FULL-LENGTH ORIGINAL RESEARCH

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Epilepsia

Regional microglial populations in central autonomic brain regions in SUDEP

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Abstract

Objective: Sudden unexpected death in epilepsy (SUDEP) may arise as a result of autonomic dysfunction during a seizure. The central autonomic networks (CANs) modulate brainstem cardiorespiratory regulation. Recent magnetic resonance imaging (MRI) studies in SUDEP have shown cortical and subcortical volume changes and altered connectivity between CAN regions, but the pathological correlate is unknown. Because neuroinflammation is both a cause and a consequence of seizures and may relate to regional brain pathology, our aim was to evaluate microglial populations in CANs in SUDEP.

Methods: In 55 postmortem cases, including SUDEP, epilepsy controls without SUDEP and nonepilepsy controls, we quantified Iba1-expressing microglia in 14 cortical and thalamic areas that included known CAN regions.

Results: Mean Iba1 labeling across all brain regions was significantly higher in SUDEP cases compared to epilepsy and nonepilepsy controls. There was significant regional variation in Iba1 labeling in SUDEP cases only, with highest labeling in the medial thalamus. Significantly higher labeling in SUDEP cases than epilepsy and nonepilepsy controls was consistently noted in the superior temporal gyrus. In cases with documented seizures up to 10 days prior to death, significantly higher mean Iba1 labeling was observed in SUDEP compared to epilepsy controls.

Significance: Our findings support microglial activation in SUDEP, including cortical and subcortical regions with known autonomic functions such as the thalamus and superior temporal gyrus. This may be relevant to cellular pathomechanisms underlying cardioregulatory failure during a seizure.

KEYWORDS

central autonomic networks, Iba1, microglia, SUDEP

Key points

- Dysfunction of central autonomic networks has been proposed in sudden unexpected death in epilepsy (SUDEP) from structural and functional imaging studies
- In a postmortem series we confirm increased Iba1-expressing microglia in cortical and subcortical regions in SUDEP compared to epilepsy and nonepilepsy controls

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- There was significant variability in microglia density across 14 brain regions in SUDEP and increased microglia also associated with recent seizures
- Increased microglia were noted in the parahippocampal gyrus, superior temporal gyrus, and pulvinar in SUDEP compared to nonepilepsy controls
- Strategic regional microglial activation in central autonomic regions may underlie acute dysfunction in SUDEP and represent a disease biomarker

1 | INTRODUCTION

One theory of the cause of sudden unexpected death in epilepsy (SUDEP) proposes that a central, irrecoverable failure of autonomic regulation of cardiovascular and respiratory function occurs around the time of a seizure. In previous postmortem (PM) studies we have identified alterations to selective neuronal and glial populations in medullary respiratory regulatory nuclei in SUDEP,¹⁻³ suggesting preexisting pathology, possibly secondary to previous seizures or episodes of seizure-related hypoxia. In a PM pathology-MRI (magnetic resonance imaging) correlative study we also observed volume reduction in the rostral medulla in SUDEP,⁴ in keeping with in vivo MRI studies^{5,6} and in support of regional alterations.

A group of supratentorial brain regions involved in autonomic modulation and control of brainstem centers has been referred to as the central autonomic network (CAN).⁷ Knowledge of their interconnectivity and function associated with specific regions continues to evolve.^{8,9} Recent functional MRI analyses from SUDEP cases, in addition to people with epilepsy considered at higher risk, have shown altered connectivity between autonomic cortical and subcortical cardiac and respiratory regulatory regions, including the thalamus, suggesting disorganization of interconnections.¹⁰⁻¹³

Structural MRI analyses from large data sets (ENIGMA-epilepsy consortium) recognize stereotypical regional patterns of cortical thinning in epilepsy syndromes.¹⁴ Regional patterns of increased or decreased MRI gray matter volumes have also been observed in SUDEP or at-risk patients, including the pulvinar, parahippocampal gyrus, and other CAN regions.^{12,15-17} There is some evidence that brain inflammatory pathways may underlie the regional cortical atrophy occurring in epilepsy,¹⁸ and there is a reciprocal relationship between seizure and neuroinflammatory activity, which includes microglial populations.^{19,20}

Our aim in a PM series was to evaluate regional microglial labeling in CAN regions in SUDEP compared to both epilepsy and nonepilepsy control groups to explore any regional variation and if neuroinflammatory processes could represent a disease biomarker relevant to localized cortical autonomic dysfunction during a seizure.

