Proteomic Profiling of the Hippocampus of Rats Subjected to the Pilocarpine Model of Epilepsy

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Article

Proteomic Profiling of the Hippocampus of Rats Subjected to the Pilocarpine Model of Epilepsy

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Abstract

Background/Objectives: High doses of pilocarpine to rats induce *status epilepticus* (SE) and reproduce the main characteristics of mesial temporal epilepsy. This model is considered highly isomorphic with the human disease, reason why it has been applied to elucidate the process of epileptogenesis. **Methods:** Two-dimensional electrophoresis (2-DE) was employed to study the hippocampal differential expression of proteins in rats exhibiting spontaneous recurrent seizures induced by pilocarpine. Two groups were studied: rats treated with pilocarpine (360mg/kg, N=6), and rats treated with saline (N=6). Both groups were analyzed 90 days after SE onset. Hippocampi homogenized in a lysis buffer were used to perform 2-DE. Interactome for differentially expressed proteins was performed using STRING database. **Results:** Protein spots analyzed by PDQuest software revealed forty proteins differentially expressed in epileptic rats compared to control ($p<0.05$), among them thirty-seven were successfully identified. LC-ESI-MS/MS results analyzed with MASCOT MS/MS ion search and IPI protein database showed twenty-nine up-regulated proteins in epileptic rats while six proteins were down-regulated and two proteins were expressed only in the control animals. The differentially expressed proteins integrated the domains of neuronal hyperexcitability, energy failure, synaptic dysregulation, and post-status epilepticus remodeling (confidence scores ≥0.90–0.99). **Conclusions:** The differentially expressed proteins showed high-confidence protein-protein interaction modules directly linked to the molecular pathogenesis of epilepsy. The simultaneous failure of the identified pathophysiological domains drives the transition from acute seizures to chronic, drug-refractory epilepsy. The protein complexes identified represent high-value, translation-ready candidate nodes for next-generation antiepileptogenic and disease-modifying therapies.

Keywords: temporal lobe epilepsy; drug-refractory epilepsy; hippocampus; pilocarpine; proteomics; metabolism; biomarkers

1. Introduction

Epilepsy is a chronic neurological disease that affect approximately 50 million people worldwide, being characterized by spontaneous and recurrent seizures that occur in the absence of disease toxic-metabolic or fever [1]. Temporal lobe epilepsy (TLE) accounts for approximately 40% of all cases of epilepsy, being a common form of focal epilepsy in humans. There are two subtypes of TLE, namely, mesial temporal lobe epilepsy (MTLE) and neocortical temporal lobe epilepsy (NTL) [2]. In the most MTLE patients, seizures originate in the limbic areas and are focal, and may be perceptual or non-perceptive [3]. Hippocampal sclerosis (HS) is the most frequent histopathological feature present in many patients with MTLE, and this change can usually be detected by magnetic

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resonance imaging—MRI [4–6]. HS is characterized by neuronal loss in specific subregions of the hippocampus and glial scar, but the etiology and the pathogenesis of the cell death is not well understood [5]. In addition, synaptic rearrangement and cell scattering in the granular layer of the dentate gyrus are frequently seen associated with HS in MTLE [7]. Seizures are often frequent and about 67-89% of patients with MTLE do not respond to antiseizure medications [8,9]. According to the International League Against Epilepsy (ILAE), the drug-resistance epilepsy (DRE) occurs as the failure of adequate trials of two tolerated, appropriately chosen, and used antiseizure medications, whether as monotherapy or in combination, to achieve sustained seizure freedom [10]. Until now, there is no antiseizure medications able to prevent seizures in patients with TLE that is efficient in preventing epileptogenesis [11]. Thus, the question is whether the epileptogenic process could be explained by common molecular and networks events that would be applied in new therapeutics. In this way, proteomics has been a powerful tool for protein profiling because it allows comparing proteomes of cells and tissues in normal and pathological conditions. Since the expression of proteins is determined, the transcriptional level can be examined to find the underlying mechanism for reduction or increase of certain gene products. For this reason, proteomics has been widely used in clinical research to identify biomarkers associated with disease.

