of fragmenting the genomic DNA, enrichment of methylated DNA (using commercial methylated DNA-binding enrichment kit), preparation of the MethylC-sequencing library in NGS platforms (e.g., Illumina, SOLiD, or Ion Torrent), and finally, assessment using bioinformatics data analysis (Fig. 1C). These methods have been carried out in rodent models of epilepsy, suggesting that altered DNA methylation patterns contribute to pathological mechanisms that induce genetic deregulation in epilepsy syndromes. In chronic epileptic rats, Kobow et al. [50] reported an increase in DNA methylation, mainly confined to gene bodies. These altered methylation patterns were inversely correlated with the gene expression, so that an increase in gene body methylation was associated with gene silencing and, conversely, a decrease in methylation was associated with increased gene expression. The calcium/calmodulin-dependent protein kinase 2 (*Camkk2*) gene that encodes a calcium-dependent protein kinase was found hypermethylated and downregulated, which may be linked to aberrant neuronal activity and seizure. Moreover, interleukin 10 receptor subunit beta (*Il10rb*) gene that encodes an interleukin receptor protein was found hypermethylated and upregulated, which could be a compensatory mechanism to limit brain damage following recurrent seizures. Administration of ketogenic diets to epileptic rats was found to have anticonvulsant activity, ameliorating DNA methylation changes [50]. Consistently, similar studies also revealed increased methylation in gene bodies and hypomethylation at nongenic regions, supporting the idea that altered DNA methylation is a general pathomechanism associated with epileptogenesis and epilepsy in these models [49,54]. Further, *in vitro* experiments correlated posttranscriptional modifications of histones (H3/H4 acetylation and H3 phosphorylation) with downregulation in the expression of two potential epilepsy-associated genes, the excitatory glutamate receptor genes glutamate ionotropic receptor AMPA type subunit 2 (*Gria2*) and glutamate ionotropic receptor NMDA type subunit 2A (*Grin2a*) [55]. Also, increases in histone H4 acetylation and in phosphorylation of histone H3 have been reported in animal models of epilepsy [56–58]. Based on these results, several studies have been developed histone deacetylase (HDAC) inhibitors as a possible therapy in epilepsy, since HDAC inhibition has a neuroprotective effect [57], but without a principal anticonvulsant action [59,60].

3. Gene expression studies

The fact that RNA-based approaches provide answers that cannot be revealed by conventional DNA-sequencing approaches have contributed to resolve great conundrums in epilepsy research.

3.1. Microarrays

The development of global gene expression profiling platforms has revolutionized research in molecular biology, allowing identification, cataloging, and measuring of vast amounts of information at a single time. The corresponding genomic changes are consistent, and hence, these techniques give us a broader perspective for understanding the disease process. The late 1990s heralded the development of several important technological advances in molecular biology, specifically the DNA microarrays that were used for comparing global gene expression in experiments at different conditions. This has led to flourish a relatively new field of comparative genomics [61]. DNA microarrays generate large libraries of mRNA sequences, enabling researchers to compile differential gene expression lists in disease states by statistical comparison of transcript frequencies between two or more conditions (Fig. 2A). It allows simultaneous monitoring of thousands of genes, thus providing a functional aspect to sequence information in a given sample. There are several types of microarray technologies currently in use, and among them, the oligonucleotide microarray technology is the most widely used for high-throughput quantitative studies of RNA expression [62]. As shown in Fig. 2A, RNA extracted from the material of interest is reverse transcribed to complementary DNA (cDNA) and then incubated with a mixture of fluorescent markers on a microarray chip that contains a predetermined set of genes. Quantification is then performed by computerized measurements of fluorescent intensities. Known limitations of microarray technology are the large amounts of material at the initial stage and the unfeasibility to identify novel genes, as it relies on prior knowledge of the targeted genes. These have largely been addressed with a two-stage hybridization process or global RNA amplification prior to running the array experiment [61,65]. The molecular mechanisms underlying epileptogenesis are thought to be associated with altered expression of gene groups [66], and the microarray has been the preferred platform for their gene expression analysis [61,67]. In the last 20 years, there have been published more than 40 large-scale gene expression studies on epileptic tissue obtained from resection of the epileptogenic zone [11,61,66–79]. Furthermore, mainly in the last decade, some works aimed to study the potential genes or pathways associated with epilepsy based on micro RNA (miRNA) expression profiles [80–84]. Numerous attempts have been made to employ transcriptional expression profiles in animal models, as well as in patients with temporal lobe epilepsy (TLE). Despite this, there is no consensus regarding common transcriptional drivers of epileptogenesis [66]. Variability among animal species, epilepsy models, different sample size, tissue sampling time-points, array platforms, and normalization algorithms, have resulted in only a few genes demonstrating a consistent expression change [66,68,70]. Thus, many studies reported the same differentially expressed genes related to epilepsy, such as tissue inhibitor of metalloproteinases 1 (*Timp-1*), signal transducer and activator of transcription 3 (*Stat3*), complement C1q chain (*C1q*), solute carrier family 6, member 1 (*Slc6a1*), and *Pcdh19* [70]. All these genes share the common characteristic of belonging to different pathways, some of which are related to epileptogenesis, such as the mitogen-activated protein kinase (MAPK) signaling pathway, the Janus kinases/signal transducer and activator of transcription proteins (JAK/STAT)09 pathway, p53 signaling pathway, and the extracellular matrix (ECM)/integrin signaling [70]. Interestingly, genes like *MAPK*, *HIF*, and *JAK/STAT* are potentially associated and upregulated under a common hypoxic condition in a HIF-dependent manner [85].

By using microarrays, our research group analyzed changes in gene expression of the IC in two audiogenic seizure strains, the WAR and

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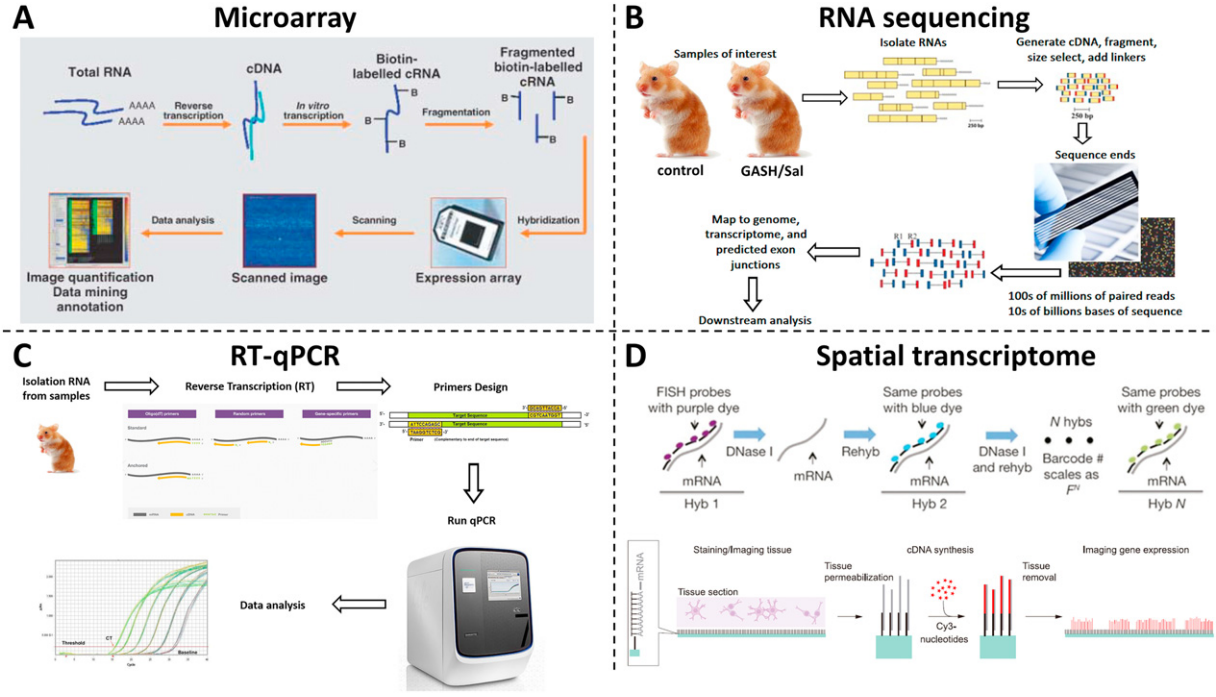

