Inflammation induced-PINCH expression leads to actin depolymerization and mitochondrial mislocalization in neurons

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Abstract

Background Diseases and disorders with a chronic neuroinflammatory component are often linked with changes in brain metabolism. Among neurodegenerative disorders, people living with human immunodeficiency virus (HIV) and Alzheimer's disease (AD) are particularly vulnerable to metabolic disturbances, but mechanistic connections of inflammation, neurodegeneration and bioenergetic deficits in the central nervous system (CNS) are poorly defined. The particularly interesting new cystine histidine-rich protein called PINCH is nearly undetectable in healthy mature neurons, but is robustly expressed in tauopathy-associated neurodegenerative diseases including HIV infection and AD. Although robust PINCH expression has been reported in neurons in the brains of patients with HIV and AD, the molecular mechanisms and cellular consequences of increased PINCH expression in CNS disease was not known.

Methods We investigated regulatory mechanisms responsible for PINCH protein-mediated changes in bioenergetics, mitochondrial subcellular localization and bioenergetic deficit in neurons exposed to physiological levels of TNFα or Tat. Changes in the PINCH-ILK-Parvin (PIP) complex association with cofilin and TESK1 were assessed to identify factors responsible for actin depolymerization and mitochondrial mislocalization. Lentiviral and pharmacological inhibition experiments were conducted to confirm PINCH specificity and to reinstate proper protein-protein complex communication.

Results We have identified MEF2A as the PINCH transcription factor in neuroinflammation and the biological consequences of increased PINCH in neurons. TNFα-mediated activation of MEF2A via increased cellular calcium induced PINCH leading to disruption of the PIP ternary complex, cofilin activation by TESK1 inactivation, and actin depolymerization. Disruption of actin led to perinuclear mislocalization of mitochondria by destabilizing the kinesin-dependent mitochondrial transport machinery resulting in impaired neuronal metabolism. Blocking TNFα-induced PINCH preserved mitochondrial localization and maintained metabolic functioning.

Conclusions These data report for the first time mechanistic and biological consequences of PINCH expression in neurons in the CNS in diseases with a chronic neuroinflammatory component. Our findings point to maintenance of PINCH at normal physiological levels as a new therapeutic target for neurodegenerative diseases with impaired metabolism.

Background

Particularly interesting new cysteine-histidine rich (PINCH) protein is non-catalytic and consists of five double zinc-finger LIM domains that bind to several different proteins including integrin-linked kinase (ILK) and Nck-2 (1). In addition to interacting with PINCH, ILK interacts through its C-terminal domain with Parvin to form ternary PINCH-ILK-Parvin (PIP) complexes in a wide-variety of cell types (2-4). However, specific functions of the PIP complex seem to be cell type dependent. Studies in mammalian cells using dominant negative mutants of PINCH, ILK, and Parvin that inhibit PINCH-ILK or ILK-Parvin interactions have provided biochemical and cellular evidence showing that the interactions among PINCH, ILK and Parvin are important for cell adhesion, spreading, motility, and extracellular matrix (ECM) assembly (3-8). Furthermore, genetic studies in invertebrate model organisms such as *C. elegans* and *Drosophila* have demonstrated that mutations in ILK (PAT- 4) (9, 10), PINCH (UNC-97) (11, 12), or Parvin (PAT-6) (13) results in defects in the assembly of integrin-actin complexes and cell-ECM attachment. The importance of the PIP complex is further manifested by studies in podocytes where the it is involved in the regulation of cell adhesion, cytoarchitecture, and apoptosis. Overexpression of the PINCH-1 binding ankyrin (ANK) region of ILK disrupted the PIP complex and compromised podocyte shape change and survival signaling (14). Moreover, forced disruption of the PIP complex in mesangial cells significantly reduced the fibronectin matrix deposition (5). Additionally, disruption of the PIP complex in TGF-β1 treated podocytes activated the P38 mitogen-activated protein kinase and promoted apoptosis (15). While the functions of the PIP complex are widely studied in non-neuronal cells, much less is known about their regulation in the CNS.

PINCH is highly expressed during development but data from our laboratory and others have shown that PINCH is nearly undetectable in healthy adult brain (16-19). However, PINCH is robustly expressed in the brains of patients with neuroinflammatory or neurodegenerative disease with a tauopathy component including HIV (16, 18, 19), Alzheimer's disease (AD) (18), and epilepsy (17). In vitro, PINCH is induced in neurons by exposure to TNFα directly, or indirectly by exposure to the HIV transactivator of transcription (Tat) protein that triggers TNFα production (16, 18, 20-22). Studies show that PINCH facilitates the formation and accumulation of hyperphosphorylated Tau (hpTau) via AKT/GSK3β signaling; whereas, blocking TNFα-induced PINCH expression with TNF-receptor antagonist or with siRNA or shRNA against PINCH reduces formation and accumulation of hpTau in neurons (16, 18). Given the pivotal role of PINCH in the PIP complex, and altered expression of

PINCH under neuroinflammatory conditions, the contribution of PINCH in the progression of neurodegenerative processes in neuroinflammatory diseases is highly likely.

The regulatory mechanisms responsible for the induction of PINCH and the role of the PIP complex in neuronal disease are unknown. This prompted us to investigate whether inflammation-induced PINCH expression alters PIP complex formation and functioning in neurons. We report here for the first time, that surrogate models of neuroinflammation (TNFα- or Tat-treated neurons) induce PINCH by calcium-dependent transcriptional regulation. Increased expression of PINCH in neurons led to disassociation of parvin from the PIP complex promoting parvin's interaction with and inactivation of TESK1 (23-28), the actin associated kinase. Inactivation of TESK1 prevented TESK1-mediated cofilin phosphorylation, thereby increasing cofilin's actin depolymerization activity (29-31). Depolymerization of actin disrupted the tubulin-kinesin-miro complex, thereby causing mislocalization of mitochondria to perinuclear regions of the neuron. Our results provide new information on the cause and consequences of induced PINCH expression in neuroinflammatory conditions. Furthermore, the data show that integrity of the PIP complex is essential in neurons to maintain actin stabilization and mitochondrial distribution.