Proteomics allows us to find proteins changed by a cell, tissue, or organism's response to internal states, external stimulations, or developmental changes, and to profile any differential protein expression [12,13]. Proteomics not only measures the amount of a given protein, but also whether there are any modifications of a protein as phosphorylation, ubiqutination, palmitoylation, oxidation, and other post-translational modifications (PTMs) [14]. Proteomics is a multidisciplinary method which is based on principles of biochemical, biophysical and bioinformatics to allow distinguishing healthy and diseased cellular processes at the protein level. Aslam et al. (2017) [15] highlighted some aspects of a target organism's proteome that can be assessed by proteomics, for example: protein identification, protein quantification, protein localization, post-translational modifications, functional proteomics, structural proteomics, and protein-protein interactions. Currently, the proteomics technique has been applied in the investigation for biomarkers associated with disease [16].

Proteomics technology applied to understanding the nervous system is called "neuroproteomics" or "neuromics". The neuroproteomics enables to study proteome of brain fragments or single cell, in cultures or isolated, and this is important to determine the dynamics of subproteome under different conditions (i.e., inflammation, oxidative stress, treatments, etc). In a global analysis, complementary studies could contribute to the understanding of complex biological networks that include protein interactions, complexity of signal and metabolic pathways that can be applied to select potential targets for specific drug therapy, and to the development of diagnosis or prognosis for neurological disorders [16].

Neurosciences have benefited greatly from the increased use of the technique proteomics in recent years. Despite this, studies of epilepsies are still modest with the application of proteomics. Experimental models that reliably reproduce the main symptoms of diseases have aroused the interest of researchers in the search for biomarkers. Studies employing human tissue are limited by the low amount obtained by surgical procedures and for ethical reasons. The use of experimental models of epilepsy can expand our knowledge regarding these mechanisms involved in epileptogenesis, allowing interfere or prevent the onset of the spontaneous seizures. Chemical convulsants such as pilocarpine [17,18] and kainic acid [19] can initiate *status epilepticus* in rodents and cause hippocampal sclerosis, memory impairment and spontaneous and recurrent seizures [20].

Despite technological advances applied to neurosciences, little is known about the cellular and molecular phenomena related to the epileptogenic process [21].

Here, we have applied proteomics method allied to interactome analysis to study the differentially expressed proteins in the hippocampal samples of rats subjected to pilocarpine induced MTLE.

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2. Results

2.1. Comparative Proteomic Analysis

In the hippocampal samples from rats studied 90 days following *status epilepticus* our results showed forty proteins differentially expressed when compared to control animals. Among them, thirty-seven proteins were successfully identified as shown on Table 1. The protein profile revealed that twenty-nine proteins were up-regulated, six were down-regulated and two were expressed only in control animals.

Table 1. Proteins differentially expressed in the hippocampus of rats subjected to pilocarpine model of temporal lobe epilepsy.

GeneCards	Protein name	Changes	IP	MW
Ldha	L-lactate dehydrogenase A chain	ø	5.5	36874
Pebp1	Phosphatidylethanolamine-binding protein 1	ø	5.2	20902
Aldoa	Fructose-bisphosphate aldolase A	⇩	9.3	39783
Pla2g4c	Cytosolic phospholipase A2 gamma (Fragment)	⇩	5.2	37522
Abcb6	ATP-binding cassette sub-family B member 6, mitochondrial	⇩	9.3	93305,18
Mdh1	Malate dehydrogenase, cytoplasmic	⇩	6	36631
Gnb1	Guanine nucleotide-binding protein G(I)/G(S)/G(T) subunit beta-1	⇩	5.4	38151
Gnb3	Guanine nucleotide-binding protein G(I)/G(S)/G(T) subunit beta-3	⇩	5.3	38125
Eno1	Alpha-enolase	⇧	6	47440
-	Enolase	⇧	10.3	34166
Eno3	Beta-enolase	⇧	7.9	47326
Eno2	Gamma-enolase	⇧	4.8	47510
Aldh5a1	Isoform Short of Succinate-semialdehyde dehydrogenase, mitochondrial	⇧	9.4	53391
Park7	Protein DJ-1	⇧	6.2	20190
Mapk1	Mitogen-activated protein kinase 1	⇧	6.5	41648
Tpi1	Triosephosphate isomerase	⇧	7.9	27345
Nsf	Vesicle-fusing ATPase	⇧	6.5	83170
Pgam1	Phosphoglycerate mutase 1	⇧	6,6	28928
Ywhag	14-3-3 protein gamma	⇧	4.6	28456