2 | METHOD

Formalin-fixed paraffin-embedded tissue blocks were selected from 55 PM cases in the archives of UCL Epilepsy Society Brain and Tissue Bank, Epilepsy Research UK Corsellis Brain Collection, and MRC sudden death brain bank (collected between 1978 and 2017; median 2015). The PM intervals and fixation times were noted. The project has ethical approval (under NRES 17/SC/0573) and cases had research consent, compliant with UK Human Tissue Act 2014. We included both nonlesional epilepsy cases (18; no identified symptomatic cause or acquired brain pathology identified following neuropathology examination) and lesional cases (21; a focal, likely epileptogenic, brain pathology identified) in addition to non-epilepsy controls (NEC, 16). Epilepsy cases were then stratified according to the cause of death as SUDEP-definite (8), SUDEP-other (possible or probable SUDEP, implementing the criteria of Nashef²¹ [17]), and non-SUDEP deaths, which served as an epilepsy control group (12). Further details of the cases are presented in Table 1 and Table S1.

From each case a maximum of 14 brain regions, corresponding to Brodmann areas (BAs) and thalamic regions, were selected from both left and right hemispheres where available: medial perirhinal/transentorhinal cortex in the parahippocampal gyrus (BA35), temporal pole (BA38), superior temporal gyrus (BA21/22/41/42), insular cortex (BA13/14), anterior cingulate cortex (BA24), parietal cortex (BA5/7), occipital cortex (BA17), anterior frontal F1/F2 cortex (BA6/8), frontobasal cortex (BA10/12), middle temporal gyrus (BA20/21), medial thalamus, lateral thalamus, medial pulvinar, and lateral pulvinar (Figure 1A). The rationale for the brain areas selected was to include regions implicated in SUDEP based on existing imaging and functional data and/ or recognized regions as part of the CAN as well as regions anticipated to be uninvolved (see Table S2 for rationale of selection). Because this was an archival study with cases obtained from different brain banks, the complete set of regional samples was not available on all cases. For example, pulvinar samples were not available in epilepsy controls or frontobasal cortex in NEC (Table 2) but nine regions of interest (ROIs) were consistently available across groups for regional analysis and comparisons (Table 2).

TABLE 1 D	stails of postmortem cases us	sed in the study and group	SC						
Main groups	Subgroups	Neuropathology	Age at onset of epilepsy (mean, range)	Duration of epilepsy (mean, range)	Recent seizure activity (R: P: O: C) ^c (percentage of cases)	Age at death (mean, range)	Gender (% male)	Fixation time Mean (range) days	PMI Mean (Range) days
Epilepsy N = 39	Lesional $N = 21$	HS ^a (11), FCD IIA (2), PMG/HET (6), contusions (3), perinatal infarct (1), meningioma (1)	12.6 (0.4-70)	44.3 (10-80)	22:56:11:11 (%)	54 (31-86)	66.7%	57 (19-227)	3.1 (1-7)
	Non-lesional $N = 18$	No focal pathology	8.7 (0.4-26)	42.2 (1-64)	33:33:9:25 (%)	46.2 (15-80)	66.7%	77 (7-476)	3.1 (1-5)
	SUDEP-definite $N = 8$	75% nonlesional	12.6 (5-26)	20 (1-31)	57: 29:14:0 (%)	31 (15-42)	62.5%	42 (21-227)	2.5 (1-5)
	SUDEP-other $N = 17$	51% nonlesional	13.3 (0.5-70)	36.1 (10-52.5)	25:50:8:17 (%)	48.4 (31-80)	76.5%	98 (7-476)	3.5 (2-6)
	Non-SUDEP Epilepsy $N = 12^{b}$	25% nonlesional	10 (0.4-22)	56 (29-80)	11:55:0:34 (%)	66 (46-86)	58.3%	34 (12-97)	3.1 (1-7)
NEC $N = 16$		NA	NA	NA	NA	50.7 (23-88)	68.8%	21 (3-94)	4.4 (2-12)
Significant differences between groups ^d		NA	n/s	Cause of death groups: p < .005	Cause of death groups $p > .0001$	Cause of death groups: p < .0001	s/u	<i>p</i> = .05	n/s
<i>Note:</i> The epilepsy including epilepsy: Abbreviations: FCI ^a HS includes cases ^b In two cases there	cases were stratified into lesions syndrome, circumstances of deat), focal cortical dysplasia; HET, with bilateral sclerosis and case was insufficient data to classify	al or nonlesional (gray cells) in NS anti-seizure medication gray matter nodular white m 13 had both HS and contusion the cause of death.	or according to the cau ns, is in Table S1. atter heterotopia; HS, ns (see Table S1); all 1	ise of death (SUDEP hippocampal sclerosi HS were International	or non-SUDEP (blue cells) a s; n/s, not significant; NA, nc I League Against Epilepsy (II	nd non-epilepsy contro at applicable; PMG, foc LAE) type 1.	ls (NEC, gree al polymicrog	n cells). Further det syria; PMI, postmor	ail of cases, em interval.

