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Proteomics and Transcriptomics of the Hippocampus and Cortex in SUDEP and High-Risk SUDEP Patients

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Dominique F. Leitner, PhD^{*1,2}, James D. Mills, PhD^{*3}, Geoffrey Pires, MS^{*2,4,5}, Arline Faustin, MD^{2,4}, Eleanor Drummond, PhD^{2,4,6}, Evgeny Kanshin, PhD⁷, Shruti Nayak, MS⁷, Manor Askenazi, PhD⁸, Chloe Verducci, BA¹, Bei Jun Chen, PhD⁹, Michael Janitz, PhD⁹, Jasper J. Anink, BSc³, Johannes C. Baayen, MD¹⁰, Sander Idema, MD, PhD¹⁰, Erwin A. van Vliet, PhD^{3,11}, Sasha Devore, PhD^{1,2}, Daniel Friedman, MD, MSc^{1,2}, Beate Diehl, MD, PhD¹², Catherine Scott, MPhil¹², Roland Thijs, MD, PhD¹³, Thomas Wisniewski, MD^{2,4,14,15}, Beatrix Ueberheide, PhD^{2,7,16}, Maria Thom, MD¹², Eleonora Aronica, MD, PhD^{3,13}, Orrin Devinsky, MD^{1,2}

¹ Comprehensive Epilepsy Center, NYU School of Medicine, New York, NY, USA.

² Department of Neurology, NYU Langone Health and School of Medicine, New York, NY, USA.

³ Department of (Neuro)Pathology, Amsterdam UMC, University of Amsterdam, Amsterdam Neuroscience, Meibergdreef 9, Amsterdam, the Netherlands.

⁴ Center for Cognitive Neurology, NYU Langone Health and School of Medicine, New York, NY, USA.

⁵ Alzheimer's and Prion Diseases Team, Paris Brain Institute, CNRS, UMR 7225, INSERM 1127, Sorbonne University UM75, Paris, France.

⁶ Brain & Mind Centre and School of Medical Sciences, Faculty of Medicine and Health, University of Sydney, Australia.

⁷ Proteomics Laboratory, Division of Advanced Research Technologies, NYU School of Medicine, New York, NY, USA.

⁸ Biomedical Hosting LLC, Arlington, MA, USA.

⁹ School of Biotechnology and Biomolecular Sciences, University of New South Wales, Sydney, New South Wales, Australia.

¹⁰ Amsterdam UMC, Vrije Universiteit Amsterdam, Department of Neurosurgery, Amsterdam Neuroscience, De Boelelaan 1117, Amsterdam, the Netherlands.

¹¹ Swammerdam Institute for Life Sciences, Center for Neuroscience, University of Amsterdam, Amsterdam, the Netherlands.

¹² Department of Clinical and Experimental Epilepsy, University College London Institute of Neurology, London, UK.

¹³ Stichting Epilepsie Instellingen Nederland (SEIN), Heemstede, the Netherlands.

¹⁴ Department of Pathology, NYU Langone Health and School of Medicine, New York, NY, USA.

¹⁵ Department of Psychiatry, NYU Langone Health and School of Medicine, New York, NY, USA.

¹⁶ Department of Biochemistry and Molecular Pharmacology, NYU School of Medicine, New York, NY, USA.

* These authors contributed equally to this work.

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Corresponding author:

Orrin Devinsky
Email: Orrin.Devinsky@nyulangone.org

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Abstract

Objective: To identify the molecular signaling pathways underlying sudden unexpected death in epilepsy (SUDEP) and high-risk SUDEP compared to epilepsy control patients.

Methods: For proteomics analyses, we evaluated the hippocampus and frontal cortex from microdissected post-mortem brain tissue of 12 SUDEP and 14 non-SUDEP epilepsy patients. For transcriptomics analyses, we evaluated hippocampus and temporal cortex surgical brain tissue from mesial temporal lobe epilepsy (MTLE) patients: 6 low-risk and 8 high-risk SUDEP as determined by a short (< 50 seconds) or prolonged (\geq 50 seconds) postictal generalized EEG suppression (PGES) that may indicate severely depressed brain activity impairing respiration, arousal, and protective reflexes.

Results: In autopsy hippocampus and cortex, we observed no proteomic differences between SUDEP and non-SUDEP epilepsy patients, contrasting with our previously reported robust differences between epilepsy and non-epilepsy control patients. Transcriptomics in hippocampus and cortex from surgical epilepsy patients segregated by PGES identified 55 differentially expressed genes (37 protein-coding, 15 lncRNAs, three pending) in hippocampus.

Conclusion: The SUDEP proteome and high-risk SUDEP transcriptome were similar to other epilepsy patients in hippocampus and frontal cortex, consistent with diverse epilepsy syndromes and comorbidities associated with SUDEP. Studies with larger cohorts and different epilepsy syndromes, as well as additional anatomic regions may identify molecular mechanisms of SUDEP.

Introduction

Sudden unexpected death in epilepsy (SUDEP) affects 1 in 1000 epilepsy patients annually and is the leading cause of epilepsy-related deaths.¹ SUDEP most often follows a generalized tonic-clonic seizure (GTCS), and excludes trauma, drowning, status epilepticus, or other causes. Most deaths are unwitnessed, occur during sleep, and the patient is found prone.

Studies on SUDEP epidemiology, risk factors, mechanisms, and prevention have advanced our understanding, although pathophysiological understanding remains limited.^{2,3} After a GTCS, prolonged (>50 sec) postictal generalized EEG suppression (PGES) may increase SUDEP risk and may be a SUDEP biomarker, as severe prolonged reduced brain activity impairs arousal, respiration, and other autonomic functions.⁴ However, we cannot predict why some low-risk patients become SUDEP patients, high-risk patients survive for decades, and other patients succumb to SUDEP despite recovering from many earlier GTCS. SUDEP patients may harbor pathogenic gene variants in brain and heart ion channels,⁵⁻⁷ but a role in SUDEP pathogenesis remains speculative. Animal models of genetic epilepsies and chemo-induced seizures implicate abnormalities in respiration, arousal, and parasympathetic hyperactivity in SUDEP pathogenesis.^{1,8-10} However, the neuropathology of SUDEP parallels findings in non-SUDEP epilepsy patients.^{11,12} Potential proteomic and transcriptional molecular signatures associated with SUDEP have not been studied.

Our study investigated the molecular signaling networks associated with SUDEP in brain regions implicated in ictogenesis,¹³ from localized proteomics in autopsy hippocampal

CA1-3, dentate gyrus, and superior frontal gyrus from SUDEP and non-SUDEP epilepsy patients and transcriptomics in hippocampus and temporal cortex from low and high-risk SUDEP (PGES < or \geq 50 seconds) epilepsy surgical tissue.

Methods

Standard Protocol Approvals, Registrations, and Patient Consents: Autopsy brain tissue and clinical information from SUDEP or non-SUDEP epilepsy patients was obtained with approval by the New York University School of Medicine Institutional Review Board (IRB). All next of kin provided written informed consent.

