Neuropathology of SUDEP

Role of inflammation, blood-brain barrier impairment, and hypoxia

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ABSTRACT

Objective: To seek a neuropathologic signature of sudden unexpected death in epilepsy (SUDEP) in a postmortem cohort by use of immunohistochemistry for specific markers of inflammation, gliosis, acute neuronal injury due to hypoxia, and blood-brain barrier (BBB) disruption, enabling the generation of hypotheses about potential mechanisms of death in SUDEP.

Methods: Using immunohistochemistry, we investigated the expression of 6 markers (CD163, human leukocyte antigen–antigen D related, glial fibrillary acid protein, hypoxia-inducible factor-1α [HIF-1α], immunoglobulin G, and albumin) in the hippocampus, amygdala, and medulla in 58 postmortem cases: 28 SUDEP (definite and probable), 12 epilepsy controls, and 18 nonepileptic sudden death controls. A semiquantitative measure of immunoreactivity was scored for all markers used, and quantitative image analysis was carried out for selected markers.

Results: Immunoreactivity was observed for all markers used within all studied brain regions and groups. Immunoreactivity for inflammatory reaction, BBB leakage, and HIF-1α in SUDEP cases was not different from that seen in control groups.

Conclusions: This study represents a starting point to explore by immunohistochemistry the mechanisms underlying SUDEP in human brain tissue. Our approach highlights the potential and importance of considering immunohistochemical analysis to help identify biomarkers of SUDEP. Our results suggest that with the markers used, there is no clear immunohistochemical signature of SUDEP in human brain. *Neurology*® 2017;88:1–11

GLOSSARY

BBB = blood-brain barrier; **GFAP** = glial fibrillary acid protein; **HIF-1α** = hypoxia-inducible factor-1α; **HLA-DR** = human leukocyte antigen–antigen D related; **IgG** = immunoglobulin G; **IO** = inferior olive; **NESD** = nonepileptic sudden death; **PH-G** = parahippocampal gyrus; **SUDEP** = sudden unexpected death in epilepsy; **UCL** = University College London; **VLM** = ventrolateral medulla.

Sudden unexpected death in epilepsy (SUDEP) is the major cause of epilepsy-related deaths.[1] The cause of SUDEP is likely to be multifactorial, with evidence for genetic susceptibility[2,3] and preterminal cardiac, respiratory, and autonomic mechanisms.[4,5]

The tissue basis of SUDEP is unknown, with only a limited number of neuropathologic studies.[6] Sudden infant death syndrome and sudden unexplained death in childhood share with SUDEP common characteristics, including incidence, sleep-associated death, prone position at death, and history of febrile seizures.[7,8] More detailed neuropathologic studies have shed some light on the mechanisms underlying these conditions. Abnormal astrogliosis in the medulla and gross asymmetry or microscopic anomalies in the hippocampus have been reported in sudden infant death syndrome.[7,9–12] MRI studies in SUDEP have highlighted hippocampal volume asymmetries[13] and atrophic changes in the brainstem,[14]

Table 1   Clinical and pathology data of individual cases

Case	PM no.	Sex/age at death, y	Age at onset of epilepsy/duration, y	Epilepsy type	Mode of death	Brain region present
SUDEP, definite						
1	EP271	M/40	36/4	Epilepsy related to alcohol abuse	NA	H, M
2	EP266	M/38	7/31	Focal (temporal) lobe epilepsy of unknown cause	NA	A, H, M
3	NA	F/18	16/2	Unclassified	NA	A, H, M
4	NA	M/53	Unknown	Unclassified. Left-sided HS and contusions found at postmortem	NA	H, M
5	NA	F/27	0.75/26	Unclassified. Left-sided HS found at postmortem	NA	H, M
6	NA	M/19	Long term	Unknown. No lesion identified on neuropathology	NA	A, H, M
7	NA	M/42	11/31	Focal structural, old surgical scar from treated brain abscess	NA	H, M
8	NA	F/58	47/11	Structural-metabolic (hemimegalencephaly)	NA	H, M
9	NA	M/41	Unknown	Unclassified	NA	H, M
10	NA	M/69	54/15	Unclassified	NA	A, H, M
11	NA	F/17	4/13	Unclassified. Febrile convulsions as child	NA	A, H, M
12	NA	F/44	5/39	Unclassified	NA	H, M
13	NA	F/33	Unknown	Unclassified	NA	A, H, M
14	EP253	M/40	Unknown	Unknown	NA	H
SUDEP, probable						
15	EP067	F/45	14/31	Genetic generalized epilepsy	NA	A, H, M
16	EP099	M/47	1.5/46	Focal (temporal) lobe epilepsy of unknown cause	NA	M
17	EP088	F/40	8/32	Focal epilepsy of unknown cause	NA	A, H, M
18	EP130	F/19	1.2/18	Focal epilepsy of unknown cause	NA	A, H, M
19	EP219	F/27	Unknown	Unknown	NA	A, H
20	EP240	F/37	4/32	Focal epilepsy of unknown cause	NA	H, M
21	EP252	M/21	Unknown	Unknown	NA	A, H, M
22	EP290	F/48	0.5/47	Focal epilepsy of unknown cause	NA	A, H, M
23	EP286	M/31	Unknown	Focal epilepsy of unknown cause	NA	A, M
24	NA	M/26	Unknown	Unknown	NA	H, M
25	EP210	M/49	12/37	Genetic generalized epilepsy	NA	H, M
26	EP304	M/50	11/39	Structural-metabolic	NA	A, H
27	EP296	M/49	4/45	Unclassified	NA	A
28	EP262	F/40	8/32	Unclassified	NA	A, H
Epilepsy without SUDEP control						
29	EP102	M/82	5/51	Focal epilepsy of unknown cause	Bronchopneumonia	H, M
30	EP133	F/51	22/29	Structural-metabolic	Metabolic derangement, possibly secondary to pneumonia	H, M
31	EP039	F/46	0.25/45	Focal epilepsy of unknown cause	Bronchopneumonia	A
32	EP047	M/60	3/57	Focal epilepsy of unknown cause	Pulmonary edema	A
33	EP213	M/66	1/65	Dravet syndrome	Bronchopneumonia	A
34	EP031	M/44	7/37	Unclassified	Pulmonary edema	H, M
35	EP135	M/83	3/77	Focal epilepsy of unknown cause	Pulmonary embolism	A, H, M