seizures (weekly to monthly) but no confirmation of seizures "The serzure frequency prior to death is as detailed in the text: R, recent, documented/witnessed serzure events in last 10 days prior to death; P, poorly controlled reported in the last 10 days; O, occasional, less than monthly seizures reported; C, well-controlled epilepsy with no seizures reported for over a year. ^dStatistical test: Kruskal-Wallis test shown for differences between the two SUDEP, epilepsy control, and NEC groups.

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FIGURE 1 Regional sampling and microglial regional immunolabelling with Iba1. A, The location of the Brodmann and subcortical regions used in the study as indicated on brain coronal slice templates from the Allen Atlas. Red rectangles indicate regions with strong evidence, blue indicates "control" areas with low evidence, and green indicates intermediate evidence for involvement in SUDEP and/or the central autonomic network (CAN) (see also Table S2). B, Iba1 labeling of ramified microglia shown was equivalent to 2% labeling index in this case and region (C) Iba1 labeling equivalent to an overall 9% labeling index, with increased ramifications, intensity, and size of cell bodies compared to B. D. Iba1 in a SUDEP case from the parahippocampal gyrus with a fixation time of over 450 days. E, A nonepilepsy control case from the parahippocampal gyrus with fixation time of 9 days. F, Bar chart of mean Iba1 index in regions of high, intermediate, or low evidence for CANs or involvement in SUDEP (as shown in A); significant variation between three regions is observed in SUDEP-definite but not the other cause of death groups (SUDEP-other, epilepsy controls [EPC], and nonepilepsy controls [NEC]). Bar approximately equivalent to 50 microns in B, 35 microns in C, and 100 microns in D and E

Sections were cut at 5 µm thickness from each block and immunostained with microglial marker Iba1 (ionized calcium binding adaptor molecule 1) (Wako, clone 019-19741, 1:250) using Roche Ventana Discovery staining system. The 709 sections were then scanned on an SCN400F digital slide scanner (Leica Microsystems) at ×40 magnification. Using Definiens image analysis system, an ROI was drawn by the researcher (AS), who was blinded to the cause of death category, at the midpoint on both sides of the gyrus (for superior, middle temporal gyrus, occipital, anterior frontal, and parietal regions) of size 2.5 mm along the pial surface to a depth of 2 mm including only gray matter but aiming to include the whole cortical thickness (Figure S1A). Single equivalent area ROIs were placed on the gyral surface (cingulate, insular, temporal pole) or the lateral and medial thalamus and pulvinar (Figure 1A). A consistent threshold for immunostaining detection was applied across all sections and the Iba1 labeling index for each ROI was calculated (Figure S1B,C).

Statistical analysis was carried out with SPSS version 25 (IBM) using nonparametric tests including Mann-Whitney and Kruskal-Wallis for between cause of death group analysis, with correction for multiple comparisons, and the

threshold for significant differences between cause of death groups being set at p < .01.

RESULTS 3

Qualitative findings 3.1

Iba1-labeled single-ramified microglia and their processes were distributed relatively evenly in the cortex, as well as scattered perivascular macrophages; enlarged and more complex/branching microglia, focal aggregates, and amoeboid forms were apparent in some ROIs (Figure 1B-E).

Quantitative findings 3.2

3.2.1 Ibal labeling in cause of death groups

Mean Iba1 labeling index across all brain regions was higher in SUDEP-definite than epilepsy controls (p = .01)and NEC (p = .006); there was no significant difference between either SUDEP-definite and SUDEP-other groups (p = .1) or non-SUDEP epilepsy cases and NEC (p = .9)