Human Brain Tissue for Proteomics: Post-mortem brain tissue from epilepsy patients who died from SUDEP or other causes was obtained through the North American SUDEP Registry (NASR), which began enrolling patients in October 2011², with approval by the New York University School of Medicine Institutional Review Board (IRB). Causes of death were classified (OD, DF) into non-SUDEP epilepsy and SUDEP (definite SUDEP, definite SUDEP plus, and probable SUDEP).^{1,2} Lifetime GTCS history was determined from interviews and medical records, representing the best estimate for each patient and as described previously for these patients.² After neuropathological review (TW, AF), brain tissue was processed into formalin fixed paraffin embedded (FFPE) blocks and sections were stained with luxol fast blue counterstained with hematoxylin & eosin (LFB/H&E). Archival time for brain tissue storage in formalin was less than or equal to three years, thus patients were chosen from those that were enrolled in NASR between July 2014 to March 2017. Patients were age and sex matched from available NASR cases. There were no significant differences in age at death ($p=0.9190$, unpaired t-test), disease duration ($p=0.7295$), disease onset ($p=0.4797$), or sex

($p > 0.9999$). Clinical and neuropathologic data on the 14 non-SUDEP epilepsy and 12 SUDEP patients are summarized in Table 1. Group sizes were determined based on the number of patients with significant findings as previously reported,¹⁴⁻¹⁶ including our earlier studies in epilepsy patients with similar methods.^{17,18}

Laser Capture Microdissection for Proteomics: FFPE brain tissue blocks containing either hippocampus (lateral geniculate nucleus level)¹⁹ or superior frontal gyrus were sectioned at 8 μm and collected onto laser capture microdissection (LCM) compatible PET slides (Leica). Sections were stained with cresyl violet to localize regions of interest for LCM²⁰ and air dried overnight in a loosely closed container. LCM was used to individually microdissect 10 mm^2 from the hippocampal CA1-3 region and superior frontal cortex (layers I-IV), and 4 mm^2 from the hippocampal dentate gyrus into LC-MS grade water (Thermo Scientific). Microdissected samples were centrifuged for 2 minutes at 14,000g and stored at -80°C . LCM was performed at 5X magnification with a LMD6500 microscope equipped with a UV laser (Leica).

Label-free quantitative MS Proteomics: Label-free quantitative MS assessed differential protein expression, as described previously.^{18,21,22} FFPE cuts were incubate in 50 mM ammonium bicarbonate (ABC) solution containing 20% (v/v) acetonitrile (ACN) for 1h at 95°C followed by 2h at 65°C . Disulfide bonds were reduced with 10 mM DTT (1h at 57°C) and alkylated with 30 mM IAA (45 min at RT in the dark). Proteins were enzymatically digested into peptides with 300 ng of trypsin (sequencing grade, Promega) overnight at RT. Digestions were quenched by acidification with Trifluoroacetic acid (TFA) and peptides were concentrated and desalted on POROSTM R2 C18 beads. Eluates were dried in a speedvac and resuspended in 0.5% AcOH. LC separation was performed

online on EASY-nLC 1200 (Thermo Scientific) utilizing a Acclaim PepMap 100 (75 μ m x 2 cm) precolumn and a PepMap RSLC C18 (2 μ m, 100A x 50 cm) analytical column. Peptides were gradient eluted from the column directly into the Orbitrap Fusion Lumos mass spectrometer using a 165 min ACN gradient (A=2% ACN 0.5% AcOH / B=80% ACN 0.5% AcOH). The flowrate was set at 200 nl/min. The mass spectrometer was operated in a data-dependent acquisition mode. High resolution full MS spectra were acquired with a resolution of 240,000, an AGC target of 1e6, with a maximum ion injection time of 50 ms, and scan range of 400 to 1500 m/z. Following each full MS scan data-dependent HCD MS/MS scans were acquired in the ion trap (scan rate rapid, AGC target of 2e4, NCE of 32). Precursor isolation window were set at 2 Da.

Proteomics Computational Analysis: MS data were analyzed as previously described.^{18,21,22} RAW MS data were processed using the MaxQuant²³ software (v. 1.6.3.4) and the SwissProt human protein database (www.uniprot.org) containing 20,421 entries. Database including a common list of common laboratory contaminants (248 entries) were also used in the search. All peptide-spectrum matches, peptide and protein identifications were filtered to get a desired FDR level below 1% (calculated using decoy database approach). For the MS/MS search enzyme specificity was set to trypsin (up to 2 miscleavages), precursor mass tolerance was set to 20 ppm with subsequent non-linear mass recalibration. Carbamidomethylation of cysteine was set as a fixed modification, protein N-term acetylation and methionine oxidation were set as a variable modifications. Match between runs (MBR) algorithm was enabled to transfer peptide feature identifications between MS runs based on LC retention time (0.7 min tolerance after initial recalibration) and precursor mass tolerance. Label-free quantification (LFQ) were

performed used built in “maxLFQ” algorithm²⁴ and normalization was performed separately for all samples within each ROI.

Data analysis was performed in Perseus framework²⁵ (<http://www.perseus-framework.org/>), R environment (<http://www.r-project.org/>), or GraphPad Prism.

Proteomics Statistical Analyses: The protein expression matrix (n=4,129) was filtered to contain only proteins that were quantified in ≥ 8 replicates in at least 1 condition (SUDEP or non-SUDEP epilepsy) in any brain region (n=2,847). Subsequently missing values were imputed from the intensity distribution simulated low-intensity protein features (width of 0.3 and downshift of 1.8 relative to measured protein intensity distribution). An unpaired two-tailed t test was performed for PCA1 in each brain region to determine significance of separation in the SUDEP and non-SUDEP epilepsy patients. All other analyses were done using nonimputed data. A Student’s two sample t-test was used to access statistical significance of the changes in protein abundance between conditions. Obtained p-values were adjusted for multiple hypothesis testing using permutation-based FDR to a cutoff of 5%. Cell type specific annotations were included in the data available on Dryad (Table e-3) and on volcano plots in Fig. 1F-H, derived from previous data.²⁶ Annotations were included when a protein had only one associated cell type after removing cerebellar annotations and when the annotation included more than one associated cell type (both excitatory and inhibitory neuron annotations) and were thus assigned a general neuron annotation, for a total of 1066 possible annotations.

Proteomics Correlation: For the correlation in protein abundance between conditions and brain regions we used averaged over replicates LFQ values. A Pearson’s correlation was calculated for proteins detected in both SUDEP and non-SUDEP epilepsy patients for

each brain region, with 2715 proteins for hippocampal CA1-3, 2464 proteins for dentate gyrus, and 2695 proteins for the frontal cortex.

Immunohistochemistry: Immunohistochemistry was performed to validate the identified protein of interest, ermin (ERMN) as previously described.^{18,27} Briefly, FFPE sections (8 μ m) were deparaffinized and rehydrated through a series of xylenes and ethanol dilutions. Heat-induced antigen retrieval was performed with 10 mM sodium citrate, 0.05% triton-x 100; pH6. Blocking with 10% normal donkey serum was followed by ERMN primary antibody (1:200, Sigma HPA038295) overnight at 4°C. Sections were incubated with donkey anti-rabbit Alexa-Fluor 647 secondary antibody (1:500, Thermofisher Invitrogen) and coverslipped.

Image semiquantitative analysis: Whole slide scanning was performed at 20X magnification with a NanoZoomer HT2 (Hamamatsu) microscope using the same settings for each slide. One image containing the hippocampal CA1-3 region was collected for each patient, 11 non-SUDEP epilepsy and 11 SUDEP patients. Images were analyzed in Fiji ImageJ to compare the amount of ERMN in SUDEP and non-SUDEP epilepsy patients. The same binary threshold was used for all images to determine the number of ERMN positive pixels in each image, which was reported as a percentage of the total image area. An unpaired t-test was performed for statistical analysis; p-value <0.05 was considered significant.

Confocal imaging was used to collect representative images of ERMN immunohistochemistry, using a Zeiss LSM800 confocal microscope with the same

settings on each slide with a Plan-Apochromat 20X/0.8 M27 objective and a pinhole of 38 μm .

RNA-sequencing datasets: Small RNA-sequencing (small RNAseq) and RNA-sequencing (RNAseq) data sets were retrieved from the European Genome-phenome Archive (accession number: EGAS00001003922) from MTLE patients undergoing surgical resection and with available PGES duration greater than 1 second.¹⁷ The patients were age and sex matched, with no significant differences in age at surgery ($p=0.6622$, unpaired t-test), disease duration ($p=0.4391$), disease onset ($p=0.4612$), or sex ($p>0.9999$). Small RNAseq and RNAseq data was retrieved for 6 patients with PGES < 50 sec, indicating a potential low-risk for SUDEP, and 8 patients with PGES \geq 50 sec, indicating a potential high-risk for SUDEP as previously described.⁴ Table 2 summarizes the clinical characteristics of these patients. PGES occurrence and duration was assessed by two epileptologists (CS, RT).