Fig. 2. General workflows for differential gene expression analysis using different techniques. Schemes show the standard workflow of microarray (A) (reproduced with permission from [62]), RNA sequencing (B), RT-qPCR (C), and spatial transcriptome studies (D) [smFISH (reproduced with permission from [63]) scRNA-Seq (reproduced with permission from [64])].

the GASH/Sal models, and compared with age-matched controls under the same stimulation conditions. The microarray results showed that the genomic profile of the WAR animals exhibited significant upregulation of 38 genes and downregulation of 47 genes when compared with Wistar rats. Comparison of gene expression profiles between stimulated control and GASH/Sal hamsters revealed the upregulation of 10 genes and the downregulation of 5 genes. Finally, the comparison of the gene expression profiles between the two audiogenic seizure models showed only one common gene, the zinc finger immediate-early growth response gene early growth response protein 3 (*Egr3*), which was upregulated in both cases. We hypothesized that their overexpression in both models might contribute to neuronal viability and development of lymphoma in response to stress associated with audiogenic seizures [11].

Microarray studies have also been shown to demonstrate significant variations even under the same experimental conditions. As mentioned below, the validation of global expression profiling results by independent methods such as quantitative real-time polymerase chain reaction (RT-qPCR), Northern/Western blotting, and immunodetection is essential [61]. On the other hand, microarray technology has certain limitations such as the low sensitivity and high false-positive rates [86]. One way of overcoming these drawbacks is to use meta-analysis methods that integrate the results of separate microarray studies and increase both the sensitivity and the reliability of measurements of gene expression changes [75,86].

3.2. RNA-Seq

The first decade of this millennium witnessed the advent of massive parallel sequencing, also known as deep sequencing or NGS. Thus, RNA-sequencing (RNA-Seq) is now the method of choice to study gene expression and identify novel RNA species. RNA-sequencing has several advantages over microarrays. First, sequencing technology is much more sensitive and quantitative than arrays. Second, RNA-Seq provides a larger dynamic range for detection of transcripts (>9000-folds) compared with standard arrays. Third, sequence data are more specific and have less background. Furthermore, sequencing experiments do not depend on the limited probes present on tiled microarrays and can, therefore, be used to challenge any location in the genome, including unannotated genes. Finally, sequencing is not limited by array hybridization chemistry, such as melting temperature, cross-hybridization, and secondary structure concerns. In conclusion, RNA-Seq directly reveals sequence identity that is crucial for analysis of unknown genes, novel transcript isoforms, and genetic variations [87,88].

Most RNA-Seq experiments are carried out on instruments that sequence DNA molecules due to the technical maturity of commercial instruments designed for DNA-based sequencing. Therefore, as shown in Fig. 2B, cDNA library preparation from RNA is a required step for RNA-Seq. Each cDNA in an RNA library is composed of a cDNA insert of certain size (previously RNA or cDNA fragmentation), and flanked by adapter sequences, as required for amplification and sequencing on a specific platform. The cDNA library preparation method varies depending on the RNA species under investigation, which can differ in size, sequence, structural features, and abundance. Moreover, because of the detection limit of most sequencers, several cDNA libraries need to be amplified by PCR before sequencing, and to correct for PCR amplification bias, using methods that eliminate PCR duplicates from sequencing results [88]. After, directly cDNA libraries, or subsequent PCR products, are sequenced using NGS, producing millions of short sequence reads, typically 30 to 500 bp in length, depending on the DNA-sequencing technology used. Finally, the reads obtained after sequencing are either aligned to a reference genome or transcriptome, or assembled de novo without genomic sequence guidance, to create a genome-scale transcription map that provides both transcriptional structure and expression level for each gene. Thus, the number of reads mapped within a gene or an exon can then be used as a measure of its abundance in the analyzed sample [87].

Increasingly available data in RNA-Seq databases will help the neuroscience community to make new discoveries at unprecedented speed and depth. However, many challenges still remain. For example, most of the read counts are produced by very highly expressed genes. Many genes of interest with low expression levels may have gone undetected because of very low read counts. These low read counts can also be quite variable, which can cause biases in differential expression analyses [87]. Up to now,

RNA-Seq had been employed at least in 10 reports to profile the entire transcriptome across multiple phases of epileptogenesis in different animal models of epilepsy, as well as changes in transcription at the chronic phase in humans [10,11,82,89–95].

Thus, the transcriptional profile of the corpus quadrigeminum, comprised the inferior and superior colliculi, in WAR animals was recently described [10]. Transcriptomic analysis of WAR (versus Wistar rats) after acoustic stimulation allowed us to identify a set of 62 and 16 differentially regulated genes using two types of statistical packages (EdgeR and DESeq), such as a gene that encodes the coenzyme acyl-CoA synthetase (*Acsm3*), G-protein coupled receptor 126 (*Gpr126*, encodes a receptor that could be associated with alterations in the acquisition or the processing of acoustic information), regulator of telomere elongation (*Rtel*, encodes a helicase that acts in the protection, stability, and elongation of telomeres), and enzyme dihidopterina reductase quinoide (*Qdpr*, encodes the enzyme dihidopterin reductase quinoid that is responsible for recycling of BH4, a molecule that acts as enzyme cofactor and is involved in the synthesis of serotonin, dopamine, and nitric oxide). Gene Ontology (GO) enrichment analysis showed that "catalytic activity" and "metabolic processes" categories were among the most represented functional categories of the genes differentially regulated in WAR, suggesting that the model presents metabolic alterations [10].

On the other hand, our research group analyzed also the transcriptome of IC of the GASH/Sal model in comparison with their control under the same stimulation conditions. We previously used the mouse probes for the microarray analyses of gene expression (GeneChip® Mouse Gene ST Array) in hamsters as the Syrian hamster (*Mesocricetus auratus*) probes were not available at that time. To confirm these results, we employed Chinese hamster probes (*Cricetulus griseus*) via transcriptomic analysis, comparing the stimulated controls with the stimulated GASH/Sal. Upon using these probes, the number of differentially expressed genes between GASH/Sal and control animals was increased [11].

In other study, Dixit et al. [91] performed transcriptome analysis of hippocampal tissues resected from patients with mesial TLE with hippocampal sclerosis (MTLE-HS), using the RNA-seq approach. Differential gene expression analysis revealed 56 significantly regulated genes in MTLE (vs. controls without epilepsy) and their possible association with epileptogenesis and/or pharmacoresistance in MTLE-HS. Gene cluster analysis identified three important hubs of genes mostly linked to the following: 1) neuroinflammation and innate immunity; 2) synaptic transmission (as a gene encodes kinesin heavy chain isoform 5A [*KIF5A*], involved in GABA(A)R trafficking, and whose deletion causes epilepsy); and 3) neuronal network modulation (as a gene encodes fibronectin [*FN1*] and proposed cerebrospinal fluid [CSF]-serum biomarker for epilepsy), which are supportive of intrinsic severity hypothesis of pharmacoresistance [91]. Furthermore, by using this same technique, a previous study reported that the status epilepticus (SE) induced changes in the hippocampal RNA expression in an animal model of pilocarpine-evoked SE [93]. These authors performed whole transcriptome profiling to identify differentially expressed mRNAs at 12 h, 10 days, and 6 weeks after evoking experimental SE, which correspond with the distinct phases of the epileptogenic process (acute, seizure silent, and spontaneous-seizure phases).

