



Adenosine kinase and adenosine receptors A₁R and A_{2A}R in temporal lobe epilepsy and hippocampal sclerosis and association with risk factors for SUDEP

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Abstract

Objective: The “adenosine hypothesis of SUDEP” (sudden unexpected death in epilepsy) predicts that a seizure-induced adenosine surge combined with impaired metabolic clearance can foster lethal apnea or cardiac arrest. Changes in adenosine receptor density and adenosine kinase (ADK) occur in surgical epilepsy patients. Our aim was to correlate the distribution of ADK and adenosine A_{2A} and A₁ receptors (A_{2A}R and A₁R) in surgical tissue from patients with temporal lobe epilepsy and hippocampal sclerosis (TLE/HS) with SUDEP risk factors.

Methods: In 75 cases, patients were stratified into high-risk (n = 16), medium-risk (n = 11) and low-risk (n = 48) categories according to the frequency of generalized seizures before surgery. Using whole-slide scanning *Definiens* image analysis we quantified the labeling index (LI) for ADK, A_{2A}R, and A₁R in seven regions of interest: temporal cortex, temporal lobe white matter, CA1, CA4, dentate gyrus, subiculum, and amygdala and relative to glial and neuronal densities with glial fibrillary acidic protein (GFAP) and neuronal nuclear antigen (NeuN).

Results: A₁R showed predominant neuronal, A_{2A}R astroglial, and ADK nuclear labeling in all regions but with significant variation. Compared with the low-risk group, the high-risk group had significantly lower A_{2A}R LI in the temporal cortex. In HS cases with severe neuronal cell loss and gliosis predominantly in the CA1 and CA4 regions, significantly higher A₁R was present in the amygdala in high-risk than in low-risk cases. There was no significant difference in neuronal loss or gliosis between the risk groups or differences for ADK labeling.

Significance: Reduced cortical A_{2A}R suggests glial dysfunction and impaired adenosine modulation in response to seizures in patients at higher risk for SUDEP. Increased neuronal A₁R in the high-risk group could contribute to perictal amygdala dysfunction in SUDEP.

Smriti Patodia and Beatrice Paradiso contributed equally to this study.

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KEYWORDS

adenosine kinase, adenosine receptors, amygdala, gliosis, temporal lobe epilepsy

1 | INTRODUCTION

Adenosine has varied physiological functions in the central nervous system (CNS) including endogenous anticonvulsant actions. Although not a conventional neurotransmitter, its binding with high affinity G protein-coupled presynaptic and postsynaptic receptors (A_1R and $A_{2A}R$) modulates synapses with predominantly inhibitory and neuroprotective effects.^{1,2} Adenosine levels rise before seizure termination^{2,3}; activation of hyperpolarizing presynaptic A_1R suppresses seizures and their spread, whereas A_1R -selective antagonists reverse this effect.² By contrast, $A_{2A}R$ neuronal activation has mainly proconvulsive actions.¹

Alteration in adenosine receptors densities and their cellular distribution has been shown in animal models of epilepsy^{1,2} and human tissues.⁴⁻⁶ An imbalance between the inhibitory and excitatory effects of adenosine through differential activation of A_1R and $A_{2A}R$ receptors may contribute to temporal lobe epilepsy (TLE).⁴ In addition to seizure termination, postictal depression, refractoriness, and respiratory depression are considered manifestations of overactivation of A_1R coupled with underactivation of $A_{2A}R$. This has led to the “adenosine hypothesis of SUDEP” (sudden unexpected death in epilepsy) as common clinical phenomena, respiratory depression, and impaired arousal⁷ could be mediated through overstimulation of these receptors in autonomic and brainstem centers in the postictal period.⁸⁻¹²

Experimental evidence also supports the adenosine SUDEP hypothesis; in a kainic acid model, with pharmacologically impaired adenosine clearance, induced seizures triggered sudden death while the adenosine receptor antagonist caffeine administered after seizure onset was associated with increased survival times.¹⁰ Similar effects were observed in seizure-induced death in DBA/2 mice, where administration of an adenosine antagonist protected from seizure-induced respiratory arrest¹³; in genetically epilepsy-prone rats inhibitors of adenosine metabolism, were associated with increased duration of postictal respiratory depression.¹⁴

Adenosine receptors have not been assessed in human tissues in relation to SUDEP. We used a large TLE surgical series, risk stratified for SUDEP similar to prior magnetic resonance imaging (MRI) studies^{15,16} based on the greatest risk factor of three or more generalized seizures (GS) occurring in the last year prior to surgery.¹⁷ We evaluated several regions, including the hippocampus, amygdala, and temporal cortex, to explore the differential expression of adenosine receptors and its major regulator, ADK, in relation to regional neuronal and glial populations.

Key Point

- The adenosine hypothesis of sudden unexpected death in epilepsy (SUDEP) proposes that adenosine stimulation in the postictal period mediates depressed arousal and respiration.
- In a temporal lobe epilepsy/hippocampal sclerosis (TLE/HS) surgical series, significantly lower astroglial adenosine A_{2A} receptor labeling in the temporal cortex in higher-risk SUDEP group suggests impaired adenosine modulation
- Amygdala stimulation mediates apnea in seizures; we found increased amygdala neuronal A_1R in the higher risk for SUDEP group of potential functional relevance.