Bioinformatic analysis of RNAseq data: Bioinformatic analysis was performed as described previously.¹⁷ Briefly, library normalization and differential expression testing was carried out using the R package *DESeq2*. The Wald test identified differentially expressed genes using a Benjamini-Hochberg adjusted p-value <0.05 for significance. Cell type specific annotations were included (Dryad table e-4, table e-5), and on volcano plots in Fig. 2C, 2E, derived from previous data.²⁶ Annotations were included when a protein had only one associated cell type after removing cerebellar annotations and when the annotation included more than one associated cell type (both excitatory and inhibitory neuron annotations) and were thus assigned a general neuron annotation, for a total of 1066 possible annotations.

A Reactome pathway enrichment analysis was performed using the R package *ReactomePA*. The differentially expressed genes from the RNAseq differential expression analysis were put into R and tested for over-representation of enriched Reactome pathways using hypergeometric testing. Pathways with a Benjamini-Hochberg corrected p-value <0.05 were considered significantly enriched.

Bioinformatic analysis of small RNAseq data: Bioinformatic analysis of the small RNAseq data was performed as described previously.¹⁷ Briefly, library normalization and differential expression testing was carried out using the R package *DESeq2*. The Wald test identified differentially expressed genes with a Benjamini-Hochberg adjusted p-value <0.05 considered significant.

RNAseq validation by qPCR: The gene expression of GDNF Family Receptor Alpha 1 (*GFRA1*) was assessed in the same cohort of samples used in the RNAseq analysis for which sufficient RNA remained (PGES < 50s, n=4, PGES ≥ 50s, n=7). PCR primers based on the reported cDNA sequences were designed using the NCBI primer design tool.²⁸ The sequences for the forward and the reverse primers of GFRA1 were 5'-TCT TCC AGC CGC AGA AGA AC-3' and 5'-AAC AGT GGG GAC AAA CTG GG-3' respectively. 700 ng of total RNA was reverse transcribed into cDNA using oligodT primers. For each qPCR reaction, a mastermix was prepared as follows: 1 µl cDNA, 2.5 µl of 2x SensiFAST SYBR Green Reaction Mix (Bioline Inc, Taunton, MA, USA), 0.2 µM of both reverse and forward primers and the PCRs were run on a Roche Lightcycler 480 thermocycler (Roche Applied Science, Basel, Switzerland). Each sample and primer pair was run in triplicates. Data quantification was performed as previously described¹⁷

relative to the reference genes, Eukaryotic Translation Elongation Factor 1 Alpha 1 (*EEF1A1*) and Chromosome 1 Open Reading Frame 43 (*C1orf43*). The normalized ratio was compared between the two groups (Mann-Whitney U test); $p < 0.05$ was considered significant.

Data availability: All data needed to evaluate the conclusions in the paper are present in the paper and on Dryad at <https://doi.org/10.5061/dryad.dfn2z3508>. Additional data related to this paper may be requested from the authors.

Results

Proteome of SUDEP and non-SUDEP epilepsy autopsy patients

The differential expression of proteins in autopsy SUDEP (n=12) and non-SUDEP (n=14) epilepsy patients was evaluated using label-free quantitative mass spectrometry (MS) in the microdissected hippocampal CA1-3 region, dentate gyrus, and superior frontal cortex, as these regions have been implicated in ictogenesis and may also be influenced by seizure activity.¹³ Patient histories are summarized in Table 1 and Fig. 1A-B. A principal component analysis (PCA) did not distinguish SUDEP and non-SUDEP epilepsy patients in any of the studied brain regions (Fig. 2A-C). The main source of variation in these patients, PCA1, did not show a significant difference when comparing SUDEP and non-SUDEP epilepsy patients in each brain region by an unpaired two-tailed t test, as depicted by a box plot in Fig. 2A-C. Lifetime GTCS burden, associated with an increased SUDEP risk,¹ was evaluated to determine whether this factor may contribute to

protein differences as seen by a separation of groups. From patients with available data (9 SUDEP and 8 non-SUDEP epilepsy patients), 55.6% of SUDEP and 62.5% of non-SUDEP epilepsy patients had > 10 lifetime GTCS, and 22.2% of SUDEP patients and 12.5% of non-SUDEP epilepsy patients had > 100 lifetime GTCS. Lifetime GTCS frequency did not contribute to group differences in the PCA (Fig.2A-C). There was no enrichment in SUDEP or non-SUDEP epilepsy patients with > 10 or > 100 lifetime GTCS by a Fisher's exact test. Further, in the PCA, there was no relationship of SUDEP status to neuropathology (focal cortical dysplasia (FCD, n = 10), hippocampal dentate gyrus dysgenesis (n = 7), hippocampal sclerosis (n = 3), and gliosis (n = 3)). Of note, microdissected regions did not necessarily contain observed FCD as it may have been present in other brain regions. Similarly, neuropathology was unrelated to SUDEP status (FCD in 50% of SUDEP patients versus 28.6% of non-SUDEP epilepsy patients, Fisher's exact test, $p = 0.4216$).

There were no significant differences in protein expression between SUDEP and non-SUDEP epilepsy patients in any brain region (Fig. 3A-C, Dryad fig. e-1A-C, table e-3). Further, a correlation of LFQ values for all proteins showed the similarity in protein expression when comparing SUDEP and non-SUDEP epilepsy patients in each brain region by a Pearson's correlation ($p < 0.0001$) with the corresponding R^2 values being ≥ 0.98 (Dryad, fig. e-1). Brain cell type specific annotation was evaluated in the 2847 identified proteins, derived from previous methods,²⁶ with 19.8% (564/2847) proteins having an annotation while the remaining 80.2% did not and were more ubiquitously expressed or cell type is unknown. Most (78.2%; 502/564) annotated proteins were generally neuronal, with excitatory neuron proteins predominating (48.1%; 271/564) (Fig. 3A-C, Dryad table e-3). Some proteins showed a trend for altered expression in

SUDEP patients ($p < 0.01$; Dryad table e-1-2), but these were not statistically significant at a 5% FDR. Several of these protein changes have been reported in epilepsy animal models and non-epilepsy patients or include proteins encoded by genes in which mutations have been previously linked to epilepsy. Yet, none of the proteins trending for altered expression in this study (Dryad table e-1-2) have been previously linked to SUDEP pathogenesis. Ermin (ERMN) had the strongest trend for difference in SUDEP with a 2.8-fold decrease in the hippocampal CA1-3 region when comparing SUDEP and non-SUDEP epilepsy patients by MS (Dryad fig. e-2A). Further, ERMN was detected in more non-SUDEP epilepsy than SUDEP patients by MS, indicating lower abundance of this protein in SUDEP. Validation of the quantitative MS findings with semiquantification of immunohistochemistry (Dryad fig. e-2B) also showed a decrease of ERMN in SUDEP patients with a 1.3-fold change but was not significant (student's unpaired t test, p -value = 0.4871). Because ERMN may play a role in myelinogenesis and myelin maintenance, we reviewed the mature oligodendrocyte marker myelin basic protein (MBP) but found no difference between SUDEP and non-SUDEP epilepsy patients in the hippocampal CA1-3 region by MS (Dryad fig. e-2C).