Continued

Case	PM no.	Sex/age at death, y	Age at onset of epilepsy/duration, y	Epilepsy type	Mode of death	Brain region present
Table 1	**Continued**					
36	EP154	M/77	5/72	Focal epilepsy of unknown cause	Bronchopneumonia	A, H, M
37	EP196	M/84	16/64	Unclassified	Bronchopneumonia	A
38	EP236	M/74	14/50	Focal epilepsy of unknown cause	Adenocarcinoma of the bowel with metastasis	A
39	EP188	M/86	14/72	Structural-metabolic	Bronchopneumonia/lung cancer	A
40	EP244	M/60	7.5/53	Unclassified	Aspiration of stomach contents	A
NESD control						
41	NA	M/75	NA	NA	Myocardial infarct	H, M
42	NA	F/35	NA	NA	Cardiac arrest	H, M
43	NA	F/85	NA	NA	Bilateral pulmonary embolism	A
44	NA	M/43	NA	NA	Acute heart failure/inferior myocardial infarct, old with recent extension	M
45	NA	M/49	NA	NA	Antero-lateral and inferior myocardial infarcts	A, M
46	SD028-08	F/44	NA	NA	Cardiovascular disease, Marfan syndrome	A, H, M
47	SD032-09	F/60	NA	NA	Cardiovascular disease; hypertensive heart disease	A, H, M
48	SD006-10	F/32	NA	NA	Cardiovascular disease; Marfan syndrome	A, M
49	SD018-11	F/50	NA	NA	Peritonitis; bowel infarction	A, H, M
50	SD029-11	F/42	NA	NA	Bronchial asthma; pulmonary congestion	A, H, M
51	SD023-12	F/50	NA	NA	Bronchial asthma; pulmonary congestion	H, M
52	SD024-12	M/44	NA	NA	Amitriptyline and dihydrocodeine toxicity	A, H, M
53	SD038-12	M/51	NA	NA	Cardiovascular disease; ruptured cardiac aneurysm	H, M
54	SD042-12	M/29	NA	NA	Suspension by a ligature	A, M
55	SD044-12	M/46	NA	NA	Suspension by a ligature	A, H, M
56	SD049-12	M/60	NA	NA	Ischemic and hypertensive heart disease	A, H, M
57	NA	F/38	NA	NA	Cardiorespiratory failure	A, H
58	NA	M/29	NA	NA	Hypertrophic cardiomyopathy	H, M

Abbreviations: A = amygdala; H = hippocampus; HS = hippocampal sclerosis; M = medulla; NA = not applicable; NESD = nonepileptic sudden death; PM = postmortem; SUDEP = sudden unexpected death in epilepsy.

For unclassified, determination was not possible from available data; for unknown, no data were available for determination.

warranting neuropathologic corroboration. Seizures spreading into the amygdala, which is functionally connected to the medulla, may cause cessation of spontaneous breathing.[15] Focal neuronal loss and gliosis have been described within amygdaloid subnuclei in SUDEP.[16]

Seizures can damage the brain through hypoxic stress[17,18] and alter blood-brain barrier (BBB) integrity[19–21] and promote inflammatory processes[22,23] through chronic or acute changes. Our aim was to identify possible neuropathologic signatures of SUDEP in 3 brain regions, the medulla, hippocampus, and amygdala, which might be considered specifically to "prime" the brain in epilepsy for SUDEP or to reflect its occurrence. We investigated neuropathologic changes using commonly used markers of inflammation, gliosis, BBB disruption, and acute neuronal injury due to hypoxia in SUDEP, epilepsy, and nonepileptic sudden death (NESD) cases. This was an exploratory study of these processes in SUDEP intended

Table 2 Primary antibodies and experimental conditions

Primary antibody target	Source	Clone or reference	Supplier	Dilution	Incubation time	Pretreatment	Secondary antibody
CD 163	Mouse mAb	EDHu-1	AbD Serotec, UK	1/2,000	Overnight at 4°C	HIER, Tris-based buffer, pH 9	Universal anti-mouse/rabbit HRP (DAKO Envision, UK)
HLA-DR	Mouse mAb	CR3/43	Dako, UK	1/100	Overnight at 4°C	HIER, Tris-based buffer, pH 9	Universal anti-mouse/rabbit HRP (DAKO Envision)
GFAP	Mouse mAb	6F2	Dako, UK	1/1,000	Overnight at 4°C	HIER, Tris-based buffer, pH 9	Universal anti-mouse/rabbit HRP (DAKO Envision)
IgG	Rabbit pAb	A0423	Dako, UK	1/20,000	60 min at RT	Proteinase K	Universal anti-mouse/rabbit biotinylated (VECTASTAIN, ABC HRP kit, Vector, UK)
Albumin	Rabbit pAb	A0001	Dako, UK	1/60,000	Overnight at 4°C	None	Universal anti-mouse/rabbit HRP (DAKO Envision)
HIF-1α	Mouse mAb	ESEE122	Abcam, UK	1/1,500	Overnight at 4°C	HIER, citrate-based buffer, pH 6	Universal anti-mouse/rabbit biotinylated (VECTASTAIN, ABC HRP kit, Vector, UK)

Abbreviations: GFAP = glial fibrillary acid protein; HIER = heat-induced epitope retrieval; HIF-1α = hypoxia-inducible factor-1α; HLA-DR = human leukocyte antigen–antigen D related; HRP = horseradish peroxidase; IgG = immunoglobulin G; mAb = monoclonal antibody; pAb = polyclonal antibody; RT = room temperature.

to generate hypotheses for further testing in larger cohorts.

