

Conserved Thalamic Astrocytic Pathology in Mice and Patients Links TBI to Epilepsy

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Abstract

Post-traumatic epilepsy (PTE) can emerge months to years after traumatic brain injury (TBI) and is a major cause of morbidity, yet the pathological processes that distinguish TBI progressing to epilepsy remain poorly defined. Here, we combined longitudinal multimodal imaging, electrophysiology, neuropathology, and human imaging and postmortem analyses to identify cross-species features associated with PTE. In a mouse model of PTE-prone TBI, injury produced widespread brain pathology, whereas animals developing PTE showed disproportionate thalamic atrophy, white-matter abnormalities with reduced optic tract fractional anisotropy, and persistent metabolic alterations. Histological analyses revealed pronounced thalamic astrocytic reactivity, reduced cytoskeletal complexity, and impaired expression of proteins involved in glutamate homeostasis. Using an early ECoG signature to prospectively stratify PTE risk, we found that astrocytic simplification and accumulation of insoluble GFAP were already present during the latent period before spontaneous seizure onset. In patients with PTE, thalamic volume loss and reduced posterior thalamic radiation fractional anisotropy independently improved PTE classification beyond clinical variables. PTE patients also showed reduced pulvinar-centered thalamocortical functional connectivity involving visual and default mode networks. Postmortem tissue further demonstrated pronounced astrocytic reactivity and reduced astrocytic complexity in cortex and thalamus. In contrast, IBA1-defined microglial reactivity did not distinguish PTE from non-PTE at the chronic time points examined. Together, these findings identify a conserved thalamocortical phenotype associated with PTE and show that astrocytic remodeling emerges before spontaneous seizures. The convergence of cellular pathology with structural, metabolic, and functional abnormalities identifies the thalamus as a candidate hub of post-traumatic epileptogenesis and provides translational markers for tracking disease progression after TBI.

Background

Traumatic brain injury (TBI) is a leading cause of mortality and morbidity worldwide, with ~ 2 million new patients each year in the United States alone; yet, no Food and Drug Administration-approved therapies exist to prevent long-term sequelae[1]. Post-traumatic epilepsy (PTE) is among the most severe complications of TBI, accounting for 5% of all epilepsies and up to 20% of acquired epilepsy cases[2, 3] with approximately 20,000 new PTE patients diagnosed each year in the United States[4]. The lifetime risk of PTE depends on TBI severity, ranging from 1.2% after mild to 16.7% after severe TBI[5]. Notably, PTE typically develops after a latency period. About 50% of cases present within 6 months (m), 65% within 1 year, and 80% within 2 years of TBI[4], providing a unique seizure-free window to be exploited for disease-modifying interventions. However, no preventive treatments are currently available as the mechanisms driving post-traumatic epileptogenesis are poorly understood. Current anti-seizure medications only control symptomatic seizures without altering disease progression[6].

Multiple brain regions, including cortex, hippocampus, and thalamus, are vulnerable after TBI and have been implicated in PTE pathogenesis in both patients[7, 8] and experimental models[9–11]. The thalamocortical circuit is of particular interest in PTE, given its key role in seizure generation and

propagation in various forms of epilepsy[12, 13], through regulation of complex excitatory and inhibitory neuronal interactions[14]. While the pathophysiology of PTE is relatively unknown[15], neuroinflammation is a consistent response observed after TBI and in epilepsy[16, 17], with chronically persistent reactive astrocytes and microglia observed in both rodents[18–20] and humans[21]. In particular, astrocytes transition from a physiological to a reactive state following injury through astrogliosis[22] which can disrupt glutamate buffering, potassium clearance, and blood–brain barrier integrity, thereby contributing to hyperexcitability and epileptogenesis[23]. Experimental and clinical evidence indicates that reactive astrocytes can actively contribute to both the initiation and progression of epilepsy[23], with astrocytic alterations observed in preclinical PTE models[24–27]. Thus, astrocytic reactivity represents a potential pathophysiological mechanism linking TBI to PTE. However, astrogliosis is also a common response to brain injury in the absence of epilepsy, making it critical to distinguish generic injury responses from astrocytic alterations specifically associated with progression to PTE. Moreover, most studies have examined astrocytes after epilepsy is established, leaving unresolved whether astrocytic pathology precedes spontaneous seizures or instead develops as a consequence of recurrent seizure activity. To address these gaps, we leveraged a clinically relevant mouse model characterized by ~ 50% PTE incidence at 5m post-injury, a seizure-free window of up to 1.5m[11, 28], along with an acute EEG biomarker that allows the stratification of mice at high- or low-risk of PTE by 7 days post-injury[28], enabling the investigation of brain tissue changes before seizure onset.

Glial fibrillary acidic protein (GFAP), the main intermediate filament of astrocytes, is a hallmark of reactive gliosis. Under normal conditions, GFAP filaments are soluble and dynamically regulated; however, under sustained or severe stress, GFAP can aggregate into insoluble forms, indicating irreversible astrocytic remodeling and cytoskeletal dysfunction[29–31]. The accumulation of abnormal GFAP, including ubiquitinated and insoluble species, is a hallmark of Alexander’s disease, a pathology characterized by seizure development[32, 33]. Insoluble GFAP species have been observed in TBI, primary tauopathies, and chronic neuroinflammatory and neurodegenerative states, but their role in PTE remains unknown[33–36]. We therefore asked whether a distinct thalamic astrocytic phenotype differentiates TBI that progresses to epilepsy from TBI that does not, whether this phenotype is already detectable before spontaneous seizure onset, and whether corresponding structural, cellular, and network abnormalities are conserved in human PTE. To address these questions, we integrated longitudinal multimodal neuroimaging and electrophysiology in a clinically relevant mouse model of PTE with an early ECoG biomarker that prospectively stratifies animals at high or low risk of epilepsy, together with human structural and functional MRI and postmortem neuropathology. This cross-species approach identifies a conserved thalamocortical phenotype associated with PTE and shows that astrocytic remodeling and accumulation of insoluble GFAP emerge during the latent period preceding spontaneous seizures.

Materials and methods

Ethics statement

All animal procedures complied with Italian legislation (D.lgs 26/2014), EU Directive 2010/63/EU, and the NIH Guide for the Care and Use of Laboratory Animals (2011) and were approved by the Mario Negri Institute Animal Care and Use Committee and the Italian Ministry of Health (Decreto no.328/2019-PR).

Human imaging analyses used data from the prospective multicenter CENTER-TBI study (ClinicalTrials.gov: NCT02210221). MRI was performed in a subset of participants at 3T and acquired longitudinally at multiple timepoints, including within 72h, ~2–3w, and 6, 12 and 24m post-injury. In this study, analyses focused on the 6m timepoint to match the timing of the animal experiments. PTE was defined as epilepsy developing within 6m post-injury, in the absence of documented seizures during hospital admission. All participants or legal representatives provided informed consent according to local regulations. Clinical data were accessed through the Neurobot platform (RRID:SCR_017004). Human brain tissue was obtained through the Pacific Northwest Brain Donor Network (University of Washington) with informed consent from legal next of kin. All procedures were approved by the University of Washington School of Medicine Compliance Office.

Mice

Eight-week-old male CD1 mice (~25g; Envigo, Italy) were housed in specific-pathogen-free facilities, maintaining a constant room temperature (23°C) and relative humidity (60 ± 5%). They had free access to food and water and followed a fixed 12-hour light/dark cycle (7 a.m.- 7 p.m.). Five mice were kept in each cage until stereotaxic surgery, after which each mouse was individually housed with environmental enrichment[37].

Experimental Design

To investigate regional vulnerability and neuroinflammation in PTE, we performed multimodal analyses across four cohorts. Cohort 1 underwent T2WI at 4m and DTI at 3w and 4m, followed by biochemical analyses at 5m. Cohort 2 underwent ¹H-MRS at 3w and 4m and DTI at 4m (**Suppl. Figure 1A**). Mice in both cohorts were implanted with cortical screw electrodes at 4m and continuously ECoG monitored up to 5m for detection of spontaneous recurrent seizures (SRSs) and classification into PTE or NoSRS. PTE incidence was 43% in **Cohort 1** (7/18 TBI mice) and 47% in *Cohort 2* (10/24 TBI mice). To determine whether astrocytic reactivity is a hallmark of PTE in our model we performed histological analyses on biobanked brain sections from sham (n = 7) NoSRS (n = 9) and PTE mice (n = 9) from a previously published study[28] (*Cohort 3*). Then, we set out to investigate astrocytic alterations at 21d post injury, before seizure development, in mice at high- or low-risk of PTE (*Cohort 4*, acute experiment, **Suppl. Figure 1B,C**). Mice were implanted with cortical screw electrodes immediately after TBI and underwent acute ECoG monitoring up to 7d post TBI. Spike and sharp wave number was quantified in the contralateral posterior electrode to classify mice at high- and low-risk of PTE[28]. Out of 36 TBI mice, 2 died; of the 34 surviving mice, 19 were classified as low-risk and 15 as high-risk.

Experimental brain injury

Mice were anesthetized with isoflurane (5% for induction, 1.5% for maintenance) in N₂O/O₂ (70/30%) and placed in a stereotaxic frame for general gas anesthesia. Subsequently, craniectomy was performed, followed by the induction of controlled cortical impact (CCI) brain injury, as detailed in previous studies[38–40]. Following the injury, the craniectomy was covered with a cranioplasty, and the scalp was sutured. Throughout the surgical procedures, mice were maintained at a body temperature of 37°C.

Detection and quantification of seizures and spiking activity

Either at 4m (after MRI analyses, *Cohorts 1 and 2*) or immediately following TBI (*Cohort 4*), mice underwent electrode implantation as previously described[11, 28] (see Suppl. Figure 1C for electrodes placement). Mice from *Cohort 4* were implanted with contralateral electrodes only.

Spontaneous seizures. Mice in *Cohorts 1 and 2* underwent continuous monitoring (24/7) at 4m post-TBI for the detection of spontaneous seizures as previously described[28]. Seizures were defined as paroxysmal events characterized by high frequency (5-10Hz) and/or multispikes complexes and/or high amplitude (0.7-1.0mV vs 0.1-0.3mV at baseline) spikes, lasting ≥ 10 s. Mice developing PTE exhibited at least one spontaneous seizure on ECoG during the recording period. NoSRS showed no spontaneous seizures during the corresponding recording time.

Spiking activity. *Cohort 4* underwent continuous monitoring during the first 7d post-injury for quantification of epileptiform spiking activity and stratification into low- and high-risk PTE groups as previously described[28]. Spikes were defined as events with an amplitude at least 2.5-fold higher than the standard deviation of the baseline and lasting 20-70ms. Sharp waves were similar to spikes but had a longer duration (70-200ms)[41]. Spiking activity was analyzed throughout the entire 24-hour period for each electrode, with automatic detection visually confirmed by two experienced researchers unaware of sample identity. ECoG analysis of seizures and spiking activity was conducted by two independent investigators. A deviation of $\leq 5\%$ from concordance was deemed acceptable; otherwise, a third person analyzed the ECoG tracing to establish consensus.

MRI acquisition and analysis

MRI was performed under isoflurane anaesthesia using a 7T Bruker BioSpec scanner. Structural MRI, DTI, and ¹H-MRS acquisitions and analyses were performed as previously described[42, 43], with full acquisition and processing details provided in the Supplementary Materials. ROI-based analyses were performed for grey and white matter regions.