Analysis of RNAseq and small RNAseq in low and high-risk SUDEP patients

To determine whether there is a pathological difference in epilepsy patients of low (PGES <50 seconds, $n=6$) and high (PGES ≥ 50 seconds, $n=8$) risk of SUDEP, RNAseq and small RNAseq analyses were performed on resected surgical frozen hippocampal and temporal cortex tissue. Patient histories are summarized in Table 2 and Fig. 4A. A t-SNE (t-distributed stochastic neighbor embedding) plot revealed that anatomical region rather than PGES segregated patients (Fig. 4B). A differential expression analysis comparing

the hippocampus of low and high-risk SUDEP patients identified 55 differentially expressed genes: 11 were decreased and 44 were increased in high-risk SUDEP patients (Fig. 4C, Dryad table e-4). Brain cell type specific annotation was evaluated in the 55 differentially expressed genes in the hippocampus, derived from previous methods,²⁶ with 14.5% (8/55) of genes having a cell type specific annotation: 4 generally neuronal, 3 excitatory neuron, and 1 inhibitory neuron. The dominant transcripts for the differentially expressed genes in hippocampus were: 37 protein-coding, 15 long non-coding RNAs (lncRNAs), and three awaiting confirmation (Fig. 4D). A Reactome pathway analysis on the 55 significant genes in the hippocampus did not reveal a significant association with any signaling pathways. Several of these genes have been associated with epilepsy human disease and have been studied in animal models, however none of the genes in Table 3 have been linked to SUDEP pathogenesis. The most significantly decreased protein-coding gene in the high-risk SUDEP patients, *GFRA1*, was validated by real time quantitative PCR (RT-qPCR, Table 3, Dryad fig. S3). In accordance with the RNAseq analysis, *GFRA1* was decreased 1.7-fold in the high-risk SUDEP patients (Mann-Whitney U test, p-value = 0.0121). In the temporal cortex, one protein-coding gene (*SLC6A5*) with an “undefined” cell type annotation was significantly decreased in the high-risk SUDEP patients, within this small group of patients (Fig. 4E, Dryad table e-5). No genes were differentially expressed in the small RNAseq analyses in the hippocampus and temporal cortex (Dryad table e-6-7).

Comparison of SUDEP Proteome to High-risk SUDEP Transcriptome

Comparing the 37 differentially expressed protein-coding genes in the RNAseq analyses to the proteomics analyses, only four (GRM2, ERC2, CRT1, AHNK2) were detected in the proteomics analyses. Two (GRM2, ERC2) were detected in most patients of the

hippocampal CA1-3 region but showed no trend in differential expression for SUDEP patients compared to non-SUDEP epilepsy patients in the proteome. Additional analysis on the fold change of proteins in the hippocampus with a $p < 0.05$ (before the FDR at 5%, $n=83$ proteins) that match RNA gene IDs ($n=83$ gene IDs) do not show a significant correlation ($p=0.3510$, $R^2=0.01075$, Pearson correlation).

Discussion

Our study compared SUDEP or high-risk SUDEP patients to epilepsy controls and revealed no differentially expressed proteins in the hippocampus and frontal cortex and limited transcriptomic changes in the hippocampus and temporal cortex. Thus, the proteome in SUDEP and transcriptome in high-risk SUDEP largely reflects other epilepsy patients, consistent with the diverse spectrum of syndromes and severities associated with SUDEP.² In the hippocampus, the few differentially expressed genes identified in high-risk SUDEP patients included a high proportion of lncRNAs (15/55, 27%). Given that we detect robust proteome¹⁸ and transcriptome¹⁷ differences in the hippocampus and cortex with similar group sizes for epilepsy and non-epilepsy control patients, our data in this study suggest that these brain regions are not especially or uniquely affected in SUDEP.

To validate the label-free quantitative MS findings, immunohistochemistry was used to confirm changes in ermin (ERMN) expression, as this protein had the strongest trend for difference in SUDEP. Immunohistochemistry results corroborated a trend in a decreased fold change of ERMN in the hippocampal CA1-3 region of SUDEP patients when

compared to non-SUDEP epilepsy patients, although this similarly was not significant. Further, *ERMN* was not significantly altered in the current RNAseq study or in our previous proteomics analyses between non-SUDEP epilepsy and controls.¹⁸ However, in our previous RNAseq study between MTLE and non-epilepsy controls, *ERMN* was decreased¹⁷ and is reportedly decreased in a murine model of status epilepticus.²⁹ Expressed by oligodendrocytes, *ERMN* regulates cytoskeleton arrangement during myelinogenesis and myelin sheath maintenance.³⁰ Myelin damage may occur after prolonged seizures and its loss may promote further seizure activity.³¹ We found that the mature oligodendrocyte marker myelin basic protein (MBP) is decreased in epilepsy patients compared to non-epilepsy control patients,¹⁸ and it is decreased in the hippocampus of an animal model of epilepsy.³² However, we found no further decrease in MBP expression in SUDEP or high-risk SUDEP patients when compared to controls in this study, nor was *MBP* different in our recent RNAseq analysis between MTLE and non-epilepsy controls.¹⁷ Overall, *ERMN* is significantly decreased in surgical MTLE versus non-epilepsy controls at the transcriptomic level¹⁷ and trending to decrease in protein expression of SUDEP versus non-SUDEP epilepsy, indicating that *ERMN* may be decreased in response to the elevated seizure activity that may be seen in refractory epilepsy that requires surgery and in some patients with SUDEP. The affect on myelination, as measured by MBP, is only apparent in these patients for protein expression rather than gene expression in epilepsy versus non-epilepsy controls with no further decrease in SUDEP. Thus, further investigation should assess the potential role of *ERMN* in epilepsy and SUDEP, and whether reduced *ERMN* may reflect the severity of pathology resulting from seizure burden in some SUDEP patients.

The RNAseq and small RNAseq analyses showed moderate changes in the hippocampus and minimal differences in the temporal cortex in high-risk compared to low-risk SUDEP MTLE patients. Interestingly, 15/55 differentially expressed genes in the hippocampus were lncRNAs. LncRNAs are an understudied transcriptomic component implicated in many neurological disorders,³³ but few studies have been done regarding their role in epilepsy or SUDEP.³⁴ Among the protein-coding genes differentially expressed in the hippocampus, *GFRA1* (GDNF Family Receptor Alpha 1) was the most decreased. GDNF (glial cell-derived neurotrophic factor) binds to GFRA1 and plays a role in neuronal survival and differentiation, including that of GABAergic interneurons.³⁵ Localized release of GDNF in the hippocampus of an animal model of epilepsy suppresses seizure activity.³⁶ Thus, decreased *GFRA1* may reflect a change in cell survival or result in reduced GDNF mediated seizure suppression in high-risk SUDEP patients. Of the top 20 differentially expressed genes (Table 3), *SGCG* (Sarcoglycan Gamma) had the largest change at a 22.0-fold increase (adjusted $p=0.0023$) in the high-risk SUDEP patients. *SGCG* is expressed in the cerebrovascular system and may localize to vascular smooth muscle cells, potentially involved in membrane contractility, stabilization, and signaling in the associated dystrophin complex affecting neurovascular coupling.³⁷ Its neural role is unknown, but aberrant cerebrovascular organization occurs in MTLE.³⁸ Additional studies are needed to determine how the altered levels of some protein-coding genes and lncRNAs we identified may affect mechanisms related to SUDEP risk.