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Epilepsy-non SUDEP Iba1 LI (SD) (N)	4.2 (2.7) N = 18	5.7 (2.2) N = 2	2.6 (1.9) N = 10	2.7 $N = 1$	4.2 (2.0) N = 6	1.3 (1.4) N = 7	1.5 (2.0) N = 13	1.5 (2.0) N = 21	4.2 (3.3) N = 5	2.8 (2.3) N = 5	I	I	4.6(2.9) N = 2	5.8 (3.0) N = 2
SUDEP (other) Ibal LI (SD) (N)	5.2 (2.8) N = 30	4.5 (1.4) N = 8	4.8(1.7) N = 21	5.4 (2.7) N = 22	4.4 (1.9) N = 14	3.6(2.1) N = 10	3.6 (1.6) N = 22	4.0(2.1) N = 23	5.1 (2.2) N = 23	3.0 (2.0) N = 23	5.0 (2.8) N = 13	5.3 (3.0) N = 12	2.2 (0.1) N = 3	
SUDEP (Definite) Ibal LI (SD) (N)	5.6(2.3) N = 14	7.1 (4.5) N = 10	5.2 (2.2) N = 17	4.0(3.4) N = 14	5.7(2.9) N = 11	3.1 (1.7) N = 11	3.5(2.2) N = 9	3.9(2.2) N = 11	7.5 (4.7) N = 8	4.5 (2.4) N = 8	5.8(2.2) N = 3	7.3 (2.1) N = 15	I	I
Non-epilepsy controls Iba1 LI (SD) (N)	2.6 (2.4) N = 27	4.9 $N = 1$	3.1 (2.2) N = 28	3.4 (3.2) N = 10	3.5 (2.3) N = 29	3.4 (3.2) N = 27	2.3 (1.5) N = 31	2.5(1.3) N = 30	3.6(2.8) N = 27	2.9(2.0) N = 30	4.5(6.4) N = 2	3.1 (3.0) N = 10	Ι	1
Epilepsy- lesional Iba1 LI (SD) (N)	4.9 (2.6) $N = 36$	5.2 (1.6) N = 10	4.0 (2.2) N = 27	6.2 (2.7) N = 12	4.4 (1.8) N = 16	1.6 (1.4) N = 12	2.7 (2.0) N = 24	3.4 (2.3) N = 33	5.7 (3.9) N = 14	2.9 (1.8) N = 14	2.4 (2.4) N = 3	5.4 (1.8) N = 7	3.2 (1.9) N = 5	5.8(3.0) N = 2
Epitepsy nonlesional Iba1 LI (SD) (N)	5.0(2.9) N = 27	6.3 (4.2) N = 13	4.9 (2.1) N = 24	4.1 (2.9) N = 25	4.8 (2.8) N = 18	3.4 (1.9) N = 19	3.2 (2.2) N = 21	3.7 (2.0) N = 25	5.4 (2.6) N = 22	3.6 (2.4) N = 22	6.3 (2.1) N = 4	6.7 (2.9) N = 21	Ι	I
All epilepsy Iba1 LI (SD) (N)	4.9 (2.7) N = 63	5.8(3.3) N = 23	4.9(2.7) N = 51	4.8(3.0) N = 37	4.6(2.4) N = 34	2.7 (1.9) N = 31	2.9 (2.1) N = 45	3.6(2.1) N = 58	5.5(3.1) N = 36	3.3 (2.2) N = 36	4.6(2.9) N = 7	6.4 (2.7) N = 27	3.2 (1.9) N = 5	5.8(3.0) N = 2
Groups/ regions	Parahippocampal gyrus (BA35)	Temporal pole (BA38)	Sup. temporal gyrus (BA21/22/42)	Insular (BA13/14)	Ant. cingulate (BA24)	Parietal (BA5/7)	Occipital (BA17)	Frontal (BA6/8)	Thalamus (medial)	Thalamus (lateral)	Pulvinar (medial)	Pulvinar (lateral)	Frontobasal (BA11/12)	Middle temporal gyrus (BA20/21)

Abbreviations: ANT, anterior; N, total number of samples for region including left and right sides; SD, standard deviation; SUP, superior. Note: Nine regions shown in bold were consistently available across the groups.