Materials and Methods

Human primary neurons

Fetal brain tissue (gestational age, 16-18 weeks) for isolation of neurons was obtained from elective abortion procedures performed in full compliance with National Institutes of Health and Temple University ethical guidelines. Neurons were provided by the Comprehensive NeuroAIDS Center at Temple University. Briefly, fetal brain tissue was washed with cold Hanks balanced salt solution (HBSS), and meninges and blood vessels were removed. Tissue in HBSS was digested with papain (0.8 mg/ml) for 30 min at 37°C. The tissue was washed with HBSS, resuspended in NM5 media (neurobasal media supplemented with 5% horse serum, 1% B27, 1% glutamax, gentamycin), and further dissociated by repeated pipetting to obtain single-cell suspensions. The cell suspension was passed through a 70-micron cell strainer and cells were counted. The single-cell suspension was plated at a density of ~3.0×10^6 cells/poly-D lysine coated 60 mm dish in NM5 media. Twenty-four hours later, cultures were refed with a complete change of neurobasal media without horse serum (NM0). Four days later, half of the media was removed and replaced with neurobasal media (NM0) supplemented with 1.25 μM FDU and 1.5 μM uridine. Purity of cultures was assessed by immunolabeling with antibodies for MAP2, GFAP and Iba-1.

Treatment conditions

After 14 days in culture, neurons were treated with 50 ng/ml recombinant Tat (101 amino acids, rTat, ImmunoDX LLC, Woburn, WA) or 50 ng/ml recombinant human tumor necrosis factor-α (TNFα) (Sigma

Aldrich, St Louis) for 48h. Concentrations used were based on results from previous publications using primary neurons and time course and dose response data for cell toxicity and PINCH expression levels (16, 18). Neurons not exposed to Tat or TNFα served as controls.

Plasmids and lentivirus

Light Switch Promoter Reporter GoClone plasmid with *lims1/pinch* (PINCH) (NM_004987) promoter sequence (SwitchGear Genomics; S712264) and the corresponding control plasmids were used for luciferase assay. The ready to use human CACNA1G siRNA lentivirus (Applied Biological Materials Inc. Richmond, BC, Canada) was used to knockdown Cav3.1 in human primary neurons. Mission® lentiviral transduction particles (TRCN0000365202) and Mission® TRC3 Human ORF lentivirus particles (TRCN0000468941) were used to knockdown (KD) and overexpress PINCH respectively. PINCH KD and overexpression was confirmed by Western blot analysis 72h post-infection.

Quantitative measurement of PINCH expression

Neurons treated with or without Tat/TNFα for 48h were harvested and total RNA was isolated using RNeasy Mini Kit (Qiagen). cDNA was generated using an iScript™ cDNA Synthesis Kit (Bio-Rad, USA). Semi-quantitative real-time PCR was performed using SYBR green reagents (LightCycler®96, Roche, USA) using PINCH-specific primers: F-5’-GCCTGTTCTACCTGCAACAC-3’, R-5’-CCTTCCTAAGGTCTCAGCTAGT-3’. GAPDH was used as control.

Western blotting

Cell extracts were prepared from neurons treated with or without Tat/TNFα using RIPA buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 0.25% deoxycholic acid, 1 mM EDTA, 1% NP-40, protease and phosphatase inhibitor cocktail, Thermo Scientific). Protein concentrations were quantified using the Pierce™ 660nm protein assay reagent. Equal amounts of protein (25 µg/well) were separated on 4-12% Bis-Tris polyacrylamide gel, transferred to a nitrocellulose membrane using iBlot 2 NC regular stacks (Thermo Scientific), and probed with antibodies specific for phospho-CAMKII (1:1000, Abcam), CAMKII (1:1000, Abcam), pP38 (1:1000, Cell Signaling), P38 (1:1000, Cell Signaling), pMEF2A (1:1000, Abcam), MEF2A (1:1000, LifeSpan BioSciences), PINCH (1:1000, BD Biosciences), GAPDH (1:5000, Santa Cruz), ILK1 (1:1000, Abcam), Parvin (1:1000, Cell Signaling), TESK1 (1:1000, Cell Signaling), pCofilin (1:1000, Cell Signaling). Cofilin (1:1000, Cell Signaling), Chronophin (1:1000, Cell Signaling), Tubulin (1:1000, Cell Signaling), Miro1 (1:1000, Abcam), Kinesin (1:1000, Abcam), Trak1 (1:1000, ThermoFisher).

ChIP, quantitative PCR, and luciferase activity

The ChIP assay was performed using Pierce™ Magnetic ChIP kit. In brief, DNA-protein complexes from neurons exposed to Tat or TNFα were crosslinked and immunoprecipitated using ChIP-validated antibodies against RNA polymerase II, MEF2A, c-Jun, Foxd1, HOXA9 and negative control, IgG. Immune complexes were extracted and analyzed by quantitative PCR using primers (5' TTCAGGCAAGGACACCCTCA3' and 3'TCCGACTGAGTCACCTCCTGG5') that flank specific regions in the PINCH promoter. Values were normalized to input DNA. Results were depicted as the fold enrichment over basal expression. Neurons were transfected with luciferase reporter plasmids (4 μg) containing the PINCH promoter sequence with binding elements for MEF2A. After 48h, neurons were lysed, and the luciferase activity was measured (LightSwitch Luciferase Assay Reagent) using a plate reader (BiotTek Cytation1).

Measurement of cytosolic Ca^{2+}

Human primary neurons grown on 50 μg/ml poly-D lysine (Sigma Aldrich, St Louis) coated 35 mm glass coverslips were exposed to Tat or TNFα for 48h. After treatment, neurons were loaded with 5 μM Fluo-4/AM for 30 min in extracellular medium at room temperature as previously described (32). Coverslips were mounted in an open perfusion microincubator (PDMI-2; Harvard Apparatus) at 37°C and imaged. Confocal images were acquired at 488 excitations using a 63X oil objective (LSM 800; Carl Zeiss, Inc.). Images were analyzed and Ca^{2+} fluorescence quantified by using ImageJ (NIH).