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Ldhb	L-lactate dehydrogenase B chain	↑	5.5	36874
RGD1565368	glyceraldehyde-3-phosphate dehydrogenase-like	↑	9.3	36045
Dpysl2	Dihydropyrimidinase-related protein 2	↑	5.8	62638
Dpysl3	Isoform 1 of Dihydropyrimidinase-related protein 3	↑	5.9	62327
Atp6v1b2	V-type proton ATPase subunit B, brain isoform	↑	5.4	56857
Car2	Carbonic anhydrase 2	↑	6.9	29267
Gfap	Isoform 1 of Glial fibrillary acidic protein	↑	5.1	49984
Tubb5	Isoform 1 of Tubulin beta-5 chain	↑	4.6	50095
Tubb2a	Tubulin beta-2A chain	↑	4.6	50274
Tubb2c	Tubulin beta-2C chain	↑	4.6	50225
Tubb3	Tubulin beta-3 chain	↑	4.6	50842
Atp5b	ATP synthase subunit beta, mitochondrial	↑	4.9	56318
Tpi1	Triosephosphate isomerase	↑	7.9	27345
LOC500959	Triosephosphate isomerase	↑	6.4	27306
Capzb	F-actin-capping protein subunit beta	↑	5.4	30952
Wdr1	WD repeat-containing protein 1	↑	6.1	66824
Atp6v1a	V-type proton ATPase catalytic subunit A	↑	5.2	68564
Cdca7l	Cell division cycle-associated 7-like protein (Cdca71)	↑	5.8	50854

[1] (Filled up arrow) up-regulated proteins; (Open down arrow) down-regulated proteins; (pilocarpine versus control rats), and (Ø) proteins expressed only in the hippocampus of control rats. MW: molecular weight; IP: isoelectric point.

2.2. Figures, Tables and Schemes In the hippocampal samples

Table 2. Cellular and molecular functions of hipocampal proteins identified by proteomic analysis applied to the animal model of temporal lobe epilepsy induced by pilocarpine.

Functions	Proteins	Key references supporting functions
Glycolysis & metabolic enzymes	Ldha, Ldhb, Aldoa, Eno1, Eno2, Eno3, Pgam1, Tpi1, LOC500959, RGD1565368, Mdh1	[23–32]
Mitochondrial ATP production & heme metabolism	Atp5b, Abcb6	[33,34]

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Proton transport & pH regulation	Atp6v1a, Atp6v1b2, Car2	[35–37]
Cytoskeleton structure, dynamics & axon guidance	Tubb5, Tubb2a, Tubb2c, Tubb3, Capzb, Wdr1, Dpysl2, Dpysl3	[38–45]
Vesicle trafficking, GPCR signaling and neurotransmission	Nsf, Gnb1, Gnb3, Pla2g4c	[46–49]
Cell signaling regulators	Mapk1, Pebp1, Ywhag, Cdca7l	[50–53]
Stress response, neuroprotection & glial structure	Park7, Gfap	[54,55]
GABA metabolism	Aldh5a1	[56]