METHODS **Standard protocol approvals, registrations, and patient consents.** The project has been approved through National Research Ethics Service Committee South Central–Hampshire B reference 12/SC/0699. Tissue from all postmortem cases was retained with era-appropriate consent.

Case selection. All SUDEP and epilepsy cases were selected either from archives in the Epilepsy Society Brain and Tissue Bank, University College London (UCL) Institute of Neurology (London, UK) or via Brain UK (http://www.southampton.ac.uk/brainuk) from the pathology department at Derriford Hospital (Plymouth, UK). Cases were categorized according to a recent scheme[24] on the basis of all available information, including detailed clinical and investigational data (e.g., MRI, EEG), and general postmortem findings, including organ histology and toxicology. NESD control tissues were obtained through the MRC Sudden Death Brain Bank, Edinburgh. We included 3 patient groups: SUDEP (definite and probable[24]), epilepsy controls, and NESD. From these cases, blocks were selected from the medulla, hippocampus, and amygdala (both sides when available). The clinical details and number of cases in each group are presented in table 1.
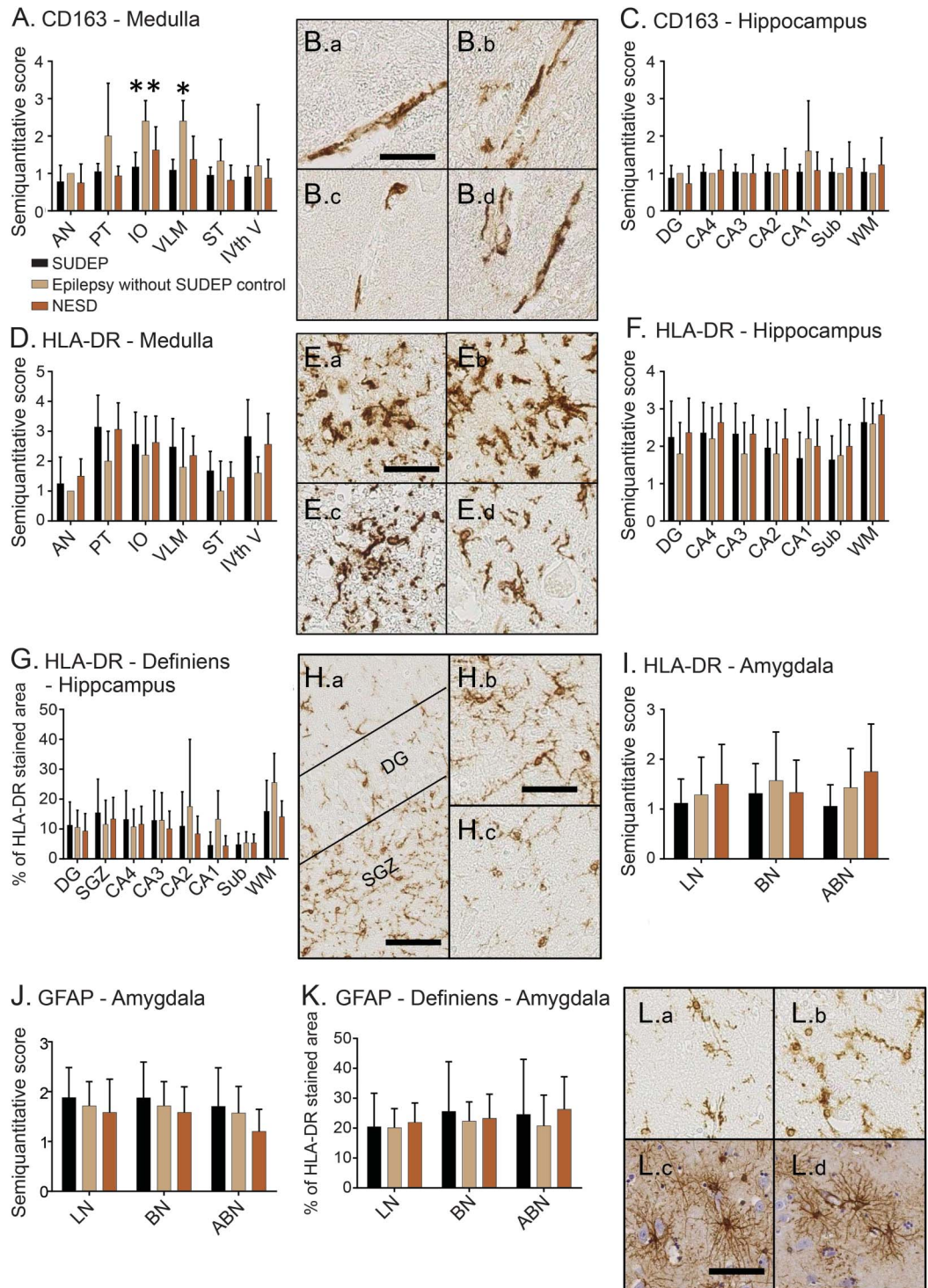
Immunohistochemistry. Primary antibodies and experimental conditions are listed in table 2. Immunohistochemistry was performed on 5-μm-thick formalin-fixed paraffin-embedded sections. Endogenous peroxidase activity was blocked with 1% hydrogen peroxide. The sections were incubated with primary antibodies in diluent buffer (Dako, UK Ltd, Ely, UK) followed by 30 minutes of incubation at room temperature with secondary antibodies. Immunocomplexes were visualized with Dako DAB+ Chromogen (Dako, Glostrup, Denmark). Sections were washed with phosphate-buffered saline (Fisher Scientific, Ltd,

Leicestershire, UK) between all steps. Negative controls were run simultaneously but without primary antibodies.