Immunofluorescence labeling for PINCH and actin

Human primary neurons grown on 50 μg/ml poly-D lysine (Sigma Aldrich, St Louis) coated 35 mm glass coverslips were exposed to Tat or TNFα for 48h. After treatment, neurons were fixed in PBS containing 4% (w/v) paraformaldehyde for 20 min at room temperature. Neurons were permeabilized with 0.5% (v/v) Triton X-100 for 15 min at room temperature and then blocked in PBS containing 3% bovine serum albumin for 1 hour. The cells were then incubated with anti-PINCH antibody (1:200 dilution; BD biosciences) overnight at 4 °C. After 3 washes with 1X PBS, neurons were incubated for 1h with Alexa Fluor 488-conjugated anti-mouse IgG (Invitrogen; 1:200 dilution) and rhodamine phalloidin (1:1000, Invitrogen) to simultaneously label PINCH and actin, respectively. After 1h incubation, neurons were washed with PBS and mounted with Vectashield containing DAPI (Vector Lab., Burlingame, CA, USA). Confocal images were obtained at 405 (DAPI) 488 (PINCH) and 561 (actin) nm excitations respectively using a 63X oil objective (LSM 800; Carl Zeiss, Inc.). Images were analyzed using ImageJ (NIH).

Analysis of neuronal mitochondria distribution

Human primary neurons grown on 50 μg/ml poly-D lysine (Sigma Aldrich, St Louis) coated 35 mm glass coverslips were exposed to Tat or TNFα for 48h. After treatment, neurons were loaded with Rhodamine 123 (1 μM) for 20 mins at 37°C and confocal images were obtained at 488 nm using a 63X

oil objective (LSM 800; Carl Zeiss, Inc.). The changes in mitochondrial distribution were assessed and compared with untreated control cells. The distance of mitochondria from neuronal nucleus was measured using image J.

Co-immunoprecipitation and Western Blot Analysis

Protein extracts were collected from neurons treated with or without Tat or TNFα using RIPA buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 0.25% deoxycholic acid, 1 mM EDTA, 1% NP-40, protease and phosphatase inhibitor cocktail, Thermo Scientific). The extracts were immunopurified with antibodies specific for Kinesin or PINCH or Parvin and Western blotted with antibodies specific for tubulin, Miro1, Kinesin, TRAK1, ILK, PINCH, Parvin, and TESK1.

TNFα ELISA

TNFα human ELISA Kit was used to quantify TNFα in media from neurons exposed to Tat for different lengths of time as per the manufacturer's protocol (ThermoFisher Scientific).

Mitochondrial Oxygen Consumption Rate

Non-target (control) shRNA and PINCH KD neurons (3×10^5) were exposed to Tat or TNFα for 48 h. Neurons were then subjected to oxygen consumption rate (OCR) measurements at 37°C in an XF96 extracellular flux analyzer (Seahorse Bioscience). Neurons were sequentially challenged with 2 μM oligomycin, 0.5 μM FCCP, and 0.5 μM rotenone plus antimycin A to measure basal and maximal OCR, ATP coupled respiration, spare capacity, and proton leak.

Statistical Analyses

Data from multiple experiments ($n \geq 3$) were quantified and expressed as Mean ± SEM, and differences between groups were analyzed using two-tailed paired Student's T-test or, when not normally distributed, a nonparametric Mann-Whitney U test. Differences in means among multiple datasets were analyzed using one-way ANOVA with the Kruskal-Wallis test, followed by pairwise comparison using the Dunn test. $P \leq 0.05$ was considered significant in all analyses. The data were computed either with GraphPad Prism version 7.0 or SigmaPlot 11.0 software.

Results

PINCH is expressed at high levels during development but is nearly undetectable in healthy adult brains or in normal mature neurons. However, previous studies have reported a dramatic increase in PINCH expression brains from HIV, AD, epilepsy and CTE patients, and in neurons exposed to the HIV protein Tat or to TNF-α (16-18). Although expression of PINCH is observed in CNS disease, the regulatory mechanisms involved in PINCH expression are unknown. Because exposure to Tat induces

TNFα production and release by neurons (16, 18, 20-22) in a time dependent manner (**Fig. 1a**) leading to a feed forward loop for TNFα production (20, 33, 34), in the present study we exposed human primary neurons to Tat or TNFα as an *in vitro* surrogate model for neuroinflammation. While PINCH expression can be regulated at transcriptional, translational or post-translational levels, we first measured the abundance of PINCH mRNA by qPCR. Similar to increased protein expression (16, 18), a significant increase in PINCH mRNA levels (**Fig. 1b**) was observed in neurons exposed to Tat or TNFα, indicating inflammation-mediated transcriptional regulation of PINCH.

Bioinformatics analysis of PINCH (*lims1/pinch*) promoter sequence predicted conserved putative binding sites for several transcription factors, including MEF2A, c-FOS, C-Jun, and AP-1 with a binding site upstream of the *lims1/pinch* transcription start site (**Fig. 1c**). To identify the transcription factor(s) that regulate PINCH expression during inflammation, we performed a ChIP assay in neurons. Neurons exposed to Tat or TNFα showed significantly increased binding of MEF2A to the *lims1/pinch* promoter (**Fig. 1d**), indicating MEF2A as a transcriptional activator of PINCH. C-Jun showed binding but binding was not regulated in response to Tat or TNFα. Because P38 is a potent kinase that activates MEF2A to enhance MEF2A-dependent gene expression (35-38), we next assessed changes in the P38-dependent cascade for MEF2A activation by Western analysis. In agreement with increased MEF2A binding to *lims1/pinch* promoter, Western analysis showed increased phosphorylation of MEF2A (**Fig. 1e, h**) in neurons exposed to Tat or TNFα and the phosphorylation was found to be mediated through calmodulin dependent protein kinase (CAMKII) signaling pathway (**Fig. 1e-h**). To further confirm MEF2A as an activator for PINCH expression, we generated luciferase reporter constructs of the *lims1/pinch* promoter with an MEF2A binding site (**Fig. 1j**). Neurons expressing the luciferase construct were stimulated with Tat or TNFα and luciferase activity was measured. Neurons expressing *lims1/pinch* promoter construct (-169-175 bp) showed increased luciferase activity in response to Tat or TNFα (**Fig. 1k**), indicating MEF2A-mediated transcriptional regulation of PINCH during inflammation.

