An integrin receptor complex mediates filamentous Tau-induced activation of primary astrocytes

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Protein aggregates formed by microtubule-associated protein Tau can be transmitted in a stereotypic pattern in human brains, which correlates with the progression of several neurodegenerative diseases collectively termed Tauopathies. This process requires Tau, released from neurons, to interact with a cell surface receptor on a target cell, but little is known about the underlying mechanisms and the downstream pathophysiological consequences particularly for Tau that engages glial cells. Using a spatially resolved proteomic mapping strategy, we identify integrin αV/β1, a heterodimer of the integrin family, as a receptor that not only mediates Tau fibril entry into astrocytes, but also activates integrin signaling upon Tau binding. We show that distinct Tau species differentially activates integrin signaling, with filamentous Tau being a more robust stimulator. This leads to differential upregulation of pro-inflammatory cytokines and chemokines. Additionally, a sub-group of neurotoxic astrocyte markers are also induced in an integrin-dependent manner preferentially by filamentous Tau, causing astrocytes to release a neurotoxic factor(s). Together, these findings establish a paradigm that astrocytes can be directly converted into a neurotoxic state by filamentous Tau via an integrin receptor, which as a sensor transducing unfolded protein pathology, may provide a new therapeutic target for Tauopathies.

Clinically documented protein aggregates such as extracellular amyloid plaques and intracellular neurofilaments (NFL) are pathological hallmarks of neurodegenerative diseases such as Alzheimer's disease (AD). NFLs are formed primarily by hyper-phosphorylated Tau, a soluble microtubule-binding protein abundantly expressed in neurons of the central nervous system (CNS) [1, 2]. The primary function of Tau is to stabilize microtubules, but its intrinsic misfolding propensity prompts it to form toxic filamentous aggregates, particularly in neurons during aging. In addition to AD, Tau-containing NFLs have been linked to brain dysfunctions in other neurodegenerative diseases such as Pick's disease and frontotemporal dementia, which are collectively termed Tauopathies [1].

An unusual characteristic of tauopathies is the prion-like propagation of Tau-containing aggregates, which seems to correlate with the decline of cognition during disease progression [1-6]. This intercellular protein propagation process may involve the release of Tau in either monomeric or aggregated forms, followed by the uptake by recipient cells (neurons or glial cells) [7]. Tau fibrils formed along this path can serve as 'seeds' once inside a target cell, converting endogenous Tau monomers into an aggregation-prone toxic conformer. Accumulating evidence suggests that both monomeric Tau and Tau aggregates can be released from neurons independent of cell death, and this process is enhanced by neuronal activities [8-10]. The mechanisms underlying Tau release are controversial. Specifically, whether Tau is predominantly released in a free soluble form [11-14] or in membrane-associated vesicles [8] is unclear. By contrast, on the uptake side, several studies showed that neurons, microglia, and astrocytes all could readily internalize

different Tau species [15-18]. In certain immortalized cells, endocytosis of Tau preformed fibrils (PFFs) is initiated upon Tau binding to heparan sulfate proteoglycans (HSPGs) on the cell surface [19-21], which cooperate with a membrane receptor to mediate the uptake of monomeric and oligomeric Tau [22], but other unidentified mechanisms also contribute to Tau uptake, particularly in primary non-neuronal cells [22, 23].

In addition to damaging endogenous Tau folding when Tau PFFs enter neurons, Tau fibrils can also influence non-neuronal cells from cell exterior. This model is particularly attractive in the complex brain microenvironment in which the fitness of neurons is tightly modulated by glial cells such as astrocytes [24, 25]. It was shown previously that activation of astrocytes by lipopolysaccharide (LPS) increases their phagocytic activity, which might mitigate Tauopathies because activated astrocytes help to clear protein aggregates [26, 27]. However, activated astrocytes might also exacerbate Tauopathies because they can contribute to neuroinflammation by releasing pro-inflammatory cytokines and chemokines [28, 29]. Notably, recent transcription profiling studies have revealed two distinct functional states of reactive astrocytes, termed as “A1” and “A2”, respectively [30]. A2 astrocytes appear to restore neuronal activities after injury; whereas A1 astrocytes not only lose their neuro-supportive function, but also gain neurotoxic activities by releasing some unknown factors.

How astrocytes are converted to the pathogenic A1 state is a key question relevant to many neurodegenerative diseases. To date, accumulating evidence suggest that microglia activated during classic inflammation response serves as an essential mediator

of astrocytic A1 conversion because factors released by reactive microglia are sufficient to activate the astrocyte A1 state [31]. However, whether the astrocyte A1 state can be achieved independent of microglia under stress or disease conditions is unknown.

Given the close link between Tau misfolding and neurodegenerative diseases, we here investigate whether harmful insults triggered by extracellular Tau aggregates can directly convert astrocytes to the A1 state via a cell surface receptor. We used a proximity-based ligation approach to identify an integrin complex as a receptor for Tau monomer and fibrils in primary astrocytes. The ligation of Tau fibrils to integrin not only initiates their internalization, but also activates integrin signaling, which in turn induces inflammation and facilitates the conversion of astrocytes into the A1 state. These findings establish a direct link between misfolding-associated proteotoxic stress and astrocyte A1 activation.

Results

HSPG-independent uptake of Tau PFFs by primary astrocytes

To study the mechanism of Tau fibril uptake in primary astrocytes, we purified Tau (the 2N4R variant) as a recombinant protein from *E. coli* (Supplementary Fig. 1a) and labeled it with a fluorescent dye (Alexa 594). Labeled protein was used to generate PFFs following a well-established protocol (Supplementary Fig. 1b) [32]. Consistent with a previous report [15], treating mouse primary astrocytes with Tau PFFs resulted in their rapid internalization, but this process was largely unaffected by heparin (Supplementary Fig. 1c, d), a competitive inhibitor that blocks Tau PFF binding to cell surface HSPGs in some cell lines [19-21]. This finding is in line with another study, which suggests that unlike immortalized cells,

primary astrocytes do not use HSPGs for Tau PFF internalization [33]. These findings prompted us to search for Tau receptor(s) in primary astrocytes.

Identification of integrin αV/β1 as a Tau interactor in primary astrocytes

Because Tau monomers could also enter primary astrocytes, albeit with reduced efficiency compared to Tau PFFs [15, 33], we presumed that Tau fibrils and monomers engaged a common receptor, but with different affinities. Therefore, we employed a proximity labeling-based proteomic mapping strategy using purified APEX2-tagged Tau monomer as bait (Figure 1a). APEX2 is an engineered peroxidase that can covalently tag proximal lysine side chains with biotin molecules in the presence of biotin-phenoxyl radicals [34]. Because the relatively large APEX2 fusion might affect the interaction of Tau with the plasma membrane, we first used Sh-SY5Y cells to characterize the binding of different Tau fusion proteins to the plasma membrane (Figure 1b). When APEX2 was fused to the C-terminus of Tau, Tau-APEX2 could bind to the cell surface (Supplementary Fig. 2a), but fusion of APEX2 to the N-terminus of Tau abolished the binding (Supplementary Fig. 2b). Thus, Tau binding to the plasma membrane appears to be mediated by a sequence near its N-terminus, which is masked by N-terminally fused APEX2.

To identify Tau receptor on astrocytes, we incubated Tau-APEX2 or APEX2 as a control with primary astrocytes and then performed biotin labeling by addition of biotin-phenol. Biotinylated plasma membrane proteins were enriched by streptavidin-affinity purification. Immunoblotting using biotin antibodies detected a major biotinylation product with Mr. of ~120kDa (Figure 1c). As expected, APEX2 alone was not able to biotinylate

this 120kDa protein (Figure 1c). Mass spectrometry analysis of the excised band identified several membrane proteins enriched in Tau-APEX2-treated cells (Supplementary Figure 2c). Interestingly, the top candidates were all cell adhesion molecules such as integrins and neural cell adhesion molecules (NCAM).

The identification of integrin as a potential Tau receptor was intriguing given that genetic variations in integrin genes had been suggested as a potential risk factor for neuroinflammation/neurodegeneration [35-37] and that α6/β1 integrin was shown to facilitate phagocytosis-mediated elimination of Aβ amyloid [38, 39]. Since αV/β1 integrin (ITGαV/β1) was the most significantly modified proteins (based on peptide count), we focused this study on the functional link between ITGαV/β1 and Tauopathy.