Recently, Jehi et al. [96] also proposed that the maturation of a new epileptic focus may explain late seizure recurrences. Using RNA-seq, they identify 29 differentially expressed genes between late-recurrence and seizure-free patient samples, concluding that late recurrences after epilepsy surgery may be influenced partly by differences in gene expression in neuroinflammatory and brain healing/remodeling pathways [96].

Finally, it is noteworthy to highlight the fact that transcriptome analysis can also be performed at the level of single cells. Unlike classical methods, which consider mixtures of heterogeneous cell populations, single-cell RNA sequencing (scRNA-Seq) provides a much more detailed view of transcription dynamics [135]. For example, the analysis of transcriptomes from single cells has revealed the substantial transcriptional heterogeneity among seemingly identical cells [97]. Recently, Skene and Grant [98] applied the expression-weighted cell-type enrichment (EWCE), a method that uses single cell transcriptomes to generate the probability distribution associated with a gene list that has an average level of expression within a cell type. Also, these authors applied EWCE to human genetic data from cases of epilepsy among other neurologic pathologies [98]. By last, as mentioned below, a study describes a method called fluorescent in situ RNA sequencing (FISSEQ), which enables not only the study of the transcriptomes of single cells but also the determination of the precise location of each transcript within the cell [99].

3.3. RT-qPCR

After data mining obtained by microarrays and RNA-Seq, the results need to be validated with highly reliable biotechniques that allow precise quantitation of transcriptional abundance of the identified genes.

The RT-qPCR technology is central to biomarker validation where potential markers need to be measured with greater accuracy and precision in larger sample sets. RT-qPCR represents the method of choice for analyzing gene expression of a moderate number of genes in anywhere from a small number to thousands of samples [100–102]. Quantitative real time PCR allows quantification of PCR products in "real time" during each PCR cycle, yielding a quantitative measurement of PCR products accumulated during the course of the reaction. Real-time reactions are carried out in a thermocycler that permits measurement of a fluorescent detector molecule, which decreases postprocessing steps and minimizes experimental error (Fig. 2C). Quantitative real time PCR is most commonly achieved through the use of fluorescence-based technologies such as probe sequences that fluoresce upon hydrolysis (TaqMan) or hybridization (LightCycler), fluorescent hairpins, or intercalating dyes (SYBR Green) [103]. The majority of analyses of RT-qPCR data use relative quantitation that is easier to measure and are of primary interest to researchers examining disease states as epilepsy. In epilepsy research, the most common method is the $2^{-\Delta CT}$ method that relies on two assumptions [104]. The first is that the reaction is occurring with 100% efficiency; in other words, with each cycle of PCR, the amount of product doubles. The second is that there is a gene (or genes) that is expressed at a constant level between the samples. This endogenous, housekeeping, or reference gene will be used to correct for any difference in sample loading [103]. Housekeeping genes such as β-actin, glyceraldehyde 3-phosphate dehydrogenase, cyclophilin, or tubulin are commonly used since they are ubiquitously expressed in cells and tissues. In a model of TLE, Crans et al. [105] have recently validated two rodent-specific short interspersed nuclear elements (SINEs) as reference genes, comparing these with other nine genes and using three types of algorithms: geNorm, NormFinder, and rank aggregation. Our research group, as mentioned above, investigated the comparison of the gene expression profiles between two audiogenic seizure models using the microarray data, showing upregulation of *Egr3* in WAR and GASH/Sal animals [11]. By using RT-qPCR studies, we confirmed the differential expression of the *Egr3* as well as in the two other early growth response proteins 1 and 2 (*Egr1* and *Egr2*), which were also upregulated in both models. Differences between microarray and RT-qPCR data might occur for the following reasons: 1) the use of different probes in the microarray and RT-qPCR experiments, which may lead to capture differential expression in splice variants; 2) differences in the methods used for the normalization of expression data; and 3) possible false positive outcomes of expression changes. In addition, lower correlations between RT-qPCR and microarray results were consistently reported for genes exhibiting small degrees of changes [106].

3.4. Spatial transcriptome

The advance in sequencing technologies makes possible the study of the genomic and transcriptomic of single cells and tissues. The

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complexity of multicellular organisms requires the design of high-throughput measurements that preserve spatial information about the tissue context or subcellular localization of the analyzed nucleic acids. Some applications of this approach are intended to measure gene expression and activity for thousands of genes in multiple regions of the brain [107–110].

The single-molecule RNA fluorescence in situ hybridization (smFISH) technique represents a powerful means of gauging individual RNA expression values from single-cell transcriptome-wide measurements. However, the number of transcripts that can be visualized simultaneously in the same sample is small (usually 1–3) because of the limited availability of fluorophores with nonoverlapping spectra, which prevents highly multiplex measurements. An improvement of this method is the so-called sequential fluorescence in situ hybridization (seqFISH), which implies increasing multiplexing by the use of spatial or sequential barcoding that is combined with super-resolution microscopy. Thus, each transcript is identified through multiple cycles of hybridization, imaging, and probe removal (Fig. 2D).

Other alternative method to visualize RNA molecules in situ is based on smFISH that uses padlock probes and rolling circle amplification as well as branched DNA probes [107,109,110]. This strategy combines smFISH and scRNA-Seq, allowing visualization and quantification of the transcription with spatial resolution in tissue sections [64].

Also, laser capture microdissection (LCM) is a powerful technology that enables the isolation of cells or small tissue regions from defined anatomical locations. Data sets derived from these samples correlate directly with the known original location, thus preserving spatial information. Nucleic acids can be extracted from LCM-captured cells and used in a variety of downstream applications, including gene expression microarrays and RNA-seq.

Other approach for spatially resolved transcriptomics based on RNA extraction from discrete tissue regions is the serial microtomy sequencing. In this method, RNA is extracted from single thin tissue cryosections and subjected to sequencing. To improve spatial resolution and quantification of this technique, a modified approach named RNA tomography sequencing method (Tomo-seq) has been developed [107,110].

The transcriptome in vivo analysis (TIVA) based on RNA extraction and sequencing of selected neurons within tissue slices allows characterization of gene expression profiles in accurate brain locations [107,110,111].

4. Proteomic

Proteomics refers to the large-scale analysis of proteins and deals with the study of protein compositions, including those derived from the posttranslational modifications, as well as protein interactions. The proteomic technologies also involve the identification and quantification of overall proteins, and hence, are well-suited for understanding the changes that occur in the epileptogenic tissue after seizures. One of the major applications of proteomics is to find useful markers for the diagnosis, treatment, and monitoring of various clinical entities. In this regard, a principal objective of the epilepsy biomarker discovery is to identify “hot lesions” in epileptogenic brain tissues that express a highly specific and sensitive biomarker signature [5]. For this reason, determining the proteomic profile differences in the epileptogenic nuclei of the audiogenic seizure models provides crucial information about the mechanisms involved in seizure susceptibility and regulation of neuronal excitability.

Unfortunately, the results from proteomics studies are very variable as they depend on the sample preparation method, the separation between nuclear and cytoplasmic components, the type of protein extraction, and the high biological variation between individuals. Finally, unlike the DNA stability, the proteome (and transcriptome) varies in space and time, thereby contributing to the great heterogeneity of results that can be found in the literature. The existing process templates for large-scale study of proteins are shown in Fig. 4.