Semiquantitative evaluation (medulla, hippocampus, and amygdala). All sections were evaluated semiquantitatively twice by one observer (Z.M. or D.O.) and independently reviewed in 10% of cases by a second observer (M.T. or Z.M.). In the medulla, 6 regions of interest on the left and right sides were identified, 4 with known autonomic/cardiorespiratory regulatory functions: (1) floor of the fourth ventricle containing the dorsal nucleus of the vagus nerve and the 10th and 12th cranial nerve nuclei, (2) solitary tract, (3) arcuate nucleus, and (4) ventrolateral medulla (VLM) containing the pre-Bötzinger complex and nucleus ambiguus. In addition, 2 control medullary regions, (5) the inferior olives (IOs), and (6) pyramidal tracts were included. These easily identified regions were used with the rationale that if there were any "global" premortem insult involving the brainstem (e.g., hypoxia, infection), molecules such as hypoxia-inducible factor-1α (HIF-1α) and human leukocyte antigen–antigen D related (HLA-DR) would be more globally upregulated in control regions and in key autonomic-regulatory regions. In hippocampal sections, 8 regions of interest were studied: (1) dentate gyrus, (2) CA4, (3) CA3, (4) CA2, (5) CA1, (6) subiculum, (7) parahippocampal gyrus (PH-G), and (8) PH-G white matter. In the amygdala, 3 regions of interest were studied: (1) lateral nuclei, (2) basal nuclei, and (3) accessory basal nuclei. Grading scales for semiquantitative evaluation of immunoreactivity scores for all markers are detailed in appendix e-1 at Neurology.org.

Quantitative analysis. To supplement the semiquantitative data, quantitative evaluation of pathologic changes was carried out in the hippocampal sections stained for HLA-DR and in the amygdalar sections stained for glial fibrillary acid protein (GFAP). Slides were digitized with a whole slide scanner (LEICA SCN400 scanner, LEICA, Cambridge, UK) at ×40 magnification. Definiens Tissue Studio 3.6 (Definiens AG, Munich, Germany) was used to outline the regions of interest on the scanned slides. Immunostaining was automatically

(A–C) Macrophage infiltration, (D–I) microglial activation, and (J–L) gliosis. (A) Semiquantitative evaluation of CD163 marker in the medulla. Expression of CD163 marker was significantly lower in sudden unexpected death in epilepsy (SUDEP) cases than in epilepsy without SUDEP controls in inferior olives (IOs) (**$p < 0.0005$) and ventrolateral medulla (VLM; *$p \leq 0.001$). (B) CD163-immunopositive perivascular macrophages are less frequent in a SUDEP case (a, c) than in an epilepsy without SUDEP control (b, d) in both VLM (a–b) and IO (c–d) regions in the medulla. (C) Semiquantitative evaluation of CD163 marker in the hippocampus. (D) Semiquantitative evaluation of human leukocyte antigen–antigen D related (HLA-DR) marker in the medulla. (E) SUDEP case presenting high immunoreactivity for HLA-DR on microglial cells in medulla in the VLM (a), floor of the fourth ventricle (IVth V; b), pyramidal tracts (PT; c), and IOs (d). (F) Semiquantitative evaluation of HLA-DR marker in the hippocampus. (G) Quantitative evaluation of HLA-DR immunostaining in the hippocampus by Definiens software. (H) Hippocampus of a SUDEP case presenting higher immunostaining for HLA-DR in subgranular zone (SGZ; a) and in CA3 (b), with

Continued

detected by Definiens software, which gave the percentage of the immunolabeled area in each region of interest. Details of threshold-based analysis of immunostaining by Definiens software are given in appendix e-1.

Statistical analysis and graphical representation. Statistical analysis was carried out with SPSS for windows (version 21, IBM Corp, Armonk, NY). GraphPad Prism software (version 6.1) was used for the graphic representation of the data. Nonparametric (Mann-Whitney U) tests were used to compare immunoreactivity scores between groups, and a stringent value of $p \leq 0.001$ was considered significant. We did not correct for multiple comparisons because this was a study intended to generate hypotheses.

RESULTS Inflammation. Immunoreactivity for CD163 (medulla, hippocampus), HLA-DR (medulla, hippocampus, amygdala), and GFAP (amygdala) was observed in all studied groups and regions of interest. Semiquantitative and quantitative (when applied) evaluation showed no difference overall between SUDEP cases and either control group in the immunoreactivity score, except for CD163 marker, which in VLM and IOs in SUDEP cases labeled fewer perivascular or parenchymal macrophages than in the epilepsy control group ($p < 0.0005$ and $p \leq 0.001$, respectively, figure 1, A and B). This differential pattern of staining was not observed when SUDEP cases were compared to the NESD group.

In the medulla and hippocampus, the expression of CD163 marker was observed mainly in perivascular macrophages (figure 1, A–C). HLA-DR was immunolabeled in cells of microglial morphology, sometimes aggregating into nodules. Particularly in the medulla, intense HLA-DR immunostaining was noted in the VLM, solitary tract, floor of the fourth ventricle, and pyramidal tracts (in SUDEP and NESD cases, figure 1, D and E). In the hippocampus in all studied groups, HLA-DR–immunopositive cells were numerous in the dentate gyrus (especially in the subgranular zone), in the CA3-CA4 subfields, and in the white matter (figure 1, F–H). Fewer immunoreactive cells were observed in the CA1 subfield and the subiculum. In the amygdala, HLA-DR immunolabeling demonstrated intense microglial staining in all studied groups and nuclei (figure 1I). In the amygdala, in both control groups, the HLA-DR immunolabeling appeared more widespread and intense than in SUDEP cases, but these observations were not supported by semiquantitative analysis.