Protein expression in the brain has rarely been studied in human SUDEP. Hippocampal HSP70 positive neurons are reportedly increased in post-mortem SUDEP patients when compared to non-SUDEP epilepsy patients, but similar to surgical epilepsy patients, suggesting this is likely related to ante-mortem neuronal injury perhaps due to a terminal

seizure in SUDEP patients.³⁹ HSP70 expression was similar in both the proteomic and RNAseq analyses among our patients. Another immunohistochemistry study found few differences in the hippocampus, amygdala, and medulla of post-mortem SUDEP compared to non-SUDEP epilepsy and non-epilepsy control patients with minimal significant changes reported for several markers of inflammation (CD163, HLA-DR, GFAP), compromised blood brain barrier (IgG, albumin), and HIF-1 α , a transcriptional regulator of cellular responses to hypoxia.¹² We found increased GFAP in the hippocampus of three epilepsy patients (3/26, 11.5%); two had gliosis independent of SUDEP status. GFAP was not increased in most non-SUDEP epilepsy patients when compared to non-epilepsy control patients,¹⁸ but it was increased in the hippocampus of one (1/14, 7.1%) epilepsy patient with hippocampal gliosis. Increased GFAP occurs in some epilepsy patients and after prolonged seizures in rodent models.⁴⁰ Further, *GFAP* was not altered in MTLE patients with high-risk of SUDEP in the current RNAseq analysis, but this gene was significantly increased in the hippocampus of MTLE patients compared to non-epilepsy controls.¹⁷

Our study had some limitations. The LCM derived label-free quantitative MS allows for detection of localized protein changes that would not be possible using bulk homogenate, however this technique detects a lower quantity of membrane proteins that are relatively insoluble with this method. Thus, we may not detect differential expression of some membrane proteins, although downstream signaling pathways reflecting their functional activity may be identified. Additional limitations include the heterogeneity of epilepsies, seizure types, and neuropathology due to available patients, and further reinforces the importance of banking various brain tissue samples from SUDEP patients. Our study was powered to identify proteomic differences across the representative SUDEP group rather

than epilepsy-subgroups. Potential pathogenic gene variants were not assessed in our patients. Our proteomics analyses were based on NASR referrals, skewed by major referral sources: the San Diego Medical Examiner Office (mainly low socioeconomic white and Hispanic patients) and direct referrals (mainly high socioeconomic white patients). For the RNAseq analyses, surgical patients had treatment-resistant MTLE. PGES duration as a biomarker of SUDEP risk has not been validated, can vary within the same patient for different seizures, and the number video EEG-recorded GTCS in each patient was limited.^{4,41,42} Thus, group differences may reflect sampling bias. Further, the number of patients used for the RNAseq temporal cortex analyses was low. Finally, further investigation is needed in brain regions implicated in SUDEP, including the brainstem, as it modulates autonomic functions and it has been suggested that seizure-induced postictal depression of arousal, respiratory, and cardiac function may occur in SUDEP.^{43,44}

In summary, in contrast to robust differences we found in proteomic and RNAseq analyses between epilepsy and non-epilepsy patients,^{17,18} there were no differences detected in the proteomic analyses of autopsy tissue from SUDEP and non-SUDEP epilepsy patients and limited transcriptomic differences comparing surgical tissue from low and high-risk SUDEP patients in the brain regions analyzed, consistent with the diverse epilepsy syndromes and comorbidities associated with SUDEP and indicating that epilepsy subtypes and additional brain regions should be examined further.

Figures and Tables:

Figure 1. SUDEP and non-SUDEP epilepsy patient history. A) Patient history is summarized for autopsy SUDEP and non-SUDEP epilepsy patients. **B)** A summary of lifetime GTCS history burden for the patients in this study with known information.

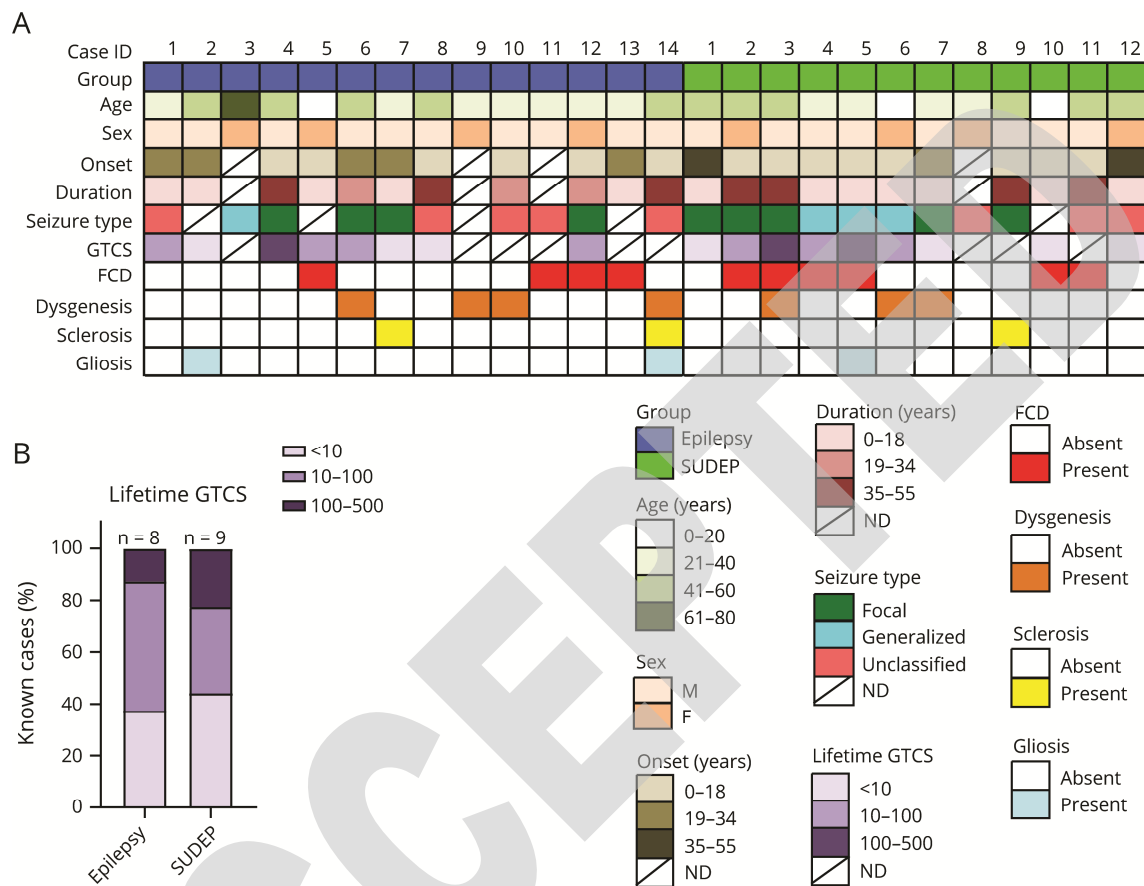


Figure 2. Proteomics PCA analyses in hippocampus, dentate gyrus, and frontal cortex of SUDEP and non-SUDEP epilepsy patients. A-C) A principal component analysis (PCA) of the proteomics analyses shows the indicated variation in each brain region of SUDEP patients (n=12) and non-SUDEP epilepsy patients (n=14). There is no separation by SUDEP status or lifetime GTCS history burden. An unpaired two-tailed t test of PCA1 between the SUDEP and non-SUDEP epilepsy groups in each brain region was not significant, as depicted by a box plot with bars indicating minimum and maximum values. ND = not determined.