4.1. Two-dimensional electrophoresis (2DE)

The two-dimensional electrophoresis (2DE) is the typical method to separate proteins in two dimensions according to their isoelectric point, molecular mass, and solubility, using a polyacrylamide gel. Then, the gel is stained using different dyes — such as Coomassie blue or silver nitrate. Another way is to use fluorescent reporters to identify the target samples as in the 2D Fluorescence Difference Gel Electrophoresis (2D-DIGE) (Fig. 4A). This technique has the advantage to directly display the relative abundances and patterns of protein isoforms in the samples. In this case, protein spots are considered as differentially expressed with statistical significance when 1) they are present at least in 70% of the gel images, 2) a 1.5-fold change as a threshold average ratio, and 3) p-values lower than 0.05. For this analysis, different software programs are employed for image capturing (i.e., EttanTM DIGE Imager, GE Healthcare), image analysis (i.e., DeCyder TM Differential Analysis Software, GE Healthcare), and image quantification and selection of quantitative changes [113].

In the samples separated by simple 2DE and DIGE, the protein spots of interest are manually excised from the stained gels, de-stained, and subsequently subjected to in-gel digestion with trypsin. After protein extraction, they are further analyzed with a mass spectrometer [high-performance liquid chromatography (HPLC)/mass spectrometry (MS)–MS or by matrix-assisted laser desorption/ionization-time-of flight (MALDI-TOF) mass spectrometer for fluorescent samples] (see next section for more information). Different spectra are analyzed with specific software (i.e., FlexAnalysis 3.3.65, Bruker Daltonics), using different algorithms (i.e., MASCOT - Matrix Science, London, UK or X! Tandem http://www.thegpm.org/tandem/).

There are many databases that can be used to identify the protein sequences in molecular studies of rodent models of epilepsy. Among them, http://kr.expasy.org or database of SwissProt 2015_06, in which 1612 Rodentia sequences are available.

This technique has been used to analyze changes in brain tissue of the WAG/Rij model after induced seizure enhancement, showing 16 differentially expressed proteins in the frontoparietal cortex and 35 proteins in the thalamus [113]. Four of them were found in both brain areas and were related to metabolism [ATP synthase subunit d, mitochondrial protein (ATP5H) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH)] and glial activation [glial fibrillary acidic protein (GFAP) and neurofilament light polypeptide (NEFL)], which are able to modulate voltage-gated calcium channels [114]. Consistently, 2DE performed in the epileptogenic nucleus of naïve GASH/Sal animals also showed alterations in proteins related to metabolism, with a significant decrease of ATP5H as well as increases in GAPDH and malate dehydrogenase (MDH1). In addition, 2D-DIGE analyses were carried out to compare the proteomic profile of baseline and postseizure states, showing nonsignificant differences in the epileptogenic nucleus of the GASH/Sal (unpublished data). The most striking differences were found between control and GASH/Sal at baseline conditions, showing an increment in the metabolism protein pyruvate kinase (PK) and ATP synthase beta subunit (ATP5F1B), along with an elevated concentration of peroxyredoxin-2 (PRDX2) in the IC. Additionally, the induction of epileptic seizures made the PRDX2 to switch from a 9.62-fold increase to a 4.05-fold decrease, which suggests an impairment of the antioxidant systems. These data are in agreement with other results in Lafora Disease mice models [115].

4.2. Mass spectrometry and MALDI-TOF

Mass spectrometry is an analytical technique that measures the mass-to-charge (m/z) ratio of ions. In this procedure, the samples are ionized into charged molecules and then subjected to an electric field in the mass analyzer, which determines the acceleration of the sample toward a detector, so that the ratio of their m/z can be measured. In the MALDI-TOF MS, the ion source is the matrix-assisted laser

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desorption/ionization (MALDI), and the mass analyzer is time-of-flight (TOF) analyzer (Fig. 4B). It is the technique that must be used when the samples are attached to a fluorescent (i.e., DIGE). In proteomics, one of the most important and difficult step is the separation of complex peptide or protein mixtures prior to their detection and data analysis. This step was done in the past using HPLC–MALDI-MS that performs offline peptide separation and spotting or fraction collection. The fact that this technique involves time-consuming manual inspection for

Table 1
Massive analysis in rodent genetic models of epilepsy shows dysregulated proteins and their corresponding functional pathway.

Reference	Model organism	Epilepsy subtype	Anatomical area of study	Proteins upregulated (symbol)	Proteins downregulated (symbol)	Classification/function
Penot et al. [136]	Kainate injection in hippocampus of mouse	MTLE	Hippocampus	IL-1beta; IL-1Ra; SOCS3; COX-2 cPLA(2)α		Cytokines, inflammatory processes apoptosis
Danis et al. [137]	Genetically modified rat GAERS	AbE	Cortex Thalamus Hippocampus	ATP5F1D; TMEM70 MBP; MIF MIF; HBB		Energy generation membrane conductance inflammatory processes Oxygen transport
Huang et al. [138]	SD rats injected intraperitoneally with lithium chloride and then pilocarpine twice	TLE	Hippocampus	HSP27 TRIP6; LIMK1; FBA, CK MBP	PPIase TUBB; ACTB; INA GADPH; SCOAL; ACO SNX3	Stress response Cytoskeleton Metabolism Membrane conductance Intracellular trafficking
Jiang et al. [139]	Phenytoin in electrical amygdala-kindled rats	RefE	Full brain	VDAC1 GADPH; TPI1; ALDOA TUBB2	VDAC2 MT-ATP; GLUD1; MDH ABCB10; NDUFA10; HSP-70 ACO; ALDH; CFL1	Membrane conductance Energy generation Mitochondrial transport Stress response metabolism cytoskeleton
Greene et al. [140]	Lithium pilocarpine-induced rat	CStE	Hippocampus	HSP-27 TUBA; EZR DPYSL2 DHPR		Stress response cytoskeleton Endocytosis production of neurotransmitters
Junker et al. [141]	Kindled rats	RefE	Hippocampus	UQCRFS1		Mitochondrial respiratory chain
Walker et al. [142]	Rats, electrically-induced status epilepticus	StE	Parahippocampal cortex Hippocampus	HSP90B1 HSPA1A IL1rapl1; ITGB2;RPS27A HMGB1; HSP90B1; HSPA1A; TMED7; TOLLIP	DNM3;IL1RAP TOLLIP; USP7	Immune and inflammatory responses
Bitsika et al. [143]	KA-MTLE mouse model	MTLE	Hippocampus	TRF; GFAP; VIM; CLU; DHRS1; FLNA; HEXB; FLNA; APOD; TGM1; CAPG; CTSZ	MAP2; CTTN; PPP1R9B; MAP1A; AAK1; BAIAP2; RPH3A; SLC6A1	Cytoskeletal proteins neuronal responses Microglia/Astrocyte Activation Inflammatory Response
Györffy et al., [113]	WAG/Rij rats	AbE	Frontoparietal cortex Thalamus	GFAP;GAPDH PSME1; UQCRC1 HNRNPA2B1 GFAP; CAPG INA; DPYSL2 MYL1; TUBB PRDX6;ENO1 MDH1; SEPT3 ATP6V1E1 PARK7; APOE	ATP5H; NEFL LMN; SUCLA2; GUK1 GFAP; ACTA2 MYH1; NEFL ATP5H; GAPDH LMN; TIP1 MDH1;HNRNPH2 ATP6V1E1	Cytoskeletal proteins Motor proteins Chaperone/oxidative stress metabolism ATP synthesis/respiratory chain signal transduction transcription/translation synaptic transmission
Liu et al. [144]	Pilocarpine rat model	TLE	Hippocampus	NEFM; NRP2: HSP90AA1; DDT HSPA1A; ANXA6; VDAC2 SEMA6B; PKM TUBA1B; SYN2 HOMER2; INA ATP5F1B; SPTA1; NRGN MSI1; COX6B1	ACTA1; TUBB2B; ACTB; ENOSF1; PDHA1; ENO1; ALDOA; PRPS1; HSPD1; SNAP25; STMN1; ARHGDIA; UBE2E2; HBA1/2 AK1; TAGLN3; NADPH; GSTM1; HBB	Cytoskeletal proteins Metabolism Stress response energy metabolism
Li et al. [145]	Pilocarpine mouse model	TLE	Dentate gyrus	PFN1; UCHL1 ARHGDIA; CA2 PPID; CRYAB SNCB; PRDX6 ENO2; LAMP2 VIM; CTSD HSPB1	ACTB; LDHB PDXP; CRYM HNRNPDL; APOA1 GRHPR	Cytoskeletal proteins Metabolism Stress response Apoptosis synaptic transmission
Wu et al. [146]	Lithium pilocarpine-induced rat treated	MTLE	Hippocampus	ACBP; CAH2 CAH5A; CH10 COF1; CX6B1 FABP7; GADPH GFAP; GLOD4 HBA; KAD1; KCRB NRGN; PDIA3; PPIA; PRDX1; TTR; UB2V2		Cytoskeletal proteins synaptic function energy metabolism mitochondrial function molecular chaperones signal regulation