2 | METHODS

2.1 | Case selection

Cases were selected from the UCL Epilepsy Society brain and tissue bank from adult patients who had undergone temporal lobectomy between 2009 and 2017 with a clinical and radiological diagnosis of suspected hippocampal sclerosis (HS). The project has ethical approval and patients provided written informed consent for participation in research. In 75 cases, tissue samples were available of the temporal lobe, hippocampal body, and amygdala. The neuropathology diagnosis was reviewed and the subtype of HS revised according to International League Against Epilepsy (ILAE) criteria.¹⁸

2.2 | SUDEP risk stratification

Clinical records were reviewed to extract data regarding seizure history, including age at onset, seizure type, and recent frequency (Table 1). Risk stratification for SUDEP was based on the number of GS in the previous 12 months at the last preoperative assessment¹⁷: Patients with ≥ 3 GS were high risk, no GS were low risk, and 1-2 GS were intermediate risk.

2.3 | Tissue preparation and immunohistochemistry

From each case, three tissue samples from the temporal lobe (taken approximately 2.5 cm posterior to the temporal

TABLE 1 Groups of TLE cases stratified according to risk of SUDEP

SUDEP risk group, N = number	Hippocampal sclerosis subtypes	Gender, F: M (%)	Side L: R (%)	Age at onset Age at surgery Duration (years, (SD))	GS (% of cases)	Focal seizures with LOA (weekly seizures) (%) of cases)	Nocturnal Sz, Episodes SE (% of cases)	IPI (% of cases)
High risk (HR), N = 16	Type 1 62.5% Type 2 18.8% Other 18.8%	56.3:43.8	62.5:37.5	13.7 (11.3) 34.9 (9.4) 21.1 (12)	100%	100% (73.3%)	31.3% 20%	FS 33.3% Other 13.4% None 53.3%
Intermediate risk (IR), N = 11	Type 1 82% Type 2 9% Other 9%	63.6:36.4	63.6:36.4	12.7 (7.1) 40 (9) 26.7 (11.7)	100%	100% (55.5%)	36.4% 36.4%	FS 30% Other 30% None 40%
Low risk (LR), N = 48	Type 1 75% Type 2 8.3% Other 16.7%	50:50	58.3:41.7	13.5 (11.8) 42.2 (11.1) 28.7 (14.8)	62.5%	80.6% (63%)	8.3% 2.1%	FS 34.1% Other 22.7% None 43.2%
Significance between: Groups ~	n/s	n/s	n/s	n/s	$P = .001^*$	n/s	$P = .02$ $P = .002$	n/s

Note: ~Kruskal-Wallis test was used to compare groups.

Abbreviations: GS, generalized seizures; IPI, initial precipitating injury; LOA, loss of awareness; SE, status epilepticus; Sz, seizures.

*The frequency of GS in last 12 months was used as the criterion to stratify groups. Hippocampal sclerosis subtypes other = type 3, no HS and cases with uncertain HS types (incomplete subfields represented in the resected specimen).

pole), the hippocampal body, and amygdala were selected. Serial sections were cut at 5 μ m thickness and an immunohistochemistry staining panel carried out using standard techniques: A₁R (ab124780, Abcam monoclonal, dilution 1:200), A_{2A}R (ab3461, Abcam polyclonal, dilution 1:1000), adenosine kinase (ADK) (LSB6272, Lifescience bio monoclonal dilution 1:500), glial fibrillary acidic protein (GFAP) (Z0334, DAKO polyclonal, 1:2500), and neuronal nuclear antigen (NeuN) (Millipore, 1:2000) using standard methods (detailed in Supplementary Files). Additional double-labeling immunofluorescence was carried out on select cases representing each risk group for A₁R, ADK, and A_{2A}R combined with GFAP and neuronal markers NeuN or MAP2 using standard methods (see Supplementary Files) and visualized with Zeiss Axio Imager Z2 fluorescent microscope (Zeiss).

The slides were scanned at 40 \times magnification using a Leica SCN400 (Leica Microsystems) and Zeiss AxioScan Zen 2.3 slide scanners. Seven regions of interest (ROIs) were defined using Definiens Tissue Studio (Definiens AG) image analysis: *ROI 1 Temporal cortex* to include the entire cortical thickness from the sulcus, adjacent to the superior temporal gyrus, to both gyral crowns, *ROI 2 Temporal cortex white matter* region of subcortical white matter underlying ROI 1 (Figure S1A), *ROI 3 dentate gyrus* to include the granule cell layer and molecular layer, *ROI 4 CA4 subfield of hippocampus* to include the region between the blades of the dentate gyrus, *ROI 5 CA1 subfield of hippocampus* to include the maximal representation of pyramidal cell layer but excluding CA2,

ROI 6 Subiculum to include its maximal representation on section (Figure S1B), *ROI 7 Amygdala* regions outlined with cytoarchitecture compatible with amygdala as identified on NeuN (Figure S1C); due to the fragmented nature of amygdala samples, it was not possible to delineate subnuclei. With Definiens Tissue Studio software, the defined seven ROIs were transposed onto the serial immunostained sections to ensure similar ROI capture across each sample; each individual section was manually edited for any slight misalignment of ROI. An intensity threshold was set for the chromogen detection for each antibody, which was applied across all cases (Figure S1D,E) and the total labeling index (LI) (percentage area of cellular immunostaining with high-intensity threshold) for each ROI was measured.