Immunohistochemistry

Mice were perfused transcardially, and brains were formalin fixed, paraffin-embedded, and sectioned (8µm thick) sagittally on a microtome and collected at 80µm intervals starting from – 0.5mm from midline.

For heat-induced antigen retrieval, sections were incubated in sodium citrate buffer pH 6.0 in a microwave for 5min at 750 Watt. After cooling to room temperature (RT), slices were incubated with 3% hydrogen peroxide and 0.25% Triton X-100 in water for 30 min at RT to inactivate endogenous peroxidases, then the sections were incubated for 1h at RT with 10% normal goat serum and 1% Triton X-100 in PBS, followed by overnight incubation at 4°C with the primary antibody (IBA- 1 1:2000; Wako, Neuss, Germany; GFAP 1:4000; Dako, Glostrup, Denmark). After washing with PBS, sections were incubated with a secondary antibody solution (1:200, biotinylated anti-rabbit Vector Laboratories, Burlingame, CA, USA) for 1h at RT, followed by incubation with the Vectastain Elite ABC reagent (Vector Laboratories, Burlingame, CA, USA). Immunoreactivity was tested using DAB as a chromogen (Dako, Glostrup, Denmark). Negative controls were run in parallel for each staining and no staining was observed.

Immunofluorescence

After antigen retrieval slices were blocked in 0.5% Triton X-100, 10% fetal bovine serum in PBS. Primary antibodies for rabbit anti-GFAP (Dako, 1:4000), guinea-pig anti-GLT-1 (Millipore, 1:1000), mouse anti-GS (Millipore, 1:2000) were incubated overnight at 4°C. Fluorescent secondary antibodies (Thermofisher, Alexa Fluor 594 conjugate of goat anti-rabbit 1:500, biotinylated anti-guinea pig 1:500 amplified with fluorescein-488 and biotinylated anti-mouse 1:500 amplified with fluorescein-488) were used. Negative controls were run without the primary antibodies and no staining was observed.

Histology acquisition and quantification

Two FFPE brain sagittal sections including the lesion core (-0.5mm, -0.58mm from midline) were analyzed per marker and animal at 20x with an Olympus BX-61-VS microscope with full acquisition and processing details provided in the Supplementary Materials. Images acquired with Nikon A1 confocal scan were acquired using an unbiased approach within the predefined ROIs, by acquiring 3 fields of views per ROI in each animal.

Stained area and cell quantification. GFAP and IBA-1 positive area was quantified across two distinct mediolateral slices in predefined ipsilateral cortical and thalamic ROIs (**Suppl. Figure 2**) and expressed as percentage of total area. GFAP+ (astrocytes) and IBA-1+ (microglia) were quantified using semi-automated image analysis in QuPath[44] (version 0.4.3).

Sholl Analysis. Sholl analysis was conducted to assess astrocyte morphology[45, 46]. Approximately ten astrocytes with clear visible processes and soma were selected within the defined regions of interest of each animal (excluding the glial scar in the ipsilateral cortex). After thresholding, each astrocyte was

skeletonized using the Skeletonize plugin in Fiji. A two-dimensional Sholl analysis was performed using the Neuroanatomy Sholl plugin[47] in Fiji, overlaying circles of increasing diameter (5µm step size) starting from the astrocyte soma. The number of intersections of the astrocyte with each circle was plotted against radial distance from the soma to assess morphology, with a higher number of intersections indicating a ramified phenotype and a lower number indicating elongated astrocytes. Astrocytic complexity was quantified as the area under the Sholl curve.

Colocalization. Colocalization of GLT-1 or GS with GFAP signal was analyzed using the JaCoP plug-in in Fiji after manually adjusting the threshold.

Biochemical analyses

Ipsilateral pericontusional cortex and thalamus were collected 21d after TBI, snap-frozen, and processed for proteins extraction in 10% weight/volume of 1X RIPA buffer containing 0.1% SDS (BioRad Laboratories, USA), protease inhibitors (Roche, Mannheim, Germany), and phosphatase inhibitors (Roche, Mannheim, Germany). Samples were lysed on ice with an automatic pestle and transferred to a rotating shaker for 90 minutes at 4°C and centrifuged for 10min at 10'000g at 4°C; pellets (insoluble fraction) were quantified using JESS Simple Western™ (ProteinSimple®, Bio-Techne, Minneapolis, MN, USA). Protein dosage performed using BCA assay (ThermoFisher), as per manufacturer's instructions.

JESS Simple Western™ was run as per manufacturer's instructions. The primary anti-GFAP antibody (1:20 BD Pharmigen, USA), and the secondary goat-anti-mouse horse-radish peroxidase antibody and enhanced chemiluminescence reagents were used as per kit's instructions. The Ab diluent, washing buffer, plates and capillary cartridges used were derived from the 12–230kDa separation module (ProteinSimple®, Bio-Techne).

Dot blot. Proteins (3µg) were loaded directly onto nitrocellulose Trans-blot transfer membranes (0.45µm; Bio-Rad), depositing each sample on the membrane by vacuum filtration, as previously described[48]. Dot blot membranes were blocked with 3% (w/v) BSA (Sigma-Aldrich) and 0.1% (v/v) Tween 20 in Tris-buffered saline, pH 7.5, and incubated with rabbit polyclonal anti-ubiquitin primary antibody (1:1000, Abcam, Cambridge, UK) and then with anti-rabbit peroxidase-conjugated secondary antibodies (1:10000, GE Healthcare). Blots were developed with the Luminata Forte Western Chemiluminescent HRP Substrate (Millipore) on the ChemiDoc™ Imaging System (Bio-Rad). Densitometric analysis was done with Image Lab 6.0 software (Bio-Rad). Immune reactivity was normalized to Ponceau Red staining (Fluka).

MRI analysis in patients

MRI acquisitions were performed in the chronic stage after TBI. MRI data were analyzed as previously described[49], with full protocols available at <https://www.center-tbi.eu/project/mri-study-protocols>.

Structural T1w and diffusion MRI. Structural and diffusion MRI data were acquired across multiple 3T scanners using harmonized CENTER-TBI protocols. Sequences were processed on a TBI-specific

pipeline optimized for trauma-related anatomical distortions[50]. The 138 anatomical regions were collapsed into 27 ROIs, normalized on whole brain volume.

Diffusion weighted images were corrected for imaging artefacts and registered to the JHU ICBM-DTI-81 atlas[51] using ANTS[52] to obtain FA and MD values for all 48 individual white matter tracts. Extremely small and potentially inaccurate tracts were manually removed leaving a total of 40 individual white matter tracts.

Diffusion data was harmonized across sites using the Combat[53, 54]. To preserve true biological variance, age, sex, time since injury, GCS-M, and the presence/absence of any reported traumatic MR abnormality were used as covariates; age-correction of individual white matter tracts was performed based on an established detrending method[55]. Patients' demographics are reported in **Suppl. Table 1**.

Resting-state fMRI. Data were available for 121 participants across 12 sites (**Suppl. Table 2**). Structural T1w and fMRI data were pre-processed using fMRIPrep[56] (v24.1.1; see Suppl. Materials). Post-processing included confound regression (six motion parameters and derivatives[57]), lesion-aware WM signal, CSF signal, DCT high-pass filter at 0.008 Hz) and spatial smoothing (6 mm FWHM). Volumes exceeding frame-wise displacement (FD) > 0.5 mm or std-DVARS > 1.5 were censored; subjects with fewer than 96 clean volumes were excluded[58]. Of 121 subjects, 28 were excluded (19 data sufficiency, 9 QC failures). Mean FD was retained as a group-level covariate. Thalamic nuclei were delineated using the Najdenovska probabilistic atlas[59], as previously used in CENTER-TBI data[60], yielding 16 bilateral ROIs (seven nuclei per hemisphere plus whole-thalamus), resampled to native BOLD space (Nilearn v0.10.4)[61]. Three FC measures were extracted: (i) within-thalamus FC (120 nuclear pairs); (ii) thalamocortical FC using the Schaefer 2018 atlas (100 parcels, Yeo 7-network[62, 63]) and (iii) corticocortical FC between three composite ROIs: MTL (bilateral temporal pole and anterior temporal default-mode parcels), ACC (bilateral salience/ventral attention medial and cingulo-opercular parcels), and posterior parietal cortex (bilateral dorsal attention posterior, default-mode parietal, and frontoparietal parietal parcels). Analyses targeted pulvinar connectivity (primary a priori ROI)[64, 65], other epilepsy-relevant nuclei (anterior, mediodorsal, centromedian)[64, 66, 67], and corticocortical connectivity[68, 69]. To restrict analyses to the chronic phase, PTE(-) participants scanned within 150 days of injury were excluded (n = 7), yielding a final sample of 86 participants (65 HC, 14 PTE(-), 7 PTE(+); **Suppl. Table 3**). FC features were harmonised using NeuroCombat[54] (Suppl. Material, **Suppl. Table 4**).

Histopathological analysis of human brain donors

Twenty-three donors with a history of TBI were included, comprising 12 PTE(-) and 11 PTE(+) cases. Thirteen control cases were obtained from uninjured patients without previous history of TBI or neurological disease (some previously published[70]). Demographics are reported in **Suppl. Table 5**. FFPE sections (20µm) from the orbitofrontal cortex (OFC) and thalamus were immunostained for IBA-1 (1:1000, Wako) and GFAP (1:1000, DAKO) using standardized automated protocols. Quantification of GFAP- and IBA-1-positive area within grey matter regions was performed using Halo image analysis

software (Indica Labs), using the Area Quantification module to quantify the percent area of staining in the grey matter. For astrocytic morphological analyses, GFAP immunofluorescence was performed in a subset of OFC and thalamic sections (n = 4 PTE(-), n = 6 PTE(+), n = 10 controls) as previously described[70]. Confocal z-stack images were reconstructed in Imaris and astrocytic cytoskeletal complexity quantified by Sholl analysis.

Statistics

One-way analysis of variance (ANOVA) was used to compare histological structural MRI, DTI and biochemical data across the three groups, followed by Tukey post-hoc test or post-hoc tests controlling for the false discovery rate. Mixed-model effect was used to compare longitudinal metabolite changes evaluated by ¹H-MRS followed by Tukey post-hoc test.

Sholl analysis data was quantified by comparing the area under the curve (AUC) obtained from the number of intersections plotted against the distance from soma of each group, and then AUCs were compared by one-way ANOVA. Human Sholl data plots were compared using repeated measures two-way ANOVA. For human imaging data, univariable analyses were performed using Mann-Whitney U tests, and logistic regression for multivariable analysis. Group differences in thalamic FC were examined using linear mixed models (hemisphere as repeated within-subject factor; subject as random intercept). Two contrasts were tested: HC versus all TBI patients (TBI-related changes) and PTE(-) versus PTE(+). Effect sizes are reported as Cohen's d; multiple comparisons were corrected using Benjamini-Hochberg FDR[71], applied separately according to each analysis family. All analyses were conducted in Python.

