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Dysregulation of PINCH signaling in mesial temporal epilepsy

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Abstract

Mounting evidence suggests that inflammation is important in epileptogenesis. Particularly interesting New Cysteine Histidine-rich (PINCH) protein is a highly conserved, LIM-domain protein known to interact with hyperphosphorylated Tau. We assessed PINCH expression in resected epileptogenic human hippocampi and further explored the relationships among PINCH, hpTau and associated kinases. Resected hippocampal tissue from 7 patients with mesial temporal lobe epilepsy (MTLE) was assessed by Western analyses to measure levels of PINCH and hyperphosphorylated Tau, as well as changes in phosphorylation levels of associated kinases AKT and GSK3 β in comparison to normal control tissue. Immunolabeling was also conducted to evaluate PINCH and hpTau patterns of expression, co-localization and cell-type specific expression. Hippocampal PINCH was increased by 2.6 fold in the epilepsy cases over controls and hpTau was increased 10 fold over control. Decreased phospho-AKT and phospho-GSK3 β in epilepsy tissue suggested involvement of this pathway in MTLE. PINCH and hpTau co-localized in some neurons in MTLE tissue. While PINCH was expressed by both neurons and astrocytes in MTLE tissue, hpTau was extracellular or associated with neurons. PINCH was absent from the serum of control subjects but readily detectable from the serum of patients with chronic epilepsy. Our study describes the expression of PINCH and points to AKT/GSK3 β signaling dysregulation as a possible pathway in hpTau formation in MTLE. In view of the interactions between hpTau and PINCH, understanding the role of PINCH in MTLE may provide increased understanding of

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mechanisms leading to inflammation and MTLE epileptogenesis and a potential biomarker for drug-resistant epilepsy.

Keywords

PINCH protein; Hyperphosphorylated Tau; Mesial temporal lobe epilepsy; Hippocampal sclerosis

1. Introduction

Mesial temporal lobe epilepsy (MTLE) is the most common form of epilepsy in adults and can result in hippocampal sclerosis [11]. A large percentage of patients suffering from MTLE have a history of a prior injury such as prolonged febrile seizure, status epilepticus, head trauma, or meningitis in the first 4–5 years of life that may play a role in epileptogenesis [6,8,54]. Pathological studies of MTLE specimens typically reveal atrophy and gliosis within the hippocampus associated with loss of inhibitory neurons and aberrant sprouting of mossy fibers with granular cell axons in the dentate gyrus [9,46]. Tau is a microtubule-associated protein that is ubiquitously expressed in the brain where it assembles and stabilizes microtubules in neuronal axons. Accumulation of hyperphosphorylated (hp)Tau is implicated in more than 20 neuropathological diseases including Alzheimer's disease (AD) [30], HIV encephalitis (HIVE) [2,40,42], chronic traumatic brain injury [45] and fronto-temporal dementia (FTD) [45,56]. The pathology associated with aberrant hpTau involves its dissociation from microtubules and accumulation of paired helical filaments (PHF) and neurofibrillary tangles (NFT) in neurons and in some cases astrocytes [7]. Recent studies in rodent and fruit fly models of epilepsy have also shown that reducing Tau decreases hyperexcitability in neural networks [15,23,41]. In a large post-mortem analysis of 138 patient with chronic epilepsy, neurofibrillary tangles were identified in mesial temporal structures with a density and distribution equivalent to Braak stages III/IV [49]. Similar findings of hyperphosphorylated tau were recently reported in resected human mesial temporal lobe tissue from patients with a prolonged history of drug-resistant epilepsy (39.5 years) [47].

Multiple kinases and phosphatases have been linked to abnormal phosphorylation of Tau [7] and may also play a role in epileptogenesis. One such protein is *Particularly Interesting New Cysteine Histidine-rich protein* or PINCH. PINCH is a highly conserved, LIM-domain only protein consisting of five double zinc finger LIM domains [28]. PINCH expression is significantly increased during development where it functions in cell migration, attachment to the extracellular matrix (ECM), and to maintain cellular polarity [32,33]. In healthy adult mammals, PINCH expression in the central nervous system is nearly undetectable [44]. However, like hpTau, PINCH is robustly expressed by neurons and to a lesser degree by astrocytes in neurodegenerative diseases that include HIV, AD, and FTD [27,40,44]. In fact, recent studies show that PINCH interacts with hpTau and may influence its clearance via interactions with the heat shock machinery [40]. Recent studies in HIV show that changes in CSF levels of hpTau are mirrored by changes in PINCH [1].