Statistical analysis was carried out with SPSS (version 22, IBM) for nonparametric tests to compare data between cause of groups (Kruskal-Wallis and Mann-Whitney tests), regions within groups (Friedman test), and clinical correlations (Spearman's rho test); P values of $<.05$ were regarded as significant and corrected for multiple comparisons as detailed in results. For graphical representation of data, GraphPad Prism 7 (University of California, San Diego) was used.

3 | RESULTS

Our 75 cases were classified for SUDEP risk as high (16), intermediate (11), and low (48). The three risk groups had

no significant differences in the ILAE HS subtypes (type 1 comprised 62.5% to 82%), gender, age at onset, age at surgery, frequency of focal seizures with impaired awareness, or history of initial precipitating injury (IPI). There was a lower frequency of nocturnal seizures ($P = .02$) and episodes of status epilepticus ($P = .002$) in low-risk compared with intermediate and high-risk groups (Table 1).

3.1 | Distribution of A₁R, A_{2A}R, and ADK in temporal lobe regions

A₁R. Temporal neocortex: Pancortical neuronal cytoplasmic labeling was observed with A₁R and membranous or nuclear labeling was not observed (Figure 1A-D). Reduced intensity of superficial compared with deep cortical neurons was seen in some cases, particularly in cases with superficial neuronal loss (Figure 1B, C). Scattered neurons in layer I were also A₁R positive (Figure 1A) and in the interstitial white matter (Figure 1E); scattered rare glial cells showed weak labeling with A₁R (Figure 1E, arrow). **Hippocampus:** The granule cells of the dentate gyrus showed variable A₁R labeling, particularly in the basal aspect (Figure 1F,G); labeling appeared less intense in dispersed granule cells (Figure 1E). Cytoplasmic labeling of residual CA4 and CA1 pyramidal neurons was confirmed with double labeling for MAP2 and NeuN (Figure 1E, inset) and labeling of horizontal interneurons in CA1 and subiculum pyramidal neurons but no labeling of astroglia (Figure 1H, inset). **Amygdala:** Intense neuronal cytoplasmic A₁R (Figure 1F) and occasional labeling of glial cells was noted in the periamygdala white matter but mainly little coexpression noted with GFAP.

A_{2A}R. Temporal neocortex: A stereotypical labeling pattern between cases was seen with A_{2A}R labeling of astroglial cell types in the subpial layer and cortical layer I (Figure 2A), with white matter astrocytes in a perivascular distribution and subcortical location in addition to endothelial labeling (Figure 2B). No neuronal labeling was evident. **Hippocampus:** There was overall marked increase in intensity of A_{2A}R labeling, particularly in the sclerotic hippocampus (Figure 2C) compared with the temporal lobe, predominantly in astroglial cell types and some endothelium (Figure 2E). The dentate granule cell layer and subgranular zone showed striking labeling with dense meshworks of glial cells and processes (Figure 2C,D) confirmed with double labeling for GFAP (Figure 2D); in some cases A_{2A}R expression dominated in basal cell layer astroglia. CA1 showed marked labeling of astroglial cells (Figure 2C), and A_{2A}R labeling was also seen in the hippocampal fissure and streams of glial cells in the white matter. In the few cases with no sclerosis, there was less intense A_{2A}R labeling of glia in CA4, CA1, and the dentate

gyrus; also reduced labeling for A_{2A}R in the dentate gyrus was apparent in type 2 compared to type 1 HS. **Amygdala:** Infrequent A_{2A}R glial cells and processes were scattered with some labeling of capillary endothelium but no neuronal labeling (Figure 2F).

ADK: All regions showed mainly nuclear labeling of small glial cells and occasional neurons with some cytoplasmic labeling, confirmed with co-localization with NeuN (Figure 2G). The superficial temporal cortex showed more prominent labeling compared with the deeper cortex, and nuclei were also prominent in the granule cell layer.

3.2 | Quantitative analysis

3.2.1 | Regional differences in expression

The highest LI in all three risk groups for A₁R was in the dentate gyrus, with significant variation across the seven ROIs ($P < .0001$) (Table 2). For A_{2A}R, the highest LI in the high- and intermediate-risk groups was in the dentate gyrus but for the low-risk group in CA1 (Table 2); significant variation across ROIs was seen for the low- and high-risk groups only ($P < .0001$). For ADK, the highest LI was in CA1 for the high- and low-risk groups and in temporal cortex for the intermediate-risk group (Table 2); significant variation between ROIs was present in the low-risk group only ($P < .0001$).

3.2.2 | Comparison of high- and low-risk SUDEP groups

There was no significant difference in A₁R or ADK LI for any region between the three risk groups. A_{2A}R LI was lower in high- than low-risk group for all regions except the amygdala, reaching significance in the temporal cortex ($P = .035$, corrected for multiple comparisons, Figure 3A).