The choice of parametric or nonparametric tests was based on the Shapiro–Wilk normality test, and the data distribution was inspected using a QQ plot. For each experiment, the figure legend reports the statistical analysis of the data. Outliers were identified using the ROUT method and excluded from analysis. Differences between groups were reported as statistically significant for $p < 0.05$. Mice omitted from data analysis are described in the figure legends, where appropriate. Statistical analyses were done with GraphPad Prism 9 (GraphPad Software, USA), using absolute values.

Results

Thalamic atrophy and optic tract injury distinguish mice that develop post-traumatic epilepsy

We first investigated whether specific brain regions are more susceptible to damage in PTE mice by analyzing structural MRI and DTI changes at 4m after TBI in *Cohorts 1 and 2* with 43% and 47% PTE incidence, respectively (daily seizure frequency 0.24 ± 0.12 , average seizures duration 59.7 ± 27.6 s) (Fig. 1A,B). High-resolution structural 3D MRI (ROIs in Fig. 1C) revealed no differences in lesion volumes between NoSRS and PTE mice (Fig. 1D). Significant atrophy was detected in the ipsilateral cortex (il-ctx), thalamus (il-thal), and striatum (il-str), and contralateral cortex (cl-ctx) in NoSRS compared to sham mice ($p < 0.01$, $p < 0.001$, $p < 0.01$, $p < 0.05$ respectively; Fig. 1E,F and **Suppl. Figure 3A-C**), indicating a clear

injury effect. Atrophy was more pronounced and widespread in PTE mice, which exhibited significant reductions in volume compared to sham in the il-ctx, il-thal, cl-ctx, and cl-thal ($p < 0.001$, $p < 0.001$, $p < 0.001$, $p < 0.01$, respectively; **1E,F** and **Suppl. Figure 3A-C**). Notably, the only area with a statistically significant difference between PTE and NoSRS was the il-thal with a further reduction of volume in PTE mice ($-16 \pm 10\%$, $p < 0.05$ vs NoSRS; Fig. 1F), identifying disproportionate thalamic atrophy as a structural feature associated with PTE rather than TBI alone. We then longitudinally assessed white matter damage at 3w and 4m (*Cohort 1*) and found that while no changes between PTE and NoSRS were present at 3w, by 4m the FA of the ipsilateral optic tract (il-OT) was significantly reduced in PTE compared to NoSRS mice (**Suppl. Figure 4**). When combining DTI analyses at 4m of *Cohorts 1 and 2*, we found more widespread white matter involvement in PTE than NoSRS mice compared to sham (Fig. 1G and **Suppl. Figure 4**) and confirmed the disproportionate white matter damage to the il-OT of PTE mice, with FA values discriminating with high accuracy PTE from NoSRS (Fig. 1H).

We then quantified DTI changes within the gray matter, to investigate microstructural alterations potentially linked to gliosis[72] (Fig. 1I-K and **Suppl. Figure 5**). Both NoSRS and PTE mice showed a similar increase in FA and AD in the il-ctx compared to sham (FA: NoSRS vs sham $p < 0.01$, PTE vs sham $p < 0.001$; AD: both $p < 0.05$ vs sham, Fig. 1J). In the il-thal, however, increased FA and AD were observed exclusively in PTE mice (both $p < 0.05$ vs sham, Fig. 1K). No differences between groups were observed in the contralateral side, aside from increased FA in the cortex (both $p < 0.05$ vs sham, **Suppl. Figure 5**).

Thalamic and posterior thalamic white-matter abnormalities distinguish PTE in patients

Having demonstrated a reduction in thalamic volumes and lower FA in the white matter tracts in mice with PTE, we next sought to recapitulate these findings in a human cohort. As in the mouse model, patients who developed PTE showed significantly reduced thalamic volumes compared to those who did not ($p < 0.01$; Fig. 2A). In a data-driven analysis of 27 brain regions, the thalamus showed the largest volume reduction between the two groups (Fig. 2B). We also mirrored the DTI findings: FA in the posterior thalamic radiation, a corresponding posterior visual-thalamic pathway, was markedly reduced in patients who developed PTE ($p < 0.001$; Fig. 2C). This tract showed the most significant DTI difference between PTE(+) and PTE(-) patients, in the context of global FA reduction throughout the white matter (Fig. 2D). Because patients who developed PTE on average had more severe injury and were imaged later than PTE(-) (~ 16 days), we evaluated whether these factors could account for the associations. Logistic regression models incorporating radiological metrics alongside clinical variables significantly outperformed models using clinical variables alone, with thalamic volume and posterior thalamic radiation FA adding explanatory value ($p = 0.01$; Fig. 2E).

Disrupted pulvinar thalamocortical connectivity identifies network dysfunction in PTE patients

FC analyses were conducted across the three levels, including within-thalamus nuclear pairs, thalamocortical seed-to-cortex connections, and corticocortical ROI pairs and are summarised below.

Within-thalamus FC. In the HC vs TBI(all) contrast, bilateral Pulvinar disconnection was observed for Pulvinar↔Anterior ($q = 0.003$; $d_L = -0.48$, $d_R = -0.59$) and Pulvinar↔mDorsal ($q = 0.020$; $d_L = -0.47$, $d_R = -0.64$). Exploratory analysis additionally identified Pulvinar↔vIVentral ($q = 0.023$; $d_R = -0.43$) and an asymmetric Pulvinar↔vAnterior interaction ($q = 0.014$; $d_L = +0.21$, $d_R = -0.49$). In the PTE(-) vs PTE(+) contrast, PTE(+)-specific Pulvinar disconnection was observed for Pulvinar↔mDorsal ($q = 0.031$) and Pulvinar↔Central ($q = 0.023$; $d_L = -0.76$, $d_R = -0.60$) in the hypothesis-driven analysis. Exploratory analyses extended this to Pulvinar↔vIDorsal ($q = 0.023$), Pulvinar↔vAnterior ($q = 0.020$; $d_L = -1.01$), the interhemispheric Pulvinar connection ($q = 0.005$; $d = -1.02$), mDorsal↔vIDorsal ($q = 0.032$; $d_L = -1.25$), and Central↔vIDorsal ($q = 0.032$; $d_L = -0.75$). Hypothesis-driven pairs among mDorsal, Anterior, and Central were null in both contrasts. Full within-thalamus results are shown in **Suppl. Table 6**.

Thalamocortical FC. No hypothesis-driven seed×ROI pairs survived FDR correction in either contrast (**Suppl. Table 7**). At the individual parcel level, exploratory L-Pulvinar analysis in the PTE(-) vs PTE(+) contrast identified 9 FDR-significant parcels (Visual network: Vis_1–8; Default network: Default_Temp_2, Default_pCunPCC_1; $d = -0.25$ to -1.62), indicating PTE(+)-specific reduction in L-Pulvinar connectivity with visual and posterior default-mode cortex (Fig. 3). The full results are reported in **Suppl. Table 7**.

Corticocortical FC. No corticocortical pairs (MTL↔ACC, MTL↔PostParietal, ACC↔PostParietal) survived FDR correction in either contrast (**Suppl. Table 8**).

All effects reflect reduced thalamocortical FC in PTE(+) relative to PTE(-), predominantly within visual and default mode network regions.

Persistent thalamic astrocytic-neuronal metabolites are altered in PTE mice

To probe the functional significance of thalamic involvement in PTE, we quantified metabolic changes using ^1H -MRS (ROIs in Fig. 4A, *Cohort 2*). At 3w post-injury, TBI induced a marked increase in the astrocyte-specific metabolite myo-inositol (mIns, $p < 0.001$), reductions in N-acetylaspartate (NAA, $p < 0.01$), and NAA/mIns ($p < 0.01$) and a trend towards elevated lactate ($p = 0.06$) compared to sham mice (Fig. 4B,E,F, **Suppl. Figure 6**). A subset of these abnormalities persisted selectively in PTE mice: mIns remained elevated at 3m ($p < 0.05$; Fig. 4B,C) discriminating PTE from NoSRS animals with high accuracy (AUC = 0.86, $p < 0.01$; 95% CI 0.7071–1.000; Fig. 4C **inset**). PTE mice also exhibited increased glutamate+glutamine (Glx, $p < 0.05$) and taurine (Tau, $p < 0.05$) at 3m (Fig. 4D, **Suppl. Figure 6**). In contrast, NoSRS mice showed partial normalization of the NAA/mIns ratio, yielding a trend toward divergence from PTE by 3m ($p = 0.07$; Fig. 4F). Metabolite–metabolite correlations further supported astrocytic dysregulation: mIns, and taurine (astrocytic osmolytes), Glx (astrocytic functionality), and NAA (neuronal marker)[73, 74] were significantly intercorrelated in PTE mice (all $p < 0.05$), whereas, glial-metabolite coupling in NoSRS mice was weaker (**Suppl. Figure 6**). Together, these data indicate persistent astrocytic metabolic disruption and impaired neuron-glia homeostasis within the il-thal of PTE mice.

Astrocytic reactivity is increased in the cortex and thalamus of PTE mice

Given the metabolic astrocytic alterations in PTE, we next assessed astrocyte reactivity histologically in biobanked sagittal sections from sham (n = 7) NoSRS (n = 9) and PTE mice (n = 9) from our prior study[28] (*Cohort 3*, ROIs in Fig. 5A). At 5m post TBI, PTE mice exhibited robust astrocytic activation, with a 2-fold increase in GFAP stained area ($p < 0.05$ vs NoSRS) and higher GFAP+ cell count (+ 100% in il-ctx, + 30% in il-thal; $p < 0.05$ vs NoSRS; Fig. 5B,C). Microglial/macrophage activation did not differ between groups (IBA-1; **Suppl. Figure 7**), indicating a predominantly astrocytic reactivity. To probe structural remodeling, we quantified astrocytic cytoskeletal complexity using Sholl analysis. In the cortex, both NoSRS (-42%; $p < 0.001$) and PTE mice (-53%; $p < 0.001$) showed reduced AUC relative to sham, with PTE mice displaying a modest but significant additional decrement (-18%; $p < 0.05$; Fig. 5D). Thalamic effects were more pronounced: NoSRS mice showed only mild reductions (-17%; $p < 0.05$ vs sham), whereas PTE mice exhibited marked loss of cytoskeletal complexity (AUC: -58% vs sham; -50% vs NoSRS; $p < 0.001$; Fig. 5E). Thalamic astrocytes in PTE mice also showed polarized and spatially coherent orientation (**Suppl. Figure 8**), consistent with increased FA and AD in the ipsilateral thalamus of PTE animals (Fig. 1).

Levels of astrocytic proteins involved in glutamate homeostasis are reduced in the thalamus of PTE mice

To determine whether the reactive, dysmorphic astrocytes in PTE mice also exhibit functional impairment, we performed double immunofluorescence for GFAP and key astrocytic markers involved in glutamate homeostasis: glutamate transporter 1 (GLT-1) and glutamine synthetase (GS). In the il-ctx, colocalization of GFAP with GLT-1 and GFAP with GS was similar in the sham, NoSRS and PTE groups. In contrast, thalamic astrocytes of PTE mice showed a significant reduction in both GLT-1 (-30%, $p < 0.05$ vs sham) and GS (-45%, $p < 0.05$ vs sham) (Fig. 6A,B). These region-specific deficits indicate that thalamic astrocytes in PTE lose key components of glutamate clearance and conversion pathways, in line with the increase in Glx/Cr observed by ^1H -MRS, supporting the presence of a locally excitotoxic microenvironment and linking astrocytic dysfunction to thalamic vulnerability in epileptogenesis.