PINCH has been shown to regulate kinases such as integrin linked kinase (ILK), GSK3 β and AKT, and protein phosphatase 1 α (PP1- α) [10,24,25,26], all of which are involved in Tau phosphorylation and de-phosphorylation (Fig. 1). PINCH-dependent AKT activation and GSK3 β inhibition occurs when PINCH binds to and inhibits the activity of PP1 α [10]. PP1 α stabilizes microtubules by maintaining the balance of Tau phosphorylation and dephosphorylation, and is considered the rate-limiting enzyme in the regulation of microtubule associated protein-microtubule interactions. Moreover, PP1 α can inhibit ILK-integrin signaling by associating with the β 1 subunit. When ILK is inactive, GSK3 β becomes active and phosphorylates Tau at residues corresponding those detected in diseases including AD and HIVE [3,25,37,42]. Changes in expression of PINCH may impact kinase and phosphatase activities and Tau phosphorylation. Given these multiple levels at which hpTau and PINCH interact, the relationship between PINCH, hpTau, AKT and GSK3 β were analyzed in hippocampus and para-hippocampal tissue from patients with intractable MTLE.

2. Materials and methods

2.1. Human brain tissue

Human brain tissue was obtained during surgery from 7 patients suffering from drug-resistant MTLE, 6 with hippocampal sclerosis and 1 with a focal cortical dysplasia without hippocampal sclerosis. The studies were conducted in accordance with the University of Southern California's and Temple University's Human Subjects Protections and Institutional Review Boards. None of these patients had other known neurological disorders such as FTD, HIVE, or AD (see below). Patients provided written informed consent based on the research protocol approved by the IRB of the University of Southern California. All samples consisted of resected tissue from the mesial temporal lobe, including hippocampus and para-hippocampal gyrus. Approximately 15–20 mm³ of the hippocampal head and body was dissected *en bloc*, and immediately placed at –80 °C or fixed in formalin until analyses. All cases had detailed clinical histories. Control tissue was obtained from 4 post-mortem age-matched patients without a clinical history of CNS disorders, CNS infections or seizures from the California NeuroAIDS Tissue Network or Alzheimer's disease Research Center tissue repositories at the University of California under the guidelines of UC San Diego Human Subjects Protections and Institutional Review Boards (Table 1).

2.2. Protein extraction from tissue

Frozen brain tissue was homogenized by mechanical disruption on ice in TNN buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.5% NP40, 1:200 protease inhibitor cocktail) (Calbiochem, San Diego, CA, USA). Once completely homogenized, samples were centrifuged at 13,000 $\times g$ at 4 °C for 5 min. The supernatant containing the protein was collected and protein concentrations were determined via the Bradford assay.

2.3. Blood collection and analyses

Whole blood from 7 volunteers enrolled in the USC Comprehensive Epilepsy Center and 5 controls with no history of seizures was collected in accordance with the University of Southern California's and Temple University's Human Subjects Protections and Institutional Review Boards. Participants provided written informed consent based on the research

protocol approved by the IRB of the University of Southern California. Seven milliliters of whole blood were collected into vacutainer tubes without anti-coagulants (Becton Dickinson Vacutainer, Franklin Lakes, NJ, USA). Tubes containing whole blood were incubated upright at room temperature for 30 min to allow clotting. The serum was separated by centrifugation at $1500\times g$ for 10 min at 20 °C. All samples were processed within 1 h after phlebotomy and serum was stored at -20 °C in 1 mL aliquots until analyses. Serum samples were subjected to Albumin/IgG depletion using the Pierce® Albumin/IgG Removal Kit according to manufacturer's instructions (Pierce Scientific, Rockford, IL, USA). An equal volume of depleted serum samples were loaded per well and assessed by standard Western analyses as described below.