To validate the interaction of Tau-APEX2 with αV/β1 integrin, we repeated Tau-APEX2-mediated biotinylation using HEK293T cells over-expressing either Flag-tagged ITGαV or GFP-tagged ITGβ1. Following cell surface biotinylation by Tau-APEX2 and streptavidin pulldown, these proteins could be detected by immunoblotting using antibodies recognizing their corresponding tags (Figure 1d, Supplementary Fig. 2d), suggesting that they could indeed be biotinylated by Tau-APEX2. Similar experiments performed in primary astrocytes further confirmed that endogenous ITGαV/β1 could be biotinylated by Tau-APEX2 but not APEX2 (Figure 1e). Importantly, after Tau-APEX2 binding to the cell surface, immunoprecipitation of Tau-APEX2 from cell extracts using a Tau specific antibody also co-precipitated endogenous ITGαV/β1 (Figure 1f). Likewise, when Tau-APEX2 was incubated with purified ITGαV/β1 comprising the ectodomains of

these proteins, immunoprecipitation using the Tau antibody also co-precipitated ITGαV/β1 (Supplementary Fig. 3). Collectively, these results suggest that biotinylation of ITGαV/β1 by Tau-APEX2 is likely a result from a direct interaction between these proteins.

αV/β1 integrin mediates Tau PFF uptake in primary astrocytes

We next tested whether Tau PFFs also interacted with endogenous ITGαV/β1 by co-immunoprecipitation. To this end, we incubated primary astrocytes in the presence or absence of Tau PFFs on ice before solubilization by a NP40-containing buffer. Immunoprecipitation using Tau antibodies precipitated Tau PFFs together with endogenous ITGαV/β1 (Figure 2A, lane 9). By contrast, ITGαV/β1 was not precipitated when Tau PFF was omitted or when a control antibody was used (lanes 7, 8). Thus, ITGαV/β1 also interacts with Tau PFFs and may serve as a receptor for both monomeric and filamentous Tau.

Since filamentous Tau is disease-promoting, we tested whether ITGαV/β1 mediates Tau PFF cell entry. We generated lentiviruses expressing small hairpin RNAs (shRNAs) that specifically knocked down ITGαV or ITGβ1 (Figure 2b, c). Strikingly, knockdown of ITGαV and ITGβ1 either individually or in combination both significantly reduced Tau PFF internalization in primary astrocytes (Figure 2d, e, f). Furthermore, knockdown of Talin, an adaptor essential for integrin signaling and integrin-mediated endocytosis [40-42] also led to a significant reduction in Tau PFF uptake (Figure 2e, f, g). The fact that knockdown of three different integrin pathway components all reduced Tau PFF uptake suggested that the internalization defect was not caused by an off-target effect.

Intriguingly, treating cells with a potent inhibitor (PF-562271) of the focal adhesion kinase (FAK), an essential integrin regulatory kinase downstream of Talin did not affect Tau PFF endocytosis (Figure 2h, i). Together, these results suggest that binding to ITGαV/β1 triggers Tau PFF cell entry, which requires Talin but is independent of the downstream integrin signaling.

Monomeric Tau and Tau PFFs differentially activate integrin signaling

The ligation of ligands such as fibronectin to integrin activates integrin signaling, changing the expression of many downstream genes. To test whether different Tau species also activated integrin signaling upon binding to ITGαV/β1, we treated primary astrocytes with monomeric Tau, Tau PFFs, or as a control, with phosphor-buffer saline (PBS) for 6 h. qRT-PCR analyses of a collection of integrin target genes (MMP9, ICAM1, MMP13 etc.) [43, 44] showed that compared to PBS-treated cells, Tau monomer and PFFs differentially activated the expression of many integrin target gene, with Tau PFF being ~2-3fold more potent than monomeric Tau (Figure 3a). As anticipated, Tau PFF-induced integrin activation could be significantly mitigated if astrocytes were first infected with lentiviruses that knocked down ITGαV and ITGβ1 (Figure 3b).

Because astrocytes purified by the conventional method also contain some microglia and oligodendrocytes, we next used immunopanning-purified primary astrocytes to further characterize Tau PFF-induced integrin signaling (Figure 3c) (also see materials and methods). Immunostaining using an antibody against an astrocyte-specific marker confirmed the identity of the purified astrocytes, which internalized Tau PFFs similarly as

conventionally purified astrocytes (Figure 3c). Like conventionally purified astrocytes, Tau PFF also activated integrin target genes in immunopurified astrocytes (Figure 3d), which was reproducibly diminished when Talin was depletion (Figure 3d). Altogether, these data suggest that like canonical integrin ligands, the interactions of Tau and Tau PFFs with ITGαV/β1 also activate integrin signaling.

Tau PFFs induce astrocytic inflammation via αV/β1 integrin

As a major immune-competent cell in the CNS, astrocytes can sense danger signals and elicit an immune response via the secretion of pro- or anti-inflammation cytokines and chemokines [28, 45]. We therefore analyzed the mRNA expression of a collection of cytokine and chemokine genes by qRT-PCR in astrocytes exposed to Tau monomer or Tau PFF, using PBS-treated cells as a reference. Intriguingly, PFF treatment caused a strong induction of several pro-inflammatory cytokines (IL1α, IL1β, IL6, TNFα) and chemokines (CCL2, CCL3, CCL4, CXCL10). By contrast, the expression of the anti-inflammation cytokines/chemokines such as IL10, TGFβ, and CLC12 was not affected (Figure 4a, b). Monomeric Tau also activated pro-inflammatory cytokines and chemokines, but consistently at lower levels (Figure 4a, b), suggesting that integrin activation by Tau might be coupled to inflammation induction. Indeed, the induction of pro-inflammatory genes by Tau PFFs was largely abolished in integrin αV/β1 knockdown astrocytes (Figure 4c). Thus, ITGαV/β1 is required for the Tau PFF-induced pro-inflammation response in astrocytes.

In the brain, astrocyte activation is usually linked to increased expression of the NO synthase (NOS2) [46], which upregulates the production of nitric oxide (NO) during

inflammation [47]. Indeed, qRT-PCR detected a significant increase in the expression of NOS2 in astrocytes following Tau PFF treatment. Like integrin target genes, NOS2 expression was also induced by Tau monomer, but by a smaller magnitude (Figure 4d). The depletion of ITGαV/β1 significantly reduced NOS2 induction by Tau PFF (Figure 4e). Because NF-κB but not STAT3 was differentially induced in an ITGαV/β1 dependent manner by Tau monomer and PFFs (Figure 4f, g), and because NF-κB is a known inflammation mediator that had been linked to integrin-induced inflammation in human umbilical vein endothelial cells [48, 49], we proposed that Tau PFF induced an inflammation response in astrocytes in part due to activation of NF-κB following ITGαV/β1 activation.

Tau PFF-induced inflammation requires Talin and FAK

To further elucidate the functional link between Tau PFF-induced integrin signaling and astrocytic inflammation, we used immuno-purified astrocytes to test the requirement of Talin and FAK in Tau PFF-induced inflammation. As expected, Tau PFF treatment induced the expression of pro-inflammation cytokines and chemokines such as IL1α, IL1β, IL6, TNFα, CCL2, CCL3, CCL4, and CXCL10 in immunopurified astrocytes, but noticeably, the induction of IL1α and IL1β by Tau PFF was much higher than that seen in conventionally purified astrocytes (Figure 5a vs. Figure 4a). This was probably due to the use of integrin antibodies during purification (Figure 3c), which might enrich for a population of astrocytes with high levels of integrin. As anticipated, the lentivirus-mediated knockdown of Talin reproducibly reduced Tau PFF-induced expression of pro-inflammation cytokines and chemokines (Figure 5a, b, c). Talin knockdown also reduced Tau PFF-induced expression

of NOS2 and NF-kB (Figure 5d, e). By contrast, STAT3 was only minimally affected by Tau PFFs, which was also not affected by Talin knockdown (Figure 5e).

To test the role of FAK in Tau PFF-induced inflammation, we pre-treated immunopurified astrocytes with the FAK inhibitor PF-562271, which almost completely abolished Tau PFF-induced expression of pro-inflammatory genes (Figure 5f-h). Thus, Tau PFF-induced inflammation requires Talin and FAK.

Tau PFF converts astrocytes into a neurotoxic state via αV/β1 integrin

Recent studies suggested that extracellular factors in the brain microenvironment can cause native astrocytes to adopt either a detrimental 'A1' state, in which astrocytes gain neurotoxic activities, or an 'A2' state in which they remain supportive for neuronal growth [30]. These states are linked to the expression of certain A1 or A2 signature genes. Since Tau PFFs activate integrin signaling and induce astrocytic inflammation, we tested whether Tau PFF-treated astrocytes resemble the neurotoxic A1 state or the neuroprotective A2 state by determining the expression of the previously characterized A1 and A2 signature genes using qRT-PCR. Interestingly, both Tau monomer and PFFs induced the expression of A1 specific genes including Gbp2, H2-D1, H2-T23, Ggta, Lipg1, Psmb2 etc., but in general, PFFs caused a higher induction of A1 genes than monomer (Figure 6a). By contrast, most A2 genes were not significantly affected with the exception of CD14, Tgm1, and Ptx3. The knockdown of ITGαV/β1 or Talin dramatically reduced the induction of A1 genes by Tau PFFs (Figure 6b, c). Likewise, pre-treating astrocytes with PF-562271 also abolished A1 gene induction (Figure 6d). Thus, Tau PFFs alter astrocytes

in an integrin-dependent manner, converting them into an A1-like state.