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TABLE 2 Data on regional Iba1 labeling



FIGURE 2 Radar plots for Iba1 labeling in cortical and thalamic regions. A, SUDEP, epilepsy-non SUDEP, and non-epilepsy controls: mean Iba1-labeling index was significantly greater (1) in the SUDEP-definite group than NEC in the parahippocampal gyrus, superior temporal gyrus, and pulvinar (p < .0001 to .003); (2) in the SUDEP-other group than NEC in the parahippocampal gyrus, superior temporal gyrus, and frontal cortex (p < .001 to .009); (3) in SUDEP-definite cases than epilepsy controls in the superior temporal gyrus (p = .005); and (4) in SUDEP-other cases than epilepsy controls in the superior temporal gyrus (p = .008) and occipital cortex (p = .002). There were no differences between SUDEPdefinite and SUDEP-other groups or the epilepsy controls and NEC groups for any brain region. B, Lesional and non-lesional cases shown for left (L) and right (R) regions: There was significant variation in Iba1 labeling between brain regions in both lesional and nonlesional groups (p < .001). No significant interhemispheric differences were noted for cases with left- or right-sided, bilateral, or no lesions. C, All SUDEP cases with lesion or nonlesional compared to all cases with hippocampal sclerosis (HS) and all cases with a malformation of cortical development (MCD): All SUDEP cases (definite and other) showed a trend for higher Iba1 labeling in the parietal cortex in nonlesional compared to lesional cases (p = .018) and higher Iba1 labeling in the insular cortex in lesional cases compared to nonlesional cases (p = .029). HS was the largest single pathology group (including two cases in SUDEP-definite and six in SUDEP-other group, three in the non-SUDEP group); there was a trend for higher Iba1 labeling noted in the parahippocampal gyrus region in HS compared to all nonlesional cases (p < .05). No significant differences were noted for the MCD group. D, Seizure activity: Cases were categorized into four groups: (1) Seizures (SZ.) in the last 10 days prior to death; (2) Active seizures occurring weekly to monthly, ie, poorly controlled seizures but no confirmed seizure in the last 10 days; (3) Less frequent seizures (less than monthly); and (4) well-controlled epilepsy with no seizures reported for over a year. A significantly higher Iba1-labeling index was observed in cases with documented seizures in the last 10 days compared to well-controlled seizures in the parahippocampal gyrus (p = .01), anterior frontal (p = .007), occipital cortex (p = .008), and lateral thalamus (p = .009). In all radar plots, mean values are shown and the standard deviations are shown in Table 2

(Table 2). Between the 14 brain regions, the Iba1-labeling index showed significant variation in the SUDEP-definite (p = .002) and SUDEP-other (p = .007), but not in epilepsy control (p = .04) or NEC groups (p = .5). The regions were grouped into three based on *high* (BA 35,13,24, thalamus, and pulvinar), *intermediate* (BA 38,21,20), and

low evidence (BA 5,17,6) for CAN network region and involvement in SUDEP (Table S2, Figure 1A). Significant differences in mean Iba1 between these three groups was observed for the definite-SUDEP group only, with lower Iba1-labeling index in regions with *low* evidence (Figure 1F).

3.2.2 | Regional Iba1 labeling in SUDEP and control groups

In definite-SUDEP cases, regional mean Iba1 labeling was highest in the medial thalamus, cingulate, parahippocampal gyrus, and superior temporal gyrus (Figure 2A). Iba1labeling index was greater in the SUDEP-definite group than NEC in all regions except the parietal cortex, reaching significance in the parahippocampal gyrus, superior temporal gyrus, and pulvinar (p < .0001 to .003) (Table 2, Figure 2A). Iba1-labeling index was higher in the SUDEP-other group than NEC in all regions except the temporal pole, reaching significance in the parahippocampal gyrus, superior temporal gyrus, and frontal cortex (p < .001 to .009) (Table 2, Figure 2A). Iba1 labeling was higher in SUDEP-definite cases than epilepsy controls in all regions, reaching significance in the superior temporal gyrus (p = .005) (Table 2, Figure 2A). Iba1 labeling was higher in SUDEP-other cases than in epilepsy controls in all regions except the temporal pole and frontobasal cortex, reaching significance in the superior temporal gyrus (p = .008) and occipital cortex (p = .002) (Table 2, Figure 2A). There were no significant regional differences between SUDEP-definite and SUDEP-other groups or the epilepsy controls and NEC groups.

3.2.3 | Pathology lesion and lateralization in SUDEP cases

We also considered the presence of an epileptogenic focal pathology identified at PM that might affect regional microglial densities. The pathologies varied in type and localization (Table 1), and there was significant variation in Iba1 labeling between brain regions in both lesional and nonlesional groups (p < .001), but no significant interhemispheric differences were noted between left- and right-sided brain lesions (Figure 2B). HS formed the largest single pathology group (two cases in SUDEP-definite, six in SUDEP-other group, and three in the non-SUDEP group); there was a trend for higher Iba1 labeling noted in the parahippocampal gyrus region in HS compared to all nonlesional cases (p < .05) (Figure 2C). Considering all SUDEP cases (definite and other) with or without a lesion, there was a trend for higher Iba1 labeling in the parietal cortex in nonlesional cases (p = .018) and higher Iba1 labeling in the insular cortex in lesional cases (p = .029) (Figure 2C).