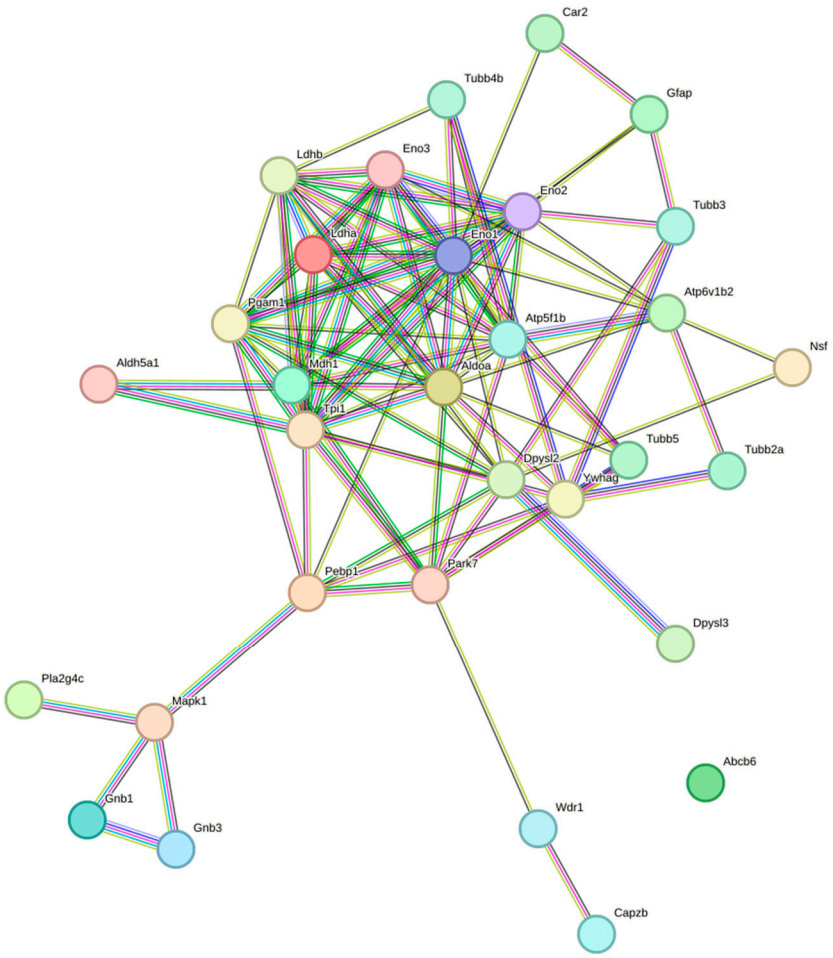

Figure 1. Schematic representation of the interactome showing the network between the hipocampal proteins identified by proteomic analysis applied to the animal model of temporal lobe epilepsy induced by pilocarpine. The presented result was based on STRING database (https://string-db.org/) [22].

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The interactome analysis revealed 100 direct interactions between query proteins with scores above the confidence threshold. The network showed several key functional clusters, classified below as major interaction modules:

Major Interaction Modules

1. Glycolytic Enzymes Cluster—Strong interactions among:

- Ldha ↔ Ldhb (score: 0.99)
- Pgam1 ↔ Eno1 (score: 0.99)
- Tpi1 ↔ Aldoa (score: 0.989)
- Eno1 ↔ Aldoa (score: 0.973)

The proteins mentioned above form a tightly connected hub around metabolic/glycolytic processes.

2. Tubulin/Cytoskeletal Module—Including:

- Wdr1 ↔ Capzb (score: 0.929)
- Tubb3 ↔ Gfap (score: 0.849)

The proteins mentioned above are important for connections to microtubule and actin regulation.

3. ATP/Energy Metabolism—Connected through:

- Atp5f1b ↔ Atp6v1b2 (score: 0.96)

Here, both ATP synthase subunit beta and V-ATPase presented high interaction scores.

4. CRMP-Related Proteins—Showing moderate connectivity:

- Dpysl2 ↔ Dpysl3 (score: 0.904)
- G-Protein Signaling—Connected module:
- Gnb1 ↔ Gnb3 (score: 0.811)
- Mapk1 interactions with Gnb1 and Gnb3