Tau PFF-treated astrocytes release neurotoxic factors

To further test whether Tau PFF converted astrocytes into a neurotoxic state, we collected conditioned medium (CM) from cells treated with either monomeric Tau, Tau PFF, or PBS. Concentrated CMs were then added to primary cortical neurons. After 72 h of incubation, neuronal cell death was monitored by a fluorescence-based assay (Figure 7a). The results showed that the medium from Tau PFF-treated cells was more toxic compared to that collected from PBS- or monomeric Tau-treated astrocytes (Figure 7b-d). By contrast, the medium from monomeric Tau-treated cells only had a slightly elevated neurotoxic activity compared to medium from PBS-treated astrocytes. Interestingly, knockdown of ITGαV/β1 alleviated the neurotoxic activity in CM from Tau PFF-treated cells (Figure 7b-d). These results suggest that Tau PFF-treated astrocytes can release a neurotoxic factor(s) in an ITGαV/β1-dependent way.

Discussion

The accumulation and propagation of Tau filamentous aggregates are observed in a broad spectrum of neurodegenerative disorders including AD [1, 50]. The progressive spreading of Tau inclusions, which was recapitulated in mouse models [4, 5, 51], is thought to take place along neuronal connections or via unconventional protein secretion followed by cell uptake [7, 52]. Tauopathies have also been detected in glial cells (both microglia and astrocytes), although endogenous Tau is not expressed in these cells. This suggests that pathological Tau inclusions may also be transmitted from neuron to glia [53-56]. Importantly, expression of

human Tau in glia in a *Drosophila* model led to neurotoxicity, suggesting a pathogenic activity for propagated Tau in glia [57]. Likewise, in a transgenic mouse model, astrocytic expression of human Tau using the astrocyte-specific GFAP (glial fibrillary acidic protein) promoter leads to neurodegeneration [58]. Collectively, these results demonstrate that the prion-like transmission of Tau aggregates from neurons to glia may contribute to neurodegeneration. However, it is unclear whether it is the internalized Tau fibrils or the interplay between a cell surface receptor(s) and the extracellular Tau prior to its cell entry that causes neurotoxicity.

In this study, we show that Tau filamentous aggregates bind to an integrin receptor complex (ITGαV/β1) on the astrocyte surface, which is not only required for Tau fibril uptake, but also regulates fibril-induced inflammation and astrocytic A1 conversion (Figure 7e). ITGαV/β1, as a member of the integrin family, is a cell surface adhesion molecule that binds to extracellular matrix proteins [59]. This activates integrin signaling, which has been linked to cancer cell invasion and proliferation [44, 59]; Through the actin-binding protein Talin and the downstream focal adhesion kinase (FAK), integrin activation alters gene expression, allowing cancer cells to adapt to the constantly changing microenvironment during metastasis. Integrins and their ligands are also widely expressed in the CNS where they support neural development [60, 61]. Moreover, several lines of evidence have hinted at roles of integrins in neurodegeneration caused by proteotoxic stress: 1) A genome-wide association study (GWAS) in a *Drosophila* model of AD identified fly homologs of the human integrin receptors ITGαM and ITGα9 as modifiers for Tau-induced neurotoxicity [35];

2) Microglia can take up fibrillar AD-associated β-Amyloid (Aβ) aggregates via ITGα6/β1 and an integrin-associated protein CD47 [38]; 3) The expression of β1 integrin is elevated in cortical astrocytes from AD patients, and in AD-related animal models β1 integrin can mediate soluble Aβ oligomers-induced astrogliosis [62]; 4) A more recent study revealed that in response to spinal cord injury, which is often associated with the release of stress-inducing factors such as misfolded proteins, astrocytes are activated via an integrin and cadherin-dependent mechanism to form astrocytic scars [63]. In light of these observations and our new findings, we propose that integrin receptors on astrocytes may help the brain to sense the extracellular protein misfolding burden, initially facilitating the clearance of misfolded protein aggregates, but eliciting a danger signaling in the form of inflammation when misfolded proteins exceed certain thresholds. However, it should be noteworthy that αV/β1integrin may not be the only astrocytic receptor for Tau, as suggested by our mass spectrometry study. The effect of filamentous Tau on cells may result from simultaneous engagement of multiple membrane receptors, which awaits further characterization.

Intriguingly, Tau fibrils and monomers can differentially activate integrin signaling, which might be due to their different oligomeric states. In support of this view, recent studies have underscored the role of liquid-liquid phase separation in transmembrane receptor signaling [64]. Specifically, the coagulation of membrane receptors into a gel-like state upon binding to a multivalent ligand can dramatically amplify the magnitude of downstream signaling. In the case of integrin, receptor clustering upon binding of a multivalent ligand is known to potentiate the outside-in integrin signaling [65].

Astrocytes, as the most abundant glial cells in the CNS, are a well-established modulator of neurodegeneration-associated inflammation [45]. Specifically, disease-associated abnormal proteins such as Aβ and α-Syn fibrils can change astrocyte physiology. For instance, different Aβ species can activate disparate astrocytic cell receptors to induce the pro-inflammatory NF-κB pathway [66-69]. Our new study shows that extracellular Tau aggregates can similarly impact astrocyte, suggesting a unified interplay between extracellular misfolded proteins and astrocytes.

Importantly, our study for the first time shows that Tau PFF-activated astrocytes resemble the neurotoxic A1 state reported recently [30]. This gain-of-function state, which has been associated with astrocytic inflammation, was suggested to play a causal role in neurodegeneration, but the upstream signals that promote astrocytic A1 conversion are unclear. In particularly, existing evidence suggests that the A1 state could only be achieved by a relayed signal elicited from microglial cells that had been exposed to an extracellular insult such as LPS. By contrast, we showed that even in the absence of microglia, misfolded Tau aggregates in the cell exterior can trigger astrocyte A1 conversion via ITGαV/β1. This suggests that astrocytic inflammation may be causal to tauopathies as opposed to a result of Tau-induced neurotoxicity. Additionally, given that neurons are usually the major source of secreted Tau, our findings raise the possibility that astrocytes may monitor the healthy state of neurons by sensing the level of misfolded Tau released by neurons. It is conceivable that Tauopathies in neurons at low levels may be mitigated by intercellular transmission and astrocyte-mediated clearance, but at high levels will

activate astrocytes, which release neurotoxic factors to cause neurodegeneration. This model opens a new venue for drug development aimed at preventing astrocytic A1 conversion in tauopathies.

Materials and methods

Animals and reagents

Wild-type C57BL/6J mice were purchased from the Jackson Laboratories. All animals were maintained in accordance with the animal care standards of the National Institutes of Health. All reagents are listed in Supplementary table 1.

Conventional primary astrocyte culture

Primary astrocyte cultures were prepared from cerebral cortices of P2-P5 C57BL/6J mice. Cortices were dissected, stripped of meninges, and digested with 0.25% trypsin at 37 °C in Hank's Balanced Salt Solution (HBSS) (Thermo Fisher) for 10 min. Trypsinization was stopped by the addition of astrocyte culture medium (DMEM/ F12 50/50 (Thermo Fisher) containing 25 mM glucose, 4 mM glutamine, 1 mM sodium pyruvate, and 10% FBS). Single-cell suspension of the digested tissue was obtained by repeated pipetting. Cells were seeded into a 75 ml flask at a density of ~4×10^5 cells/cm^2 and cultured in astrocyte culture medium at 37 °C in a humidified 5% CO_2 incubator. Monolayers of glial cells were obtained 7-10 days after plating. To remove microglia, cultures were gently shaken, and the floating cells (microglia) were removed, resulting in more than 95% pure astrocytes. The remaining astrocytes were incubated for 72 h before being infected with lentivirus or other experiments. Before experiments, astrocytes were dissociated by trypsinization and then reseeded at 4×10^5 cells

cm^2 or 1.5×10^5 cells per well in 24-well or 1.5×10^6 cells per well in 6-well or 2×10^5 cells cm^2 in Labtek imaging dish in DMEM F12 50/50 containing 10% FBS and 1% penicillin-streptomycin. At the start of each experiment, the medium was changed to regular DMEM without serum and antibiotics.

Primary astrocyte culture purified by immuno-panning

Astrocytes were purified by immune-panning from P2-P5 C57BL/6J mice and cultured as previously described [70]. Briefly, cortices were digested by Trypsin at 37 °C and then mechanically dissociated to generate single-cell suspension, which was incubated in successive negative immune-panning plates to remove macrophage and microglia cells before positive selection for astrocytes by an ITGβ5-coated panning plate. Isolated astrocytes were cultured in a defined, serum-free base medium containing 50% neurobasal, 50% DMEM, 100 U/ml penicillin, 100 μg/ml streptomycin, 1 mM sodium pyruvate, 292 μg/ml L-glutamine, 1 x G5 supplement. This medium was supplemented with the astrocyte-required survival factor HBEGF (Peprotech, 100-47) at 5 ng/ml as previously described [71].