Astrocytic reactivity and morphological simplification distinguish PTE in human brain donors

To evaluate whether neuroinflammation is a clinically relevant feature of PTE in patients, we quantified GFAP and IBA-1% stained area in orbitofrontal cortex (OFC) and thalamus (Fig. 7A,B) from brain donor tissues (Ctl n = 13; PTE- n = 12, PTE+ n = 11; key demographics, TBI characteristics and other histopathological features of the donors are in **Suppl. Table 5**). Consistent with our murine data, microglial/macrophage activation did not differentiate PTE+ from PTE- donors: IBA-1 stained area in the thalamus was comparable across groups, and although both TBI groups showed elevated IBA-1 in the

OFC, levels did not differ between PTE⁻ and PTE⁺ donors (**Suppl. Figure 9**). Thus, chronic microglial reactivity is unlikely to constitute a pathological hallmark of PTE.

In contrast, astrocytic reactivity was markedly elevated only in PTE⁺ donors. GFAP-positive area increased ~ 2.5-fold in the OFC and ~ 2-fold in the thalamus relative to controls (both $p < 0.01$; Fig. 7C), identifying selective and region-consistent astrocytosis as a distinguishing feature of clinical PTE. Morphological analysis further revealed significantly reduced astrocytic complexity in both regions exclusively in PTE⁺ donors ($p < 0.001$, Fig. 7D). These findings demonstrate that astrocytic reactivity, and not microgliosis, is a conserved, cross-species hallmark of PTE, underscoring the translational relevance of astrocyte-centered mechanisms in post-traumatic epileptogenesis.

Decreased cytoskeletal complexity and insoluble GFAP accumulation precede spontaneous seizures in mice at high risk of PTE

Having confirmed that astrocytic pathology and thalamic involvement are hallmarks of established PTE, we next asked whether these alterations emerge before the onset of spontaneous recurrent seizures. We leveraged our previously validated ECoG biomarker of spiking activity, which stratifies mice into low- or high-risk for developing PTE as early as 1w post-TBI[28] (Fig. 8A,B; *Cohort 4*). Nineteen mice were classified as low-risk and 15 as high-risk based on spiking activity measured at 1w post-injury in the contralateral posterior electrode. Of those, 21 were allocated to biochemical analysis and 13 to histology (Fig. 8A). At 21d post TBI, both low- and high-risk mice showed increased astrocytic activation relative to sham in il-ctx and il-thal ($p < 0.001$ for both areas), but no differences in GFAP positive area between risk groups were detected (Fig. 8C,D). However, Sholl analysis revealed a clear divergence in thalamic astrocyte morphology: cytoskeletal complexity was reduced in low-risk (AUC: -25%; $p < 0.01$) and further diminished in high-risk PTE mice (-50%; $p < 0.001$) compared to sham, with a significant decrease in high-versus low-risk PTE mice (-20%; $p < 0.05$, Fig. 8E). Thus, morphological simplification, but not bulk GFAP area, distinguishes mice that later develop PTE.

Biochemical analysis corroborated these early structural changes. Analyses of the insoluble fraction showed increased GFAP levels both in the il-ctx (+ 30%, $p < 0.05$) and in the il-thal (+ 100%, $p < 0.05$) of high-risk compared to low-risk PTE mice (Fig. 8F,G, **Suppl. Figure 10**). Notably, insoluble GFAP in the il-thal correlated strongly with early spiking activity ($R^2 = 0.43$, $p < 0.01$), whereas cortical levels did not ($R^2=0.12$, $p > 0.05$). GFAP accumulation in the insoluble fraction suggests this cytoskeletal protein undergoes aggregation, thus positioning thalamic GFAP aggregation as an early marker of post-traumatic epileptogenic progression. Supporting an aggregation-prone state, thalamic tissue from high-risk mice also showed elevated ubiquitin levels (+ 140%, $p < 0.01$ vs low-risk), consistent with impaired proteostasis[32]. Together, these findings demonstrate that thalamic astrocytic structural remodeling and accumulation of insoluble GFAP are already present during the latent period in mice prospectively identified as being at high risk of PTE. Their emergence before spontaneous seizure onset argues against these abnormalities being solely a consequence of recurrent seizures.

Discussion

PTE remains one of the most disabling long-term consequences of TBI, yet the pathological processes that distinguish injuries progressing to epilepsy remain poorly understood. By integrating experimental models, multimodal neuroimaging, and human tissue analyses, we show that (i) thalamic atrophy and disruption of thalamic projection pathways are associated with PTE, (ii) thalamocortical functional connectivity is reduced in patients with PTE, (iii) thalamic astrocytes display persistent reactivity with reduced cytoskeletal complexity, (iv) astrocytic proteins involved in glutamate homeostasis are selectively reduced in the thalamus, and (v) morphological simplification and insoluble GFAP accumulation emerge before spontaneous seizures in high-risk PTE mice. Together, these findings identify the thalamus as a site of pathological convergence in PTE and establish astrocytic remodeling as an early component of the disease trajectory rather than simply a consequence of recurrent seizures.

A key finding of this work is the identification of the thalamus as a vulnerable node in post-traumatic epileptogenesis. Lesion size alone did not distinguish PTE from NoSRS mice, consistent with prior work[11] indicating that gross injury burden does not necessarily determine epileptogenesis. DTI further demonstrated pronounced microstructural disruption within the optic tract and posterior thalamic radiation, pathways that traverse thalamic nuclei and link the thalamus to cortical sensory regions. This visual pathway is known to be vulnerable following TBI in both experimental models[10, 75–77] and patients[78]. Notably, the optic tract passes through thalamic regions and projects to the visual cortex via the optic radiation. After the chiasm, the fibers continue as optic tracts that reach the lateral geniculate nucleus, a key thalamic relay. Although some clinical reports have suggested a link between visual tract damage and epilepsy[79], this association has not been systematically evaluated in the context of PTE. Here, using multivariable models, we show that incorporating thalamic volume and posterior thalamic radiation FA significantly improves PTE classification beyond clinical covariates, underscoring their translational potential as imaging markers of PTE-associated pathology.

Pulvinar-centered thalamocortical functional disconnection further supports the concept that PTE is a network disorder involving disruption of large-scale corticothalamic communication. We identified reduced pulvinar connectivity predominantly within visual and posterior default mode network regions in PTE patients, together with impaired within-thalamus connectivity between pulvinar and mediodorsal/central nuclei. The pulvinar is increasingly recognized as a critical hub for synchronization of distributed cortical activity and seizure propagation in focal epilepsy[64, 80–82]. The preferential involvement of pulvinar-centered networks is particularly notable given the role of the pulvinar in coordinating long-range corticothalamic synchronization and integrating attentional and sensory processing across distributed cortical networks[65]. The convergence of structural abnormalities in posterior thalamic pathways with altered pulvinar-centered connectivity therefore suggests coordinated structural-functional disruption of thalamocortical circuits in PTE. Given the relatively small PTE(+) resting-state fMRI sample and the exploratory nature of the parcel-level analyses, these findings require validation in larger cohorts. Nevertheless, they extend the cross-species structural observations by identifying a corresponding network-level phenotype in human PTE.

Thalamic pathology in experimental TBI includes progressive atrophy, blood-brain barrier disruption, and neuroinflammation[83–85]. In patients, persistent thalamic inflammation has been associated with damage to thalamocortical white matter tracts, while experimental studies have shown that secondary thalamic astrocytic and microglial responses after focal cortical injury reflect the connectivity of the injured corticothalamic circuits[86, 87]. Previous clinical studies have reported acute thalamic damage as a prognostic biomarker of PTE[9, 10, 88]. Moreover, thalamic atrophy has been observed across several epilepsy syndromes[89], highlighting the importance of thalamocortical circuits in epileptogenesis. Given the central role of thalamo-cortical circuits in seizure generation and propagation[12, 13], our data support the thalamus as a key site of pathological convergence during post-traumatic epileptogenesis and a potentially measurable indicator of disease progression. Having identified the thalamus as a key locus of pathological changes, we probed metabolic alterations associated with experimental PTE using ¹H-MRS. We detected early thalamic metabolic changes in all TBI mice, including elevated mIns and reduced NAA, consistent with gliosis[90], neuronal dysfunction[73], and mitochondrial impairment[91]. Critically, only mice that developed PTE displayed persistent mIns and Glx increases, with sustained reductions in NAA. These signatures are consistent with astrocytic dysfunction[90], impaired glutamate metabolism[74], and progressive disruption of neuron–astrocyte homeostasis[73], processes that have been implicated in epileptogenesis[11, 92, 93]. Although constrained by a single-voxel approach, these findings highlight ¹H-MRS as a promising, non-invasive biomarker to monitor PTE associated metabolic alterations longitudinally and warrant further testing in human cohorts.

Astrocytic pathology was markedly increased in the thalamus and cortex of both PTE mice and patients. In contrast to the persistent astrocytic phenotype, IBA1-defined microglial reactivity did not distinguish PTE from non-PTE at the chronic time points examined. This does not exclude an earlier or functionally distinct contribution of microglia to epileptogenesis but identifies persistent astrocytic pathology as the glial feature most consistently associated with established PTE across species[94, 95]. These findings extend previous preclinical reports of cortical and hippocampal astrocytic reactivity in experimental PTE[24–27] and underscore the translational relevance of astrocytic responses.

In addition to increased astrocytic reactivity, astrocytes in both experimental and clinical PTE exhibited reduced cytoskeletal complexity, particularly in the thalamus. Previous work identified similar changes in hippocampal astrocytes in PTE mice[25] and epileptic rats[96]. Here we demonstrate that thalamic astrocytes undergo comparable structural deterioration in mice and humans, implicating morphological simplification as a conserved feature of PTE. Interestingly, DTI analyses revealed increased FA and AD selectively in the thalamus of PTE mice, likely reflecting more coherent but less branched astrocytic organization[97]. Whether gray matter DTI, or emerging MRI modalities[98], can be used to monitor gliosis in patients requires further exploration.

Reactive astrocytes can lose essential homeostatic functions or acquire maladaptive phenotypes[22], including impaired glutamate buffering, potassium clearance, and maintenance of blood-brain barrier integrity[99], each of which could contribute to network hyperexcitability and seizure susceptibility[23, 93,

100]. Consistent with this concept, we found that GLT-1 and GS, two key astrocytic regulators of glutamate uptake and metabolism, were selectively reduced in thalamic astrocytes of PTE mice. These region-specific deficits, together with increased Glx detected by ¹H-MRS, support disrupted astrocytic glutamate homeostasis in the thalamus. Together with the structural simplification observed in thalamic astrocytes, these findings support a model in which astrocytes adopt a dysfunctional phenotype that may contribute to epileptogenesis following TBI[24–26, 101].