2.4. Western analyses

Equal amounts of protein from brain tissue (30 µg) or an equal volume of IgG/albumin depleted serum (10 µl) were loaded per well into 4–12% Bis-Tris pre-cast mini or midi-gels (Invitrogen, Carlsbad, CA, USA), separated by electrophoresis and transferred onto nitrocellulose membranes. Membranes were blocked in 5% nonfat milk in Tris-buffered saline with 0.1% Tween-20 (TBST) for 30 min before incubation with primary antibodies. Primary antibodies were used at 1:1,000 for Western analyses unless otherwise indicated. Antibodies included: PINCH1, (BD, Rockville, MD, USA), pAKT serine 473 (Cell Signaling, Danvers, MA, USA), total AKT (Cell Signaling), pGSK3β-S9, pGSK3β-Y216 (Cell Signaling), total GSK3β (SCBT, Santa Cruz, CA, USA), GAPDH (1: 5,000) (SCBT), or Grb-2 (Cell Signaling). Anti-Tau antibodies from Thermo-Fischer (Thermo-Fischer, Pittsburgh, PA, USA) included: HT7 against human total Tau, AT8 (detects phospho- S202, -T205, -S199, -S208) and AT100 (detects phospho-S121, -T214) against PHF-Tau. Other hpTau specific antibodies included phospho-S262 and -S396 (Abcam, Cambridge, MA, USA). Membranes were incubated with primary antibodies for either 2 h at room temperature or overnight at 4 °C, washed 3X in 1X TBST and incubated with the appropriate secondary anti-mouse or -rabbit antibodies (1:10,000; Abcam) for 1 h at room temperature, and developed with ECL or ECL PRIME (Amersham Pharmacia Biotech, Piscataway, NJ, USA). Band intensities were calculated using ImageJ software [43] and normalized to loading controls, Grb-2 or GAPDH.

2.5. Immunohistochemical labeling

Formalin-fixed, paraffin-embedded hippocampal tissue from MTLE ($n = 3$) and control participants ($n = 3$) were processed for immunolabeling. Five µm serial tissue sections were rehydrated through ethanol to water, processed in citrate buffer for antigen retrieval and blocked with 5% normal horse serum in PBS, 0.1% BSA for 2 h. Slides were incubated with appropriate pairs of primary antibodies at 1:200 in PBS, 0.1% BSA overnight in a humidified chamber at room temperature. Primary antibodies included anti-PINCH (BD), anti-Tau S396 (Thermo-Fischer), MAP2 (Cell Signaling), neurofilament (Covance, San Diego, CA, USA), GFAP (Abcam), and Iba-1 (Abcam). After primary antibody incubation, slides were rinsed three times with 1X PBS, and then incubated with combined fluorescein isothiocyanate (FITC)-conjugated secondary antibody (1:500) and Texas-red isothiocyanate (TRITC)-tagged secondary antibodies (1:500) (Thermo-Fisher) for 2 h at room temperature in the dark. After washing 3 times with 1X PBS, sections were cover slipped with an

aqueous based mounting media containing DAPI for nuclear labeling (Vector Laboratories), visualized with the Leica DMI6000 and analyzed with Leica Application Suite (LAS) deconvolution software.

2.6. Statistical analyses

Statistical analyses of band densities from Western blots measured with ImageJ were conducted using the Prism 4 GraphPad software program (GraphPad Software, Inc.). Data were analyzed either by one-way ANOVA with post hoc analyses or one-tailed paired student's T-test where appropriate and results were considered significant if $p < 0.05$, $n = 3$.

3. Results

3.1. PINCH and hpTau levels are significantly increased in resected hippocampal tissues from MTLE patients

To assess if PINCH and hpTau are elevated in epilepsy, Western analyses were conducted on proteins collected from resected hippocampal tissue of patients undergoing surgical intervention for intractable temporal lobe epilepsy and compared to levels in hippocampal tissue from controls brains. Both PINCH (PINCH, $p = 0.00014$) and hpTau levels were increased significantly in epilepsy cases ($n = 7$) compared normal age-matched control tissues ($n = 4$), (Fig. 2A, C–E). Three different hpTau antibodies that recognize phosphorylation of specific amino acid residues were utilized and include S396 ($p = 0.0056$), AT8 ($p = 0.0044$) and AT100 ($p = 0.0114$), indicating that in MTLE tissues Tau is phosphorylated at sites specifically associated with pathology [4,52].