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subsequent MS analysis resulted in very low separation efficiencies [116]. Currently, there are multiple automated liquid chromatography (LC)/MS platforms that allow the separation of proteins and MS analysis automatically [117]. The automation of procedure has yielded the separation of thousands of proteins in each sample and has become the regular method of choice when it is desired to produce a high-level proteomic profile in the sample. The main limitation of the LC/MS is the fractionation of the sample prior to the MS analysis, which makes that the large amount of proteins produced by the LC separation can quickly saturate the capacity of the spectrometer detector.

4.3. Massive analysis

Another approach, especially useful for the analysis of complex protein mixtures, is to carry out a global analysis, the so-called Shotgun proteomics. This technique refers to the use of bottom-up proteomics techniques in identifying proteins in complex mixtures by using a combination of LC/MS. The most common method of shotgun proteomics starts with the digestion of proteins in the mixture and subsequently the separation of the resulting peptides by LC. Tandem MS is then used to identify the peptides (tandem MS or MS–MS) (Fig. 4C). This sequential MS provides detailed information on the sequence of the peptide, which is compiled, constituting the primary technique for the genesis of the "peptide trace". Using this technique, several authors describe a list of proteins related to changes in the brain of different epilepsy models (Table 1).

In the GASH/Sal model, some dysregulated proteins are still unknown, 5.63% are upregulated and 22.22% are downregulated. In general, there is a great variability of proteins that could be classified in different functional categories (Fig. 3) according to the PHANTHER method [118].

4.4. Isobaric tags for relative and absolute quantitation (iTRAQ)

Mass spectrometry analysis can also be used directly to quantitatively explore the proteome, applying isobaric tags for a relative and absolute quantification (iTRAQ)-based proteomics approach to identify differentially expressed proteins. This technique requires the "tagging" in N-terminal position of previously digested protein samples. Each experimental group is tagged with a different reporter, then samples are intermixed, separated through LC, and analyzed with MS–MS. The N-terminal reporters are then ionized and relative-quantified. A fundamental disadvantage of this tool is the high cost due to the tagging process and the powerful spectrometers that are necessary [119]. Using this technique, a series of cardiac tissue proteins from Wistar in acute and chronic epilepsy rat models have been described. Three of these proteins, the receptor for activated protein kinase C1 (RACK1), the aldehyde dehydrogenase 6 family member A1 (ALDH6A1), and the glycerol uptake/transporter 1 (Hhatl), were identified as playing crucial roles in cardiac injury during epilepsy [120].

4.5. Protein modifications

Proteomics is capable of profiling posttranslational protein modifications such as phosphorylation, acetylation, hydroxylation, methylation, glycosylation, AMPylation, lipidation, ubiquitination, and deamidation, as well as splicing-dependent alterations in protein expression patterns. Since these posttranslational mRNA and protein modifications define unique functional features in a cell phenotype, they are highly relevant and have to be considered in epilepsy research. Spectrophotometry techniques have become very sophisticated in recent times and allow visualizing the posttranslational changes. Mass spectrometry techniques can be used to profile posttranslational modified (PTM) peptides in a digested sample. The PTMScan® technology combines antibody enrichment of PTM-containing peptides with HPLC–MS/MS and allows identification and quantification of hundreds to thousands of even the lowest abundance peptides (Fig. 4D). All in all, this technique provides a more focused approach to peptide enrichment than other strategies and makes possible to determine novel protein sites that are phosphorylated, ubiquitinated, acetylated, methylated, or protease cleaved (cell signaling technology- https://www.cellsignal.com/contents//simplifying-proteomics/proteomics). Independently of the proteomics tools used, all results need to be validated before any potential biomarker could be subjected to further analysis. The most common method for biomarker validation is the immunodetection.

5. Immnunodetection

Epilepsy research using rodents to model epilepsy has made significant progress thanks to a variety of techniques that specifically detect and identify antigens (or proteins) in epileptic samples. Common methods of immunological detection in epilepsy research are western blot analysis, immunostaining, and protein microarrays. All of them are based on the existence of specific antibodies (Fig. 4E).

5.1. Western blot analysis

This technique – also known as electroblotting [121] or immunoblotting [122] – is a rapid and sensitive assay that combines the resolution of gel electrophoresis and the principles of immunological recognition of an antigen [123]. By using a western blot analysis, researchers are able to separate and identify specific proteins from a mixture of proteins extracted from complex biological samples such as serum, cells, or tissue. In this technique, a mixture of proteins is separated based on molecular weight, and by protein type, through gel electrophoresis. The results are then transferred to a membrane producing a band for each protein. The membrane is then incubated with labeled

Notes to table 1

Epilepsy subtypes: AbE—absence epilepsy; CStE — convulsive status epilepticus; MTLE — mesiotemporal lobe epilepsy; RefE — refractory epilepsy; StE — status epilepticus; TLE — temporal lobe epilepsy.