When considering type 1 HS cases only, A₁R in the amygdala was significantly different across the risk groups ($P = .034$, corrected for multiple comparisons), with mean values higher in both medium- and high-risk group compared with the low-risk group, reaching significance for the medium-risk group ($P = .006$, corrected for multiple comparison, Figure 3B). There was also significantly lower A_{2A}R in the temporal cortex in the high- than in low-risk groups ($P = .032$, corrected for multiple comparisons, Figure 3A) but no differences were noted for ADK.

Because A₁R is expressed mainly in neurons and A_{2A}R in astroglia, we stained for NeuN and GFAP to evaluate differences in neuronal loss or gliosis between regions and risk groups. There was significant variation across ROIs for both GFAP and NeuN LI ($P < .0001$), but there was no

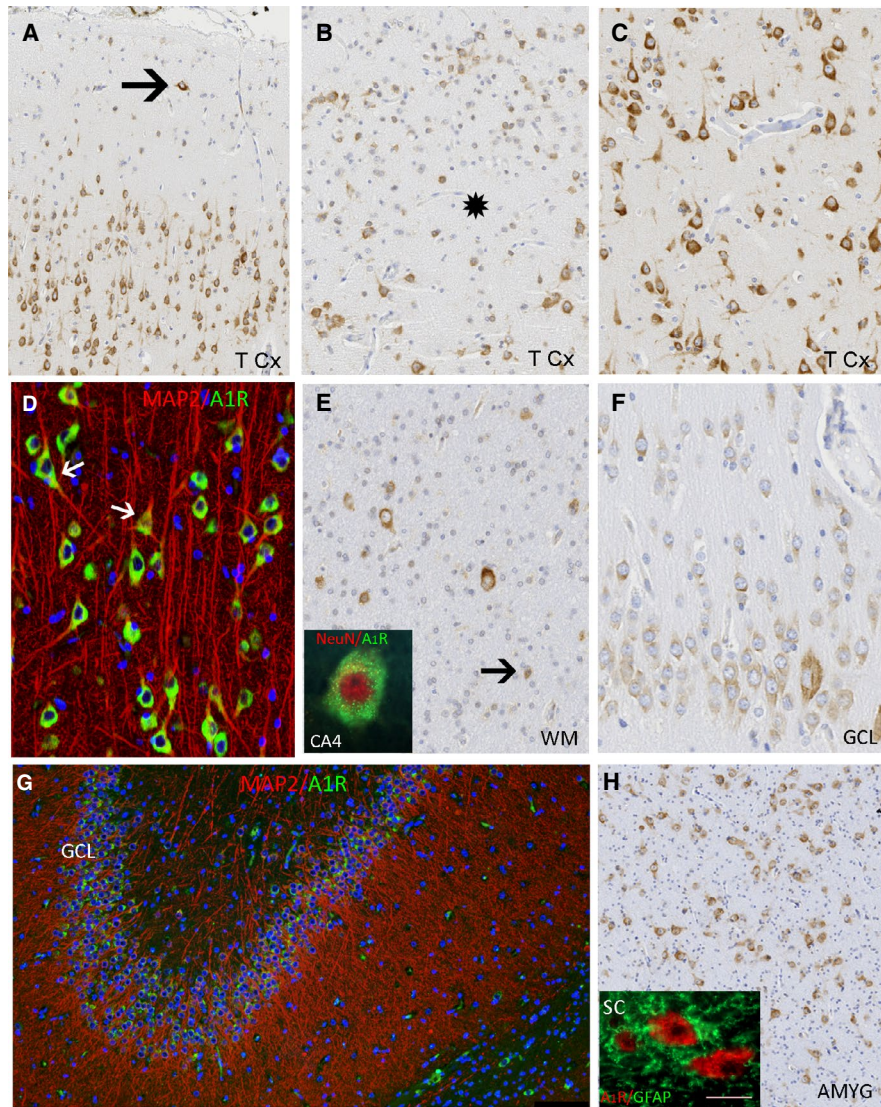


FIGURE 1 Patterns of A₁R labeling in regions of interest in temporal lobe epilepsy (TLE) cases. A, Superficial temporal neocortex showing predominant neuronal labeling including scattered layer I horizontal neurons (arrow). B, In some cases, reduced intensity of superficial cortical neurons was noted, particularly in cases with temporal lobe sclerosis and regions of neuronal loss (asterisk); however, labeling of glial cells in these regions was not increased. C, Deeper cortical layers showed more uniform and intense neuronal labeling. D, A₁R granular cytoplasmic labeling associates with MAP2-positive cortical neurons, with MAP2 located mainly in the dendrites (arrows). E, White matter interstitial neurones were positive; inset shows A₁R labeling of neuronal nuclear antigen (NeuN)-positive CA4 neuron. F, Granule cells showed more variable patterns prominent in basal cytoplasmic aspect of basal cells and somewhat diminished in more dispersed cells; inset shows double labeling with MAP2-positive granule cells and apical dendrites. G, Low power view of the dentate double-labelled with MAP2 confirming the location of A₁R in the granule cell layer. H, Labeling of amygdala neurons was intense; inset shows subiculum with A₁R neuronal labeling, separate from adjacent glial fibrillary acidic protein (GFAP)-positive processes. There was no significant glial labeling for A₁R in any subfields. Bar = approximately equivalent to 200 μ m in A, B, C, & H; 75 μ m in D-F; 150 μ m in G; equivalent to 20 μ m for insets in E and H. Abbreviations: AMYG, amygdala; GCL, granule cell layer; SC, subiculum; T Cx, temporal cortex; WM, white matter