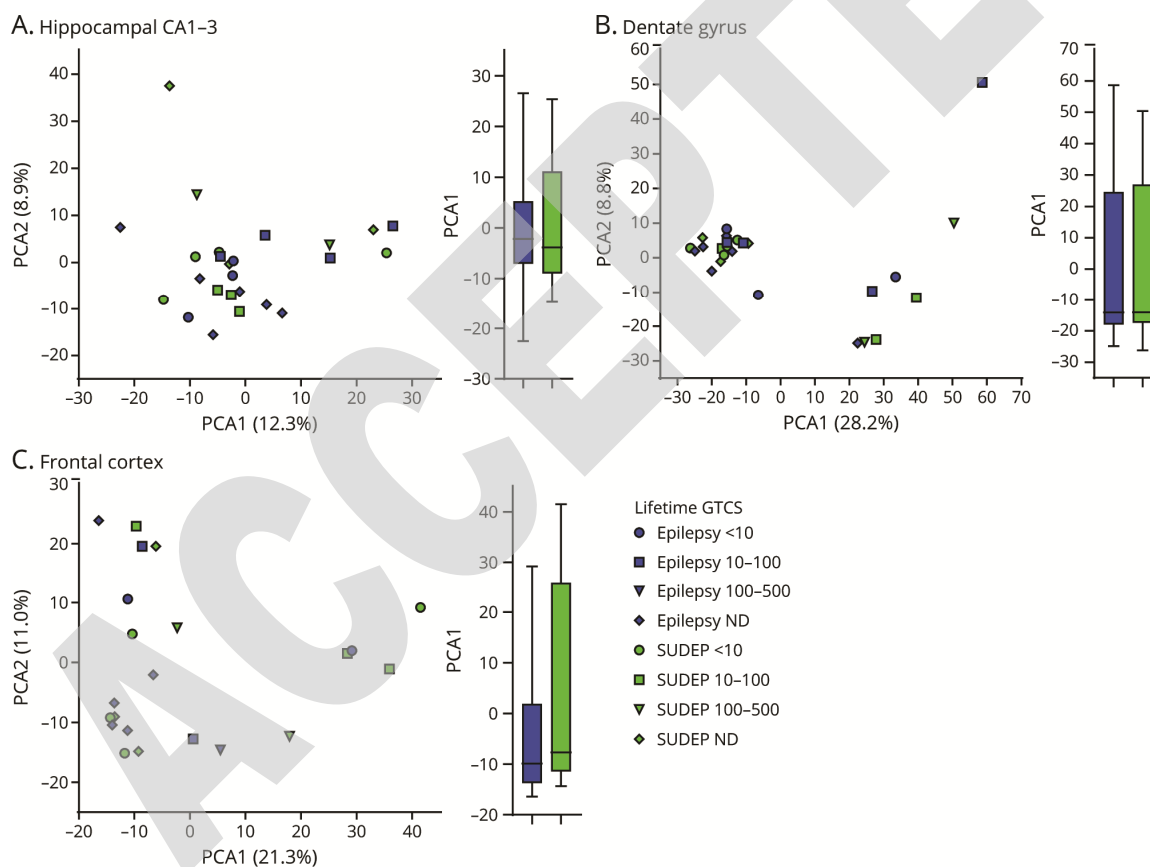
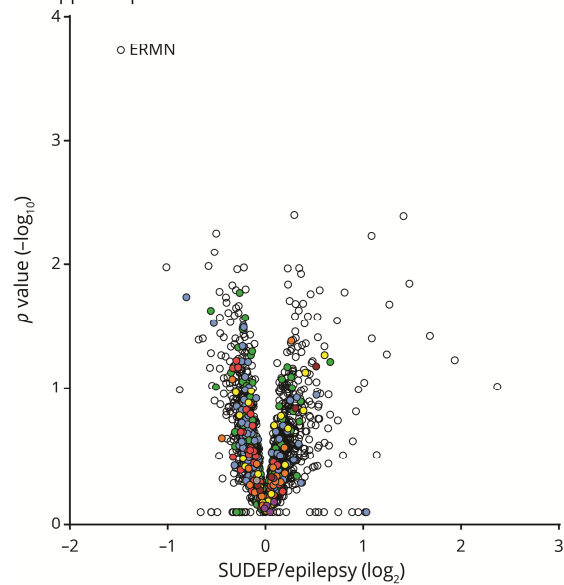
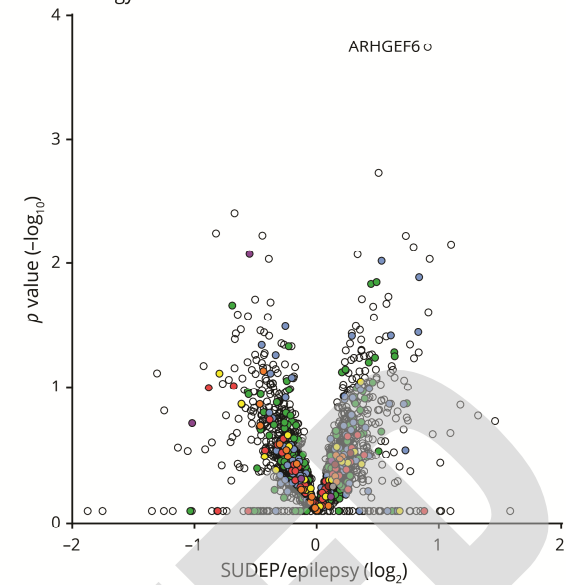


Figure 3. Proteomics analyses in hippocampus, dentate gyrus, and frontal cortex of SUDEP and non-SUDEP epilepsy patients. A-C) Volcano plots indicate that there are no significantly different proteins in the hippocampal CA1-3 region, dentate gyrus, or frontal cortex of SUDEP and non-SUDEP epilepsy patients as determined by a student's two tailed t-test with permutation correction at a 5% FDR. The top proteins with the lowest p values in each brain region are noted. Cell type specific protein annotation is included, with the most predominant listed in decreasing order in the legend. Proteins annotated "General – Neuron" have both excitatory and inhibitory neuron annotations.

A. Hippocampal CA1-3



B. Dentate gyrus



C. Frontal cortex

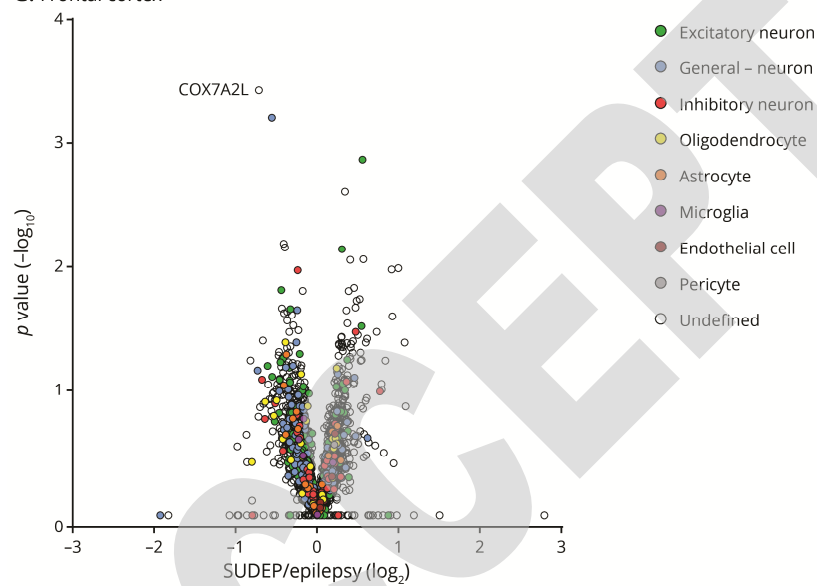


Figure 4. RNAseq in hippocampus and temporal cortex with low and high-risk SUDEP, as determined by PGES. A) Patient history is summarized for low and high-risk SUDEP patients. **B)** The t-SNE (t-distributed stochastic neighbor embedding) plot of RNAseq data shows separation by brain region rather than SUDEP risk status. **C)** A volcano plot shows the results of differential expression analysis of the hippocampus from low-risk (n=4) and high-risk (n=8) SUDEP patients. Eleven genes were decreased and 44 genes were increased in hippocampus of high-risk SUDEP patients. The Wald test identified differentially expressed genes using a Benjamini-Hochberg adjusted p-value <0.05 for significance. Cell type specific gene annotation is included, with the most predominant listed in decreasing order in the legend. Genes annotated “General – Neuron” have both excitatory and inhibitory neuron annotations. **D)** Biotypes of differentially expressed genes are depicted in the hippocampus for high-risk SUDEP compared to low-risk SUDEP patients. Of the 55 differentially expressed genes 67.3% were protein-coding genes, 27.3% were long non-coding RNAs, and 5.5% are yet to be experimentally confirmed (TEC). **E)** A volcano plot shows the results of differential expression analysis in the temporal cortex from low-risk (n=2) and high-risk (n=3) SUDEP patients. One gene was decreased and no genes were increased in the temporal cortex. The Wald test identified differentially expressed genes using a Benjamini-Hochberg adjusted p-value <0.05 for significance.