Finally, by leveraging an acute ECoG biomarker of PTE risk[28], we demonstrate that astrocytic alterations arise before the onset of spontaneous seizures. High-risk PTE mice exhibited reduced astrocytic complexity, resembling the pathology observed in chronic PTE, suggesting that astrocytic structural impairment may contribute to epileptogenesis rather than result from seizure activity. Notably, we detected an accumulation of insoluble GFAP in high-risk mice, particularly within the thalamus suggesting aggregation of this cytoskeletal protein. GFAP aggregation and post-translational modification are well-documented injury responses with established neurotoxic potential[33, 102–105]. Pathogenic GFAP inclusion formation is characteristic of Alexander disease[32], a disease often associated with epilepsy, where astrocytes display impaired morphology, accumulation of ubiquitinated proteins, and disrupted glutamate regulation[32, 104, 105]. The parallels with our findings in high-risk PTE mice suggest that early proteostatic dysfunction and GFAP aggregation may contribute to astrocytic pathology during epileptogenesis. GFAP aggregation may reflect localized proteinopathy or a broader proteasomal deficit[32, 106], potentially linked to the reductions in mIns/NAA observed by ¹H-MRS. Whether this localized proteinopathy directly drives PTE or reflects broader astrocytic dysfunction remains to be determined. Characterizing the biochemical and structural properties of these aggregates and their role in epileptogenesis will be key to defining their mechanistic role in PTE and evaluating whether they represent viable therapeutic targets.

Limitations

Limitations should be considered. First, although prospective risk stratification establishes that astrocytic abnormalities precede spontaneous seizures, the observational nature of the study does not demonstrate that astrocytic remodeling is necessary or sufficient for PTE development. Direct manipulation of the identified astrocytic pathways will be required to establish causality. Second, IBA1-based analyses cannot exclude earlier, transient, or functionally distinct microglial contributions. Third, the experimental studies were conducted in male mice, and the generalizability of the findings across sexes remains to be determined. Finally, the human analyses are observational, and the relatively small PTE(+) resting-state fMRI sample requires replication of the functional connectivity findings in larger prospective cohorts.

Conclusions

In conclusion, our findings identify a conserved thalamocortical phenotype associated with PTE across experimental and human TBI. Structural and white-matter abnormalities, persistent metabolic disruption,

altered pulvinar-centered connectivity, and pronounced astrocytic remodeling converge on the thalamus, while prospective risk stratification demonstrates that key astrocytic abnormalities emerge during the latent period before spontaneous seizures. These findings distinguish PTE-associated astrocytic remodeling from generic post-injury astrogliosis and identify the thalamus as a candidate site of pathological convergence during post-traumatic epileptogenesis. The cross-species alignment of cellular pathology with clinically accessible imaging measures provides a translational framework for identifying and monitoring PTE-associated disease progression and for testing whether thalamocortical astrocytic dysfunction represents a modifiable component of epileptogenesis.

Abbreviations

¹H-MRS

proton magnetic resonance spectroscopy

ACC

anterior cingulate cortex

AD

axial diffusivity

ANOVA

analysis of variance

AUC

area under the curve

BOLD

blood-oxygen-level-dependent

CCI

controlled cortical impact

CD1

CD-1 mouse strain

CENTER-TBI

Collaborative European NeuroTrauma Effectiveness Research in Traumatic Brain Injury

Cr

creatine

CSF

cerebrospinal fluid

DTI

diffusion tensor imaging

ECoG

electrocorticography

EEG

electroencephalography

FA

fractional anisotropy
FC
functional connectivity
FDR
false discovery rate
FFPE
formalin-fixed, paraffin-embedded
FWHM
full width at half maximum
GCS-M
Glasgow Coma Scale motor score
GFAP
glial fibrillary acidic protein
GLT-1
glutamate transporter 1
Glx
glutamate plus glutamine
GS
glutamine synthetase
HC
healthy controls
IBA-1
ionized calcium-binding adaptor molecule 1
MD
mean diffusivity
MRI
magnetic resonance imaging
mIns
myo-inositol
NAA
N-acetylaspartate
NoSRS
no spontaneous recurrent seizures
OFC
orbitofrontal cortex
PTE
post-traumatic epilepsy
ROI
region of interest
TBI

traumatic brain injury
WM
white matter
Ctl
control
ctx
cortex
il-
ipsilateral
OT
optic tract
thal
thalamus
PTE+
TBI patients with PTE
PTE-
TBI patients without PTE
m
months
w
weeks
d
days
h
hours.

Declarations

Ethics approval and consent to participate

All animal procedures complied with Italian legislation (D.lgs 26/2014), EU Directive 2010/63/EU, and the NIH Guide for the Care and Use of Laboratory Animals (2011) and were approved by the Mario Negri Institute Animal Care and Use Committee and the Italian Ministry of Health (Decreto no.328/2019-PR). Human imaging analyses used data from the prospective multicenter CENTER-TBI study (ClinicalTrials.gov: NCT02210221). All participants or legal representatives provided informed consent according to local regulations. Clinical data were accessed through the Neurobot platform (RRID:SCR_017004). Human brain tissue was obtained through the Pacific Northwest Brain Donor Network (University of Washington) with informed consent from legal next of kin. All procedures were approved by the University of Washington School of Medicine Compliance Office.

Consent for publication

Not applicable

Competing interests

FM, IL, EN, EOL, RDS, SFC, AL, LL, EM, SH, KS, FK, EM, AV, VB, TR, LL, KKW, ALN, DKM, ERZ report no competing interests. WEH holds equity in Gryphon Bio and Owl Therapeutics. VFJN holds investigator led-grants with Abbot and Roche, unrelated to this project.

Supplementary material

Supplementary material is available online.

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Author Contribution

Author contributionsconceptualization: FM, IL, EOL, AV, TR, LL, KKW, WEH, ALN, DKM, ERZdata collection and analysis: FM, IL, EN, RDS, KC, SFC, AL, LL, EM, SH, KS, FK, EM, TR, VFJN, EAS, ALN, DKM, ERZfunding: EOL, LL, KKW, WEH, DKM, ERZmanuscript writing: FM, IL, EN, AV, VB, ALN, DKM, ERZ

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Data Availability

All preclinical data from this manuscript will be made available to reviewers upon request during the peer-review process and will be publicly available upon publication. Clinical data from the CENTER-TBI study are accessible with a study proposal submission on the CENTER-TBI website (<https://www.center-tbi.eu/data/study>).

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Figures

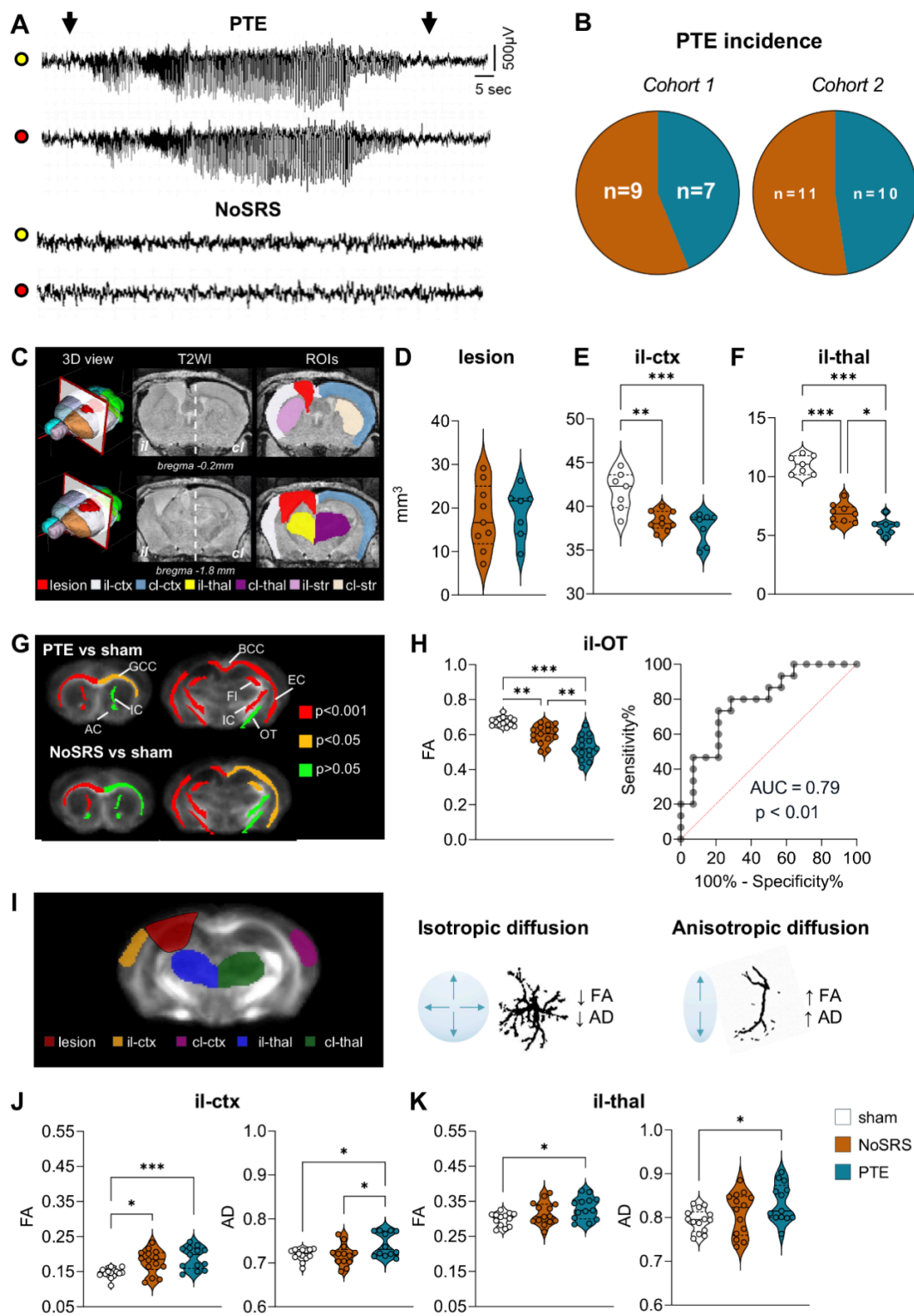


Figure 1

PTE mice display exacerbated thalamic atrophy, white matter damage, and gray-matter microstructural changes compatible with gliosis (A) Representative ECoG tracings of seizure activity from a PTE mouse 5m post-TBI and of physiological activity from a NoSRS mouse. (B) Percentages of NoSRS (orange) PTE (blue) mice, in Cohorts 1 & 2 at 5m post-TBI. (C-F) Gray matter T2WI, Cohort 1, 4m post-TBI (C) Schematic representation of the selected ROIs. (D-F) Volumetric analyses of the lesion, il-ctx and il-thal (G-H) White

matter DTI, *Cohorts 1 & 2*, 4m post-TBI. **(G)** Representative coronal section showing the areas with a significant reduction of FA in PTE and NoSRS mice compared to sham. **(H)** Violin plot depicting FA values in the il-OT in sham, NoSRS and PTE mice. ROC curve obtained by comparing FA values in the il-OT in PTE vs NoSRS mice. **(I-K)** Gray matter DTI, *Cohorts 1 & 2*, 4m post-TBI. **(I)** Schematic representation of the selected ROIs and of DTI gray matter metrics in relation to glial microstructural changes. **(J,K)** FA and AD measures in the **(J)** il-ctx **(K)** il-thal. Data are mean $\pm$ SEM. *** $p < 0.001$, ** $p < 0.01$, * $p < 0.05$, One-way ANOVA, Tukey post-hoc test.