3.2. PINCH and hpTau co-localize in some neurons in MTLE tissues

We next assessed patterns of hpTau and PINCH protein expression in resected mesial temporal lobe tissues, including hippocampus and para-hippocampal gyrus from MTLE patients. Double immunofluorescence labeling of tissues revealed both regional and cell-type specific patterns of expression. The dentate gyrus showed an even and ubiquitous distribution of hpTau (green), especially in the hilus, molecular and polymorphic layers, with the granular cell layer being largely spared (Fig. 3A). Double immunolabeling and higher magnification show regions where hpTau fills the extracellular space, with low levels of PINCH (red) detected in association with cell bodies in the molecular and granular layers (Fig. 3B), again with sparing of the dentate gyrus granular layer.

In CA1/2 regions, hpTau (green) is detected in molecular, pyramidal and polymorphic cell layers, again with sparing of the dentate gyrus granular layer as shown in the suprapyramidal blade outlined in white (Fig. 3C). Double immunolabeling of PINCH (red) and hpTau (green) indicate co-localization (arrowheads, Fig. 3D, insets 1, 2) in the CA1/2 region with PINCH being primarily cell-associated and hpTau both extracellular and intracellular. PINCH and hpTau co-localize and appear to associate with cellular bodies and processes of small cells morphologically similar to neurons (Fig. 3D, F, insets). In some cases, PINCH and hpTau co-localize with structures reminiscent of tangles (Fig. 3E, arrowhead). These results show that while both PINCH and hpTau are highly expressed in the hippocampus of MTLE patients, and may co-localize, distinct regional distribution patterns are observed.

3.3. PINCH is expressed by neurons and astrocytes in MTLE

Previous studies in other neurodegenerative diseases including HIVE and AD confirm hpTau and PINCH expression primarily by neurons and to a lesser extent, by astrocytes [27,40,44]. Cell-type specific immunolabeling of PINCH and hpTau in MTLE indicated that PINCH is expressed by both neurons (Fig. 4A) and astrocytes (Fig. 4B). PINCH was detected in the soma of large MAP2 positive neurons located in the hilus region of the dentate gyrus, with labeling of smaller cells that are not MAP2 positive (Fig. 4A, arrowheads). PINCH was robustly expressed by GFAP positive astrocytes in the CA1/2 region with significant co-localization with hpTau (Fig. 4B, insets). These data show clear evidence of PINCH production by both neurons and astrocytes in MTLE.

3.4. hpTau is detected largely in the extracellular space and in association with neurons

Neurons appear to be the primary cell type expressing hpTau (Fig. 5A), with little hpTau co-localizing with astrocytes (Fig. 5B, C). Pyramidal neurons positive for both hpTau and neurofilament are observed in the CA1/2 region. As expected, hpTau is not detected robustly in neurofilament positive axons (Fig. 5A, insets arrow), but is associated with the neuronal soma (Fig. 5A, insets arrowhead). However, some hpTau associated with axonal processes is observed in damaged/dystrophic axons (Fig. 5A, inset 1, arrow). In addition, a large portion of hpTau appears to be extracellular (Fig. 5A, asterisk). hpTau is also detected in regions with highly reactive astrocytes (Fig. 5B, arrowheads) in the molecular layer of the dentate gyrus. Accumulated hpTau is detected the polymorphic layer of the dentate gyrus as well (Fig. 5B, asterisk). Less common were hpTau associations with cellular processes positive for GFAP (Fig. 5C, inset 1, arrowhead). In the CA1/2, hpTau appears largely extracellular, with few hpTau positive cell bodies (Fig. 5C, inset 2, arrow) and processes (Fig. 5C, arrowhead).