Because increased cytosolic Ca^{2+} (cCa^{2+}) activates the calcium/calmodulin-dependent kinase (39), we next asked if increased phosphorylation of CAMKII and the downstream signaling cascade for PINCH expression is the result of elevated cCa^{2+} in neurons exposed to Tat or TNFα. Measurement of cCa^{2+} using Fluo-4-AM showed significantly increased Ca^{2+} fluorescence in neurons exposed to Tat or TNFα (**Supplementary Figure 1a**). Because Voltage-gated Ca^{2+} channels play important roles in neuronal excitability. Since, the low-voltage-activated T-type Ca^{2+} channel, Cav3.1 is involved in triggering αCaMKII activation (40), we transiently knocked down Cav3.1 in neurons and as expected, transient knock down (KD) of Cav3.1 (**Supplementary Figure 1c, d**) in neurons normalized the elevated cCa^{2+} even after exposure to Tat or TNFα (**Supplementary Figure 1b**). Given the reduced Ca^{2+} influx in Cav3.1 KD, we next asked whether the expression of PINCH and the relative upstream

cascade is disrupted in Cav3.1 KD neurons exposed to Tat or TNFα. In line with decreased cCa^{2+} levels, phosphorylation of P38 and MEF2A were significantly decreased (**Supplementary Figure 1c, 1e, f**) an accompanied by decreased PINCH expression (**Supplementary Figure 1c, g**). To further confirm that decreased activation of MEF2A in Cav3.1 KD neurons blocked Tat or TNFα induced PINCH expression, we performed ChIP and luciferase assays. Consistent with decreased phosphorylation of MEF2A, the binding of MEF2A to *lims1/pinch/pinch* promoter was decreased in Tat or TNFα treated Cav3.1 KD neurons (**Supplementary Figure 1h**) These results were further confirmed by decreased luciferase activity in Cav3.1 KD neurons exposed to Tat or TNFα (**Supplementary Figure 1i**). Taken together, these data confirm that under conditions of neuroinflammation, PINCH expression is transcriptionally regulated by MEF2A by a cCa^{2+}-dependent regulatory cascade.

PINCH interacts with integrin-linked kinase (ILK) and α-Parvin forming a heterotrimeric complex called the PIP complex. The PIP complex assembles in the cytosol to signal actin cytoskeletal rearrangements and growth factor signaling through its interaction with Nck2 (1, 41, 42). It was recently reported that forced disruption of the PIP complex either by TGF-β1 or by overexpression of the PINCH-binding ANK-repeat domain of ILK in podocytes decreases podocyte-matrix adhesion and foot process formation and induces apoptosis (14, 15). Given the pivotal role of the PIP complex in regulating cell adhesion, cytoarchitecture and cell survival (14), we asked whether the increased expression of PINCH alters the PIP complex formation and cytoskeletal arrangements that can likely contribute to neurodegeneration. To test whether increased PINCH expression alters PIP formation, we immunoprecipitated PINCH from control neurons or neurons exposed to Tat or TNFα. Western analysis confirmed that ILK-1 co-immunoprecipitated with PINCH; whereas, the α-Parvin was disrupted from the PIP complex in neurons treated with Tat or TNFα (**Fig. 2a, i**). Because the PIP complex provides crucial links between integrins and the actin cytoskeleton, we next asked whether the disruption of the PIP complex alters actin organization. Immunofluorescence labelling in neurons exposed to Tat or TNFα, showed disruption of the F-actin filaments compared to untreated neurons (**Fig. 2b-d**).

Specifically, actin filament dynamics and reorganization are tightly regulated by a variety of actin-binding proteins of which cofilin plays a definitive role (43, 44). Cofilin binds to actin and promotes the disassembly of actin filaments. The activity of cofilin is inhibited by phosphorylation of serine 3 (43, 44). We therefore tested whether Tat or TNFα exposure activated (i.e., dephosphorylated) cofilin in neurons. We observed decreased levels of cofilin phosphorylation in neurons exposed to Tat or TNFα (**Fig. 2e, f**), suggesting increased cofilin activity accompanies actin depolymerization. The testicular protein kinase 1 (TESK1) and haloacid dehalogenases chronophin phosphorylate and dephosphorylate cofilin, respectively (45-51). We next sought to determine whether the decreased phosphorylation is due to differential expression of chronophin. However, chronophin levels were unaltered in Tat- or

TNFα–treated neurons (**Fig. 2e, g**), suggesting a possible role for kinase in regulating cofilin activity in Tat or TNFα treated neurons.

α-Parvin binding to TESK1 inhibits its activity, thereby activating cofilin for actin depolymerization. Moreover, interactions of α-Parvin with TESK1 or ILK were mutually exclusive (28). Because we observed α-Parvin disruption from the PIP complex upon increased PINCH expression (Fig. 2a), we examined the interaction of α-Parvin with TESK1 and ILK. Immunoprecipitation of α-Parvin from neurons treated with or without Tat or TNFα followed by Western analysis showed that TESK1 co-immunoprecipitated with α-Parvin, whereas the ILK-1 was disrupted from the Parvin-TESK1 complex in the presence of Tat or TNFα (**Fig. 2h, i**). These results suggest that increased expression of PINCH could disrupt the PIP complex, thereby promoting inactivation of TESK1 by interaction with α-Parvin with TESK1 followed by cofilin activation to reduce actin polymerization (**Fig. 2i**).

The dynamic organization of the actin cytoskeleton is crucial for numerous cell processes (52). For example, actin waves from the cell body to the tip of the neurites generate flow that transiently widens neurite shafts. Stochastically, this creates the space needed for microtubules to polymerize and create tracks for kinesin-based transport of cargo/organelles (53). Mitochondria are one such organelle that travels along the cytoskeleton using microtubules for long-distance trafficking to supply ATP to meet the high energy demands of neurons. However, the regulatory connections between actin and tubulin for proper mitochondrial distribution has only recently been reported (54-58). Given the actin reorganization observed in neurons exposed to Tat or TNFα (Fig. 2b, c), we examined mitochondrial distribution in control and in neurons treated with Tat or TNFα. Confocal images of neurons exposed to Tat or TNFα labeled with Rhodamine- 123 showed aggregation of mitochondria, often clustered around the nucleus (**Fig. 3a, b**), unlike mitochondria in control neurons that were evenly distributed throughout the neuronal soma and processes (**Fig. 3a, b**). Taken together thus far, these data show that neurons exposed to inflammatory signals, robustly express PINCH in a Ca^{2+}-dependent manner, leading to disruption of the PIP complex and actin, and consequently mitochondrial mislocalization.