GFAP-immunopositive astrocytes were numerous in all studied groups and amygdala subnuclei (figure 1, J–L) as either isolated cells with frequent multiple strongly labeled processes or patchy areas of gliosis.

BBB integrity. Immunoreactivity for immunoglobulin G (IgG; medulla, hippocampus, amygdala) and albumin (medulla, hippocampus) was observed in the cytoplasm of neurons, astrocytes, and small cells with the morphology of oligodendroglia in all studied groups and regions of interest, indicating a compromised BBB. In the medulla and amygdala, there was no difference overall between SUDEP cases and either control group in the immunoreactivity score (figure 2, A–C, G, and H).

In the hippocampus, prominent immunolabeling of a proportion of pyramidal neurons and astroglial cells was observed for both markers in all studied groups and subfields (figure 2, D–F). The dentate granule cell layer showed a prominent mosaic-like pattern of labeling of neurons and dendritic processes (figure 2E, a and e). The only differences were noted in the CA1 subfield and PH-G, with lower IgG immunoreactivity observed in SUDEP cases compared to NESD controls (both $p \leq 0.001$, figure 2E, b, f– h).
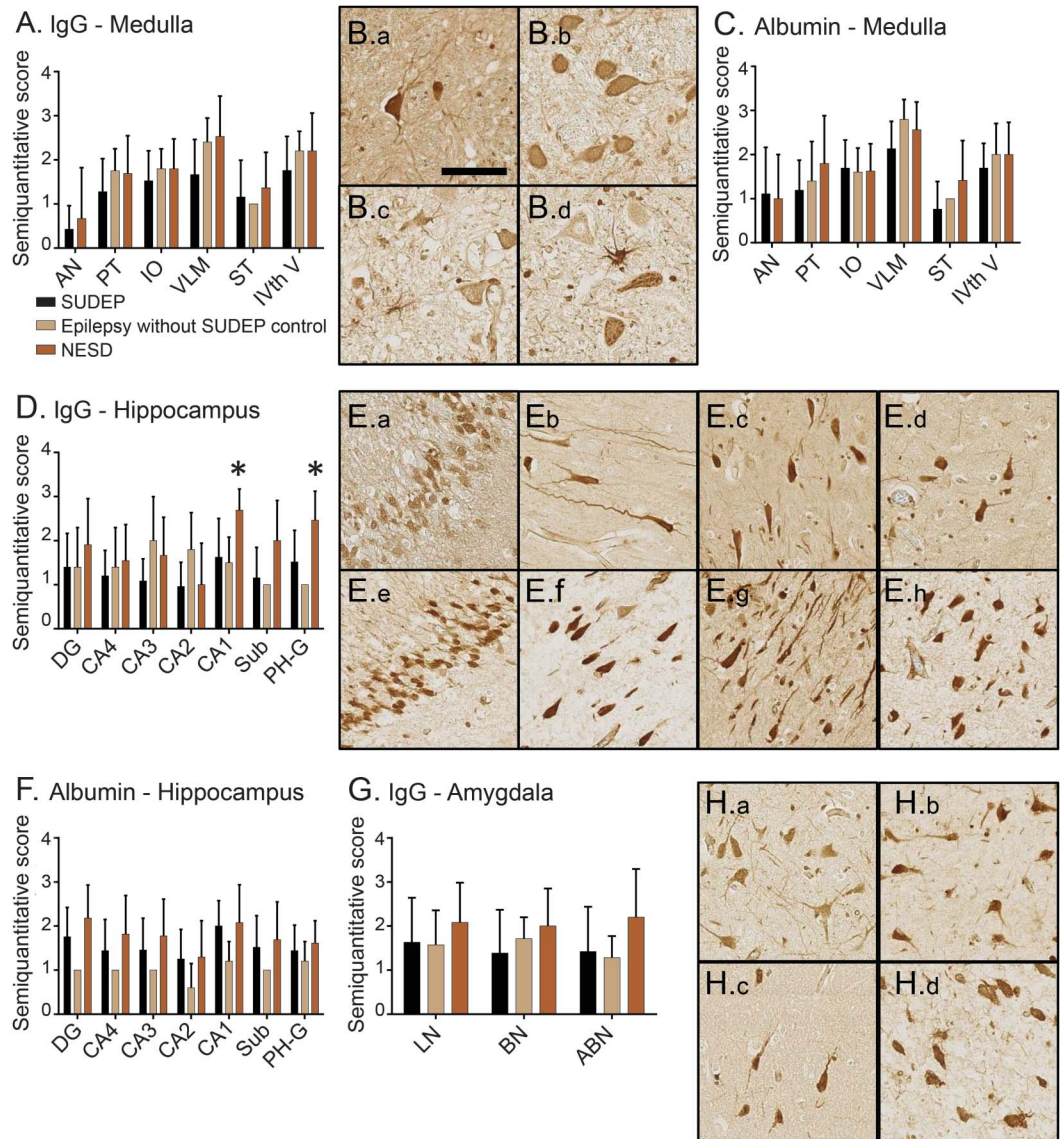
Acute neuronal injury. No significant differences were noted between SUDEP cases and either control group in any studied regions of interest for HIF-1α immunolabeling except for the hippocampus, where fewer HIF-1α–immunopositive cells were noted in PH-G in SUDEP cases and the epilepsy control group than in NESD controls (both $p \leq 0.001$, figure 3, C and D, e and f).