AD: axial diffusivity; AC: anterior commissure; CC: corpus callosum; cl: contralateral; ctx: cortex; EC: external capsule; FA: fractional anisotropy; FI: fimbria of the hippocampus; IC: internal capsule; il: ipsilateral; NoSRS: no spontaneous recurrent seizures; OT: optic tract; thal: thalamus; PTE: post-traumatic epilepsy; ROI: region of interest; T2WI: T2-weighted imaging; TBI: traumatic brain injury

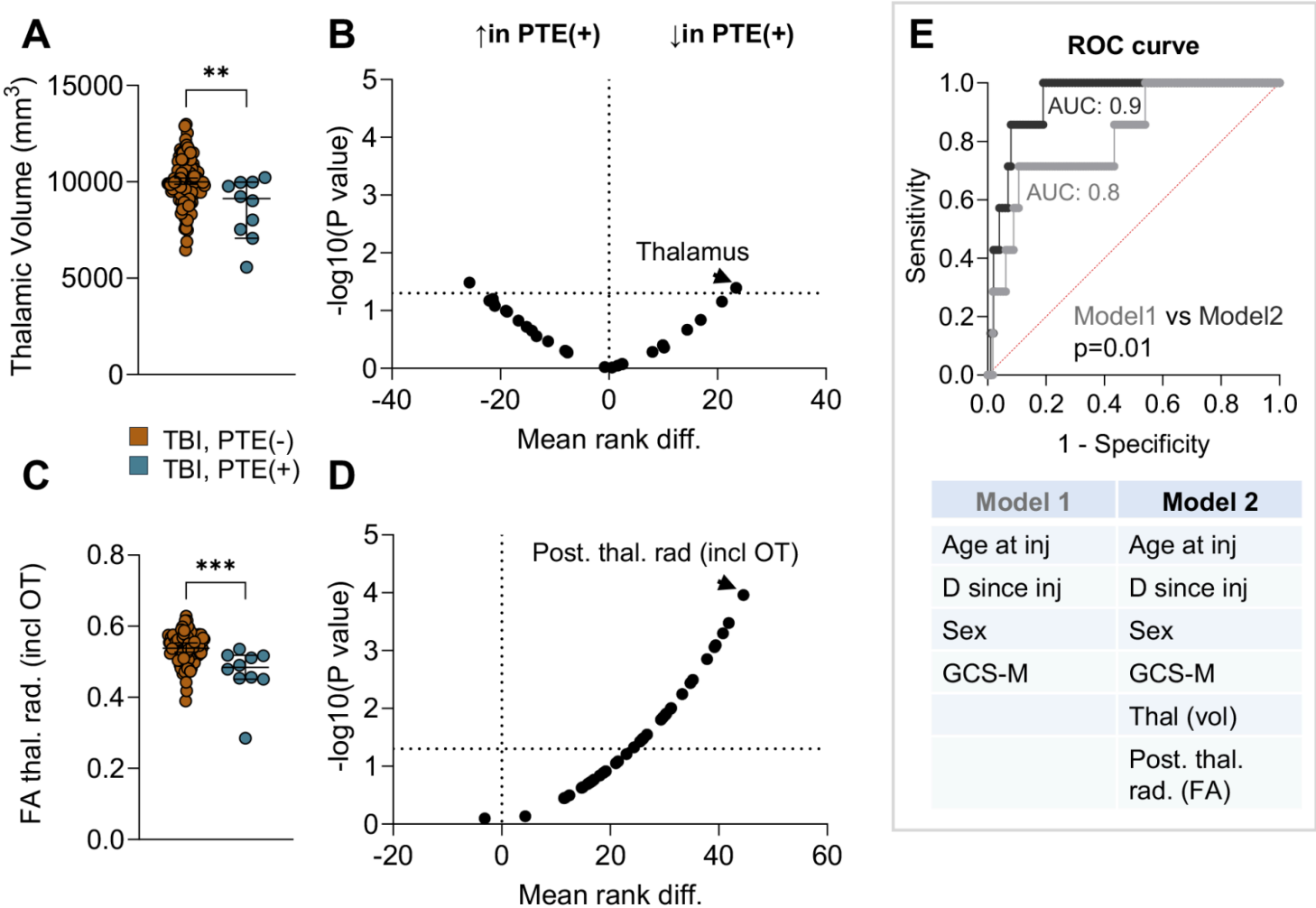


Figure 2

Pre-clinical imaging findings are recapitulated in human PTE patients. **(A)** Quantification of thalamic volume (mean of both thalami) in PTE(-) and PTE(+) Mann-Whitney test. **(B)** Volcano plot of differences in brain volume across 27 brain areas, highlighting the thalamus (black arrow) as the region with the

greatest reduction in TBI PTE+. Multiple Mann–Whitney tests. **(C)** Quantification of FA value in the thalamic radiation including the optic tract (mean of left and right tracts) in TBI PTE(-) and PTE(+), Mann-Whitney test. **(D)** Volcano plot of differences in mean FA valued across 40 brain areas, highlighting the thalamic radiation (black arrow) as the area with the most reduced FA in PTE(+). **(E)** Multiple logistic regression analysis comparing Model1(light grey) and Model2 (dark grey).

D: days; FA: fractional anisotropy; GCS-M: Glasgow coma scale, motor; incl: including inj: injury; mm: millimeter; OT: optic tract; ROC: receiver operating characteristic curve; PTE: post-traumatic epilepsy; post: posterior; thal: thalamus; TBI: traumatic brain injury; vol: volume.

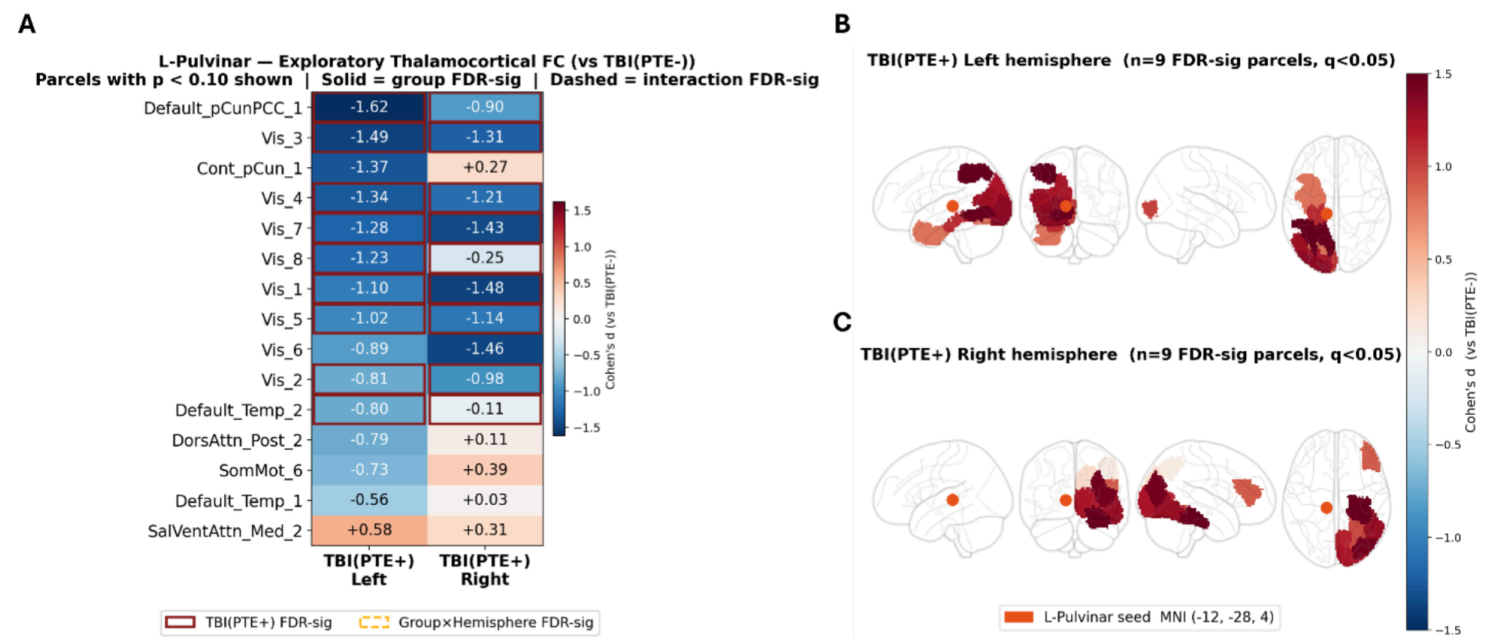


Figure 3

L-Pulvinar thalamocortical FC is reduced in PTE patients. **(A)** Heatmap showing Cohen's d effect sizes for L-Pulvinar seed-based FC across cortical parcels with $p < 0.10$, separately for left and right hemisphere FC estimates. Parcels with a solid red border survived group-level FDR correction ($q < 0.05$). **(B,C)** Glass brain projections of FDR-significant parcels ($q < 0.05$, $n = 9$) coloured by Cohen's d for the left **(B)** and right **(C)** hemisphere FC estimates. The orange sphere denotes the L-Pulvinar seed location (MNI: -12, -28, 4).

FC: functional connectivity; FDR: false discovery rate; MNI: montreal neurological institute; pCunPCC: precuneus/posterior cingulate cortex network; Temp: temporal cortex network; DorsAttn_post: posterior dorsal attention network; SomMot: somatomotor network; SalVentAttn_med: salience/ventral attention network; Vis: visual network; PTE: post-traumatic epilepsy.