Primary neuron cultures

Primary neuron cultures were prepared from cerebral cortices of P0-P1 C57BL/6 mice. Cortices were dissected, stripped of meninges, and digested with 0.25% trypsin at 37 °C in HBSS for 10 min. Trypsinization was stopped by the addition of FBS. A single-cell suspension of the digested tissue was obtained by repeated pipetting. Cells were seeded into a 24 well plate at a density of 1.5×10^5 cells per well and cultured in DMEM culture medium supplemented with 10% fetal bovine serum (Thermo Fisher), 100 U/ml of penicillin, 0.1 mg/ml

of streptomycin (Thermo Fisher), 25 mM glucose, 4 mM glutamine, and 1 mM sodium pyruvate at 37 °C. At 24 h after seeding, the medium was changed with Neurobasal medium (Thermo Fisher) supplemented with B-27 (Thermo Fisher) containing 0.5 mM glutamine. Cells were cultured at 37 °C in a humidified chamber supplemented with 5% CO2. Cultures were used for experiments from 7 to 10 days after seeding.

Purification of Tau protein

The recombinant human (Tau 2N4R isoform), containing 2 N-terminal inserts and 4 microtubule binding repeats was isolated as previously described [32]. Briefly, the Tau protein was expressed using the pET29b vector in the *E.coli* strain *BL21*. From a culture volume of 10 liter, the cell pellet was resuspended in ice cold cell resuspension buffer (20 mM MES, 1 mM EGTA, 0.2 mM $MgCl_2$, 5 mM DTT, 1 mM PMSF). Cells were disrupted by sonication, and NaCl was added to a final concentration of 500 mM. Cell suspension was boiled for 20 min, which denatured almost all proteins except for Tau. The denatured proteins and insoluble cell debris were sedimented by centrifugation at 127,000g for 40 min at 4 °C. The supernatant was dialyzed overnight twice in the cation exchange chromatography buffer A (20 mM MES, 50 mM NaCl, 1 mM EGTA, 1 mM $MgCl_2$ with 2 mM DTT, 0.1 mM PMSF at 4 degree under constant stirring. Cleared the dialysate by centrifugation at 127,000g for 40 min at 4 °C. The clear supernatant was loaded onto a cation-exchange chromatography column. The bound Tau protein was eluted with a linear gradient of 0-60% final concentration of the cation exchange chromatography buffer B (20 mM MES, 1 M NaCl, 1 mM EGTA, 1 mM $MgCl_2$, 2 mM DTT, 0.1 mM PMSF) over 6 column volumes. Fractions containing Tau were pooled and concentrated

by an ultrafiltration device (Millipore 10 kDa MW cutoff) to a final volume of 1 ml. Concentrated Tau protein was further fractionated by a size exclusion column in a gel filtration buffer (PBS with 1mM DTT), flash-frozen in liquid nitrogen and stored at -80 °C. 50 μM purified Tau was labeled with Alexa Fluor 594 dye following the manufacturer's instruction (Thermo Fisher).

Preparation of paired helical filaments from Tau proteins

The condition for Tau polymerization is: 50 μM purified Tau protein, 12.5 μM heparin, a protease inhibitor cocktail (10 μg/ml leupeptin, 5 μg/ml chymostatin, 3 μg/ml elastatinal, and 1 μg/ml pepstatin), and 2 mM DTT in phosphate buffer saline (PBS). This solution was incubated at 37 °C for two weeks. Tau polymerization is induced by the addition of heparin. 1mM fresh DTT was added into the solution every 24 h to maintain reducing condition since disulfide bond formation in the four-repeat domain of Tau reduces polymerization efficiency. After polymerization, Tau filaments were dialyzed extensively with PBS to remove heparin and DTT. Transmission electron microscopy was used to check PHF assembly.

Electron microscopy (EM) analysis of Tau filaments

5 mg/ml Tau filaments were diluted 10-fold in PBS and loaded onto an EM grid. The grid was then washed with pure water and stained with 3% uranyl acetate for 1 min and then imaged with a Morgagni 268 transmission electron microscope.

Tau internalization assay

Tau filaments were sonicated as described previously before the experiments [72]. Primary astrocyte culture was treated with 200 nM Tau filament-Alexa Fluor 594 for 2 hours in the astrocyte culture medium. In the case of inhibitor treatment, culture was pretreated with 10 μM

PF262271 for 2 hours. Cells were either directly imaged using a Zeiss LSM780 confocal microscope or fixed with 4% paraformaldehyde in PBS before imaging.

RNA isolation and gene expression analysis.

RNA isolation was performed using a RNeasy Mini Kit (Qiagen) according to the manufacturer's protocol. RNA concentration was measured using Nanodrop-1000. Complementary DNA synthesis was performed using an iScript™ Reverse Transcription Supermix for RT-qPCR (Bio-Rad) according to the manufacturer's instructions, with a minimal input of 200 ng total RNA. Quantitative PCR (qPCR) was performed using a CFX96 Touch Real-Time PCR Detection System (Bio-Rad) using cDNA amount equivalent to 1–2 ng total RNA during cDNA synthesis. SsoAdvanced Universal SYBR Green Supermix (Bio-Rad) and a 2 pmol/ml mixture of forward and reverse primers were used for 45 cycles of gene amplification. The primers used for qPCR are listed in Supplementary Table 2. GAPDH mRNA was used as an internal reference. The CFX manager software was used to analyze the qPCR results. To calculate fold changes, we used PBS-treated samples as a reference.

Lentivirus production and infection

For Lentivirus production, two 15 cm dishes of HEK293FT cells were seeded at 40% confluence. On the next day, 1 h prior to transfection, the medium was replaced with 13 ml pre-warmed Opti-MEM medium (Thermo Fisher). Transfection was performed using Lipofectamine 2000 and the PLUS reagent (Thermo Fisher). For each dish, 6.8 μg pCMV-VSV-G, 10.1 μg psPAX2 (Addgene), 13 μg gene-specific lentiviral shRNA plasmids and 135 μl of PLUS reagent (Thermo Fisher) were added to 4 ml Opti-MEM as mixture A, which is then

mixed with mixture B containing 68 μL lipofectamine 2000 and 4 ml Opti-MEM. The complete mixture was incubated for 20 min at room temperature and then added to cells. After 6 h, the medium was changed to 25 ml D10 medium (DMEM medium with 10% FBS and 1% Bovine Serum Albumin) with antibiotics (penicillin/streptomycin, 10 U/ml) for virus production. After 60 h of incubation, virus-containing medium from two culture dishes were combined and centrifuged at 3,000 rpm at 4 °C for 10 min to pellet cell debris. The supernatant was filtered through a 0.45 μm low protein-binding membrane (Steriflip HV/PVDF, Millipore). To concentrate lentivirus, the cleared supernatant was ultracentrifuged at 24,000 rpm for 2 hours at 4 °C using the JA25.50 rotor (Beckman). The virus was resuspended overnight in 180 μL D10 medium at 4 °C. Virus was aliquoted, flash-frozen in liquid nitrogen and stored at −80 °C. For infection, 10 μl concentrated virus was directly added to astrocytes cultured in 6 well. The medium was changed 24 hours after infection, and the knockdown efficiency was evaluated by qRT-PCR 96 h post-infection.

Cell lines and DNA transfection

HEK293T and SH-SY5Y cells were purchased from ATCC. HEK293FT cells were from Thermo Fisher. HEK293T and HEK293FT Cells were maintained in Dulbecco's Modified Eagle's Medium (DMEM, Corning) containing 10% fetal bovine serum (FBS) and antibiotics (penicillin/streptomycin, 10 U/ml). SH-SY5Y cells were maintained in DMEM/ F12 50/50 (Thermo Fisher) containing 10% fetal bovine serum (FBS) and antibiotics (penicillin/streptomycin, 10 U/ml). All cell lines were maintained at 37 °C. Cell transfections were performed using TransIT-293 reagent (Mirus) for HEK293T or Lipofectamine 2000

(Thermo Fisher) for SH-SY5Y cells following the manufacturer's instructions.

Immunoblotting

Immunoblotting was performed using a standard protocol. Proteins were separated in NuPAGE (4%–12%) Bis-Tris gels (Thermo Fisher) and transferred onto nitrocellulose membranes (Bio-Rad). The target protein was detected by specific primary antibodies followed by secondary horseradish peroxidase (HRP)-conjugated antibodies (for less abundant proteins) (Sigma) or fluorescence-labeled secondary antibodies (for abundant antigens) (Thermo Fisher). For immunoblotting with HRP-conjugated secondary antibodies, the signal was detected by the enhanced chemiluminescence method (ECL) using the Immobilon western chemiluminescent HRP substrate (Millipore) and recorded by a Fuji LAS-4000 imager. The intensity of the detected protein bands was quantified by ImageGauge v3.0 software. For immunoblotting with fluorescence-labeled secondary antibodies, membranes were scanned using a LI-COR Odyssey scanner. The intensity of the protein bands was quantified by the Odyssey software.