To understand how disruption of actin may contribute to mitochondrial mislocalization, we assessed changes in interactions among tubulin, kinesin, Trak and mitochondrial Rho GTPase 1 (Miro1) (**Fig. 3c, d**). Kinesin is a microtubule-based molecular motor protein essential for the anterograde mitochondrial transport via an adaptor complex of kinesin-Trak-Miro proteins (59, 60). Trak forms a bridge between kinesin and Miro1. Co-immunoprecipitation and Western blot analyses indicated that exposure of neurons to Tat or TNFα disrupted the interaction between Trak and Miro1, while the interactions between Trak and kinesin, and kinesin and tubulin were preserved (**Fig. 3c, d**). These data suggest actin depolymerization disrupts the localization of mitochondria by disrupting the Trak/Miro1 interaction (**Fig. 3c, d**). To assess the bioenergetic capacity of mislocalized mitochondria in neurons exposed to Tat or TNFα, we assessed the oxygen consumption in all conditions. Neurons

exposed to Tat or TNFα were bioenergetically impaired as evidenced by reduced oxygen consumption rate (OCR) (**Fig. 3e-g**), decreased spare capacity (**Fig. 3h**) and diminished ATP coupled respiration (**Fig. 3i**).

To confirm that increased PINCH expression is ultimately responsible for actin depolymerization, mitochondrial mislocalization and impaired neuronal bioenergetics, we (i) exogenously overexpressed PINCH (**Fig. 4**), or (ii) knocked down PINCH (**Fig. 5**), or (iii) knocked down Cav3.1 (Supplementary **Fig. 2**). Exogenous expression of PINCH in neurons (**Fig. 4a**) disrupted the PIP complex (**Fig. 4b**), reduced phosphorylation levels of cofilin (**Fig. 4c**) by increasing the interaction of Parvin with TESK1 (**Fig.4d**), thus inactivating its activity and consequent disruption of actin (**Fig. 4e-g**). Depolymerization of actin resulted in mitochondrial mislocalization (**Fig. 4h and 4i**) by disrupting the Kinesin-Trak-Miro complex (**Fig. 4j**). Next, we knocked down PINCH with shRNA (**Fig. 5a, b**). In neurons infected with non-target shRNA, Tat and TNFα exposure induced mitochondrial mislocalization to the neuronal soma (**Fig. 5c, d**), whereas knocking down PINCH blocked Tat and TNFα-induced mitochondrial mislocalization (**Fig. 5c, d**). Likewise, KD of PINCH also prevented Tat and TNFα-induced reduction in OCR and ATP coupled respiration (**Fig. 5e-i**). Because we observed knocking down Cav3.1 in neurons prevented Tat- or TNFα-mediated induction of PINCH (**Supplementary Fig. 1c, g**), we next assessed the effect of Cav3.1 KD on actin and mitochondrial distribution in neurons treated with or without Tat or TNFα. Knocking down Cav3.1 restored actin polymerization (**Supplementary Fig. 2a-c**) by increasing the cofilin phosphorylation (**Supplementary Fig. 2d**). In addition, Tat/TNFα-mediated disruption of the Kinesin-Trak-Miro complex (**Supplementary Fig. 2e**) and mitochondrial mislocalization to the neuronal soma (**Supplementary Fig. 2f, g**) was prevented by Cav3.1 KD.

Our studies identified the regulatory mechanisms for and the biological consequences of increased PINCH expression during neuroinflammation. We have shown PINCH expression to be transcriptionally regulated through a Ca^{2+}-dependent kinase cascade and that increased PINCH is involved in the disruption of PIP complex, actin depolymerization, mitochondrial mislocalization to the neuronal soma by disrupting the kinesin-Trak-Miro complex, and bioenergetic crisis. Targeting PINCH expression to maintain levels at baseline could be a new therapeutic strategy for the treatment of neurodegeneration.

Discussion

Numerous studies have investigated the role of the actin cytoskeleton in various aspects of neurobiology. Many actin-regulatory proteins are mutated in neurological disorders linking actin cytoskeletal dynamics to normal CNS development and function (61-63). In addition, the extracellular matrix (ECM) play key roles as guidance molecules during CNS development, and are implicated in the

maintenance of stable neuronal connections and regulations of synaptic plasticity (62, 64). At the molecular level, cell-ECM adhesion is mediated by a network of integrin (transmembrane adhesion receptors) and a selective group of integrin-proximal cytoplasmic proteins. PINCH, ILK and Parvin are the cytoplasmic components, which link integrins to the actin cytoskeleton and signaling proteins (25, 65, 66). Through two direct interactions, one mediated by the PINCH N-terminal LIM1 domain and the ILK N-terminal ANK-repeat domain, and other mediated by the ILK C-terminal domain and the α-Parvin calponin homology 2 domain these cytoplasmic components form a ternary protein complex, the PIP complex.

PINCH (*lims1*/*pinch* gene) is non-catalytic and consists of five double zinc-finger LIM domains that bind to several different proteins including integrin-linked kinase and Nck-2 (1). Limited information exists regarding the role of PINCH; however, it appears to be critical for cell migration. Primarily, genetic analyses in *C. elegans* demonstrated that mutation of the PINCH homolog, UNC-97 caused locomotor defects. This was manifested by an uncoordinated movement phenotype, suggesting that this PINCH homolog was functionally important for muscle attachment and assembly and for proprioceptive neuronal function (11, 12). Later, PINCH was identified to be expressed abundantly in Schwann cells and dorsal root ganglia neurons post-injury to sciatic nerve (67) and at high levels during development to regulate cell migration and neuronal polarity (68-70). However, in healthy adult brains PINCH was nearly undetectable (18, 19) but is induced in CNS diseases and in cell and animal models of neurodegeneration. Our studies published in 2008 showed for the first time that PINCH was robustly detected in the brains and cerebrospinal fluid (CSF) of HIV patients (19). Importantly, our earlier studies reported an increase in levels of hpTau in *in vitro* neuronal cultures where PINCH was overexpressed or induced by exposure to TNFα (16, 18). In fact, blocking PINCH expression with shRNA in neurons exposed to hpTau inducing agents decreased levels of hpTau (18). Likewise, in a tauopathy mouse model, PINCH was shown to be significantly increased in several brain regions, where it was undetectable in wildtype mice (18). Our findings were then expanded to AD, frontotemporal dementia and mesial temporal epilepsy patients' brains (17, 18). Until recently, the overlap of increased PINCH and hpTau levels was unclear. Our current data indicate that along with actin depolarization and mitochondrial mislocalization, microtubule interactions with various protein components (Miro1, Trak, kinesin) are altered. In this context, it is possible that these disruptions also contribute abnormal Tau hyperphosphorylation and dissociation from the tubulin cytoskeleton. Understanding these potential interactions warrants further investigation into how alterations in Tublin, Trak, miro1 and kinesin impact Tau.