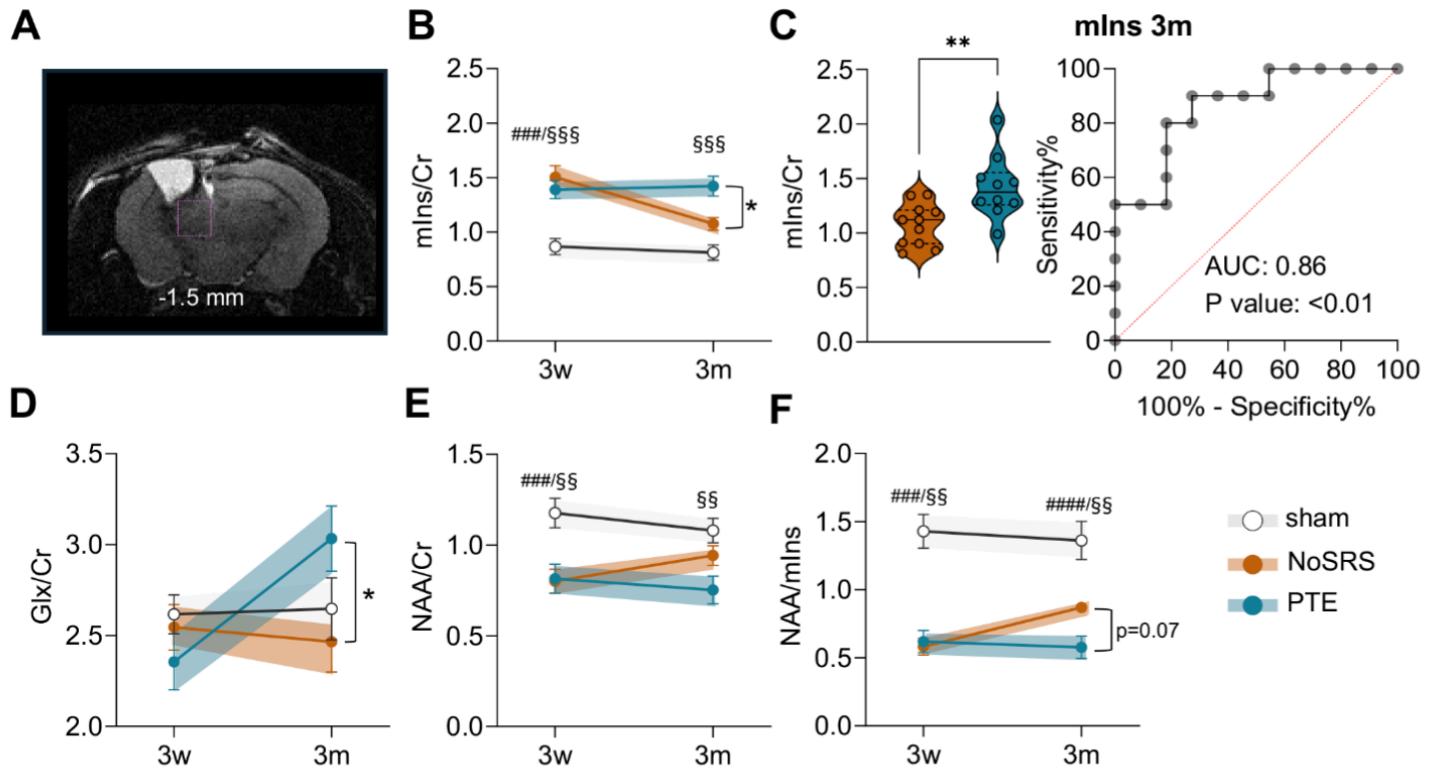


Figure 4

¹H MRS reveals alterations in thalamic neuron-astrocyte integrity in PTE mice. (A) Region of interest is indicated by the pink box; All ¹H MRS metabolites are expressed as ratio relative to creatine. Quantification of (B,C) mIns, (D) Glx, (E) NAA and (F) NAA/mIns levels by in vivo ¹H MRS at 3w and 3m post injury. Insert in panel (C) shows ROC analysis with mIns levels 3m after injury. Data are mean ± SEM. p<0.0001, ###p<0.001, #p<0.05 NoSRS vs sham, §§ p<0.01 PTE vs sham, *p<0.05 PTE vs NoSRS; Mixed model effect, Tukey post-hoc test

¹H MRS: one-proton magnetic resonance spectroscopy; AUC: area under the curve; Cr: creatinine; Glx: glutamate+glutamine; il: ipsilateral; m: months; mIns: myo-Inositol; mm: millimeters; NAA: N-acetyl aspartate; NoSRS: no spontaneous recurrent seizures; PTE: post-traumatic epilepsy; TBI: traumatic brain injury; thal: thalamus; w: weeks

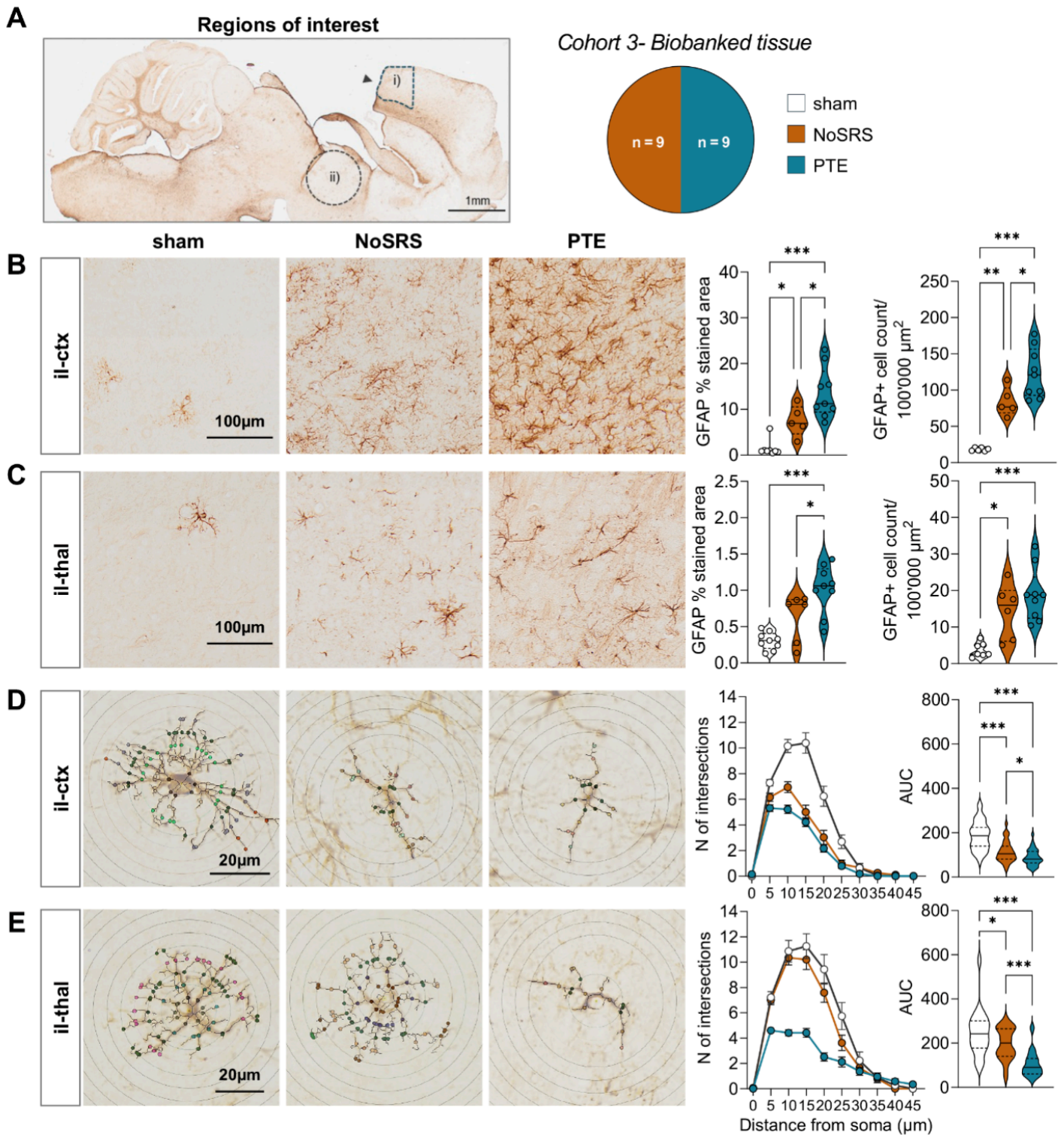


Figure 5

Astrocytic reactivity in the cortex and thalamus of PTE mice. (A) Schematic representation of the selected ROIs and PTE incidence. **(B-C)** Representative images, quantification of GFAP stained area and GFAP+ cell count in the il-ctx and il-thal 5m post-injury. **(D-E)** Representative images, quantification of astrocytes' cytoskeletal complexity by Sholl analysis and quantification of the AUC in the il-ctx and il-thal. Data are mean $\pm$ SEM. *** $p < 0.001$, ** $p < 0.01$, * $p < 0.05$ One-way ANOVA, Tukey post-hoc test.

AUC: area under the curve; ctx: cortex; GFAP: glial fibrillary acidic protein; il: ipsilateral; NoSRS: no spontaneous recurrent seizures; PTE: post-traumatic epilepsy; ROI: region of interest; thal: thalamus; TBI: traumatic brain injury.

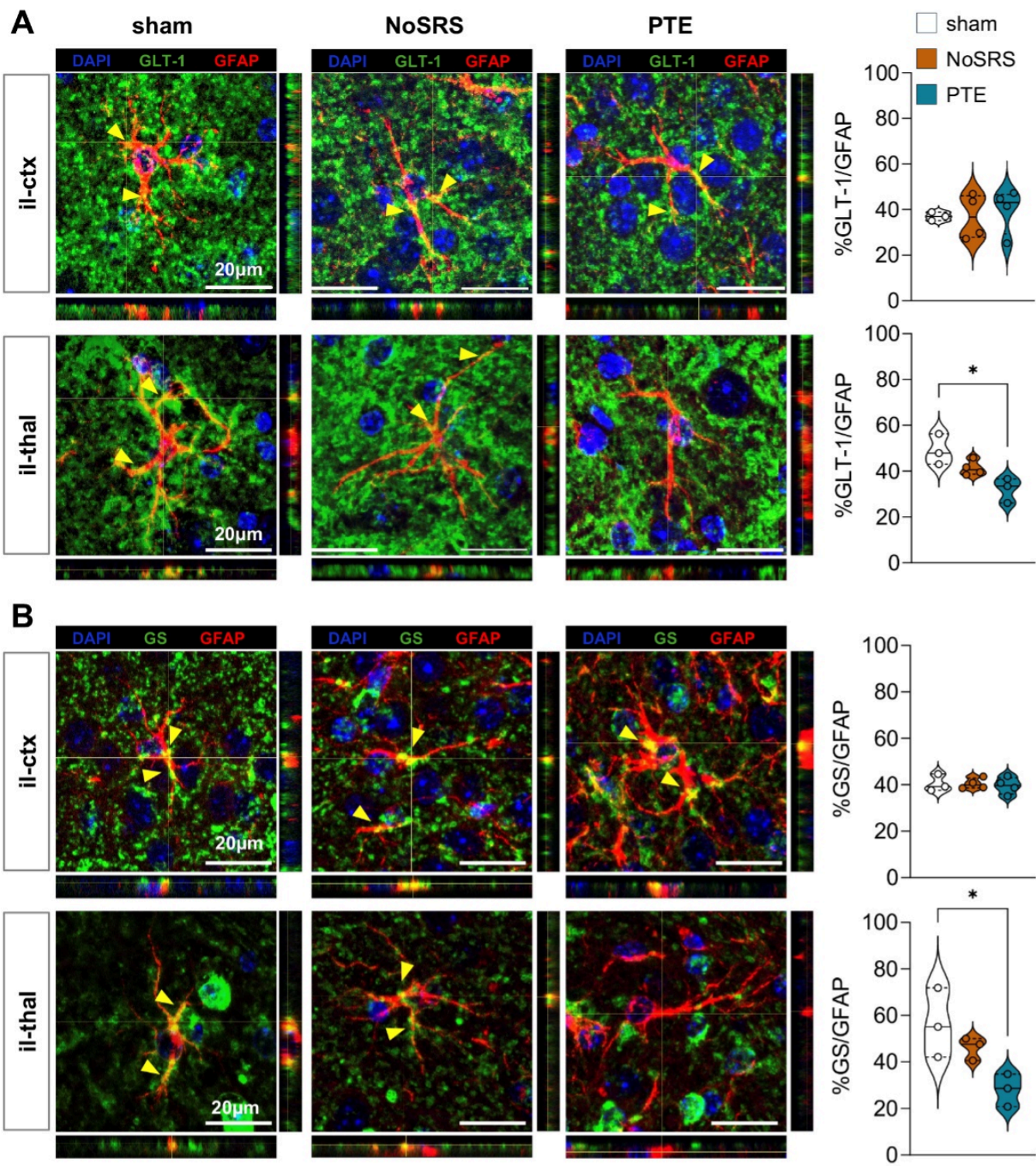


Figure 6

Astrocytic surface functional antigens. (A) IF staining of GLT-1 and GFAP shows comparable colocalization of GLT-1 and GFAP signal in the cortex (above) but significantly reduced GLT-1 colocalization in GFAP+ astrocytes in the thalamus of PTE mice only. **(B)** Similarly, colocalization of GS with GFAP remains consistent in the cortex but is markedly diminished in the thalamus of PTE mice. Data are mean $\pm$ SEM, * $p < 0.05$, one-way ANOVA, Tukey post hoc.

ctx: cortex; GFAP: glial fibrillary acidic protein; GLT-1: glutamate transporter-1; GS: glutamine synthetase; IF: immunofluorescence; il: ipsilateral; NoSRS: no spontaneous recurrent seizures; PTE: post-traumatic epilepsy; thal: thalamus.