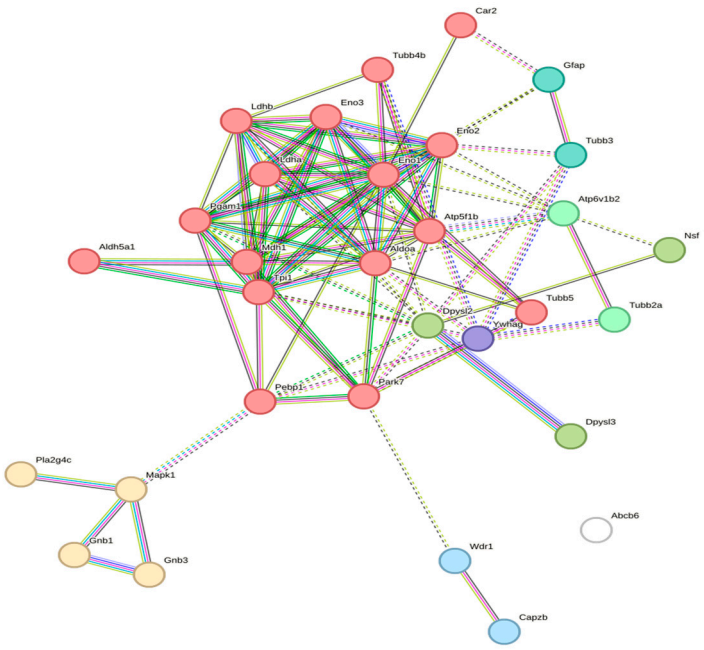

Figure 2. Clustered interactome visualization of the hipocampal proteins identified by proteomic analysis based on STRING database (https://string-db.org/) [22]. Different colors represent different protein clusters. Red: NAD metabolism, glycolysis and gluconeogenisis; Gold: thrombin and PAR Signaling; Olive: CRMP in Sema3A signaling and hydantoinase; Green: vesicular transport and microtubules; Blue: neuronal cytoskeleton; Cyan: actin filament regulation; Purple: 14-3-3 Adaptor (Isolated).

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The clustering analysis of the interactome revealed that the hipocampal protein network is segregated into 7 distinct functional modules as shown on Table 3.

Table 3. Hipocampal protein network presenting seven distinct functional modules as part of the clustering interactome of proteins identified by proteomic analysis applied to the animal model of temporal lobe epilepsy induced by pilocarpine.

Cluster	Proteins	Functional Annotation	Count
1 (Red)	Pebp1, Pgam1, Eno1, Tpi1, Park7, Atp5f1b, Tubb5, Eno3, Tubb4b, Eno2, Ldha, Aldoa, Ldhb, Mdh1, Aldh5a1, Car2	NAD Metabolism; Glycolysis/Gluconeogenesis	16
2 (Gold)	Mapk1, Gnb3, Gnb1, Pla2g4c	Thrombin Signaling; PAR Signaling	4
3 (Olive)	Dpysl2, Nsf, Dpysl3	CRMP in Sema3A Signaling; Hydantoinase	3
4 (Green)	Atp6v1b2, Tubb2a	Vesicular Transport; Microtubules	2
5 (Blue)	Tubb3, Gfap	Neuronal Cytoskeleton	2
6 (Cyan)	Wdr1, Capzb	Actin Filament Regulation	2
7 (Purple)	Ywhag	14-3-3 Adaptor (Isolated)	1

According to the results shown on Table 3, the cluster 1 dominates with 16 proteins, forming the largest functional module centered on energy metabolism. The cluster 2 (MAPK/G-protein signaling) represents transmembrane signaling and lipid metabolism. The clusters 3-6 represent specialized modules for neuronal signaling, transport, and cytoskeletal dynamics, while Ywhag (14-3-3γ) was found relatively isolated, acting as a potential hub protein with broader multi-module interactions. Potential hub proteins are proteins predicted to have a high number of interactions within a biological network, suggesting Ywhag (14-3-3γ) could be a critical control point.

3. Discussion

Among the down-regulated proteins we found enzymes related to the carbohydrate metabolism and ATP synthesis, reflecting disturbances in the energetic metabolism. These data are in line with findings reported by other authors [57,58]. The gene encoding the malate dehydrogenase was reported as a factor involved with the generation of generalized idiopathic epilepsy [57]. Altered proteins such as phospholipase A2, fructose-bisphosphate aldolase and enolase, have been reported by other authors associated with neuropsychiatric mechanisms [59–61]. However, phospholipase A2 can also participates in neurogenesis processes [62].

The guanine nucleotide–binding protein (G proteins) was down-regulated in the hippocampi of the pilocarpine-induced epilepsy. This is an important finding considering the wide role of this protein in the signal transduction by hormones, neurotransmitters, chemokines, and autocrine and paracrine factors [63].

In another study using lithium-pilocarpine model, we identified 24 proteins in the hippocampal samples of rats, but only 7 were differentially expressed compared to control rats, namely, 4 were up regulated and 3 were downregulated [64]. The interactome analysis revealed that the proteins are

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mainly related to glycolysis (14%) and to inflammation processes mediated by chemokine and cytokine signaling (5%). We also found proteins associated to Huntington's (5%) and Parkinson's disease (5%) and associated to fructose and galactose metabolism (4.80%). In addition, minor changes (2%) were also observed in several other pathways [64].