Abbreviations: AAK1 — AP2-associated protein kinase 1; ACBP — Acyl-CoA-binding protein; ACO — aconitate hydratase; ACTA1 — alpha actin-1; ACTA2 — alpha actin-2; ACTB — beta actin; ALDH — Aldehyde dehydrogenase; AK1 — Adenylate kinase isoenzyme 1; ALDOA — Aldose A; ALK — Anaplastic lymphoma kinase; ANXA6 — Protein disulfideisomerase A6 precursor; APOA1 — apolipoprotein A1; APOE — Apolipoprotein E; ARHGDIA — Rho GDP — Dissociation Inhibitor Alpha; ATP5F1B — ATP synthase subunit beta; ATP5F1D-ATP Synthase F1 Subunit Delta; ATP5H — ATP synthase subunit D; ATP6V1E1 — ATPase H + Transporting V1 Subunit E1; BAIAP2 — Brain-specific angiogenesis inhibitor 1-associated protein 2; CA2 — carbonic anhydrase 2; CAH5A — Carbonic anhydrase 5A; CAPG — Macrophage-capping protein; CD28 — CD28 Antigen; CFL1 — Cofilin 1; CH10 — 10 kDa heat shock protein; CK — creatine kinase; CLU — Clusterin; COF1 — Cofilin-1; COX2 — cycloxygenase-2; COX6B1 — Cytochrome c oxidase subunit Vlb isoform 1; cPLA(2)α — group IVA cytosolic phospholipase α2; CRYAB — crystallin, alpha B; CRYM — crystallin, mu; CTSD — cathepsin D; CTSZ — Cathepsin Z; CTTN — Src substrate cortactin; CX6B1 — Cytochrome c oxidase subunit Vlb isoform 1; DDT — D-Dopachrome Tautomerase; DHPR — dihydropteridine reductase; DHRS1 — Dehydrogenase/reductase SDR family member 1; DNM3 — Dynamin 3; DPYSL2 — dihydropyrimidinase-related protein-2; ENO1 — enolase 1; ENO2 — enolase 2; ENOSF1 — Enolase Superfamily Member 1; EZR — ezrin; FABP7 — Fatty acid-binding protein; FAS — Fas Cell Surface Death Receptor; FBA — fructose-bisphosphate aldolase A; FLNA — Filamin A; GADPH — glyceraldehyde-3-phosphate dehydrogenase; GAERS — Genetic Absence Epilepsy Rats from Strasbourg; GDNF — Glial cell line derived neurotrophic factor; GFAP — Glial fibrillary acidic protein; GLOD4 — Glyoxalase domain-containing protein 4; GLUD1 — Glutamate dehydrogenase 1; GRHPR — glyoxylate reductase/hydroxypyruvate reductase; GSTM1 — Glutathione S-Transferase Mu 1; GUK1 — Guanylate kinase 1; HBA1/2 — Hemoglobin subunit alpha-1/2;HBB — Hemoglobin subunit beta 1; HEXB — Beta-hexosaminidase subunit beta; HMGB1 — High Mobility Group Box 1; HNRNPA2B1 — Heterogeneous nuclear ribonucleoprotein A2/B1; HNRNPDL — Heterogeneous Nuclear Ribonucleoprotein D Like; HNRNPH2 — Heterogeneous nuclear ribonucleoprotein H2; HOMER2 — Homer Scaffold Protein 2; HSP27 — heat shock protein-27; HSP90AA1 — Heat shock protein HSP 90-beta; HSP90B1 — HSPA1A- Heat shock 70 kDa.

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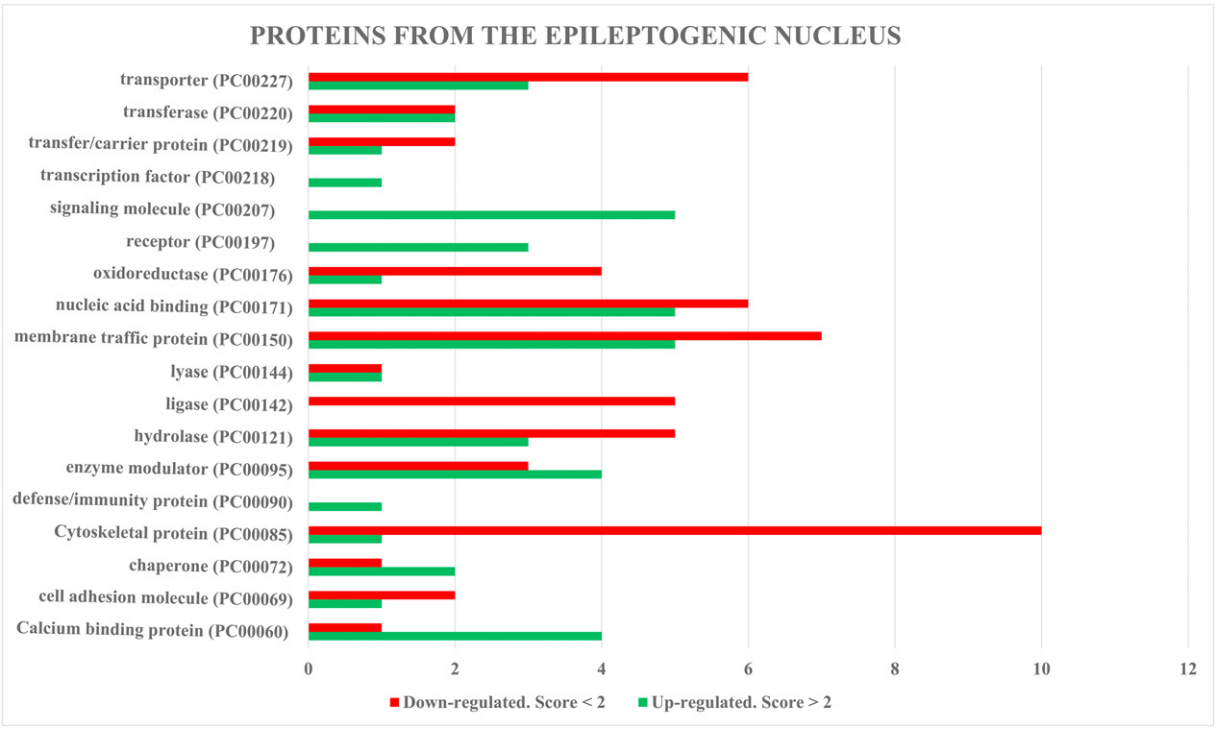

Fig. 3. Variation of protein expression between GASH/Sal and control animals in the epileptogenic nucleus (inferior colliculus) and the corresponding categorical classification. The plot displays proteins with ±2-fold change compared with controls. https://www.ebi.ac.uk/reference_proteomes

antibodies specific to the protein of interest. Since the thickness of the band corresponds to the amount of protein present, western blot analysis provides a relative comparison of protein levels, but not an absolute measure of quantity [123]. Detection, specificity, and sensitivity are crucial for accurate and quantitative analysis in western blotting that strongly depends on the quality of the available antibodies.

5.2. Immunostaining

Together with the western blot analysis, immunostaining is one of the gold standard protein analytical techniques that selectively identify proteins in cells (so-called immunocytochemistry) or in tissue sections (so-called immunohistochemistry) by exploiting the principles of immunological recognition of an antigen. Both immunostaining techniques are powerful microscopy-based techniques for the detection of specific proteins within individual cells and tissue samples and, hence, add valuable information about the immunolocalization and distribution of a target protein in cell compartments and tissues. Immunostaining is developed following a variety of protocols that involve exposure of fixed cells or tissues to primary antibodies directed against one or more proteins of interest. Bounded primary antibodies are then detected directly if they are conjugated to a label (direct detection method) or using commercially available secondary antibodies directed against the invariant portion of the primary antibody (indirect detection method). Two principal methodologies exist to visualize antigen–antibody complexes: immunofluorescence using fluorophore-conjugated antibodies or chemiluminescence using antibodies coupled to horseradish peroxidase [124]. A good example of immunohistochemistry applied in rodent models of epilepsy is the study of the association between activation immediate early genes and seizure activity. The WAR and GASH/Sal are two genetically epileptic prone rodents that showed overexpression of the immediate-early growth response genes (*Egr1*, *Egr2*, and *Egr3*) in the IC (the epileptogenic focus) after an ictal event [11]. López-López et al. [11] used immunohistochemistry to complement the results of RT-qPCR analysis of the *Egr3* gene, determining the distribution of EGR3 protein in the brain tissue of control and seizure-prone animals. Therefore, comparison between different samples obtained from control and epilepsy-prone rodents is of paramount importance and, hence, needs to be based on objective data in order to obtain accurate and reproducible results. As in western blot analysis, the quantitative immunostaining performed on tissue sections by means of digital photomicroscopy and image analysis can be used to estimate the number or density of immunopositive structures within the tissue sample as well as the immunostaining intensity across the region of interest. To compare multiple specimens, immunostaining and image acquisition should be performed in parallel for the entire set. Identical reagents and processing should be used, with identical image acquisition settings and exposure times. The most commonly used image-processing analysis software for quantitative immunostaining is the Java-based NIH ImageJ software. ImageJ is in the public domain (available at http://rsb.info.nih.gov/nih-image), supports a wide image formats, and can be expanded with the installation of more than 150 plugins that are additional tools for facilitating scientific image analysis including quantitative immunostaining. Fig. 5 shows an example of semiquantitative immunostaining analysis of transthyretin (TTR) protein using the ImageJ software. Mutant forms and overexpression of *Ttr* gene in brain tissue have been associated with seizure as well as abnormalities in behavior and movement [125]. Also, overexpression of *Ttr* could be directly related with membrane depolarization [126] and loss of GABA receptor activity [127] and thereby increasing the risk of seizures. As is the case of immediate early genes, an overexpression of *Ttr* gene might contribute to the altered brain gene expression profiles associated with seizure susceptibility in genetic rodent models of epilepsy. Since a gap between the mRNA and protein levels exists because of various levels of regulation, the results obtained by gene expression (RT-qPCR) need to be correlated with the protein levels. The western blot and immunostaining analysis in the IC of the GASH/Sal model showed that TTR protein levels are higher in the GASH/Sal model than in age-matched controls, confirming the alterations in the gene expression profiles.