Our current data uncover regulatory mechanisms for the expression of PINCH in neuroinflammatory conditions as well as define the biological consequences of PINCH expression in neurons. In this study, we have shown PINCH to be transcriptionally regulated by a P38-dependent MEF2A activation cascade (**Fig.1**). Our data show that induced inflammation of neurons increases the

cytosolic Ca^{2+} (cCa^{2+}) through the activation of Cav3.1 (**Supplementary Fig.1**). Cav3.1 is the low-voltage-activated T-type Ca^{2+} channel that is involved in triggering calcium/calmodulin-dependent kinase (CaMKII) activation (40). The increase in cCa^{2+} in neurons exposed to Tat or TNFα activated CAMKII, which in turn phosphorylates P38. P38 is a potent kinase for MEF2A, phosphorylating the regulatory threonine sites and enhancing MEF2A-dependent gene expression (35-38). MEF2A has a diverse array of functions in development, neurological diseases, and cancer (71, 72). Importantly, increased expression of both MEF2A and PINCH are associated with invasive tumorigenesis (4, 72-74), thereby, supporting the potential role of MEF2A in PINCH transcription.

After defining the regulation of induced PINCH expression in a neuroinflammatory condition, we next assessed the biological consequences of PINCH overexpression. Our data suggest that induced expression of PINCH, disrupted the PIP complex (**Fig. 2a**) and exhibited altered actin cytoskeleton organization (**Fig. 2b and 2c**). Further, our mechanistic insight showed disruption of PIP complex to alter the phosphorylation and activity of cofilin and thus increased cofilin activity triggered actin depolymerization (**Fig. 2h and 2i**). The cellular phenotypes induced by PINCH expression and disruption of the PIP complex are remarkably like those resulting from gene inactivation of PINCH/ILK/parvin. Importantly, mouse cells lacking ILK exhibited altered actin cytoskeleton organization, impaired cell–ECM adhesion and spreading, and reduced proliferation rate (75, 76). These findings suggest that the formation of PIP complexes in neurons is likely essential to maintaining the cytoskeletal architecture.

The cytoskeleton, in addition to affecting mitochondrial morphology also plays a crucial role in maintaining mitochondrial distribution throughout the cell, facilitating organelle transport to areas with high metabolic demands (77). Treatment of cells with latrunculin A (LatA), an inhibitor for actin polymerization results in aggregation of mitochondria (78). Because we observed actin to be depolymerized, we assessed the cellular distribution of mitochondria. Like LatA treatment, depolymerization of actin resulted in mislocalization of mitochondria to the perinuclear region in neurons exposed to Tat or TNFα (**Fig. 3a and 3b**) reminiscent of loss of kinesin KIF5, dynein or their mitochondrial receptors Miro1/2. In this context, we next assessed whether actin depolymerization destabilized the microtubule-based transport of mitochondria, as Miro1/2 the mitochondrial receptor for motor proteins interplay both microtubule and actin-based mitochondrial movement (59, 79). Treatment of Tat/TNFα disrupted the Kinesin-dependent transport of mitochondria (**Fig. 3c and 3d**). A variety of diseases such as AD, Parkinson's disease, Charcot-Marie Tooth 2A disease and Huntington's disease have been linked to disruption in mitochondrial dynamics and morphology, though the mechanism(s) driving these disruptions are unknown (80).

Conclusions

Given the lack of reported mechanistic links among PINCH, neuroinflammation, neurodegeneration and mitochondrial dysfunction, our study is the first to uncover an important regulatory mechanism for PINCH expression and the pathological consequences of increased PINCH in neurons. These data have identified the transcription factor responsible for PINCH induction in neuroinflammatory conditions and the biological consequences of increased PINCH expression in neurons. Given that AD and neuroHIV share pathological features including cognitive impairment with chronic neuroinflammation, TNFα plays an important role in neurodegenerative processes. Further studies into potential strategies to inhibit PINCH induction during inflammation are warranted. Several lines of investigation into these approaches are underway that include blocking PINCH over expression at both transcriptional and translational levels utilizing TNFR and calcium influx antagonists and miRNA. The goal of these studies is to drive PINCH expression back to physiological levels to alleviate bioenergetic deficits and cytoskeletal disruptions observed in chronic neuroinflammatory diseases.

List of Abbreviations

AD Alzheimer's disease

ANK ankyrin

CAMKII calmodulin dependent protein kinase

Cav3.1 low-voltage-activated CaV3.1 calcium channel

cCa^{2+} cytosolic calcium

ChIP chromatin immunoprecipitation

CNS central nervous system

ECM extracellular matrix

GFAP glial fibrillary associated protein

HBSS Hank's balanced salt solution

HIV human immunodeficiency virus

hpTau hyperphosphorylated Tau

ILK integrin linked kinase

KD knockdown

LatA latrunculin A

lims1 LIM zinc finger domain containing 1 gene

MAP2 microtubule associated protein 2

MEF2A myocyte enhancer factor 2A

NM0 neurobasal media

PCR	polymerase chain reaction
PINCH	particularly interesting new cystine histidine-rich protein
PIP	PINCH-ILK-Parvin
Tat	transactivator of transcription
TESK1	testis associated actin remodeling kinase 1
TNFα	tumor necrosis factor alpha
TGF-β1	transforming growth factor beta 1

Declarations

Ethical Approval and Consent to Participate

Not applicable

Consent for Publication

Not applicable

Availability of Supporting Data

Data sharing is not applicable to this article as no datasets were generated or analyzed during the current study.

Competing interests

The authors declare no competing interests.