Here, the identified high-confidence protein-protein interaction modules were directly and compellingly linked to the molecular pathogenesis of epilepsy, specifically in the domains of neuronal hyperexcitability, energy failure, synaptic dysregulation, and post-status epilepticus remodeling. Below, the relationship between the interactome modules or clusters revealed for hipocampal proteins of rats submitted to the induction of temporal lole epilepsy by pilocarpine and the pathophysiology of epilepsy are described:

1. Glycolytic enzymes hub—central to seizure-induced metabolic crisis

The tightly interconnected glycolytic metabolon-Ldha–Ldhb, Pgam1–Eno1, Tpi1–Aldoa, Eno1–Aldoa are highly relevant to epilepsy. Acute energy demand during seizures increases glucose utilization up to 300%, while oxidative phosphorylation collapses, forcing a switch to massive lactate production [65,66]. Physical clustering of glycolytic enzymes into metabolons that channel substrates is well-documented and dramatically enhances flux under stress [66,67]. The shift from LDH1 (H4) to LDH5 (M4) isozymes and the high level of Ldha/Ldhb heterotetramers are hallmarks of epileptic foci and post-status epilepticus hippocampus [69,70]. Therapeutic relevance: stiripentol inhibits LDH, shifting metabolism towards oxidative phosphorylation [71]; 2-deoxyglucose, by inhibiting glycolysis, has anticonvulsant properties [72].

2. Cytoskeletal module (Wdr1–Capzb, Tubb3–Gfap)—loss of dendritic spine and epileptogenesis

Wdr1-mediated actin severing via Aip1 and cofilin promotes rapid dendritic spine collapse within hours of status epilepticus, and this contributes to delayed hyperexcitability [73,74]. Capzb modulates actin barbed-end dynamics, and its interaction with Wdr1 suggests a pre-assembled actin disassembly complex that is activated by seizure-induced Ca^{2+}/calcineurin signaling [75]. Reactive gliosis and physical coupling of neuronal microtubules with astrocytic GFAP intermediate filaments impair K^+ and glutamate clearance in epileptic tissue [76,77].

3. Interaction of ATP synthase ↔ V-ATPase (Atp5f1b ↔ Atp6v1b2)—ionic homeostasis and acidosis

Seizure activity results in profound extracellular acidosis (pH ~6.8) through lactate and CO_2 [78]. The brain-enriched V-ATPase B2 subunit Atp6v1b2 is required for neuronal pH recovery and its genetic disruption decreases seizure threshold [79]. Physical or functional coupling of mitochondrial ATP synthase with V-ATPase at organelle contact sites regulates local ATP/pH homeostasis [80]. Acidosis activates ASIC1a channels and pannexin-1 hemichannels, generating a feed-forward excitotoxic loop [81,82]. Carbonic anhydrase inhibitors (topiramate, zonisamide) exert part of their anticonvulsant effect by modulating brain pH [83].

4. CRMP family (Dpysl2 ↔ Dpysl3)—axonal sprouting and network reorganization

Hyperphosphorylation of CRMP2 (Dpysl2) by Cdk5/GSK3β after status epilepticus drives aberrant mossy fiber sprouting in temporal lobe epilepsy [84,85]. CRMP2 and CRMP4 form heteromeric complexes; their interaction modulates tubulin binding and axonal guidance [86]. Lacosamide, which is an anticonvulsant, directly binds CRMP2 and thereby stabilizes its inactive conformation [87].

5. The G-protein/MAPK1 signaling node: control of inhibitory tone and excitability

Gβ1 and Gβ3 isoforms mediate GABA_B, adenosine A1, cannabinoid CB1, and opioid receptor inhibitory signaling in hippocampus [88,89]. Chronic epilepsy is associated with the desensitization of Gβγ-dependent GIRK currents and reduced inhibitory tone [90]. Convergence of Gβγ on the Raf–MEK–ERK (Mapk1) cascade links neuromodulatory receptors to long-term plasticity changes in epileptogenesis [91,92].