Please cite this article as: J.R. Bosque, R. Gómez-Nieto, S. Hormigo, et al., Molecular tools for the characterization of seizure susceptibility in genetic rodent models of epilepsy, Epilepsy & Behavior, https://doi.org/10.1016/j.yebeh.2019.106594

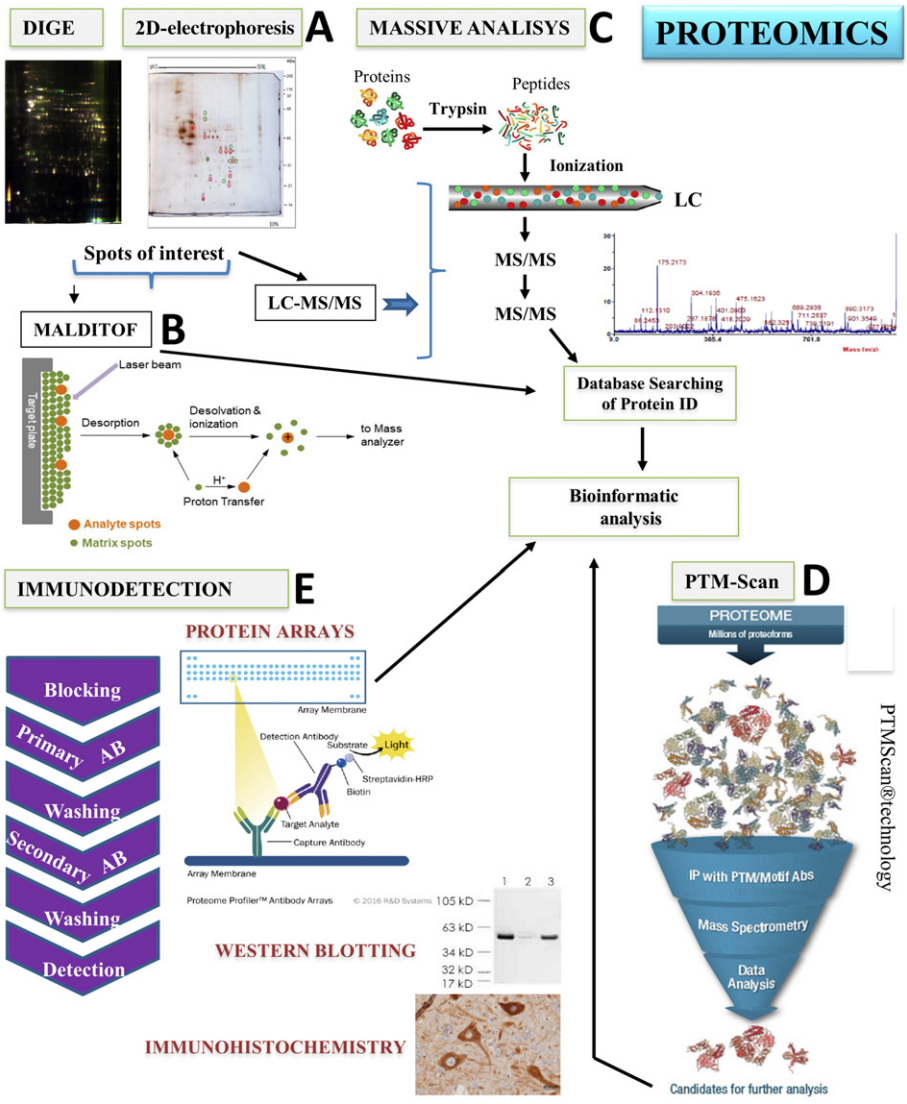

Fig. 4. Experimental workflow for proteomic studies. A. Types of bidimensional electrophoresis. B. MALDI-TOF mass spectrometry (image from https://www.creative-proteomics.com/technology/maldi-tof-mass-spectrometry.htm [112]). C. Conventional massive protein analysis: digestion of protein with trypsin, separation of peptides by LC followed by tandem mass spectrometry. D. PTM-Scan. Posttransductional modifications (image obtained from https://www.cellsignal.com/contents/_/simplifying-proteomics/proteomics). E. Immunodetection: Steps for protein immunodetection (wide arrows) and different techniques based on antigen–antibody recognition: Protein arrays (reproduced with permission of R&D Systems), Western blotting and immunohistochemistry. Abbreviation: AB — antibody; DIGE — Fluorescence Difference Gel Electrophoresis; ID — Identity; LC — liquid chromatography; MS — Mass spectrometry; PTM — posttranslational modified.

5.3. Protein microarrays

Protein microarray is a relatively new technology used for the characterization of large numbers of proteins in parallel [128]. Protein microarrays include three classes: analytical, functional, and reverse-phase protein microarrays that provide a powerful tool in quantifying and profiling proteins. It is also a high-throughput method for tracking the interactions and activities of proteins as well as determining their function and protein posttranslational modifications [129,130]. There are multiple protein microarrays designed to identify a large number of proteins as well as specific protein microarrays that are used for diagnostic assays in humans. Despite the considerable investments made by several companies, there are few commercial protein microarrays for rodents. The few protein microarrays available so far are aimed at the identification of signaling pathways (Mouse AKT Pathway Phosphorylation Array C1, # AAH-AKT-1-2, RayBiotech, Inc.) or specific proteins, such as inflammatory proteins. Among the latter, it should be noted that the cytokine array is used for the parallel determination of the relative levels of selected cytokines and chemokines (Mouse Cytokine Array# ARY006, R&D Systems, Inc.; mouse Th1/Th2/Th17 cytometric bead array kit, BD Biosciences, San Diego, CA, USA). This type of protein microarrays is very useful to determine modifications of certain interleukins associated with seizures as well as the effects of different anticonvulsant compounds [131].