3.5. Key kinases in hpTau formation are dysregulated in MTLE

Next, we explored the potential relationships among levels of PINCH, hpTau and phosphorylation states of AKT and GSK3 β , two important kinases involved in Tau hyperphosphorylation (Fig. 1). AKT is active when it is phosphorylated at serine 273 and phosphorylates GSK3 β at serine 9, rendering the latter inactive. Levels of pAKT at serine 473 and pGSK3 β at serine 9 in epilepsy tissues are significantly reduced ($p = 0.0019$ and $p < 0.0001$, respectively) (Fig. 3A–C). Thus, GSK3 β that is not phosphorylated at serine 9 is active and coincides with increased hpTau levels (Fig. 2C–E). On the other hand, phosphorylation of GSK3 β at tyrosine 216 activates it, but levels are not significantly different in epilepsy cases compared to control (Fig. 6A, D). Taken together these results show that hpTau and PINCH are increased significantly in hippocampal tissues from MTLE patients and that AKT/GSK3 β signaling pathways are altered as well. These data link for the first time, PINCH and hpTau in epilepsy and illustrate dysregulation of the AKT/GSK3 β pathway.

3.6. PINCH is detected in the serum from epilepsy patients but not in control participants

Compromise of the blood brain barrier (BBB) in seizures has been extensively studied although there is debate as to whether seizures cause BBB disruption or if BBB disruption

leads to seizures (for review see [38]). Western analyses of the serum of 7 well-characterized patients with MTLE (4 medically controlled with AED and 3 with medically intractable epilepsy) enrolled at the USC Comprehensive Epilepsy Center tested positive for PINCH immunoreactivity in blood (Fig. 7A, B); whereas, controls ($n = 5$) were negative.

Taken together these results show that PINCH is increased in the brains and blood of MTLE patients but is absent in normal controls.

4. Discussion

By analyzing mesial temporal lobe structures resected from a relatively young cohort of patients with drug-resistant epilepsy, we have demonstrate that PINCH and hpTau levels are significantly increased in epileptogenic tissues and co-localize in neurons. Accumulation of hpTau is a common feature of many neurodegenerative diseases including HIVE, AD, and FTD, and has only recently been reported in mesial temporal tissues of post-mortem studies or patient with a prolonged course of drug-resistant epilepsy [49,47]. The present study confirms and extends the finding of hpTau to a younger cohort of patients with a shorter duration of epilepsy, and demonstrates the correlation of hpTau with PINCH and associated kinases such as phospho-AKT and phospho-GSK3 β in epileptogenic mesial temporal tissues. Our data show that both hpTau and PINCH are significantly increased in MTLE tissues compared to controls and are accompanied by changes in AKT/GSK3 phosphorylation levels that have been shown to promote Tau hyperphosphorylation [7]. Finally, we have also shown that while PINCH is not present in the serum of control individuals, it is readily detectable in serum of patients with chronic epilepsy.

Recently identified as a binding partner of hpTau, the PINCH protein [1,27,40,44] is involved in protein recruitment, protein complex assembly and shuttling throughout the cell (for review, see [29]). During brain development, PINCH is expressed at high levels and functions in cytoskeletal organization and communication between the cell and the extracellular matrix [32,33]. PINCH is nearly undetectable in healthy adult brain tissue, and appears to be induced by injury or inflammation [27,44]. PINCH is a regulator in part of Tau phosphorylation since *in vitro* studies showed that shRNA knockdown of PINCH decreased the levels of hpTau even after prior treatment with okadaic acid, a well-known inducer of hpTau [40]. Further studies revealed that PINCH and hpTau bind one another, and that this interaction may be important for the regulation of hpTau's interaction with heat shock machinery [40]. In other studies, changes in the levels of PINCH and hpTau in CSF of HIV patients were shown to correlate directly with one another [1]. Taken together, results from this study and others support an important role for PINCH in tau phosphorylation.