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Author contributions

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Figure Legends

Figure 1. PINCH is transcriptionally regulated by MEF2A under inflammatory conditions.
(**a**) Quantification of TNFα production by neurons exposed to Tat at different time points. (**b**) Quantification of PINCH mRNA levels in neurons untreated or exposed to Tat or TNFα for 48 h. (**c**) Bioinformatic analysis of *lims1/pinch* promoter sequence predicted a conserved putative binding site for different transcription factors (TF). Inset: Sequences show conserved binding sites for MEF2A, C-FOS, C-Jun, and AP-1. (**d**) ChIP-assay was performed in neurons untreated or exposed to Tat or TNFα. Antibodies specific for MEF2A, c-Jun, Foxd1, and HoxA9 were used to immunoprecipitate the chromatin and the fold enrichment of *lims1/pinch* promoter relative to the matched input control was quantified by qPCR. (**e**) Representative Western blot of lysates from neurons untreated or exposed to Tat or TNFα and probed with antibodies specific for phospho-CamkII, CamkII, phospho-P38, P38, phospho-MEF2A, MEF2A, PINCH and GAPDH. (**f-i**) Quantification of relative protein abundance of phospho-CamkII/CamkII (**f**), phospho-P38/P38 (**g**), phospho-MEF2A/MEF2A (**h**), and PINCH/GAPDH (**i**) and quantified from (**e**). (**j**) Schematic representation of the *lims1/pinch* promoter-luciferase constructs. The MEF2A consensus response element at −169-175 base pairs (bp) (TATTATA) is shown in oval. (**k**) Neurons transfected with control and *lims1/pinch* luciferase constructs were untreated or exposed to Tat or TNFα for 48 h and luciferase activity was measured. Data represents Mean ± SEM; $^*P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$; n = 3-5 (one-way ANOVA).

Figure 2. Increased PINCH expression disrupts the PIP complex and actin cytoskeleton.

(**a**) Cell lysates from control, Tat or TNFα treated neurons were immunoprecipitated with anti-PINCH antibody. Following immunoprecipitation, total cell lysates (input) and immunoprecipitated materials (IP) were subjected to Western blot analysis. Samples were probed with antibodies specific for PINCH (top left), ILK1 (top right), α-Parvin (bottom left), and TESK1 (bottom right). (**b**) Changes in cellular actin cytoarchitecture were observed using confocal microscopy in neurons untreated or exposed to Tat or TNFα. Neurons were fixed, permeabilized and labeled with anti-PINCH and stained with phalloidin, the dye that stains actin filaments. Representative confocal images show depolymerization of actin in human neurons exposed to Tat/TNFα. (**c and d**) Quantification of the PINCH fluorescence (c) and the actin filament length (d). (**e**) Representative Western blot for lysates from neurons untreated or exposed to Tat or TNFα and probed with antibodies against phospho-Cofilin, Cofilin, Chronophin, and GAPDH. (**f and g**) Quantification of relative protein abundance of phospho-Cofilin/Cofilin (**f**), and Chronophin/GAPDH (**g**) and quantified from (**e**). (**h**) Cell lysates from control, Tat or TNFα treated neurons were immunoprecipitated with anti-Parvin antibody. Following immunoprecipitation, total cell lysates (input) and immunoprecipitated materials (IP) were subjected to Western blot analysis. Samples were probed with antibodies specific for Parvin, TESK1, and ILK1. (**i**) Schematic representation of PIP complex regulation of cofilin phosphorylation and actin depolymerization. Data represents Mean ± SEM; **P <0.01; ***P <0.001; n = 3-5 (one-way ANOVA).

Figure 3. **Increased PINCH expression results in perinuclear localization of mitochondria.**
(**a**) Neurons untreated or exposed to Tat or TNFα for 48h were monitored for cellular mitochondrial distribution using confocal microscopy. Neurons were stained with dihydrorhodamine (DHR123) and changes in mitochondrial distribution was observed. Representative confocal images show perinuclear localization of mitochondria in human neurons exposed to Tat or TNFα. (**b**) Quantification of the distance (μm) of mitochondrial from the nucleus. (**c**) Cell lysates from control, Tat or TNFα treated neurons were immunoprecipitated with antibodies against kinesin. Following immunoprecipitation, total cell lysates (input) and immunoprecipitated materials (IP) were subjected to Western blot analysis. Samples were probed with antibodies specific for Kinesin (top left), Tubulin (top right), Trak1 (bottom left), and Miro1 (bottom right). (**d**) Schematic representation shows the disruption of Kinesin-Trak-Miro complex in neurons exposed to Tat or TNFα. (**e-i**) Neurons were untreated or exposed to Tat or TNFα for 48h and oxygen consumption rate (OCR) was measured. Untreated neurons were used as control. After measurement of baseline OCR, neurons were sequentially exposed to oligomycin (a), carbonyl cyanide-4-(trifluoromethoxy)phenylhydrazone

(FCCP) (b), rotenone and antimycin A (c). Representative traces of OCR in control and neurons exposed to Tat/TNFα (**e**). Quantification basal (**f**), maximal (**g**), spare capacity (**h**), and ATP coupled respiration (**i**) in control and neurons treated with Tat/TNFα. Data represents Mean ± SEM; **P <0.01; ***P <0.001; n = 3-5 (one-way ANOVA).

Figure 4. Exogenous expression of PINCH mimics the effects of Tat or TNFα exposure in neurons.