In the medulla, positive cytoplasmic neuronal labeling and a perisomatic synaptic immunolabeling pattern in the surrounding neuropil were seen in all groups studied (figure 3A). A high intensity of staining was observed in the majority of cases in the VLM (figure 3B, b and e) and in the dorsal nucleus of the vagus nerve in the floor of the fourth ventricle (figure 3B, c and f). In comparison, the 12th cranial nerve nucleus showed less intense or absent immunoreactivity (figure 3B, d and g). There was also immunolabeling in the arcuate nucleus. The IO nuclei showed a prominent band-like immunoreactivity of the neuropil, but pyramidal and white matter tracts were not labeled.

Figure 1 legend, continued:
lower immunoreactivity in the CA1 (c). (I) Semiquantitative evaluation of HLA-DR immunostaining in the amygdala. (J) Semiquantitative evaluation of glial fibrillary acid protein (GFAP) immunostaining in the amygdala. (K) Quantitative evaluation of GFAP immunostaining in the amygdala by Definiens software. (L) HLA-DR–immunopositive microglial cells in lateral nuclei (LN) of the amygdala are less abundant in a SUDEP (a) than in a nonepileptic sudden death (NESD; b) case, but expression of GFAP-immunopositive astrocytes is not different in a SUDEP (c) and a NESD (b) case. Scale bar in B, E, H.b, H.c, L.a, L.b = 50 μm; in H.a, L.c, L.d = 100 μm. ABN = accessory basal nuclei; AN = arcuate nucleus; BN = basal nuclei; DG = dentate gyrus; ST = solitary tract; Sub = subiculum; WM = white matter.

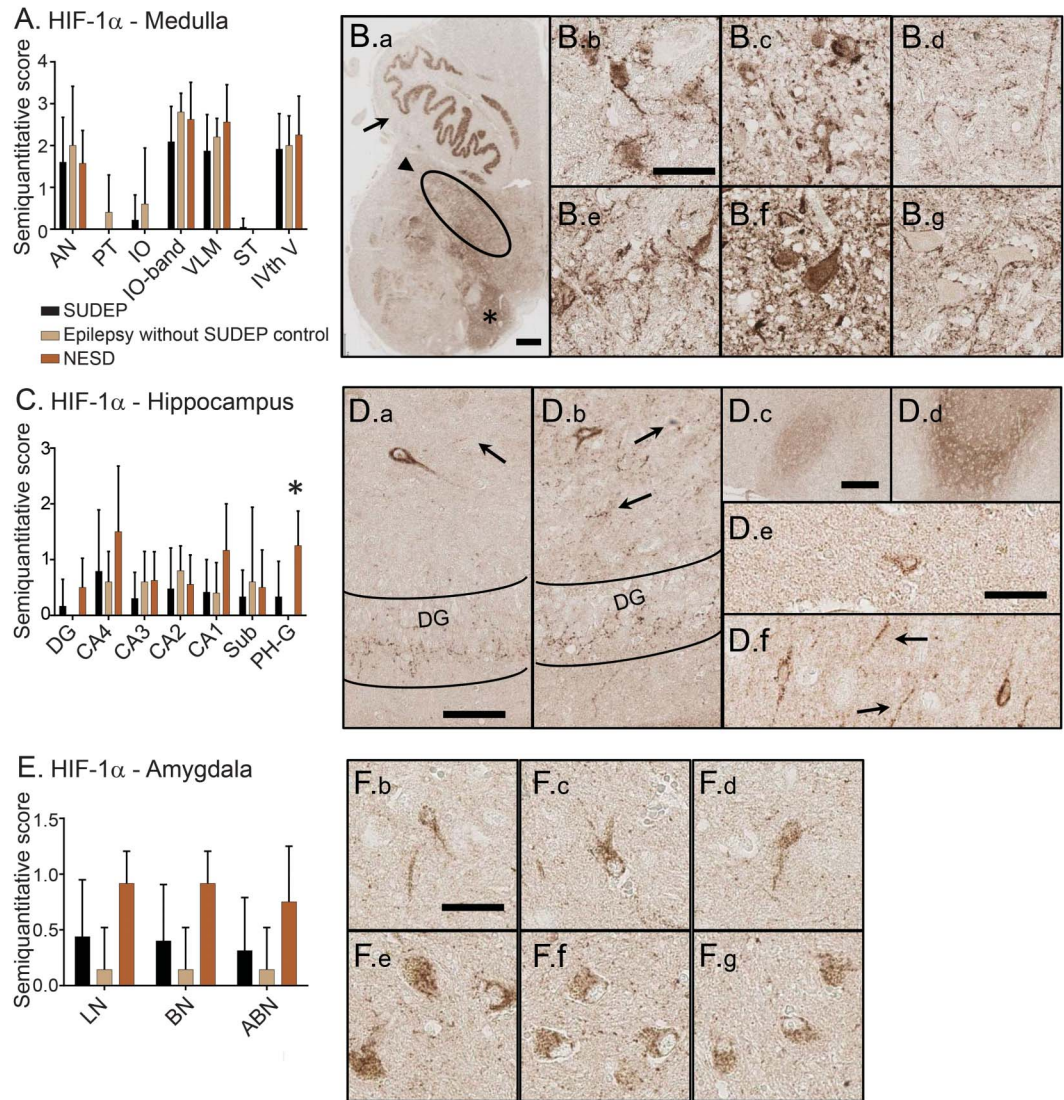**Figure 2   BBB impairment in SUDEP and control cases**