Cell surface binding assay and biotinylated samples preparation for MS proteomics

Cells were washed with pre-cold PBS two times to remove serum completely. We prepared 200 nM Tau-Apex2 or Apex2 proteins in serum-free pre-cold F-12 medium (5 ml for each 15 cm petri dish). We incubated cells with these proteins for 30 minutes at 4℃ with gentle shaking. After incubation, we removed the unbounded proteins by thoroughly washing the cells 3 times, each with 6 ml pre-cold F12 medium. We then incubated cells in 500 μM Biotin-phenol (BP) in

F-12 serum-free medium (10 ml for one 15 cm petri-dish) pre-warmed to 37 ℃. After 30 minutes of incubation, we added freshly prepared H_2O_2 stock solution (100 mM) into the cells to achieve a final concentration of 1 mM. Cells were incubated further at room temperature for 4 min. The medium was removed and cells were washed twice with the quencher solution (10 mM sodium ascorbate, 5 mM Trolox, and 10 mM sodium azide in DPBS.), followed by two washes with DPBS, and then resuspended in the quencher solution. Collect Cells were pelleted by centrifugation at 3,000 g for 10 min at 4℃, lysed in 7 ml PBS-based lysis buffer with 0.5% NP40, a protease inhibitor cocktail, 1 mM DTT, 10 mM sodium azide, 10 mM sodium ascorbate and 5 mM Trolox). After incubation on ice for 40 min, cell extracts were cleared by centrifugation at 15,000 g for 10 min at 4℃. Cleared supernatant was incubated with 50 µl pre-washed streptavidin beads, gently rocked at 4 ℃ for 1 h. The beads were extensively washed with PBS containing 0.05% NP40. Bound proteins were eluted in 50 µl 1x sample buffer.

Survival/cell toxicity assay.

Astrocytes were infected with shRNA-expressing lentivirus on 72 h after plating. Cells were grown for a total of 7 days in serum-free medium supplemented with 5 ng/ml HBEGF18 in 6 well plate. Cells were then treated with 200 nM Tau PFFs or an equivalent volume of DPBS (as a control) for 6 h, then washed extensively to remove remaining Tau filaments. To further ensure that no Tau filaments were carried over to conditioned medium, cells were trypsinized and then re-seeded into a new 6 well plate, grown for an additional 48 h. At this time, the conditioned medium was collected and a protease inhibitor cocktail was added to inhibit protein degradation. Conditioned medium was concentrated ~40 times by Amicon Ultra-15 Centrifugal

Filter Units with 30 kDa cutoff filter (Millipore, UFC903024), and then added to primary neuron cultures at 5 days in vitro (plated at 1.5×10^5 cells per well in a poly-D-lysine-coated 24-well plate). The viability assay was performed 72 h later using the Live/Dead Kit (Thermo Fisher Scientific, L3224).

Image processing and statistical analyses

Image processing was done using the Fiji software. Images were converted to individual channels and regions of interest were drawn for intensity measurement. For dead cell counting, single channel images were converted to black and white. A constant threshold was applied to all the images for dot counting. Statistical analyses were performed using either Excel or Prism 8. Values are the mean ± SD, calculated by GraphPad Prism 8, and *p*-values were calculated by the GraphPad Prism 8 or by Student's t-test using Excel. Images were prepared with Adobe Photoshop and graphs were plotted by GraphPad Prism 8.

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Author contributions

P. Wang, and Y. Ye designed the research, analyzed the data. P. Wang performed the experiments. P. Wang and Y. Ye wrote the paper together.

Competing interests

The authors declare no competing financial interest.

Figure 1

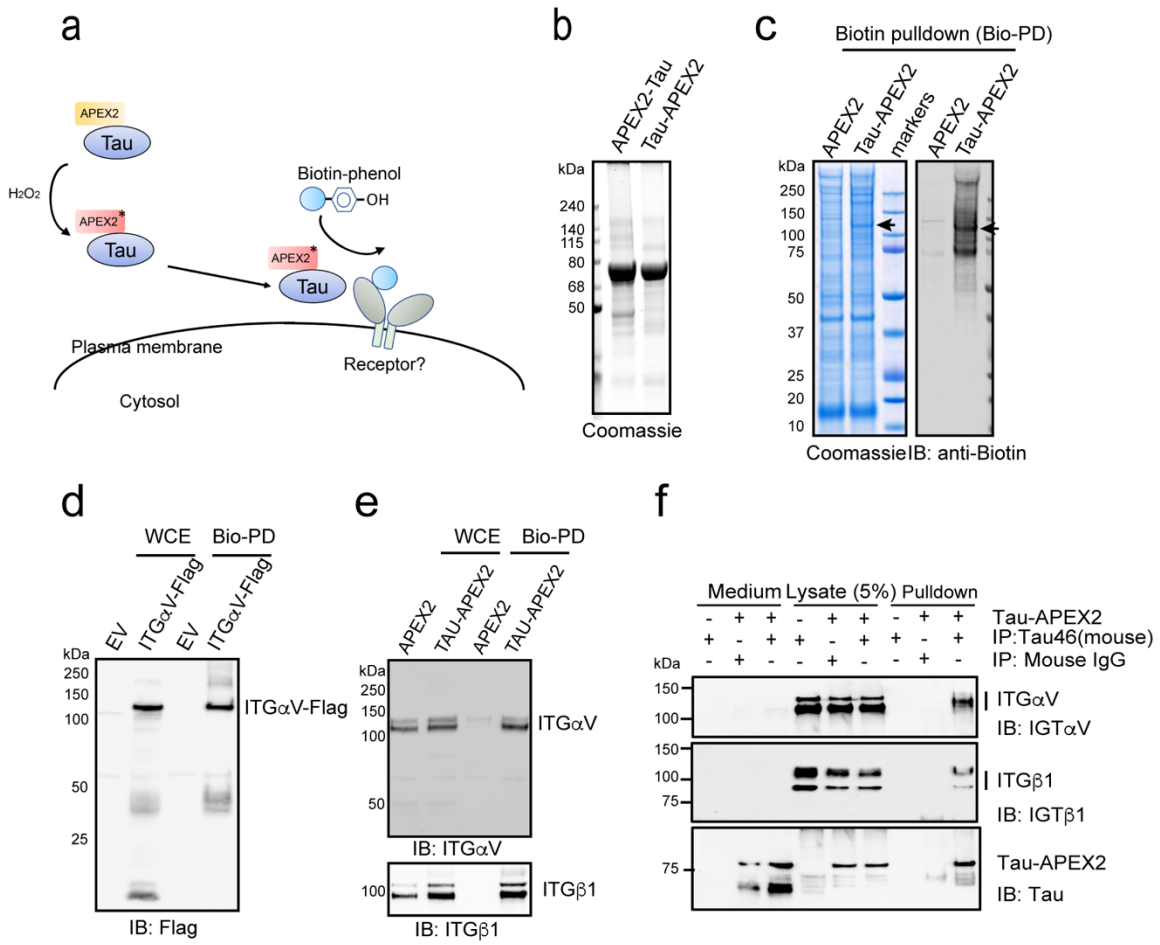


Figure1. Identification of integrin αV/β1 as a Tau interactor in primary astrocytes.
(**a**) Schematic overview of the proximity-based proteomic mapping strategy. Briefly, Tau-APEX2 (200 nM) is bound to the cell surface first. Biotinylation was initiated by incubating cells with biotin-phenol (BP) in the presence of H_2O_2 for 4 mins. Biotinylated proteins were then purified by affinity chromatography using streptavidin resin.
(**b**) Coomassie blue staining of purified Tau proteins.
(**c**) Coomassie blue staining (left) and immunoblotting (IB) analysis (right) of biotinylated proteins purified from primary astrocytes incubated with APEX2 or Tau-APEX2. The arrow indicates a 120kDa protein detected in the sample from Tau-APEX2-treated cells.
(**d**, **e**) Validation of Tau-APEX2-mediated biotinylation of ITGαV/β1. **d**, Tau-APEX2 was incubated with HEK293T cells transfected with either an empty vector (EV) or ITGαV-Flag. Cell lysates were either directly analyzed by immunoblotting or first subjected to biotin pulldown (Bio-PD) before immunoblotting. **e**, Cell lysates from primary astrocytes treated with either APEX2 or Tau-APEX2 were analyzed directly by immunoblotting or first

subjected to biotin pulldown before immunoblotting.

(**f**) Co-immunoprecipitation of Tau-APEX2 and ITGαVβ1. Astrocytes were treated with Tau-APEX2 or PBS as a control. Cells were lysed in an NP40-containing lysis buffer. Cell lysates were subjected to immunoprecipitation with either a control IgG or a Tau specific antibody (Tau-46). Both cell lysates and precipitated proteins were analyzed by immunoblotting. Where indicated, a fraction of the remaining medium was also analyzed by immunoblotting to show input.