PINCH is a key modulator of several kinases involved in Tau phosphorylation and de-phosphorylation such as integrin linked kinase (ILK), AKT and protein phosphatase 1 α (PP1- α) [10,24,25,26], and is therefore critical for maintaining neuronal structure and fitness (Fig. 1). For example, the LIM1 domain of PINCH binds to ILK in the intracellular compartment and to $\beta 1$, $\beta 3$ integrin at the cell surface to ensure proper communication among the cytoskeleton, focal adhesions and the ECM. In the absence of PINCH, ILK

cannot localize to focal adhesions [13,17,31,55,57,58], thereby disrupting the cytoskeleton, cell migration and attachment.

Studies of resected tissues have provided valuable information regarding cellular networks involved in MTLE [9,11,12,46,50,51,34]. However, the advanced stage of neuropathology in these tissues limits their use to address mechanisms that may contribute to seizure onset and epileptogenesis.

Of the 7 cases studied by Western analyses, 1 case (Figs. 2A, 3A, arrow) showed focal cortical dysplasia with no evidence of hippocampal sclerosis. Incidentally, this participant also represented the case with fewest years living with seizure disorder (6 years) (Table 1). Even in the absence of sclerosis, levels of PINCH and hpTau were elevated at levels similar to cases with sclerosis. These results suggest that changes in PINCH and hpTau may be generally related to the process of epileptogenesis rather than the development hippocampal sclerosis per se. Moreover, decreased levels of phosphorylated AKT (ser273) indicating decreased activity, and of phosphorylated GSK3 β (ser9) indicating increased activity (Fig. 3) are observed in both sclerotic and non-sclerotic cases pointing to ongoing dysregulation of these pathways that may contribute to increased hpTau.

Many studies have addressed possible connections between chronic seizure disorder and rapid brain aging that may increase risk for AD or other dementias [5,14,16,18,19,20,21,22,35,36,39,48]. One large post-mortem study by Thom et al. reported that hippocampal sclerosis in chronic epilepsy was linked to moderate asymmetrical Tau accumulation rather than changes associated with Braak staging [49]. More recently, a pattern of Tau pathology similar to both Alzheimer's dementia and chronic traumatic encephalopathy was observed in study of resected hippocampi from "elderly" patients with chronic epilepsy who underwent mesial temporal lobe resection between 50 and 65 years of age [47]. Interestingly, the extent of Tau pathology correlated with post-operative verbal learning measures. In this context, previous studies addressing PINCH in neurodegenerative disease such as HIV, have proposed that along with hpTau accumulation, PINCH may also contribute to rapid brain aging. Results from our study support these findings, but also demonstrate similar patterns of Tau/PINCH pathology earlier in the course of chronic epilepsy. Given the multiple levels at which hpTau and PINCH interact, our findings that PINCH is highly expressed in MTLE may lead to increased understanding of underlying cellular mechanisms leading to neuronal injury and epileptogenesis.

Given the correlation between PINCH and hpTau, our finding that PINCH is readily detectable in serum derived from patients with epilepsy, yet absent from the serum of control subjects, is particularly interesting. Most intriguing is the possibility that PINCH may serve as a biomarker in epilepsy, similar to other proposed molecules shown to be elevated in patient with epilepsy such as matrix metalloproteinase-3 (MMP-3) and circulating micro-RNAs [53].

Several limitations exist within the current study. First, as with all studies conducted on resected surgical tissues, transoperative variables such as anesthesia duration and drugs, hemodynamic changes, time of hippocampal vascular supply coagulation were uncontrolled.

Second, times between last seizure and surgery may have been variable among the MTLE cases. Third, times between tissue collection and freezing and biochemical assays were different. Finally, tissues from controls were collected post-mortem and differences in tissue preservation may exist. However, given the consistency across groups our data provide strong evidence of dysregulation of the PINCH-AKT-GSK3 β signaling pathway that may have contributed to significantly increased hpTau levels in MTLE.

Our study provides evidence of PINCH's involvement in MTLE and points to dysregulation of AKT/GSK3 β signaling as likely contributing pathways in hpTau formation in these cases. Changes in blood levels of PINCH may provide a surrogate marker for PINCH levels in the brain and, by correlation, hpTau levels. Given the interactions between hpTau and PINCH, understanding the role of PINCH in MTLE may provide increased understanding of inflammatory mechanisms leading epileptogenesis.