significant difference in NeuN or GFAP LI for any ROIs between the three risk groups; this was also the case when considering type 1 HS cases alone. Across all cases, there was a positive correlation with NeuN and A₁R LI in all ROIs ($P < .01$ to $P < .000$) apart from the dentate gyrus and CA1. There was a positive correlation with GFAP LI and A_{2A}R for all hippocampal and amygdala ROIs ($P < .05$ to $.001$) but not for temporal cortex ROIs. There were no

significant correlations between ADK and GFAP labeling in any region.

3.2.3 | Clinical correlations

There was a negative correlation between duration of epilepsy and A₁R LI (ROI1, $P = .04$ and ROI2, $P = .03$) and

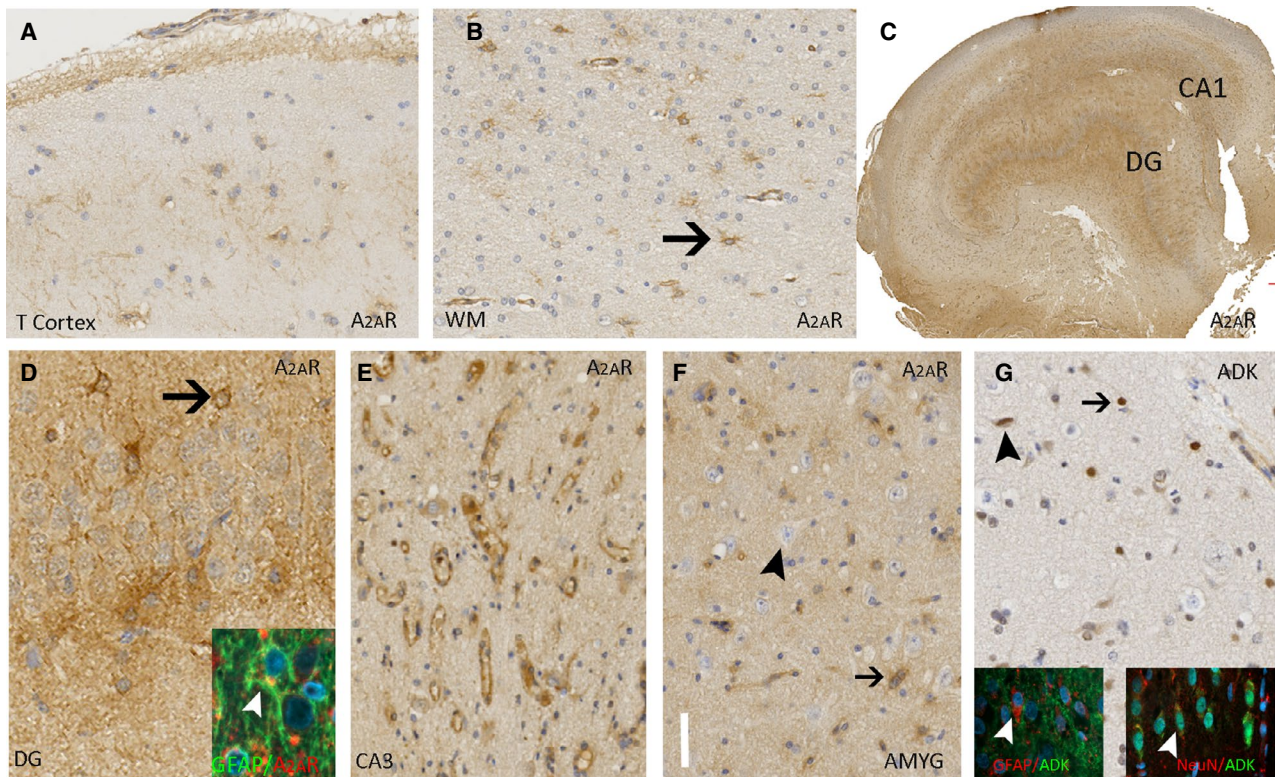


FIGURE 2 Patterns of $A_{2A}R$ and adenosine kinase (ADK) labeling in regions of interest in temporal lobe epilepsy (TLE) cases. A, The temporal neocortex showed a relatively stereotypical labeling pattern with $A_{2A}R$ between cases with astroglial-like cells in subpial layer and layer I showing cytoplasmic expression. B, In the white matter, scattered astrocytes with perivascular distribution (arrows) and endothelial labeling was noted with $A_{2A}R$. No neuronal labeling was apparent. C, Marked increase in intensity of labeling in hippocampus compared with the lobe was noted with $A_{2A}R$, predominantly in astroglial cell types in CA1 and the end folium particularly (case shown is high-risk SUDEP group with type 1 hippocampal sclerosis). D, The dentate granule cell layers showed variable labeling intensity of dense meshworks of glial cells (arrow) and processes; inset shows cytoplasmic co-localization of GFAP and $A_{2A}R$ in cells in the dentate gyrus (case shown is low risk for SUDEP group with type 2 hippocampal sclerosis). E, Shows focal intense labeling of endothelium in stratum pyramidale for $A_{2A}R$. F, The amygdala showed less-frequent glial cells and processes (arrow) and endothelium labeling, but neuronal cells appeared negative (arrowhead). G, ADK in the amygdala showing nuclear labeling pattern in glial and occasional scattered cytoplasmic labeling in neuronal cells. Inset bottom left shows lack of cell co-localization with GFAP and ADK in the dentate gyrus and bottom right shows focal cell co-localization with NeuN and ADK in subiculum. Bar in F equivalent to approximately 75 μm in A, B, 500 μm in C, 20 μm in D-G. Abbreviations: DG, dentate gyrus; T Cortex, temporal cortex; WM, white matter

ADK LI (ROI1, $P = .03$) but a positive correlation with $A_{2A}R$ LI (ROI2, $P = .04$) in temporal lobe ROIs. In patients with a history of nocturnal seizures, lower $A_{2A}R$ labeling occurred in CA4 ($P = .02$), CA1 ($P = .001$), and amygdala ROIs ($P = .019$) (Figure 3C); it was also significantly lower in CA1 in patients with a history of status epilepticus compared with those without this history ($P = .02$).