Figure 2

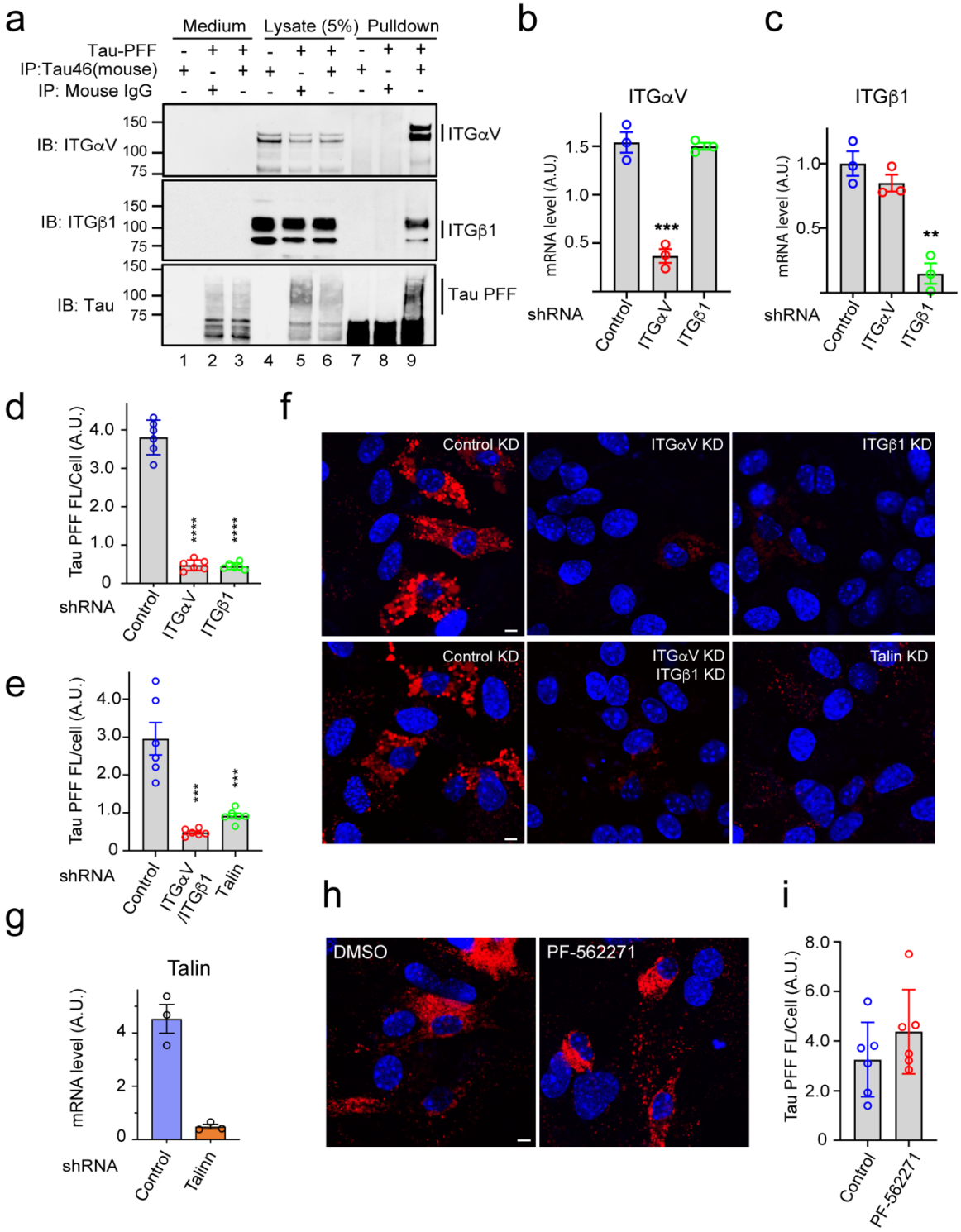


Figure 2. Integrin αV/β1 and Talin are required for Tau PFF uptake in astrocytes. (**a**) Co-immunoprecipitation of Tau PFF and ITGαV/β1. Astrocytes were treated with Tau PFFs or PBS as a control. Cell lysates were subjected to immunoprecipitation with either

a control IgG or a Tau specific antibody (Tau-46). Both lysates and precipitated proteins were analyzed by immunoblotting. Where indicated, a fraction of the medium collected after the binding was also analyzed by immunoblotting.

(**b, c**) Quantification (mean ± SD) of mRNA expression by qRT-PCR performed 72 h post lentiviral infection confirmed the knockdown efficiency of ITGαV (**b**), β1 (**c**). A.U. arbitrary unit. n= 3 independent experiments. Unless otherwise specified, asterisks indicate *p* values from two-tailed, unpaired t-test, * <0.05, ** <0.01, *** <0.001 and **** <0.0001.

(**d-g**) Astrocytic uptake of Tau PFFs is dependent on ITGαV/β1 and Talin. **f**, Representative images of lentivirus-treated astrocytes incubated with Alexa594 (Red) labeled Tau-PFF (200 nM) for 2 hours. Cells were stained with Hoechst to label the nuclei (blue). Scale bar, 5 μm. **d**, **e**, Quantification of the relative Tau PFF fluorescence intensity/cell. Mean± SD. Dots represent the average fluorescence intensity/cell from images (>20 cells) acquired from three independent experiments.

(**g**) As in **b**, except that Talin mRNA expression was determined after Talin knockdown.

(**h**, **i**) FAK is not required for Tau PFF uptake. **h**, Representative images of DMSO or PF-562271-treated astrocytes (10 μM) incubated with labeled Tau-PFF (200 nM) after 2 hours. **i**, Quantification of Tau PFF uptake in **h**. Mean± SD, Dots represent the average fluorescence intensity/cell from images acquired in two independent experiments.

Figure 3

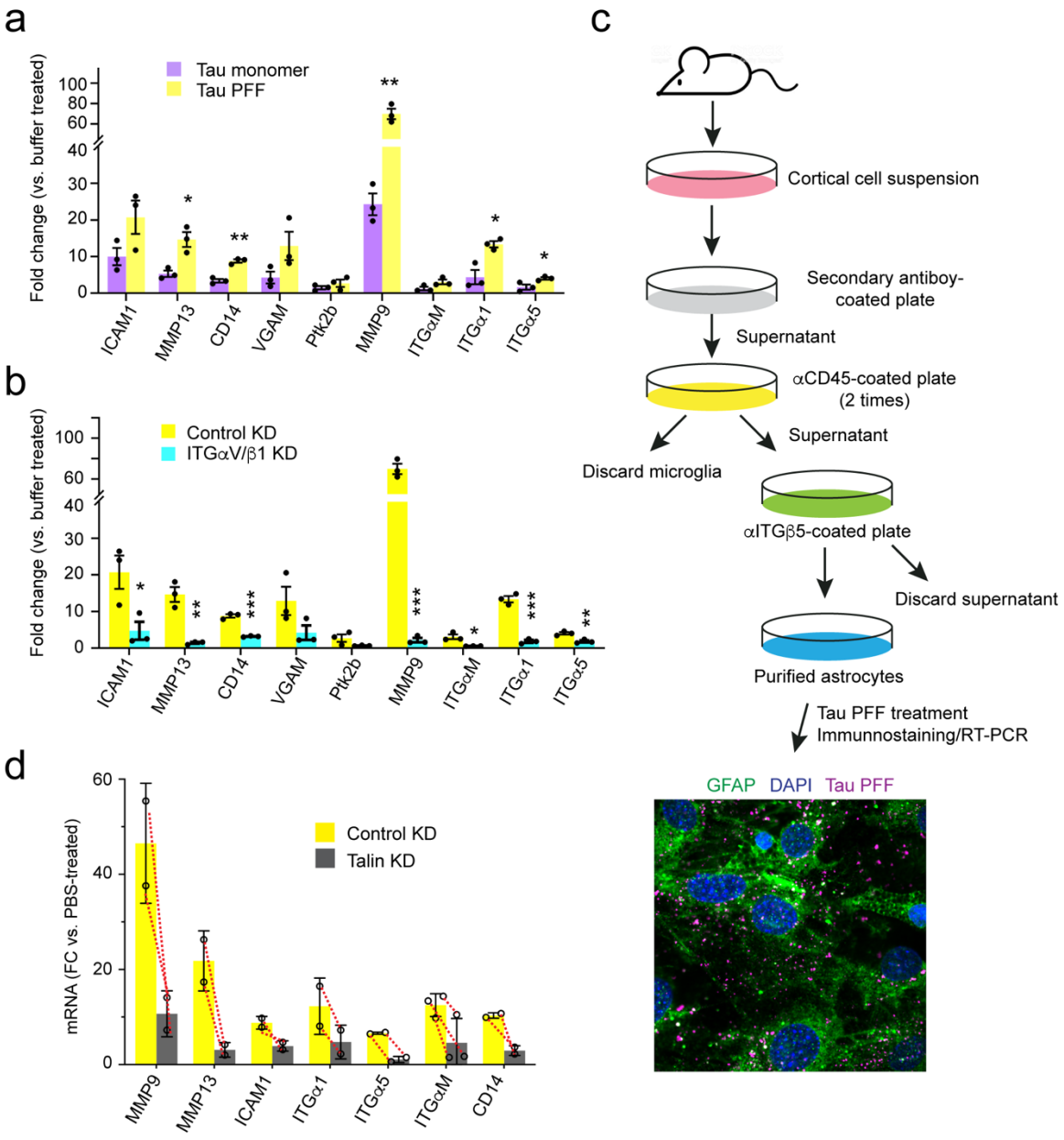


Figure 3. Monomeric and filamentous Tau differentially activate integrin signaling. (**a, b**) Tau PFF activates integrin signaling in an ITGαV/β1 dependent manner. qRT-PCR analyses of the expression of a collection of integrin target genes in primary astrocytes. Cells were first infected with either control or ITGαV/β1 shRNA for 72 h and then treated with PBS, monomeric Tau, or Tau PFFs (200 nM) for 6 hours. Shown are fold changes (FC) normalized to PBS-treated samples. (**a**) A comparison between Tau monomer and PFF. (**b**) A comparison of Tau PFF treatment in control vs. ITGαV/β1 knockdown cells. Mean ±SD, n=3 independent experiments, each with two technical repeats.