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Fig. 1.
Proposed PINCH signaling pathway involved in hpTau accumulation in MTLE.

Fig. 2.

PINCH and hpTau levels are significantly increased in MTLE. (A) Representative Western blot shows increased PINCH and hpTau in epilepsy ($n = 7$) versus control ($n = 4$) tissues. Arrow indicates the case with out sclerosis. Membranes containing 20 μ g/lane of total protein were probed with anti-PINCH antibody, and anti-Tau antibodies: S396, AT8 and AT100 and HT7 and GAPDH as a loading control. (B) Graphic representation of fold change of PINCH over GAPDH, $*p = 0.0014$ by unpaired t-test. (C) Graphic representation of fold change of hpTau (S396) over total Tau (HT7), $*p = 0.0056$ by unpaired t-test. (D) Graphic representation of fold change of hpTau (AT8) over total Tau (HT7), $*p = 0.0044$ by unpaired t-test. (E) Graphic representation of fold change of hpTau (AT100) over total Tau (HT7), $*p = 0.0114$ by unpaired t-test.

Fig. 3.

Patterns of PINCH and hpTau expression in the hippocampus from representative MTLE cases. (A) hpTau expression pattern (green) in the dentate gyrus. (B) Double immunolabeling for PINCH (red) and hpTau (green) in the dentate gyrus. Inset shows a PINCH immunoreactive cell at the junction of the molecular and granular layers. (C) hpTau pattern (green) in the CA1/2 region of the hippocampus. The white line indicates the suprapyramidal blade of the dentate gyrus. (D) Double immunolabeling of PINCH (red) and hpTau (green) in the CA2 region of the hippocampus. Insets indicate some areas of hpTau/PINCH co-localization (arrowheads) with cellular processes. (E) PINCH (red) and hpTau (green) double immunolabeling in the CA1/2 region of the hippocampus. Arrowhead indicates extracellular accumulation of PINCH and hpTau. Inset 1, PINCH labeling of small neurons. Inset 2, PINCH/hpTau co-localization in a small cell. (F) PINCH and hpTau co-localization in long cellular processes in the CA1/2 region, arrows, insets). Nuclei are labeled blue with DAPI. spb, suprapyramidal blade of dentate gyrus; h, hilus; ipb, infrapyramidal blade of dentate gyrus; pml, polymorphic layer; ml, molecular layer; gl, granular layer; pl, pyramidal layer. Scale bars $\approx 40 \mu\text{m}$.

Fig. 4. PINCH expression in neurons and astrocytes in the hippocampus from representative MTLE cases. (A) Double immunolabeling of PINCH (green) and MAP2 (red) in large neurons in the hilus region of dentate gyrus. The white-hatched line indicates the junction of image stitching. PINCH is detected in MAP2 negative smaller cell bodies and processes (arrowheads). (B) PINCH (red) and GFAP (green) co-localize in the CA1/2 region of the hippocampus (insets 1 and 2). Nuclei are labeled blue with DAPI. Scale bars $\approx 40 \mu\text{m}$.

Fig. 5.

hpTau expression in neurons and astrocytes in the hippocampus from representative MTLE cases. (A) Double immunolabeling of hpTau (green) and neurofilament (red) in the CA1/2 region. Asterisk indicates largely extracellular hpTau. Insets indicate hpTau in neuronal soma (arrowheads) with little to no detection in the axon (arrow). (B) Double immunolabeling of astrocytes (GFAP, green) and hpTau (red). Reactive astrocytes (arrowheads) are adjacent to hpTau, but no co-localization is observed. Asterisk indicates accumulation of hpTau in the polymorphic layer of the dentate gyrus. (C) In the CA1/2 region, GFAP (red) and hpTau (green) co-localization is minimal (inset 1, arrowhead) and two clearly distinct cell populations are labeled (inset 2). Nuclei are labeled blue with DAPI. Scale bars $\approx 40 \mu\text{m}$.

Fig. 6.