(A–C) Medulla, (D–F) hippocampus, and (G–H) amygdala. (A) Semiquantitative evaluation of immunoglobulin G (IgG) marker in the medulla. (B) Fewer IgG-immunopositive neurons and astrocytes were present in ventrolateral medulla (VLM; a) and the floor of the fourth ventricle (IVth V; c) in a sudden unexpected death in epilepsy (SUDEP; b) than in a nonepileptic sudden death (NESD; d) case. (C) Semiquantitative evaluation of albumin marker in the medulla. (D) Semiquantitative evaluation of IgG marker in the hippocampus. Expression of IgG marker in SUDEP cases in CA1 and parahippocampal gyrus (PH-G) was significantly lower than in NESD controls (*both $p \leq 0.001$). (E) Lower expression of IgG marker in the hippocampus of a SUDEP (a–d) than in a NESD (e–h) case in mosaic-like pattern of expression in the dentate gyrus (DG; a, e), pyramidal neurons in CA1 (b, f), subiculum (Sub; c, g) and PH-G (d, h). (F) Semiquantitative evaluation of albumin marker in the hippocampus. (G) Semiquantitative evaluation of IgG marker in the amygdala. (H) Fewer IgG-immunopositive cells were present in lateral nuclei (LN) in the SUDEP (a) than in the NESD (b) case and in accessory basal nuclei (ABN; c and d, respectively). Scale bar for B, E, H = 100 μm. AN = arcuate nucleus; BBB = blood-brain barrier; BN = basal nuclei; IO =inferior olive; PT = pyramidal tracts; ST = solitary tract.

In the hippocampus and amygdala, the pattern shared across the groups was of scattered cytoplasmic immunolabeling in single neurons in all subfields and nuclei (figure 3, C–F). Moreover, in the hippocampus, in SUDEP cases and NESD controls, a granular labeling pattern of staining was noted on fine processes at synapses within the dentate gyrus and molecular layer (figure 3D, a and b) and within the pyramidal cell layer of CA2. Synaptic-like staining was also prominent in the layer II nodules in the subiculum (figure 3D, c and d) and in all amygdaloid nuclei. The epilepsy control group did not show this prominent synaptic-like staining pattern.

DISCUSSION SUDEP is rare, but it is the most devastating outcome in epilepsy. A number of risk factors

Figure 3 Immunohistochemistry patterns of neuronal and synaptic staining for HIF-1α in SUDEP and control cases

A. HIF-1α - Medulla

SUDEP
Epilepsy without SUDEP control
NESD

C. HIF-1α - Hippocampus

E. HIF-1α - Amygdala

(A) Semiquantitative evaluation of hypoxia-inducible factor-1α (HIF-1α) immunolabeling in the medulla. (B) HIF-1α expression in the medulla of a sudden unexpected death in epilepsy (SUDEP) case (a) demonstrating band-like pattern in inferior olives (IOs; arrow) and strong immunolabeling in ventrolateral medulla (VLM; arrowhead) and floor of the fourth ventricle (IVth V) in the dorsal vagal nuclei (asterisk). Positive neurons in VLM (b, e) and IVth V dorsal vagal nuclei (c, f) were less frequent in a SUDEP case (b, c) than in a NESD case (e, f). Absence of immunopositive neurons in IVth V 12th cranial nerve in both SUDEP (d) and NESD (g) cases. (C) Semiquantitative evaluation of HIF-1α immunolabeling in the hippocampus. Expression of HIF-1α marker in SUDEP cases in parahippocampal gyrus (PH-G) was significantly lower than in NESD controls (*$p \leq 0.001$). (D) HIF-1α expression in the hippocampus demonstrating single immunopositive neurons and perisomatic immunolabeling (arrows) in the CA4 subfield and prominent synaptic-like staining in the dentate gyrus (DG) in a SUDEP (a) and a NESD (b) case. Less HIF-1α immunolabeling in layer II nodules in the subiculum (Sub) in a SUDEP than in a NESD (d) case. HIF-1α expression in the PH-G presenting fewer immunopositive cells and perisomatic labeling (arrows) in SUDEP (e) than in NESD (f) case. (E) Semiquantitative evaluation of HIF-1α immunolabeling in the amygdala. (F) HIF-1α expression in the amygdala shows fewer immunopositive neurons in a SUDEP (a–c) than in a NESD (d–f) case in lateral nuclei (LN; a, d), basal nuclei (BN; b, e), and accessory basal nuclei (ABN; c, f). Scale for B.a = 1 mm; B.b–B.g = 100 μm; D.a, D.b = 100 μm, D.c, D.d = 200 μm, D.e, D.f = 50 μm; F = 50 μm. AN = arcuate nucleus; PT = pyramidal tracts; ST solitary tract.

and terminal pathophysiologic phenomena have been determined. The cause remains unknown, although genetic factors may play a role.[2,25] Associated acute neuropathologic alterations have been poorly investigated. We report a detailed analysis of postmortem brain tissue from people who have died of SUDEP, looking for acute cellular alterations in comparison to a variety of controls. With the markers used, there is limited evidence for a specific or stereotypical pathologic cellular reaction pattern occurring in people who have died of SUDEP in terms of inflammation, gliosis, BBB disruption, or acute neuronal injury due to

hypoxia. The only significant differences were shown in the medulla for CD163 and in the hippocampus for IgG and HIF-1α markers.

In the medulla, lower immunolabeling of CD163 was noted in VLM and IOs in SUDEP cases than in epilepsy controls, but these differences were not seen when SUDEP cases were compared to NESD controls. CNS macrophages are heterogeneous; resident microglia of myeloid lineage origins populate the CNS during embryonic development, while perivascular, meningeal, and choroid plexus macrophages of bone marrow origin are renewed from circulating monocytes.[26] Perivascular macrophages are distinguished from microglia by expression of the CD163 marker.[27] They occupy a strategic location at the BBB and have antigen recognition, processing, and presenting properties, supporting a possible role in the regulation of perivascular inflammation in the CNS.[27] CD163-immunoreactive macrophages have been identified in the parenchyma in several inflammatory diseases such as HIV-related encephalitis, multiple sclerosis, and Alzheimer disease.[27–29] The low expression of this marker observed in SUDEP would suggest as a hypothesis that acute macrophage-derived immunoreaction in the medulla is not important in SUDEP.