(**c**) A schematic overview of the immuno-panning strategy for purification of astrocytes using anti-ITGβ5 antibodies. Briefly, cortical cell suspensions were passed successively over secondary antibody-coated and anti-CD45-coated plates to remove macrophages and microglia, and then plated in a positive selection panning plate for astrocytes (anti-ITGβ5 coated). The lower panel shows a representative immunofluorescence image of purified astrocytes treated with Tau PFF Alexa594 (200 nM) (Magenta) 1h and then stained with anti-GFAP antibodies (Green) and Hoechst (Blue).
(**d**) Talin is required for Tau PFF-induced integrin signaling. As in **b**, except that immunopurified astrocytes were first infected with either control- or Talin shRNA-expressing lentivirus and then treated with Tau PFF. Mean±SD n=2 independent experiments except for ITGαM, in which n=3. Dashed lines indicate experimental pairs.

Figure 4

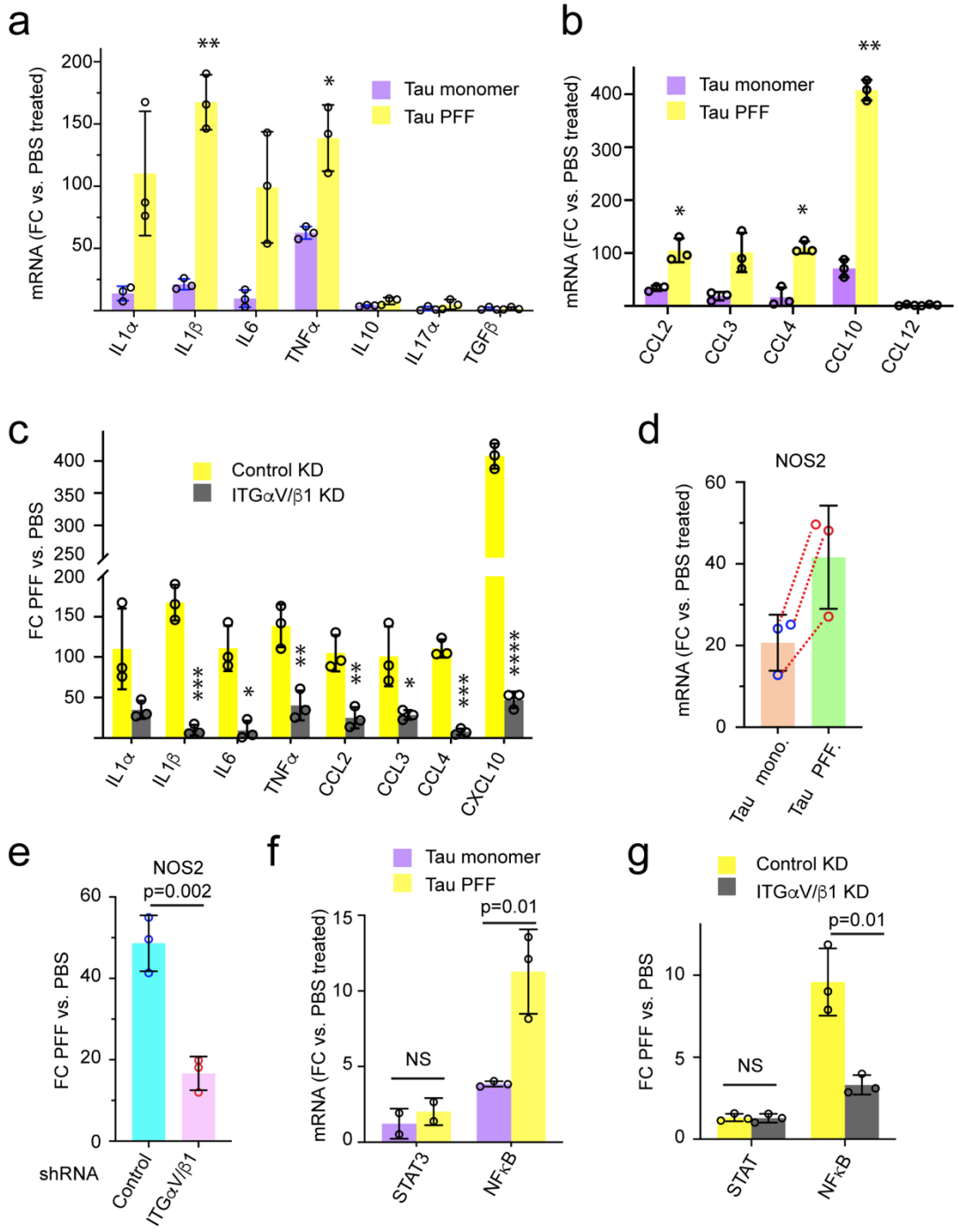


Figure 4. Tau PFFs induce inflammation in primary astrocytes via ITGαV/β1.
(**a**, **b**, **c**) Tau PFF induces astrocytic inflammation in an ITGαV/β1 dependent manner. qRT-PCR analyses of the expression of inflammation-associated cytokine and chemokine genes in primary astrocytes. Cells were first infected with either control shRNA (**a, b**) or control and ITGαV/β1 shRNA (**c**) for 72 h and then incubated with PBS, monomeric Tau

or Tau PFFs (200 nM) for 6 hours. Shown are fold changes (FC) normalized to PBS-treated cells. Mean ±SD, n= 3 independent experiments, each with two technical repeats. (**d**, **e**) Tau PFF induces NOS2 expression in an ITGαV/β1 dependent manner. **d**, As in **a**, except that NOS2 mRNA was analyzed. Mean ±SD, n=2. Dashed lines indicate experimental pairs. **e**, As in **c**, except that NOS2 mRNA was analyzed.
(**f**, **g**) Tau PFF activates the expression of NF-κB, but not STAT3 via ITGαV/β1. **f**, As in **a**, except that STAT3 and NF-κB were analyzed. **g**, As in **b**, except that STAT3 and NF-κB were analyzed.