3.2.4 | Clinical correlations and seizure frequency

There was no significant difference in the mean age at onset of epilepsy, but significantly shorter duration of epilepsy (p = .004) and younger age at death (p < .001) in SUDEPdefinite compared to other groups (Table 1). In NEC there was a positive correlation between Iba1 labeling index across all regions and age at death (p < .001) (Figure S2A), whereas in epilepsy cases there was a negative correlation between Iba1 labeling index and both age at death (p < .001) and duration of epilepsy (p < .001) (Figure S2B,C). However, when factoring for seizure activity (see below) using multiple regression analysis, there was no relationship between Iba1 labeling and duration of epilepsy or age of death in the epilepsy group.

There was insufficient detail on all cases regarding seizure types for further meaningful analysis (Table S1). Regarding seizure frequency prior to death, we categorized cases as follows: (1) documented/witnessed seizure events in last 10 days prior to death, (2) poorly controlled seizures (weekly to monthly) but no confirmation of seizure reported in the last 10 days, (3) less than monthly seizures reported, and (4) wellcontrolled epilepsy with no seizures reported for over a year. In nine cases the data available on seizure number in the last year were insufficient to categorize. There were significant differences in mean Iba1 labeling across all regions between these four groups, with highest mean values in cases with documented seizures in the last 10 days (p < .0001) (Figure S3A). Higher Iba1 labeling index was observed in cases with documented seizures in the last 10 days compared to wellcontrolled seizures in all regions, reaching significance in the parahippocampal gyrus (p = .01), anterior frontal (p = .007), occipital cortex (p = .008), and lateral thalamus (p = .009) (Figure 2D). Significantly more cases had recent seizures documented in the last 10 days in the SUDEP groups than in epilepsy controls (p < .0001) (Table 1). However, higher mean Iba1-labeling indices were observed in definite-SUDEP cases than in epilepsy controls, with recent seizures in the last 10 days and also in SUDEP-other than epilepsy controls in the well-controlled epilepsy group (p < .05) (Figure S3B). In the cases with recent seizures, higher mean Iba1 labeling was observed in SUDEP than in epilepsy controls in all regions except the parahippocampal gyrus and lateral thalamus, although differences were not significant (Figure S3C).

There was no significant difference in the gender ratios between SUDEP and other groups, with higher representation of males in all groups (Table 1); there was no significant difference in Iba1-labeling index between male and female patients in the epilepsy or NEC groups.

3.2.5 | Fixation time and postmortem interval effects

Effects of Iba1 immunostaining with fixation were tested on a time series from a surgical sample of normal temporal cortex, with incrementally increased fixation times from 1 day to 5 months; no discernible diminution in immunostaining was noted with longer fixation (Figure S1D). There was variation in the fixation times between cause of death groups (Table 1) but no significant correlation between postmortem interval (PMI) and fixation time on mean Iba1-labeling index across all regions in the NEC group.

4 | DISCUSSION

Identification of acute or subacute cellular pathology involving CANs in SUDEP could provide further insight into mechanisms leading to death as well as correlations with regional alterations reported on functional and structural neuroimaging. In this PM series we have shown increased mean cortical and subcortical microglial numbers in SUDEP compared to control groups as well as increased variability between brain regions. The most consistent evidence emerged for the parahippocampal gyrus, superior temporal gyrus, and thalamic regions, all with known autonomic regulatory functions. Furthermore, we observed a significant positive association between microglial labeling and a history of seizures occurring in the period prior to death. Microglia may therefore represent a disease activity biomarker for the localized regional autonomic dysfunction implicated in SUDEP.