(**a**) Quantification of relative protein abundance (PINCH/GAPDH) and representative Western blot for lysates from neurons with or without exogenous PINCH expression and probed with anti-PINCH and -GAPDH. (**b**) Cell lysates from control and PINCH overexpressing (OE) neurons were immunoprecipitated with antibody specific for PINCH. Following immunoprecipitation, total cell lysates (input) and immunoprecipitated materials (IP) were subjected to Western blot analysis. Samples were probed with antibodies specific for PINCH, ILK1, α-Parvin, and TESK1. (**c**) Quantification of relative protein abundance (phospho-Cofilin/Cofilin) and representative Western blot for lysates from neurons with or without exogenous PINCH OE and probed with antibodies specific for phospho-Cofilin and Cofilin. (**d**) Cell lysates from neurons with or without exogenous PINCH OE were immunoprecipitated with antibody specific for Parvin. Following immunoprecipitation, total cell lysates (input) and immunoprecipitated materials (IP) were subjected to Western blot analysis. Samples were probed with antibodies specific for Parvin, TESK, and ILK1. (**e**) Changes in cellular actin cytoarchitecture were observed using confocal microscopy in neurons with or without exogenous PINCH OE. Neurons were fixed, permeabilized and stained with anti-PINCH and phalloidin, the dye that stains actin filaments. Representative confocal images show depolymerization of actin in neurons exogenously expressing PINCH. (**f and g**) Quantification of PINCH fluorescence (**f**) and actin filament length (**g**). (**h**) Cellular mitochondrial distribution was observed in neurons with or without PINCH expression using confocal microscopy. Neurons were stained with dihydrorhodamine (DHR123) and changes in mitochondrial distribution were observed. Representative confocal images show perinuclear localization of mitochondria in neurons exogenously expressing PINCH. (**i**) Quantification of the distance of mitochondrial distribution from the nucleus. (**j**) Cell lysates from neurons with or without exogenous PINCH expression were immunoprecipitated with antibodies specific for kinesin. Following immunoprecipitation, total cell lysates (input) and immunoprecipitated materials (IP) were subjected to Western blot analysis. Samples were probed with antibodies specific for Kinesin, Tubulin, Trak1, and Miro1. Data represents Mean ± SEM; ***P <0.001; n = 3-5 (one-way ANOVA).

Figure 5. Cav3.1 KD blocks PINCH induction and preserves neuronal mitochondrial distribution.

(**a**) Cellular actin architecture was observed in Scr siRNA and Cav3.1 knockdown (KD) neurons untreated or exposed Tat or TNFα for 48h using confocal microscopy. Neurons were fixed, permeabilized and labeled with anti-PINCH and stained with phalloidin. Representative confocal images show preserved actin polymerization in Cav3.1 KD neurons exposed to Tat or TNFα. (**b and c**) Quantification of the PINCH fluorescence (b) and the actin filament length (c). (**d**) Quantification of relative protein abundance (phospho-Cofilin/Cofilin) and representative Western blot for lysates from Scr siRNA and Cav3.1 KD human neurons untreated or exposed to Tat or TNFα and probed with anti-phospho-Cofilin and -Cofilin. (**e**) Cell lysates from Scr siRNA and Cav3.1 KD neurons untreated or exposed to Tat or TNFα were immunoprecipitated with anti-kinesin antibody. Following immunoprecipitation, total cell lysates (input; left) and immunoprecipitated materials (IP; right) were subjected to Western blot analysis. Samples were probed with antibodies against Tubulin, Kinesin and Miro1. (**f**) Mitochondrial distribution was observed in Scr siRNA and Cav3.1 KD neurons untreated or exposed to Tat or TNFα for 48 h using a confocal microscopy. Neurons were stained with dihydrorhodamine (DHR123) and changes in mitochondrial distribution were observed. Representative confocal images show preserved mitochondrial distribution in Cav3.1 KD neurons exposed to Tat or TNFα. (**g**) Quantification of the distance (μm) of mitochondrial distribution from the nucleus. Data represents Mean ± SEM; ***P <0.001; n = 3-5 (one-way ANOVA).

Supplementary Figure Legends

Supplementary Figure 1. Elevated cytosolic Ca^{2+} activates MEF2A through P38 phosphorylation and facilitates PINCH expression.

(**a**) Neurons untreated or exposed Tat or TNFα for 48 h were loaded with Fluo-4 AM (5 μM) to measure cytosolic Ca^{2+} levels. Quantification of Fluo-4 fluorescence at baseline levels. Three independent experiments were performed. Each dot represents mean fluorescence of ~10 cells/field and 5 fields/experiment were quantified. (**b**) Scr siRNA and Cav3.1 KD human neurons untreated or exposed to Tat or TNFα for 48 h were loaded with Fluo-4 AM (5 μM) to measure cytosolic Ca^{2+} levels. Fluo-4 fluorescence quantified as in (a). (**c**) Representative Western blot for lysates from Scr siRNA and Cav3.1 KD neurons untreated or exposed to Tat or TNFα for 48 h and probed with antibodies against Cav3.1, phospho-P38, P38, phospho-MEF2A, MEF2A, PINCH and GAPDH. (**d-g**) Quantification of relative protein abundance of Cav3.1 (**d**), phospho-P38 (**e**), phospho-MEF2A (**f**) and PINCH (**g**) quantified from (c). (H) ChIP-assay was performed in Scr siRNA and Cav3.1 KD neurons untreated or exposed to Tat or TNFα for 48 h. Anti-MEF2A antibody was used to immunoprecipitate the chromatin

and the fold enrichment of *lims1/pinch* promoter relative to the matched input control was quantified by q-PCR. (I) Luciferase activity was measured in Scr siRNA and Cav3.1 KD neurons transfected with *lims1/pinch* luciferase construct after treatment with or without Tat or TNFα for 48 h. Data represents Mean ± SEM; **P <0.01; ***P <0.001; n = 3-5 (one-way ANOVA).

Supplementary Figure 2. Ablation of PINCH expression preserves neuronal mitochondrial distribution.

(**a**) Cell lysates from neurons infected with non-target or PINCH shRNA viral particles were Western blotted with anti-PINCH and -GAPDH. Representative Western blot analysis shows knock down of PINCH in neurons infected with shRNA viral particles. (**b**) Densitometric quantification of PINCH levels in neurons. (**c**) Mitochondrial morphology was observed in non-target shRNA and PINCH KD neurons treated with or without Tat or TNFα for 48h using confocal microscopy. Tat or TNFα treated neurons were stained with dihydrorhodamine (DHR123) and changes in mitochondrial morphology were observed. Representative confocal images show preserved mitochondrial distribution in PINCH KD neurons exposed to Tat or TNFα. (**d**) Quantification of the distance of mitochondrial distribution from the nucleus. (**e-i**) Measurement of OCR in non-target shRNA and PINCH KD neurons exposed to Tat or TNFα (**e and f**) Representative traces of OCR in non-target shRNA (**e**) and PINCH KD (**f**) neurons. (**g-i**) Quantification of basal (**g**), maximal (**h**), and ATP-coupled respiration (**i**) in non-target shRNA and PINCH KD neurons exposed to Tat or TNFα.