In the hippocampus, differences were noted in the PH-G, with lower IgG and HIF-1α immunoreactivity, and in the CA1 subfield, with lower IgG immunoreactivity, in SUDEP cases compared to NESD controls. One of the consequences of BBB dysfunction is leakage of blood-borne cells into the brain. Indeed, a passive accumulation of various proteins could lead to aggregation, which perturbs neuronal function and survival.[30,31] IgG uptake has been associated with several signs of neuronal dysfunction.[32] IgG binding may also be a response to the cell death. Such processes have been reported in aging brain.[33] Moreover, in the injured brain, naturally occurring IgG autoantibodies have been shown to bind specifically to dying neurons and were suggested to promote phagocytosis and removal of injured neurons.[31] Hypoxia changes several processes in neuronal physiology,[34] many aspects of which involve HIF-1α.[35] A small number of cells immunoreactive to HIF-1α were recently reported in postmortem samples from patients with epilepsy.[36] HIF-1α has a relatively short half-life of only several minutes, and because it is rapidly degraded in normoxia, there is essentially no detectable level of HIF-1α protein under these circumstances.[37] Amongst NESD cases, 16 of 18 had documented hypoxic/ischemic conditions as a cause of death and presented intense HIF-1α immunolabeling in all studied brain areas. Therefore, the findings of only occasional HIF-1α–immunopositive cells in SUDEP cases and lower IgG immunoreactivity

suggest the relative lack of BBB impairment or hypoxia associated with SUDEP cases in this series compared to other sudden death cases. This leads to the hypothesis that prolonged impaired cerebral perfusion/oxygenation is not always a critical process in the mechanism of death in SUDEP. These observations may seem paradoxical given the recent finding from the MORTEMUS (Mortality in Epilepsy Monitoring Unit) study[5] documenting terminal cardiorespiratory arrest in SUDEP. There are a number of possible reasons for our observations, including the possible heterogeneity of underlying causation in SUDEP whatever the terminal event, that hypoxia does not affect the whole brain to equal severity, or that there is reduced metabolic demand in tissue due to postictal generalized electric suppression. That HIF-1α immunoreactivity is seen in NESD tissue argues against insensitivity of the assay as a technical explanation.

We qualitatively evaluated IgG and albumin immunoreactivity in overall cell populations (including neurons and astrocytes) without cell-specific distinction. However, it has been reported that parenchymal accumulation of albumin, particularly in astrocytes, induces downregulation of inward-rectifying Kir4.1 potassium channels and impairs gap junction coupling, leading to an increase in extracellular potassium level, precipitating epileptiform activity.[38,39] High extracellular potassium and glutamate concentrations and severe hypoxia/anoxia can also trigger spreading depolarization. When spreading depolarization affects structures responsible for breathing control, it may worsen hypoxia, thus generating a vicious circle resulting in shutdown of cardiorespiratory centers and flattening of the EEG.[40] Therefore, functional dysregulation of the neuronal, glial, and microvascular networks at the BBB may be of relevance in the pathophysiology of sudden death, and we suggest that this area needs further investigation.

There are limitations of our study. The circumstances of life, death, and postmortem conditions in humans often vary significantly between cases, in contrast to studies in animal models in which premortem and postmortem conditions can be controlled. We used a number of SUDEP and control cases collected over many years. Moreover, we also used tissue from other centers around the United Kingdom. In such studies, premortem and postmortem variations are inevitable, for example, with respect to postmortem intervals and tissue processing differences between centers and over time. The cohort used in the current study is not a systematically collected population-based sample and therefore may not be representative of all SUDEP. We had a limited number of controls in our study, especially epilepsy

controls. Another potential limitation is the different tissue sampling between cases in that sections of medulla, hippocampus, and amygdala were not all at identical levels. However, we used adjacent sections stained with Luxol fast blue and cresyl violet to enable proper orientation of each section, and we examined only the sections in which each region of interest was entirely present. Another constraint is that not all 3 brain regions were available from each patient, which may have affected the comparison between the studied groups. We emphasize that our study was intended to generate hypotheses and that further work is needed. We examined a limited number of antigens, which may not represent all aspects of inflammation, BBB integrity, and hypoxic stress. We could not use most "omics" techniques because all material available in this study was fixed and embedded without details on premortem and agonal conditions. We are aware that use of omics techniques may provide information for a larger number of genes and gene products involved in mechanisms underlying pathology, which cannot be obtained with traditional techniques. However, cases of SUDEP with brain tissue available for research are very limited, and we attempted to make the most of the limited resources available. Finally, we note that none of our SUDEP cases were from acute monitoring units, so the immediate preterminal period was not electrophysiologically documented.

This study represents a starting point to explore the neuropathology and cellular alterations associated with SUDEP. We did not find any distinct pathognomonic brain processes to support activation of inflammatory, BBB impairment, or cell stress/hypoxia pathways. However, further investigation of the role of glioneuronal interactions and the BBB impairment may be worthwhile.

AUTHOR CONTRIBUTIONS

Zuzanna Michalak: study concept and design, acquisition, analysis and interpretation of data, study supervision, writing the manuscript. Dima Obari: acquisition, analysis and interpretation of data. Matthew Ellis: acquisition and analysis of data. Maria Thom and Sanjay M. Sisodiya: study concept and design, critical revision of manuscript for intellectual content.

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DISCLOSURE

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