Levels of phosphorylated AKT and GSK3 β are significantly decreased in MTLE compared to control. (A) Representative Western blot shows decreased pAKT, pGSK3 β -S9 in epilepsy ($n = 7$) versus control ($n = 4$) tissues. Arrow indicates the case with out sclerosis.

Membranes containing 20 μ g/lane of total protein were probed with anti-pAKT serine 473 (ser273), total AKT, pGSK3 β serine 9 (ser9), pGSK3 β tyrosine 216 (tyr216), total GSK3 β antibodies and GAPDH antibody as a loading control. (B) Graphic representation of the ratio of pAKT to total AKT (** $p = 0.0019$), and pGSK3 β -S9 to total GSK3 β ($p, 0.0001$) normalized to GAPDH by unpaired t-test. No significant differences (n.s.) were observed in levels of pGSK3 β at tyrosine 216 (Tyr216).

Fig. 7.

PINCH is detectable in serum from epilepsy patients. (A) Ponceau red staining of the membrane shows even loading. (B) Western blot shows PINCH levels in epilepsy serum samples ($n = 7$) versus control ($n = 4$). Membranes containing 10 μ l/lane of IgG/Albumin depleted serum were probed with anti-PINCH antibody.

Table 1

Clinical information for cases in the study.

Epilepsy	Sex, race	Neuropathology	Age at surgery	Age at first seizure	Years of seizure	Seizure type	Seizure burden	Risk factors	No. of AEDS	Failed AEDS	Radiology results	Surgery type	Surgery outcome	Pathology
	MH	Hippocampal sclerosis	33	9	24	Focal w/ aloe (CPS)	1–3/day	Fébrile szs	3 depakote, lamictal, topamax	1 unknown in mexico	RMTS	R temp selective	1 yr post op sz free ILAE class 1	Hippocampal sclerosis
	FH	Hippocampal sclerosis	56	25	31	Focal w/ aloe (CPS)	1–3/day	None known	2 tripleptl, keppra	3 vimpat, topamax, depakote	R cys szs with mild edema	Small right hippocampal cyst, multiple areas of stable flair including right insular, likely secondary to VPS, NCC	1 yr post op class 1	Hippocampal sclerosis
	MH	Hippocampal sclerosis	50	17	33	Focal w/ aloe (CPS)	1–3/month	None known	1 tegretol	3 dilantin, keppra, depakote	LMTS	L temp selective	1 yr post op class 1	Hippocampal sclerosis
	MH	Focal cortical dysplasia no sclerosis	28	22	6	Focal w/ aloe (CPS)	1–3/day	None known	2 keppra, depakote	1 dilantin	R cortical dysplasia	R temp standard following phase2	1 yr post op class 3, one sz	Focal cortical dysplasia, no sclerosis
	MH	Left temporal sclerosis	44	3	41	Focal w/ aloe (CPS) +GTC	1–6/week, +1–3 month	Head trauma aged 3	2 keppra, vimpat	2, depakote, keppra	LMTS	L temp selective	1 yr post op class 1	Left temporal sclerosis
	MH	Hippocampal sclerosis	51	9	42	Focal w/ aloe (CPS)	1–6/week	Head trauma aged 9, second head trauma no age noted	2 depakote, vimpat	6 phenobarbital, dilantin, tegretol, vimpat, topamax, keppra	BMTS, R cortical dysplasia, R temp pole atrophy	R temp selective	1 year post op class 2, only auras	Hippocampal sclerosis
	MH	Hippocampal sclerosis	45	14	31	Focal w/ aloe (CPS)	1–6/week	None known	1 keppra	4, primidone, tegretol, depakote, topamax	RMTS, left bone enhancing lesion	R temp selective	1 yr post op class 1	Hippocampal sclerosis
	Controls													
	FH	None	30	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
	FH	None	30	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
	MW	None	53	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
	F	None	26	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A

M-Male, F-Female, H-Hispanic, W-White, B-Black.

cps: Complex partials;szs: simple partial szs, generalized tonic clonic.

cps: Complete partial seizure.

ILAE classification: class 1 sz free, class2 only auras, class 3 1–3 szs per year, may have auras.