As discussed above, our study have revealed a tightly interconnected molecular network that recapitulates a core epileptogenic axis consistently described across diverse acquired and genetic

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epilepsy models [93,94]. This axis integrates four major pathophysiological domains whose concurrent failure drives the transition from acute seizures to chronic, drug-refractory epilepsy:

1. Energy-buffering collapse—physical clustering of glycolytic enzymes into a functional metabolon (Ldha–Ldhb, Eno1–Pgam1–Aldoa–Tpi1) [67,68] coupled with direct interaction between mitochondrial ATP synthase and neuronal/astrocytic V-ATPase (Atp5f1b ↔ Atp6v1b2) [80];
2. pH-homeostasis breakdown—seizure-induced acidosis and impaired proton extrusion via V-ATPase [77,78], leading to sustained activation of acid-sensing ion channels (ASICs) and pannexin hemichannels [81,82];
3. Loss of inhibitory tone—desensitization or uncoupling of Gβγ-mediated signalling (Gnb1/Gnb3–Mapk1 node) from GABA_B, adenosine A1, and cannabinoid receptors [88–90];
4. Pathological structural plasticity—dysregulation of actin (Wdr1–Capzb) [73,74] and microtubule (CRMP2–CRMP4, Tubb3–Gfap) networks [76,84–86] that drives dendritic spine loss, aberrant mossy fibre sprouting, and reactive gliosis.

The physical nature of these interactions (confidence scores ≥0.90–0.99) indicates that these processes are not merely co-dysregulated but are structurally pre-wired to fail together under sustained ictal stress. This molecular convergence explains the notorious refractoriness of chronic epilepsy to therapies that target only one domain (e.g., ion channels or synaptic transmission) [93] and underscores why multi-node, disease-modifying strategies are required.

The protein complexes identified here represent high-value, translation-ready candidate nodes for next-generation antiepileptogenic and disease-modifying therapies, including:

(a) small-molecule or peptide disruptors of pathological CRMP oligomerization [85,87],
(b) stabilizers of the glycolytic metabolon or mitochondrial–V-ATPase tethering [68,80],
(c) allosteric modulators of Gβγ signalling [89],
(d) pH-buffering or V-ATPase-enhancing compounds [79,83].

Targeting these physically linked hubs may offer a rational path toward interrupting the self-reinforcing epileptogenic cascade at its structural roots rather than merely suppressing its symptomatic output [92,93].

4. Materials and Methods

4.1. Animals

Adult male Wistar rats, weighing approximately 250 g, were housed under standard controlled conditions (12/12-h light/dark cycle; 20–22 ◦C; 40–60% humidity) with food and water offered ad libitum. All animal procedures were conducted per national and international legislation (Guidelines of the National Council for the Control of Animal Experimentation; NIH Guide for Care and Use of Laboratory Animals) and were approved by the Ethical Committee of our University (CEUA 1770/2006). Efforts were made to minimize the number of animals used and to avoid their suffering.

4.2. Pilocarpine Protocol

Seizures were induced by pilocarpine, as reported previously [95]. Briefly, rats were injected with pilocarpine hydrochloride (360 mg/kg, Merck, Darmstadt, Germany), administered intraperitoneally (i.p.) 20 min after the subcutaneous (s.c.) injection of methyl-scopolamine. Methyl-scopolamine (1 mg/kg, s.c., Merck, Darmstadt, Germany) was used to minimize peripheral consequences of pilocarpine such as diarrhea, piloerection, and olfactory and gustatory automatisms associated with salivation, eye blinking, vibrissae twitching, and yawning that usually start 5–10 min after pilocarpine injection [95].

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4.3. Groups

Following treatments, rats were randomly divided into two groups: Saline: rats injected with saline; PILO: rats treated with pilocarpine. All rats treated with pilocarpine exhibited the sequence of behavior changes described by Turski et al. [95] and Leite et al. [96]

4.1. Sample Preparation

Hippocampi were dissected 90 days after status epilepticus induction at 4 ◦C. About 100 mg (wet weight) of hippocampal tissue was homogenized for protein extraction using a buffer (5 µL/mg tissue) consisting of 7 M urea, 2 M thiourea, 4% (*w*/*v*) CHAPS, 10 mM DTT, 1 mM EDTA, 1 mM PMSF, 0.2 mM Na2VO3 and 1 mM NaF. After sonication in an ice-bath, the suspension was centrifuged at 12,000× *g* for 40 min at 4 ◦C to remove cellular debris. The protein concentration of the samples was determined by using the Bradford method in the supernatants [97]