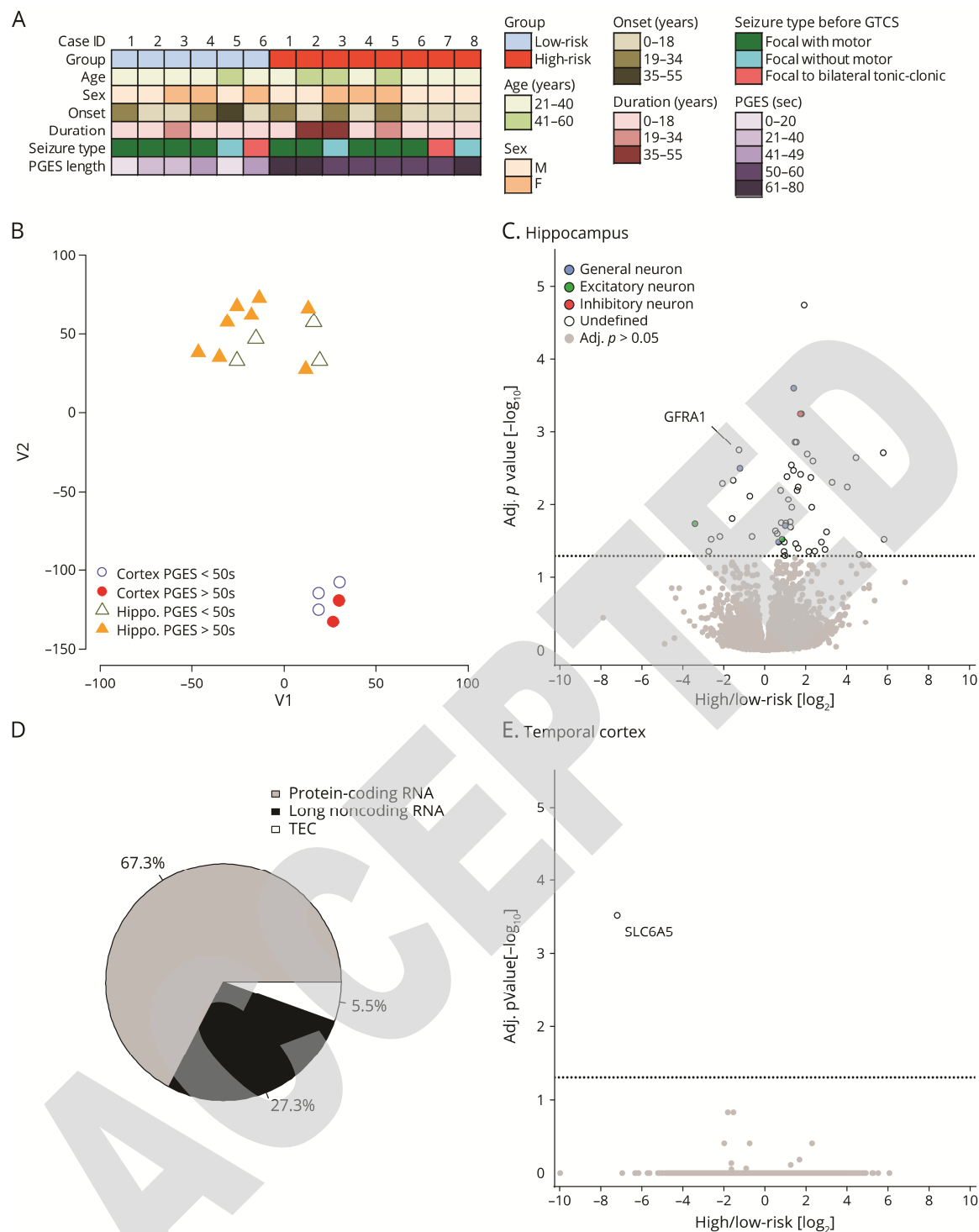


Table 1. Epilepsy and SUDEP Patients in Proteomics Analyses

Table 1. Epilepsy and SUDEP Patients in Proteomics Analyses

ID	Age (yr)	Sex	Age of onset (yr)	Disease Duration (yr)	Seizure Type	Total Lifetime GTCS	COD & SUDEP Status	PMI (hr)	Relevant Neuropathology	Brain Region
<u>Epilepsy</u>										
1	36	M	29	8	Unclassified	10-100	overdose/intoxication	20		HP, DG, FC
2	54	M	28	1	ND	<10	accident/trauma	<24	mild gliosis, contusion, disorganization	HP, DG
3	64	F	ND	ND	Generalized, Unclassified	ND	overdose	18		HP, DG
4	50	M	0.5	49.5	Focal	100-500	choking on foreign object drowning	15		FC
5	9	F	1.5	8	ND	10-100		30	FCD IIA	HP, DG
6	45	M	25	20	Focal	10-100	suicide	27	dysgenesis	HP, DG
7	36	M	24	12	Focal	<10	drowning	48	sclerosis	HP, DG, FC
8	45	M	2	43	Unclassified	<10	suicide	<48		HP, DG, FC
9	24	F	ND	ND	ND	ND	drowning	<48	dysgenesis	HP, DG, FC
10	28	M	5	22	Unclassified	ND	accident/trauma	<48	dysgenesis	HP, DG, FC
11	22	M	ND	ND	Unclassified	ND	drowning	<48	FCD IA	HP, DG, FC
12	34	F	1.5	32	Focal	10-100	pulmonary embolism	13	FCD IB	HP, DG, FC
13	32	M	19	10	ND	ND	ethanol intoxication and clobazam overdose	19	FCD IIA, Wernicke's encephalopathy	HP, DG, FC
14	49	M	0.6	48.4	Unclassified	ND	aspiration	43	dysgenesis, sclerosis, gliosis, hemisphere atrophy	HP, DG, FC
<u>SUDEP</u>										
1	48	M	46	2	Focal	<10	definite SUDEP	<72		HP, DG, FC
2	45	F	10	35	Focal	10-100	definite SUDEP plus definite SUDEP	49	FCD IA	HP, DG, FC
3	48	M	0.8	42	Focal	100-500	definite SUDEP	<48	FCD IA, dysgenesis	HP, DG, FC
4	27	M	13	14	Generalized	10-100	probable SUDEP	<48	FCD IIA	HP, DG, FC
5	32	M	18	10	Generalized, Unclassified	100-500	probable SUDEP	<48	mild FCD IIA, gliosis	HP, DG, FC

6	20	F	9	11	Generalized, Unclassified	10-100	definite SUDEP	<48	dysgenesis	HP, DG, FC
7	28	M	27	1	Focal	<10	definite SUDEP	25	dysgenesis	HP, DG, FC
8	30	F	ND	ND	Unclassified	ND	definite SUDEP	23		HP, DG, FC
9	55	M	5	50	Focal	ND	definite SUDEP plus definite SUDEP	<48	sclerosis, infarct	HP, DG, FC
10	20	M	9	11	ND	0	definite SUDEP	<48	FCD IIA	HP, DG, FC
11	44	M	4	40	Unclassified	ND	definite SUDEP	<48	FCD IIA	HP, DG, FC
12	49	F	41	9	Unclassified	<10	definite SUDEP	<24	venous angioma	HP, DG, FC

yr = year, hr = hours, ND = not determined, GTCS = generalized tonic-clonic seizure, COD = cause of death, PMI = post-mortem interval, FCD = focal cortical dysplasia, HP = hippocampus, DG = dentate gyrus, FC = frontal cortex, dysgenesis = dysgenesis of the hippocampal dentate gyrus, sclerosis = hippocampal sclerosis