4.1 | Detection of and normal distribution of microglia

Microglial activation is integral to a myriad of neuropathological conditions, including epilepsy. Iba1 serves as a sensitive and specific marker of resting and activated microglia.^{20,22} Similar to HLA-DR and CD68, Iba1 labels all phenotypes of microglia from ramified and amoeboid forms to macrophages, and is particularly suited for structural studies of normal cortex in the absence of focal pathology.²³ PM data on comparative variation in microglial density within the normal adult human brain are limited; one study using a stereological approach of frontal, parietal, medial temporal, occipital, insular. and cingulate cortex did not show spatial differences in microglial distribution.²⁴ In the current study we also did not identify significant regional differences in Iba1 labeling between cortical and subcortical brain regions in nonepilepsy controls. Available sensitive positron emission tomography (PET) microglial ligands also show diffuse low-level labeling in the normal brain.²⁵ Brain-wide gene expression data (from the atlas of the Allen institute), however, reveals regional enrichment of gene expression related to microglia (including the Iba1 gene, AIF1), and this was recently shown to correspond to regions that are vulnerable to cortical atrophy in epilepsy.¹⁸ Indeed, genes that determine the regional variability of cortical thickness in the normal human

brain also determine local microglia development and therefore susceptibility to a range of insults, including inflammation.²⁶ This suggests that seizure-related regional activation of resting, resident microglia may be a disease biomarker of cortical vulnerability in epilepsy, and if involving specific CAN regions, relevant to SUDEP.

4.2 | Microglial responses in epilepsy

In a previous quantitative PM study of microglia in the neocortex in epilepsy cases we observed regional variation in microglia using CD68, with highest labeling in the temporal pole,²⁷ as also observed in the epilepsy-control group in the current series with Iba1. In a further study of the medulla, amygdala, and hippocampus regions in SUDEP, we did not identify increased activated HLA-DR-positive microglia relative to that in epilepsy and nonepilepsy control groups.²⁸ However, this study used a semi-quantitative method that may have reduced sensitivity to detect subtle differences in cell numbers or to detect active cell morphologies compared to image analysis used in the present study. In addition, although Iba1 labels both activated and resting microglia, there is not a complete overlap with HLA-DR-labelled activated microglia.²³ As such, we cannot exclude that microglial populations in neocortical regions show enhanced plasticity in response to seizures compared to brainstem and limbic microglia. Indeed, an important observation in the current study was the association of increased microglia in patients with seizures reported in the last 10 days. Recent seizures occurring in the period prior to a SUDEP are not uncommon,²⁹ including in PM studies,³⁰ and may "prime the brain, leading to a critical decompensation during the final fatal seizure. The time course of microglial responses following experimental status using ¹¹C-PK11195 PET ligand, and confirmed on histology, showed an increase from baseline levels at 2 days, peaking at 7 days and remaining elevated for 3 weeks, including in the piriform cortex and thalamus.³¹ In the current series, more cases in the SUDEP groups had recent seizures recorded in the last 10 days compared to epilepsy controls; however, within this group with recent seizures significantly higher Iba1 was still observed in SUDEP compared to epilepsy cases without SUDEP. This could indicate an enhanced microglial response to seizure activity in SUDEP. This study was underpowered to detect regional differences in SUDEP cases with recent seizures compared to epilepsy controls; however, further studies of regional microglia responses and activation states in response to seizures are clearly warranted in SUDEP. In previous studies in SUDEP, we have recognized regional alteration in neuromodulators, including neuropeptidergic, adenosine, catecholaminergic, and serotonergic systems,^{1-3,30,32} which may reflect network changes; as these neuromodulators also regulate microglial cells and

their activation,³³ it is not inconceivable that these processes are inter-related. Activated microglial cells are also known to be increased in focal epilepsy pathologies, as focal cortical dysplasia, and correlate with duration of epilepsy,³⁴ with evidence of regional localization of the inflammation on C¹¹ PK11195 PET.³⁵ In this current series we included PM cases with regional focal pathology likely to represent the seizure focus, but no significant differences in Iba1 labeling compared to nonlesional epilepsy cases was observed.

4.3 | Pathological evidence for CAN involvement in SUDEP

The parahippocampal gyrus (BA35) showed cortical thinning in the ENIGMA-epilepsy cohort of 2149 individuals with epilepsy, which did not focus on SUDEP cases.¹⁴ In MRI studies of SUDEP, however, increased gray matter volumes have been observed in the right parahippocampal gyrus¹⁷ and bilateral increased volumes in a further SUDEP series.³⁶ In a recent morphometric PM study of 197 cases we also noted asymmetries in the length of the parahippocampal gyrus along the subiculum, which was greater on the right than on the left side in SUDEP but not in epilepsy or nonepilepsy controls.³⁷ Regarding autonomic function, the parahippocampal gyrus in addition to other mesial temporal regions has direct connections to brainstem autonomic regulatory nuclei.^{38,39} In addition, stimulation of the anterior parahippocampal gyrus region induces transient apnea, supporting a role in breathing modulation.⁴⁰ In the current study, Iba1 labeling was increased in the parahippocampal gyrus in SUDEP cases compared to nonepilepsy controls but with no evidence of difference between the right and left sides. In cases with hippocampal sclerosis we also noted higher labeling in this region. Iba1 activation in this region, particularly in the presence of hippocampal sclerosis, may indicate ongoing subacute pathology in this region and potentially relevant to acute autonomic dysfunction culminating in SUDEP.