4.2. Two-Dimensional Gel Electrophoresis (2-DE)

Five hundred micrograms of protein from hippocampal samples (PILO and control) were used for 2-DE. Linear (17 cm) pH 3–10 IPG strips (BioRad Laboratories, Hercules, CA, USA) were used for the first-dimension electrophoresis. The active rehydration was carried out for 12 h at 50 V. Isoeletric focusing (IEF) was performed using a Protean IEF cell (BioRad Laboratories, Hercules, CA, USA). After IEF, the strips were equilibrated in a buffer consisting of 50 mM Tris-HCl (pH 8.8), 6 M urea, 34% glycerol, 2% SDS, 1% DTT and 0.001% bromophenol blue. After 15 min, the stirring strips were equilibrated in a second buffer which did not contain DTT and had an additional 2.5% iodoacetamide relative to the first buffer. The equilibrated strips were then placed onto second dimension gels (12% SDS-PAGE). SDS-PAGE was performed using a Protean II xi Cell (BioRad Laboratories, Hercules, CA, USA) with a standard Tris-Glycine-SDS buffer, with a current setting of 20 mA/gel for 1 h, followed by 60 mA/gel until the bromophenol blue dye reached the end of the gel. The gels were stained using the Coomassie blue method [98]. The electrophoretic runs were made in duplicates.

4.3. Image Analysis for Proteome Determination

Stained gels were scanned by a GS-800 calibrated densitometer (BioRad Laboratories, Hercules, CA, USA), normalized to the background, and analyzed by PDQuest 2D-gel software (Version 8.0.1, BioRad Laboratories, Hercules, CA, USA). From selected gels, spots of interest were cut from the gel for mass spectrometry identification. For greater reliability, the identification was made in duplicates.

4.4. In-Gel Digestion

Excised protein spots were subjected to in-gel trypsin digestion. The spots were briefly washed by adding and removing a solution containing 100 mM (NH4)2CO3 with 50% acetonitrile until total discoloration. The gel fragments were dehydrated with 50 µL of pure acetonitrile in a vacuum centrifuge. Once fully dried, the gel fragments were rehydrated in 5 µL of digestion buffer consisting of 50 mM ammonium (NH4)2CO3 (pH 8.0) and 0.5 mg of trypsin, (Sigma-Aldrich, SP, Brazil) for 20 min at room temperature. A volume of 100 µL of 50 mM (NH4)2CO3 was added to all tubes and the samples were incubated overnight at 37 ◦C. The reaction was stopped by adding 50 µL of 0.1% trifluoroacetic acid (TFA). The samples were dehydrated in a vacuum centrifuge, re-suspended in 0.1% TFA, and analyzed by LC-ESI-MS/MS.

4.5. Nano-LC-ESI-MS/MS Analysis

An aliquot (4.5 µL) of digested proteins was injected into analytic columns C18 (1.7 µm), BEH 130 (100 µm × 100 mm), RP-UPLC (nanoAcquity UPLC, Waters), coupled with nano-electrospray tandem mass spectrometry on a Q-Tof Ultima API mass spectrometer (MicroMass/Waters), at a flow rate of 600 nL/min. A Symmetry C18 (180 µm × 20 mm) trapping column was used for sample

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desalting at a flow rate of 5 µL/min for 2 min. The gradient was 0–50% acetonitrile in 0.1% formic acid over 45 min. The instrument was operated with an MS positive mode data continuum acquisition from m/z 100–2KDa at a scan rate of 1 s and an interscan delay of 0.1s. Database searches for peptide identification from LC MS-MS experiments were completed with a Mascot Distiller v.2.3.2.0, 2009 (Matrix Science, Boston, MA, USA) using carbamidomethyl–cys as fixed modification (monoisotopic mass 57.0215Da). Lysine and/or arginine methylation, lysine acetylation, methionine and/or tryptophan oxidation were used as variable modification (monoisotopic mass 15.9949) and 0.1 Da MS and MSMS was applied as fragment tolerances. NCBI protein database was used for protein identification.

4.6. Interactome

An interactome was generated using the STRING database (https://string-db.org/) [22] to identify biological functions and the respective genes that are likely involved with these functions in the network of proteins of interest.

4.7. Statistics

The PDQuest software analyzed the statistical differences of optical density in the proteomic study. Statistical significance was defined at $p < 0.05$.

5. Conclusions

The proteins identified here by proteomic analysis when submitted to analysis of interactome clustering revealed a common core epileptogenic axis described in multiple models to illustrate why epilepsy becomes refractory through the simultaneous failure of energy buffering, pH homeostasis, inhibitory signalling and structural plasticity. The physical interactions found provide high-value candidate nodes for next-generation disease-modifying therapies.

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