Table 2. Epilepsy Patients with Low or High-Risk of SUDEP in RNAseq Analyses

Table 2. Epilepsy Patients with Low or High-Risk of SUDEP in RNAseq Analyses

ID	Age at Surgery (yr)	Sex	Age of onset (yr)	Disease Duration (yr)	Seizure Type Prior to GTCS	PGES length (sec)	Brain Region
<u>PGES < 50 sec</u>							
1	34	M	19	15	Focal with motor	3	TC
2	22	M	7	15	Focal with motor	37	TC
3	33	F	9	24	Focal with motor	24	HP, TC
4	33	F	22	11	Focal with motor	43	HP
5	58	M	51	7	Focal without motor	2	HP
6	29	F	13	16	Focal to bilateral tonic-clonic	49	HP
<u>PGES ≥ 50 sec</u>							
1	30	M	21	9	Focal with motor	62	HP, TC
2	53	M	0	53	Focal with motor	73	HP, TC
3	55	F	20	35	Focal without motor	51	HP
4	32	F	15	17	Focal with motor	52	HP
5	45	F	23	22	Focal with motor	52	HP
6	37	M	8	11	Focal with motor	54	HP
7	25	M	18	7	Focal to bilateral tonic-clonic	51	HP
8	25	M	17	8	Focal without motor	62	HP

PGES = postictal generalized EEG suppression, GTCS = generalized tonic-clonic seizure, yr = years, sec = seconds, HP = hippocampus, TC = temporal cortex

Table 3. Top 20 Significant Protein-coding Genes in Hippocampus of High vs Low-Risk SUDEP Patients

Table 3. Top 20 Significant Protein-coding Genes in Hippocampus of High vs Low-Risk SUDEP Patients

Ensembl Gene ID	Gene ID	Gene Name	UniProt ID	Adjusted p Value	Fold Change	Related References	
<u>Increased</u>							
ENSG00000164082.14	GRM2	Glutamate Metabotropic Receptor 2	Q14416	0.00002	3.80	Increased protein in epilepsy; ¹⁸ GRM2 knockout mice are NMDA toxicity resistant thus GRM2 activation may be damaging to neurons exposed to toxic insults; ⁴⁵ decreased transcript in MTLE with sclerosis ⁴⁶ and in models of status epilepticus ^{47,48}	
ENSG00000137766.16	UNC13C	Unc-13 Homolog C	Q8NB66	0.00026	2.67		Increased transcript in status epilepticus murine model ²⁹
ENSG00000082293.12	COL19A1	Collagen Type XIX Alpha 1 Chain	Q14993	0.00057	3.33		
ENSG00000164112.12	TMEM155	Transmembrane Protein 155	Q4W5P6	0.00057	3.47		
ENSG00000152784.15	PRDM8	PR/SET Domain 8	Q9NQV8	0.00142	2.79	Gain-of-function mutation results in myoclonus epilepsy with Lafora bodies ^{7,49}	
ENSG00000027001.9	MIPEP	Mitochondrial Intermediate Peptidase	Q99797	0.00142	2.92		
ENSG00000102683.7	SGCG	Sarcoglycan Gamma	Q13326	0.00229	22.01	Increased transcript in status epilepticus murine model ²⁹	
ENSG00000033867.16	SLC4A7	Solute Carrier Family 4 Member 7	Q9Y6M7	0.00287	2.47		
ENSG00000164638.10	SLC29A4	Solute Carrier Family 29 Member 4	Q7RTT9	0.00412	2.12		
ENSG00000171126.7	KCNQ3	Potassium Voltage- Gated Channel Modifier Subfamily G Member 3	Q8TAE7	0.00566	3.09		
<u>Decreased</u>							
ENSG00000151892.14	GFRA1	GDNF family receptor alpha-1	P56159	0.00180	2.39	Localized GDNF release in animal models of epilepsy suppresses seizure activity ^{36,50}	
ENSG00000108018.15	SORCS1	Sortilin Related VPS10 Domain Containing Receptor 1	Q8WY21	0.00318	2.32		
ENSG00000146070.16	PLA2G7	Phospholipase A2 Group VII	Q13093	0.00461	2.91	Decreased transcript in status epilepticus murine model ²⁹	
ENSG00000005981.12	ASB4	Ankyrin Repeat And SOCS Box Containing 4	Q9Y574	0.00507	4.18		
ENSG00000185567.6	AHNAK2	AHNAK Nucleoprotein 2	Q8IVF2	0.00752	1.65		
ENSG00000140557.11	ST8SIA2	Alpha-2,8- sialyltransferase 8B	Q92186	0.01550	3.02		
ENSG00000152595.16	MEPE	Matrix Extracellular Phosphoglycoprotein	Q9NQ76	0.02729	4.56		
ENSG00000177106.14	EPS8L2	EPS8 Like 2	Q9H6S3	0.02729	1.54		
ENSG00000189127.7	ANKRD34 B	Ankyrin Repeat Domain 34B	A5PLL1	0.02963	6.12		
ENSG00000224982.3	TMEM233	Transmembrane protein 233	B4DJY2	0.04331	6.65		

Appendix 1. Authors

Name	Location	Contribution
Dominique F. Leitner, PhD	NYU School of Medicine	Data collection and analysis, drafting and revision of manuscript
James D. Mills, PhD	Amsterdam UMC	Data collection and analysis, drafting and revision of manuscript
Geoffrey Pires, MS	NYU School of Medicine	Data collection and analysis, drafting and revision of manuscript
Arline Faustin, MD	NYU School of Medicine	Clinical data collection, drafting and revision of manuscript
Eleanor Drummond, PhD	University of Sydney	Data collection and analysis, drafting and revision of manuscript
Evgeny Kanshin, PhD	NYU School of Medicine	Data collection and analysis, drafting and revision of manuscript
Shruti Nayak, MS	NYU School of Medicine	Data collection and analysis
Manor Askenazi, PhD	Biomedical Hosting LLC	Data analysis, drafting and revision of manuscript
Chloe Verducci, BA	NYU School of Medicine	Clinical data collection, revision of manuscript
Bei Jun Chen, PhD	University of New South Wales	Data analysis
Michael Janitz, PhD	University of New South Wales	Data analysis
Jasper J. Anink, BSc	Amsterdam UMC	Clinical data collection
Johannes C. Baayen, MD	Amsterdam UMC	Clinical data collection
Sander Idema, MD, PhD	Amsterdam UMC	Clinical data collection
Erwin A. van Vliet, PhD	Amsterdam UMC	Data collection
Sasha Devore, PhD	NYU School of Medicine	Revision of manuscript
Daniel Friedman, MD, MSc	NYU School of Medicine	Clinical data collection, drafting and revision of manuscript
Beate Diehl, MD, PhD	University College London	Clinical data collection
Catherine Scott, MPhil	University College London	Clinical data collection
Roland Thijs, MD, PhD	Stichting Epilepsie Instellingen Nederland	Clinical data collection
Thomas Wisniewski, MD	NYU School of Medicine	Clinical data collection, drafting and revision of manuscript
Beatrix Ueberheide, PhD	NYU School of Medicine	Data collection and analysis, drafting and revision of manuscript
Maria Thom, MD	University College London	Clinical data collection, drafting and revision of manuscript
Eleonora Aronica, MD, PhD	Stichting Epilepsie Instellingen Nederland	Design and conceptualized study, clinical data collection, drafting and revision of manuscript

Orrin Devinsky, MD	NYU School of Medicine	Design and conceptualized study, clinical data collection, drafting and revision of manuscript
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ACCEPTED

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