Iba1 labeling was also greater in the superior temporal gyrus in SUDEP cases compared to epilepsy and nonepilepsy controls. Cortical thinning, including progressive atrophy,⁴¹ has been demonstrated in this region in epilepsy with MRI,¹⁴ and in a recent study of patients with severe ictal-hypoxia during generalized seizures, considered a risk-factor for SUDEP, lower volumes in the temporal pole and right superior temporal gyrus was observed.¹⁵ There are few data on specific autonomic functions associated with the superior temporal gyrus but parasympathetic autonomic regulation reported⁷ of relevance to SUDEP. The association of increased Iba1 labeling with both regions of atrophy as well as increased volume on MRI shown in SUDEP is unclear but may relate to different stages in disease progression.

There is also emerging evidence for a role of the thalamus in SUDEP. We noted significantly greater Iba1 labeling in definite SUDEP cases than in nonepilepsy controls in the pulvinar region; the medial thalamus showed highest Iba1 labeling in SUDEP and, in patients with recent seizures, significantly higher labeling in the lateral thalamus was observed compared to patients with well-controlled seizures. This supports the notion of seizure-related subacute pathology in this region as a potential vulnerability factor for SUDEP. In an MRI series of patients with SUDEP, Wandschneider et al. noted decreased gray matter volumes bilaterally in the pulvinar regions as one of the key observations¹⁷ supported by further studies showing reduced volume of the left posterior and medial thalamus in SUDEP³⁶ and also associated with severe ictal-hypoxia during generalized seizures.¹⁵ Altered functional connectivity between thalamus and cortex has been associated with generalization of seizures, a SUDEP risk-factor.⁴² Functional MRI also shows reduced connectivity between the left and right thalamus, brainstem, and cingulate cortex,¹³ and particularly between the putamen, with the cingulate cortex in epilepsy patients considered at risk for SUDEP.¹¹ In a retrospective functional MRI (fMRI) analysis comparing actual SUDEP cases with those considered at high-risk, the medial thalamus showed proportionally more inter-nodal connectivity, suggesting that this may be a critical region.¹⁰ The thalamus is known to have roles in respiratory control during hypoxia⁴³⁻⁴⁵ as well as sympathetic autonomic regulatory functions.⁷ There are direct efferent projections from pre-Bötzinger complex neurons to thalamic nuclei, in particular the dorsomedial region.⁴⁶ PM data on the thalamic subregional pathology in epilepsy are relatively limited,⁴⁷ the pulvinar not being previously studied. More detailed analysis of regional microglial thalamic activation as well as other neuroinflammatory pathways is clearly warranted to interrogate cellular processes that could underpin SUDEP.

There are several limitations to this PM study. Not all regions were available in all cases and, because the samples were obtained from different brain banks, there were differences in fixation times, processing methods, and PM intervals, which may differentially influence the immunolabeling intensity. However, we did not observe any statically differences in Iba1 labeling with fixation time or PM interval. The lesional pathologies were heterogenous in terms of their nature and localization in this relatively small cohort. Furthermore, clinical data on recent seizure control as well as seizure types and lifetime number of convulsive seizures are not available in all cases. Regional differences in cortical microglial populations and activation in the elderly brain can be associated with degenerative pathology⁴⁸; we did notice an increase in Iba1 labeling with age in the control group but not in the epilepsy cohort, and we cannot exclude a superimposed effect of age-related pathology.

In summary, accumulating data indicate preexisting cortical and subcortical pathology from imaging studies in patients with SUDEP, many localizing with CAN regions. This is the first neuropathological study investigating cellular correlates and we have identified upregulation of Iba1 microglia in SUDEP, particularly in temporal lobe and thalamic regions and associated with recent seizures. This could indicate strategic regional neuroinflammatory pathway activation as a risk factor and biomarker for SUDEP, which merits further investigation and validation.

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CONFLICT OF INTEREST

None of the authors has any conflict of interest to disclose. We confirm that we have read the Journal's position on issues involved in ethical publication and affirm that this report is consistent with those guidelines.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

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