6. Crosslinking

It is noteworthy to review the functional classification of genes or proteins involved in epilepsy. The gene ontology classification of cellular components, biological processes, and molecular functions has been used for functional classification by the majority of authors. Four functional groups have been described so far as the most representative in epilepsy (neuronal signaling, immunity/inflammation, transcriptional regulation, and signal transduction), and two pathways showed enrichment: the chemokine signaling pathway and Toll-like receptor signaling pathway. Moreover, additional processes and pathways that are linked

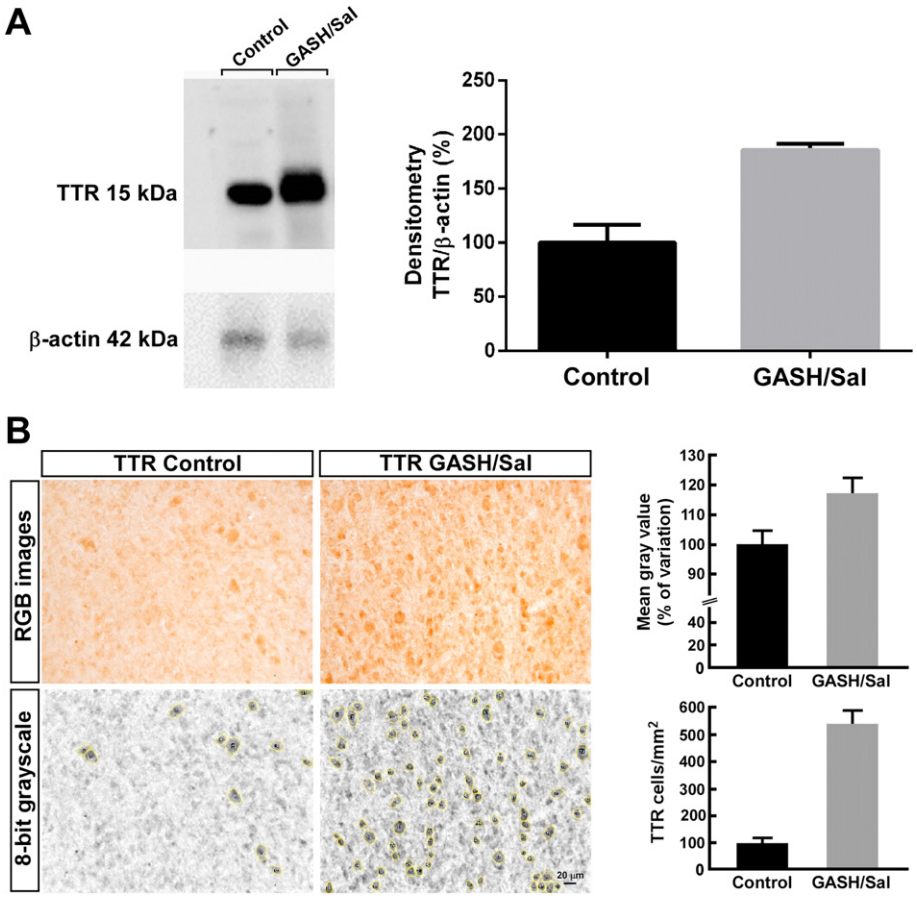

Fig. 5. Semiquantitative immunostaining analysis of transthyretin (TTR) protein in the inferior colliculus of the GASH/Sal. A. Western blot analysis of TTR protein with antibodies to TTR and β-actin. The western blot band intensities were measured with ImageJ software. The histogram shows a semiquantitative analysis of TTR protein levels, which were normalized to β-actin levels. The intensity (mean ± standard error) was normalized to wild-type values (control), which were set equal to 100%. Note that protein levels of TTR differed between control and GASH/Sal animals. B. High-magnification photomicrographs illustrating TTR immunoreactivity in the central nucleus of the IC (scale bar represents 20 μm and is the same for all panels). The RGB-color images corresponding to control and GASH/Sal animals were converted to 8-bit images that contained a grayscale of pixel intensities ranging from 0 (black) to 255 (white). The densitometric procedure for the evaluation of TTR immunostaining was performed by using ImageJ software. The upper histogram shows percentages of variation (mean ± standard error) for mean gray value of the immunostaining that were used as an indicator of changes in protein levels. The lower histogram shows the number of TTR-immunolabeled cells per mm^2. Note the increased density of immunostained cells in the GASH/Sal compared with the control animal.

to these two pathways include ubiquitin-mediated proteolysis, complement cascade, JAK–STAT signaling pathway, cellular growth and differentiation, cell survival, migration, apoptosis, Reactive Oxygen Species (ROS) production, Nitric oxide (NO) induction, MAPK signaling pathway, leukocyte transendothelial migration, and regulation of cytoskeleton system [11,66]. There are several computer-based tools to establish relationships between differentially expressed genes or proteins with epilepsy. These web interfaces integrate database that is used to determine co-occurrence between different annotations, identifying those that are statistically significant. These interactions are of special interest for proteins, because most of them work in conjunction with other proteins. This is especially useful to determine potential associations in cellular signaling pathways. Among the multiple tools on the web, Table 2 shows the most used to search functional or structural associations between genes, proteins, and metabolic pathways.

7. Conclusions

Advances in molecular tools have improved substantially the genomic and proteomic studies on specific genes and proteins associated with epilepsy, allowing comprehensive research of rodent seizure models.

Since genetic variations, gene expression changes, and protein alterations are causes of specific epilepsy phenotypes, the experimental designs in rodent models of epilepsy should incorporate complementary analysis for verifying results obtained at each stage of the flow information from DNA to RNA to protein levels. To achieve this goal, a good experimental design should be developed with careful consideration for determining the most appropriate approach, taking into account that no one technique is applicable to every set objective, and each has its own strengths and weaknesses.

Development of these research methods and their application to animal model of epilepsy might led to a rapid increase in our understanding of the complex mechanisms underlying epileptogenesis and seizure generation in the immediate future. In this regard, comparative molecular approaches to determine the differences between rodent seizure models and their wild-type counterparts provide valuable information on molecular events that contribute to seizure susceptibility and regulation of neuronal excitability.

The rapid identification of these molecular alterations, as facilitated by genomic and proteomic technologies, will shed light not only on the molecular pathways mediating epileptogenesis, but also on the development of novel applications for the diagnosis, prognosis, and treatment of seizures.

Declaration of competing interest

The authors declare no conflicts of financial and nonfinancial interests.

Please cite this article as: J.R. Bosque, R. Gómez-Nieto, S. Hormigo, et al., Molecular tools for the characterization of seizure susceptibility in genetic rodent models of epilepsy, Epilepsy & Behavior, https://doi.org/10.1016/j.yebeh.2019.106594

Table 2
Web-based interfaces and database collections used to query functional and structural associations between genes, proteins and metabolic pathways.

Gene/Protein interactions	Functional analysis.
http://akt.ucsf.edu/	http://amp.pharm.mssm.edu/Enrichr/
http://biocyc.org/	http://bioinfo.vanderbilt.edu/webgestalt/
http://david.abcc.ncifcrf.gov/	http://genecodis.cnb.csic.es
http://pid.nci.nih.gov/	http://gtlinker.cnb.csic.es/-
http://string-db.org	http://mimi.ncibi.org/MimiWeb/main-page.jsp
http://www.csuchico.edu/ge/	http://www.pantherdb.org/
http://www.cuny.edu/pathways	https://pathcards.genecards.org/
http://www.elsevier.com/online-tools/pathway-studio/biological-database	https://www.ensembl.org/Multi/Search/New?db=core
http://www.genecards.org	
http://www.genome.jp/kegg/	
http://mimi.ncibi.org/MimiWeb/main-page.jsp	
https://www.ncbi.nlm.nih.gov/	
https://www.wikigenes.org	
Pathological phenotypes	**pathways**
https://ve.genecards.org/#input	http://highered.colorado.gov/Academics/Transfers/gtPathways/
https://discovery.lifemapsc.com/gene-expression-signals/high-throughput-disease	http://www.cuny.edu/pathways
http://www.informatics.jax.org/humanDisease.shtml	http://www.ebi.ac.uk/biomodels-main/BIOMD0000000286
https://www.malacards.org/	http://www.elsevier.com/online-tools/pathway-studio/biological-database
https://www.genome.jp/kegg/pathway.html#disease	http://www.genemania.org
	http://www.genmapp.org/go_elite/
	https://pathcards.genecards.org/
	https://www.genome.jp/kegg/tool/map_pathway1.html
	https://www.uniprot.org/

Acknowledgments

This study was supported by the Regional Government of Castilla and León [#SA070P17 (IP-López DE), and #GRS 1838/A/18 (IP-Gonçalves)] and by SAF2016-78898-C2-2R (IP- Merchán).

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