4 | DISCUSSION

In our TLE patients, we identified dominant neuronal expression of adenosine A_1R , expression of $A_{2A}R$ in regions of astrocytosis, and predominant nuclear ADK expression. In patients at high risk for SUDEP, we observed a significant reduction in $A_{2A}R$ in the temporal cortex and increased amygdala A_1R ; this could implicate regional cellular imbalances in adenosine

signaling as a vulnerability factor for SUDEP. The role of the purine nucleotide adenosine in seizure modulation is supported by extensive animal and human studies.^{2,19} Experimental evidence suggests that adenosine may precipitate or contribute to the progression from seizure to SUDEP postictally,^{10,14,17,20} although this has not been investigated using human tissues.

4.1 | $A_{2A}R$, ADK, and astrocytosis in epilepsy

Epilepsy is associated with altered cellular distribution of adenosine receptors and ADK that may occur as primary or secondary adaptive phenomena.^{1,2} Earlier studies utilizing human tissues found enhanced neuronal expression of A_1R ²¹ and upregulation of $A_{2A}R$ in Rasmussen's encephalitis, with $A_{2A}R$ expression in lesional astrocytes, endothelium, as

TABLE 2 Labeling index (LI) and standard deviation (SD) for A₁R, A_{2A}R, and ADK in seven regions of interest in the temporal lobe in the three SUDEP risk groups

Group/Region	ROI1/Cortex			ROI2/WM			ROI3/DG			ROI4/CA4			ROI5/CA1			ROI6/SUBIC			ROI7/AMYG		
	A ₁ R LI	A _{2A} R LI	ADK LI × 10 ⁻²	A ₁ R LI	A _{2A} R LI	ADK LI × 10 ⁻²	A ₁ R LI	A _{2A} R LI	ADK LI × 10 ⁻²	A ₁ R LI	A _{2A} R LI	ADK LI × 10 ⁻²	A ₁ R LI	A _{2A} R LI	ADK LI × 10 ⁻²	A ₁ R LI	A _{2A} R LI	ADK LI × 10 ⁻²	A ₁ R LI	A _{2A} R LI	ADK LI × 10 ⁻²
Low risk, N = 48	0.039 (0.02)	0.005 (0.01)	0.1 (0.1)	0.0048 (0.007)	0.007 (0.023)	0.023 (0.052)	0.1 (0.09)	0.046 (0.05)	0.073 (0.1)	0.008 (0.01)	0.052 (0.06)	0.083 (0.18)	0.007 (0.01)	0.061 (0.09)	0.15 (0.26)	0.026 (0.03)	0.041 (0.09)	0.064 (0.14)	0.017 (0.01)	0.0025 (0.005)	0.046 (0.05)
Intermediate risk N = 11	0.044 (0.03)	0.04 (0.11)	0.18 (0.3)	0.004 (0.004)	0.08 (0.25)	0.071 (0.19)	0.063 (0.04)	0.09 (0.13)	0.062 (0.12)	0.0065 (0.003)	0.076 (0.11)	0.026 (0.02)	0.0065 (0.008)	0.071 (0.12)	0.10 (0.20)	0.02 (0.01)	0.07 (0.12)	0.041 (0.05)	0.025 (0.01)	0.004 (0.005)	0.057 (0.12)
High risk, N = 16	0.043 (0.02)	0.002 (0.003)	0.08 (0.09)	0.003 (0.002)	0.002 (0.003)	0.025 (0.037)	0.13 (0.18)	0.037 (0.04)	0.056 (0.094)	0.12 (0.24)	0.026 (0.029)	0.044 (0.065)	0.006 (0.008)	0.032 (0.045)	0.085 (0.05)	0.02 (0.14)	0.017 (0.003)	0.041 (0.04)	0.028 (0.034)	0.003 (0.009)	0.046 (0.04)

Note: Significant differences between risk groups shown in bold.