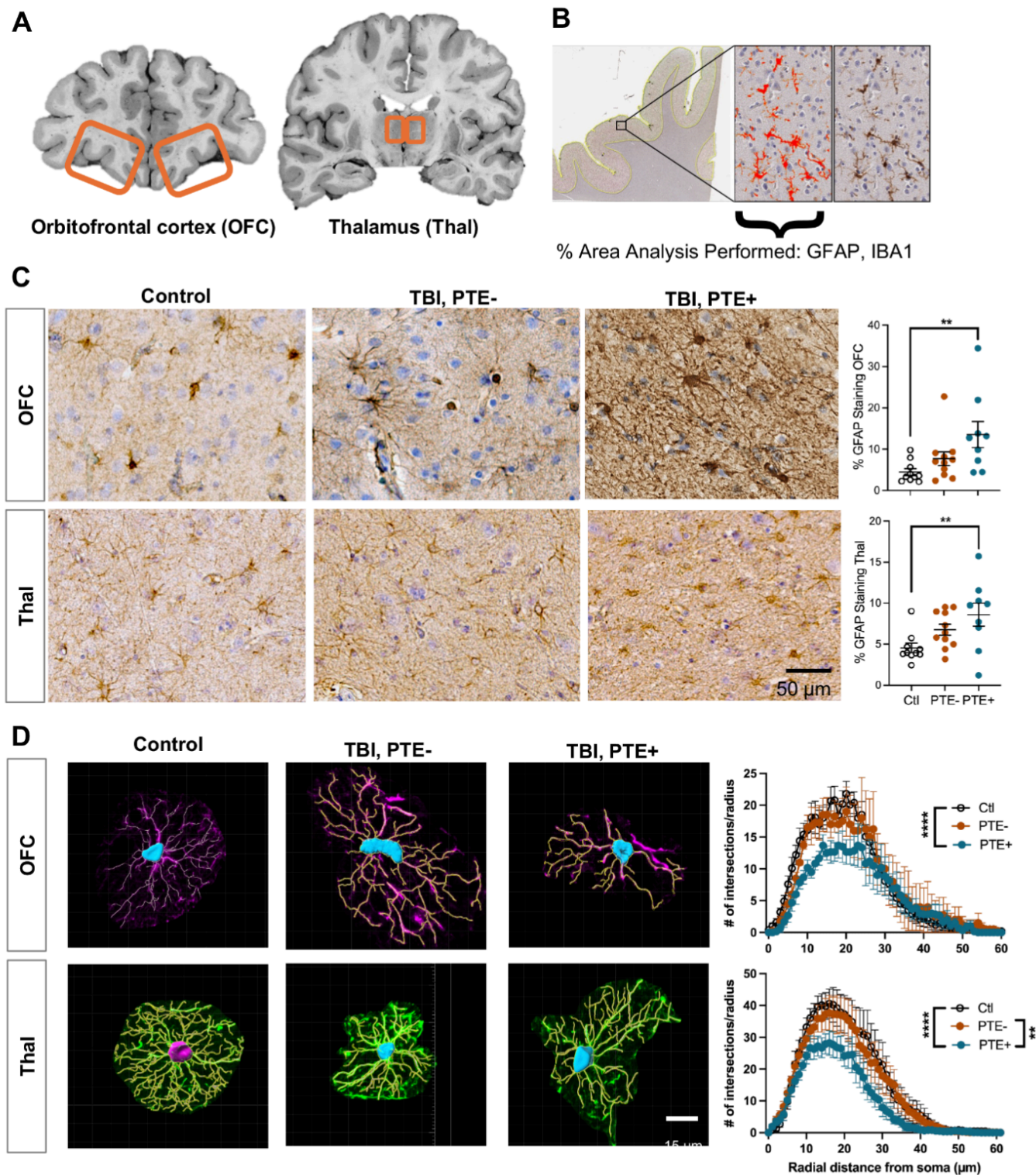


Figure 7

Astrocytic reactivity and decreased cytoskeletal complexity occur in human brain donors with PTE. (A) Schematic representation of the selected ROIs **(B)** Representative image demonstrating the Halo image analysis protocol to determine percentage area of staining (on IBA-1 stain) **(C)** Representative images and quantification for GFAP immunostains across Ctl, TBI PTE- and TBI PTE+ in the OFC (above) and Thal (below) ** $p < 0.01$, one-way ANOVA, post hoc test controlling for the false discovery rate

(D)Representative Imaris reconstruction of the soma (blue) and filaments (grey/yellow) and quantification of astrocytes cytoskeletal complexity by Sholl analysis across Ctl, PTE(-) and TBI PTE(+) in the OFC (above) and Thal (below) (OFC: ****p < 0.0001, distance x TBI effect, repeated measures two-way ANOVA, Ctl vs PTE(+); Thal: ****p < 0.0001, distance x TBI effect, repeated measures two-way ANOVA, Ctl vs PTE(+); Thal **p<0.01, for distance x PTE effect, repeated measures two-way ANOVA, PTE(-) vs PTE(+) conditions.

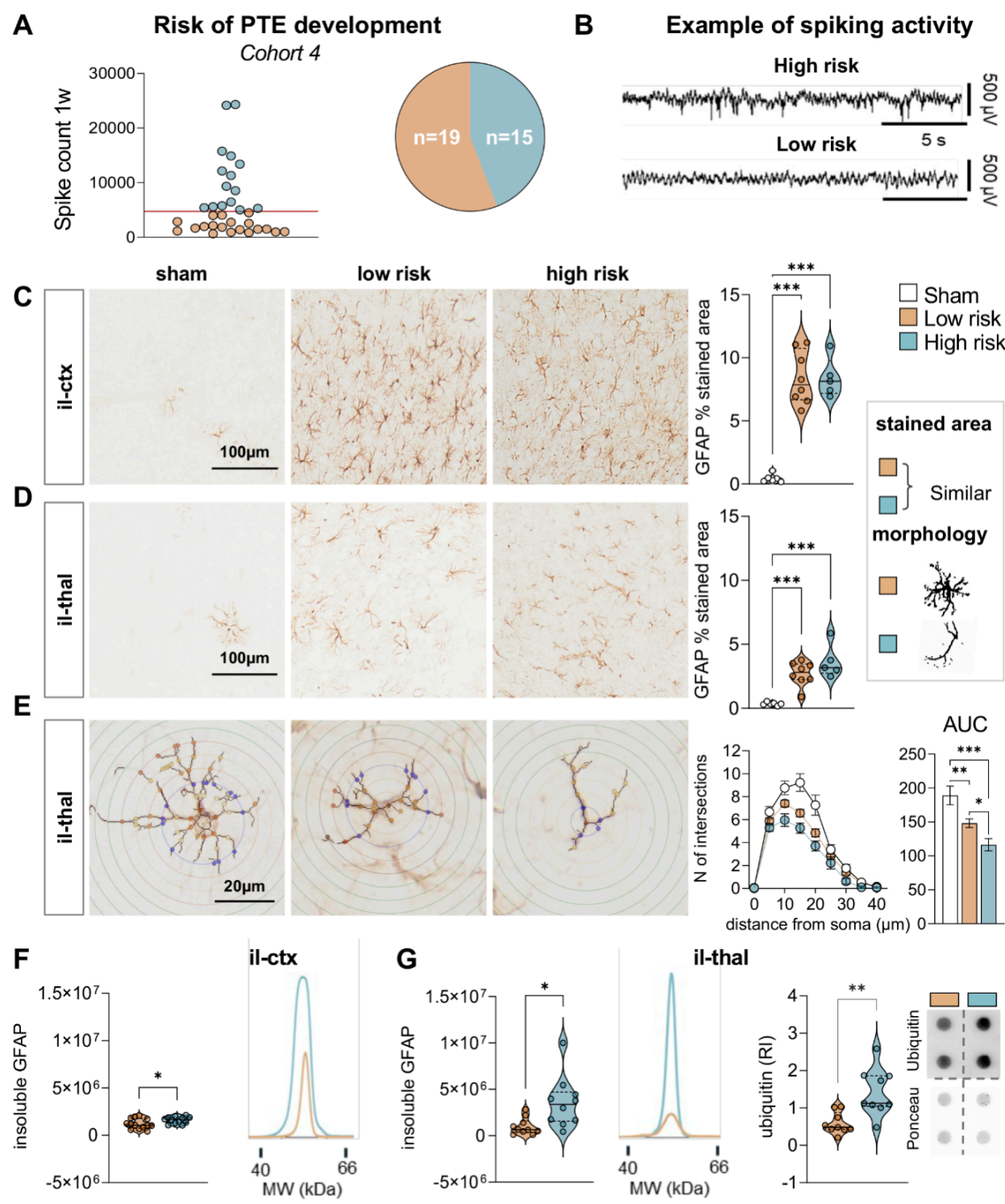


Figure 8

Decreased cytoskeletal complexity and increased GFAP aggregation within thalamus of

high-risk mice. (A) Spike and sharp wave count at day 7 in Cohort 4 and relative classification into high- and low-risk of PTE based on our previously identified spiking threshold (red line). (B) Examples of spiking activity at day 7 in the contralateral electrode of a high-risk (above) and low-risk (below) PTE mice. (C,D) Representative images and quantification of GFAP stained area and GFAP+ cell count in the (C) il-ctx and (D) il-thal 21d post-injury. (E) Representative images, quantification of astrocytes cytoskeletal complexity by Sholl analysis and quantification of the AUC in the il-thal at 21d. (F) Biochemical analyses and representative JESS spectra of insoluble GFAP levels, in the il-ctx of low-risk and high-risk mice. (G) Biochemical analyses and representative JESS spectra of insoluble GFAP levels in the il-thal of low-risk and high-risk mice; quantification and representative images of total ubiquitin levels in the insoluble fraction of the il-thal of low- and high-risk mice. Data are mean $\pm$ SEM. *** $p < 0.001$, ** $p < 0.01$, * $p < 0.05$. One-way ANOVA, Tukey post-hoc test. For F and G unpaired t-test.

AUC: area under the curve; ctx: cortex; GFAP: glial fibrillary acidic protein; il: ipsilateral; MW: molecular weight; RI: relative intensity; thal: thalamus

Supplementary Files

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- [Suplmaterials.pdf](#)