well as neurons.⁵ In TLE, A_{2A}R expression was primarily co-localized with hippocampal GFAP-positive astrocytes but not neuronal markers, showing increased receptor expression compared with postmortem controls.⁴ Astrocytic A_{2A}R regulates cellular Na⁺/K⁺ ATPase, glutamate uptake and inflammatory cytokine release, and receptor upregulation was observed following varied brain insults.²² In our study, A_{2A}R expression was predominantly astroglial, supported by the correlation between A_{2A}R and GFAP staining in mesial temporal regions; there was also a positive correlation with duration of epilepsy in the temporal lobe regions. The relative reduction of A_{2A}R in the temporal cortex in the high SUDEP risk group, despite no quantitative differences in gliosis between the risk groups, suggests impaired adaptation and adenosine modulation as well as glial dysfunction in response to seizures as a vulnerability factor for SUDEP. Our study was limited to the temporal cortex and it would be important to extend a similar study to other cortical regions, for example, the insular and cingulate cortex, that have been structurally and functionally implicated in recent clinical and neuroimaging studies in SUDEP.²³⁻²⁶ Furthermore, A_{2A}R receptors have high density on normal cerebral microvessels in contrast to A₁R,²⁷ and A_{2A}R modulates blood-brain barrier permeability.²⁸ Our previous postmortem study found no evidence for acute blood-brain barrier insufficiency in SUDEP postmortem brains.²⁹ Nevertheless, in the present series, intense endothelial A_{2A}R expression was observed in some cases, although this was not separately quantified; it is of potential relevance to the significant microvascular dysfunction in focal epilepsy³⁰ and warrants further investigation.

Astrocytes regulate the adenosine cycle during and after seizures: Following high levels of neuronal activity, cytoplasmic ATP is released through exocytosis or hemi-channels (“gliotransmission”)³¹; then following degradation, glial reuptake of adenosine occurs via nucleotide transporters with phosphorylation by cytoplasmic ADK. Astroglial ADK is the major metabolic clearance route for adenosine² and represents a candidate treatment target in epilepsy. Glial proliferation and astrocytosis occurs in most surgical epilepsy specimens with myriad pathophysiological contributions, such as recycling neuromodulators.^{32,33} ADK upregulation was reported in the balloon cells of focal cortical dysplasia,⁶ reactive astrocytes in Rasmussen’s encephalitis,²¹ and in TLE.³⁴ In contrast to these reports, we did not demonstrate significant cytoplasmic glial ADK in our series or a correlation with gliosis as measured by GFAP labeling. This discrepancy may be explained by our antibody, which was selected for its optimal labeling in temporal lobe fixed-tissue samples but has preferential recognition of the long isoform of ADK. ADK isoforms are developmentally regulated in the brain; the short isoform of ADK preferentially locates to the cytoplasm for adenosine clearance, whereas the long isoform is nuclear, with mainly epigenetic regulatory roles.³⁵

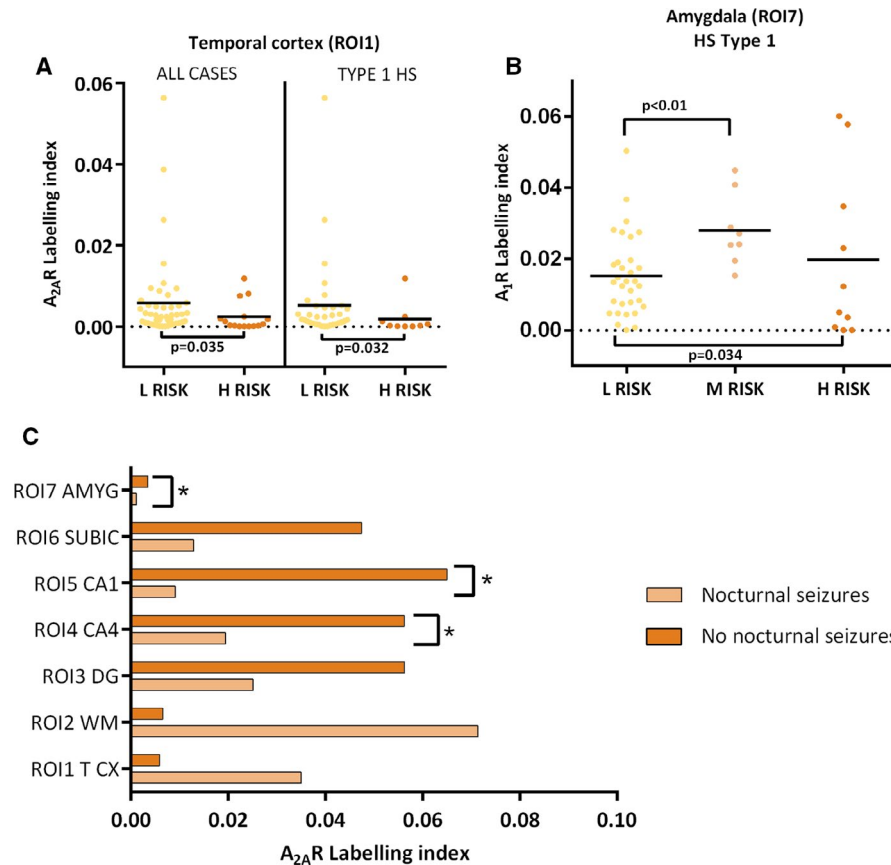


FIGURE 3 Graphs of regional A₁R and A₂A R labeling in relation to SUDEP risk, hippocampal sclerosis and nocturnal seizures. A, Scatter graph of A₂A R labeling index shown in high and low risk groups, in all cases and with type I HS only, both showing significantly lower A₂A R in the temporal cortex (ROI1) in high risk group. B, Scatter graph of A₁R labeling index in the amygdala (ROI7) which was higher in high risk and medium risk compared to low SUDEP risk group. C, A₂A R regional labeling in cases with nocturnal seizures showed lower labeling in hippocampal and amygdala ROI; *significant in CA4 ($P = .02$), CA1 ($P = .001$) and amygdala ROI ($P = .019$).

Abbreviations: AMYG, amygdala; DG, dentate gyrus; H, high; HS, hippocampal sclerosis; L, Low; M, medium; TCX, temporal cortex; ROI, region of interest; SUBIC, subiculum; WM, white matter

4.2 | A₁R expression and the amygdala

High A₁R binding densities occur in CA1,³⁶ intermediate to high concentrations in the amygdala, and lower levels in brainstem.³⁷ In the cortex, A₁R neuronal labeling was more intense in layer V than superficial cortex,³⁸ consistent with our observations. In quantitative studies of TLE tissues, A₁R labeling in the hippocampus and dentate gyrus was reduced compared with nonepilepsy controls,³⁹ whereas another study of the amygdala found similar A₁R-binding densities in TLE patients and controls.⁴⁰ Here, we observed increased A₁R in the amygdala in the high-risk group when we stratified cases into type 1 HS alone. The extent of amygdala sclerosis associated with HS is a relevant variable,⁴¹ but we did not observe any difference in NeuN labeling in any region, as a measure of neuronal number, between the risk groups. In addition, we found lower A₂A R labeling in amygdala and mesial temporal regions in patients with nocturnal seizures, a risk factor for SUDEP⁴²;

together this supports amygdala A₁R/A₂A R imbalance in cases with higher SUDEP risk.

There is accumulating clinical evidence for amygdala involvement in SUDEP; MRI studies revealed increased right-sided gray matter volumes¹⁶ and clinical studies record apnea following electrical stimulation of the amygdala.⁴³⁻⁴⁵ Of interest, tolerance to hypoxia is mediated through A₁R,⁴⁶ and patients with apnea undergoing amygdala stimulation had no symptoms of dyspnea,⁴⁵ which could be an adenosine-mediated effect. Furthermore, evidence supports brainstem spreading depolarization as a final postictal event in SUDEP⁴⁷; in the CACNA1A model of SUDEP, spreading depolarization extended through subcortical regions and the amygdala before brainstem extension.⁴⁸ There is evidence that adenosine may be responsible for prolonged depression of synaptic transmission after spreading depolarization via A₁R receptor activation.⁴⁹ Of note, the neuronal labeling we observed included cytoplasmic labeling and is likely also detecting internalized A₁R nonfunctioning receptors, in addition to functional membrane receptors. A₁R

receptors have been shown in culture to have a fast recycle time of 60–120 minutes from internalization back to the neuronal membrane.⁵⁰ In the current study, one interpretation is that cytoplasmic A₁R labeling correlates with increased nonfunctional receptor manifesting as reduced suppression of amygdala activity in high risk cases. A limitation of the resolution of immunohistochemistry is that it is not possible to discriminate functional membranous from nonfunctional receptors or receptor binding and this requires further investigation through other methods.

Further limitations of the study include the use of tissues derived from surgical cases in which clinical risk factors for SUDEP were used, rather than tissue from SUDEP cases. Our risk stratification was based on a single, albeit strong, risk factor (frequency of generalized convulsions) and depends on the accuracy of the clinical records and database. The use of surgical tissue limits us to the examination of hippocampus, temporal cortex, and amygdala; postmortem tissues would be required to study other autonomic (eg, insula, hypothalamus) and brainstem regions and further extended studies in SUDEP cases compared with nonepilepsy controls is essential. Nevertheless, the main advantage of initiating SUDEP studies in surgical tissues is the uniformity of the underlying pathology, fixation times (lack of variable postmortem intervals, and tissue degradation), and that identical ROIs can be studied. We have only applied immunohistochemistry in this study and these initial findings should be validated and confirmed with additional methodologies to assess functional receptor status. Furthermore, there is considerable literature proposing serotonin as an important neuromodulator in SUDEP,²⁰ which could act synergistically with adenosine; this will be addressed in future studies in these HS/TLE cases.

In summary, in a series of risk-stratified epilepsy surgical cases, reduced cortical A_{2A}R in glial cell types could indicate impaired adenosine modulation in response to seizures in patients at higher risk for SUDEP. Increased amygdala neuronal A₁R in the high-risk group could contribute to peri-ictal amygdala dysfunction in SUDEP and warrants further investigation.

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CONFLICT OF INTEREST

The authors have no conflicts of interest to declare. 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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