Figure 5

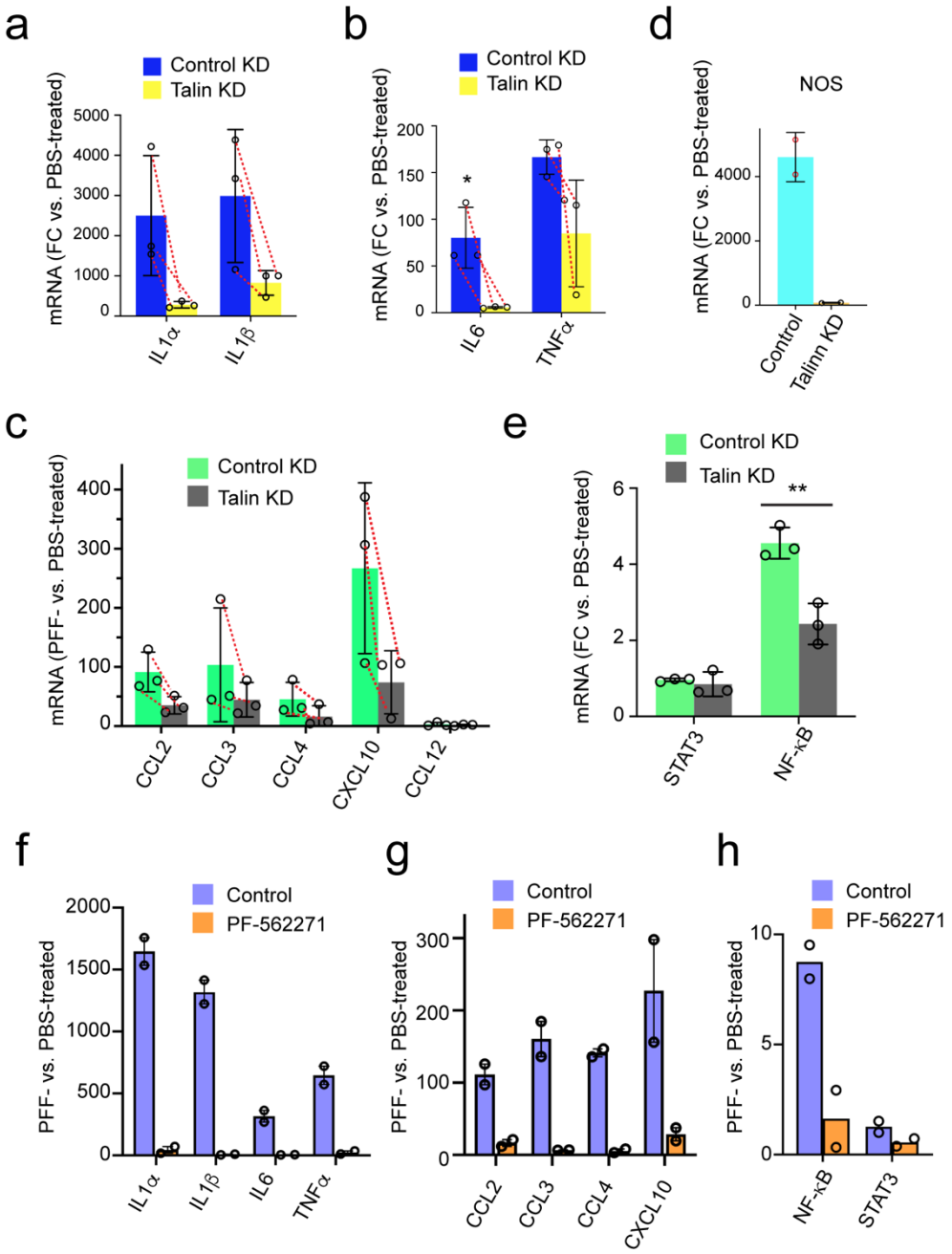
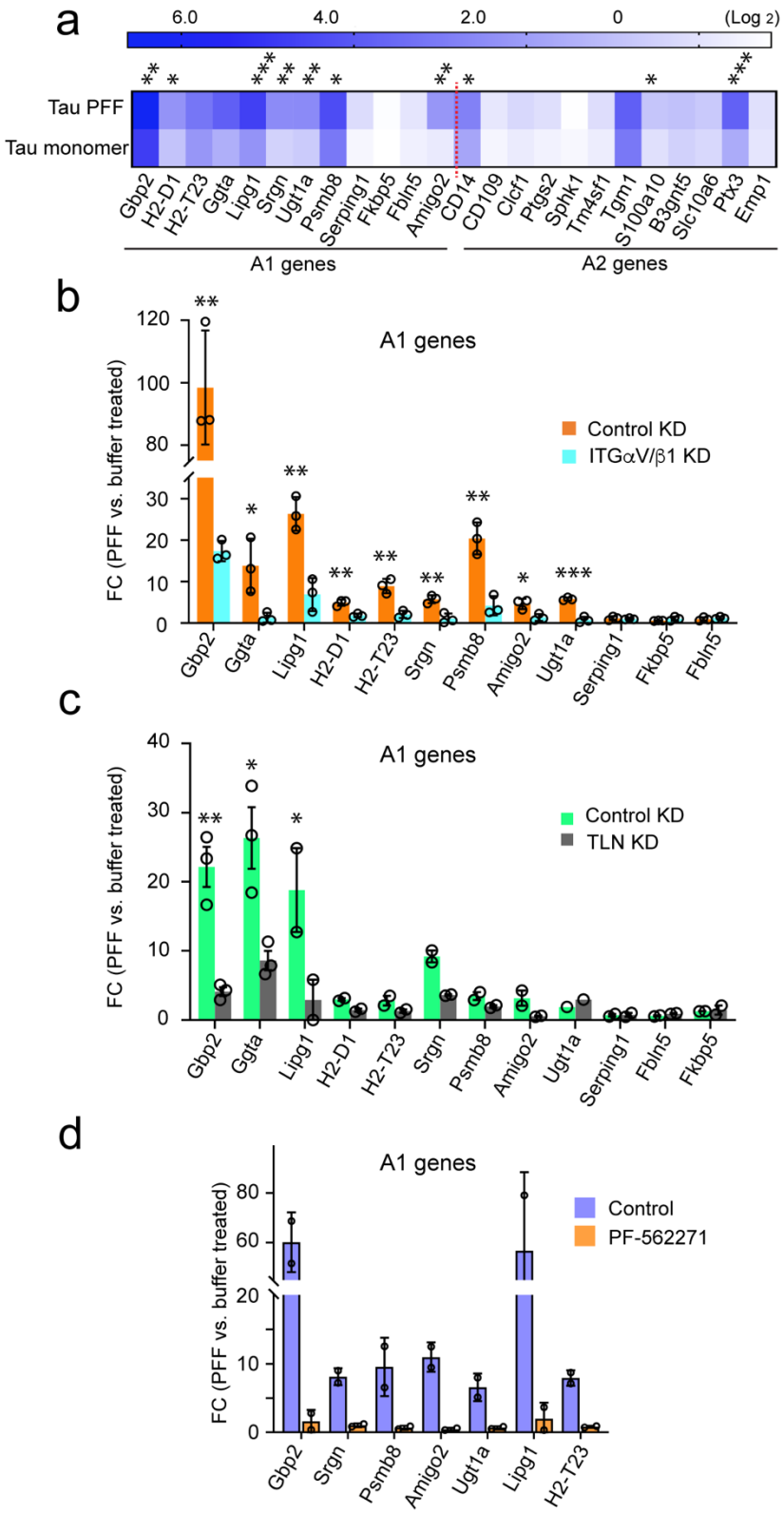


Figure 5. Tau PFF-induced inflammation requires Talin and FAK.
(**a**, **b**, **c**) qRT-PCR analyses of the expression of pro-inflammation-related cytokine genes (**a**, **b**) and chemokine genes (**c**) in immuno-purified astrocytes. Cells were infected with either a control or Talin-specific shRNA prior to the treatment with either PBS or Tau PFF (200 nM) for 6 hours. Fold changes (FC) were normalized to PBS-treated samples. Mean ±SD, n=3 independent experiments, each with 2 technical duplicates. Dashed lines indicate experimental pairs.

(**d**) As in **a**, except that NOS2 mRNA was analyzed. Mean ±SD, n=2 independent experiments.
(**e**) As in **a**, except that STAT3 and NF-κB were analyzed.
(**f**, **g**) qRT-PCR analyses of the expression of the indicated pro-inflammation cytokine (**f**) or chemokine (**g**) genes in immunopurified astrocytes that were treated with DMSO or PF-562271 (2 μM) for 1h before incubation with PBS or Tau PFF (200 nM) for 6h. Fold changes (FC) were normalized to PBS-treated samples. Mean ±SD, n=2 independent experiments, each with 2 technical duplicates.
(**h**) as in **f**, except that STAT3 and NF-κB were analyzed.

Figure 6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Tau PFF
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Ugt1a
Psmb8
Serping1
Fkbp5
Fbln5
Amigo2
CD14
CD109
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Ptgs2
Sphk1
Tm4sf1
Tgm1
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B3gnt5
Slc10a6
Ptx3
Emp1
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A2 genes
b
A1 genes
FC (PFF vs. buffer treated)
Control KD
ITGαV/β1 KD
c
A1 genes
FC (PFF vs. buffer treated)
Control KD
TLN KD
d
A1 genes
FC (PFF vs. buffer treated)
Control
PF-562271

Figure 6. Tau PFF converts astrocytes to a neurotoxic state in an integrin-dependent manner.

(**a**) A heat map shows the expression of A1 and A2 signature genes in Tau monomer- and PFF-treated primary astrocytes (200 nM, 6h), which is normalized to PBS-treated cells. The heat map values are the average of 3 independent experiments. Asterisks indicate *p* value from unpaired t-test between PFF and Tau monomer-treated samples.

(**b**) The expression of the indicated A1 genes were analyzed by qRT-PCR from PBS- or Tau PFF-treated (200 nM, 6h) astrocytes, which had been infected with lentiviruses (72 h) expressing control or ITGαV/β1 shRNAs. Fold changes (FC) were normalized to PBS-treated samples. Mean ±SD, n=3 independent experiments, each with 2 technical duplicates.

(**c**) As in **b**, except that immunopurified astrocytes infected with control- or Talin shRNA-expressing lentiviruses were used.

(**d**) As in **c**, except that cells were treated with DMSO or PF-562271 (10μM, 1h) instead of lentivirus prior to PBS or Tau PFF treatment. Mean ±SD, n=2 independent experiments, each with 2 technical duplicates.

Figure 7

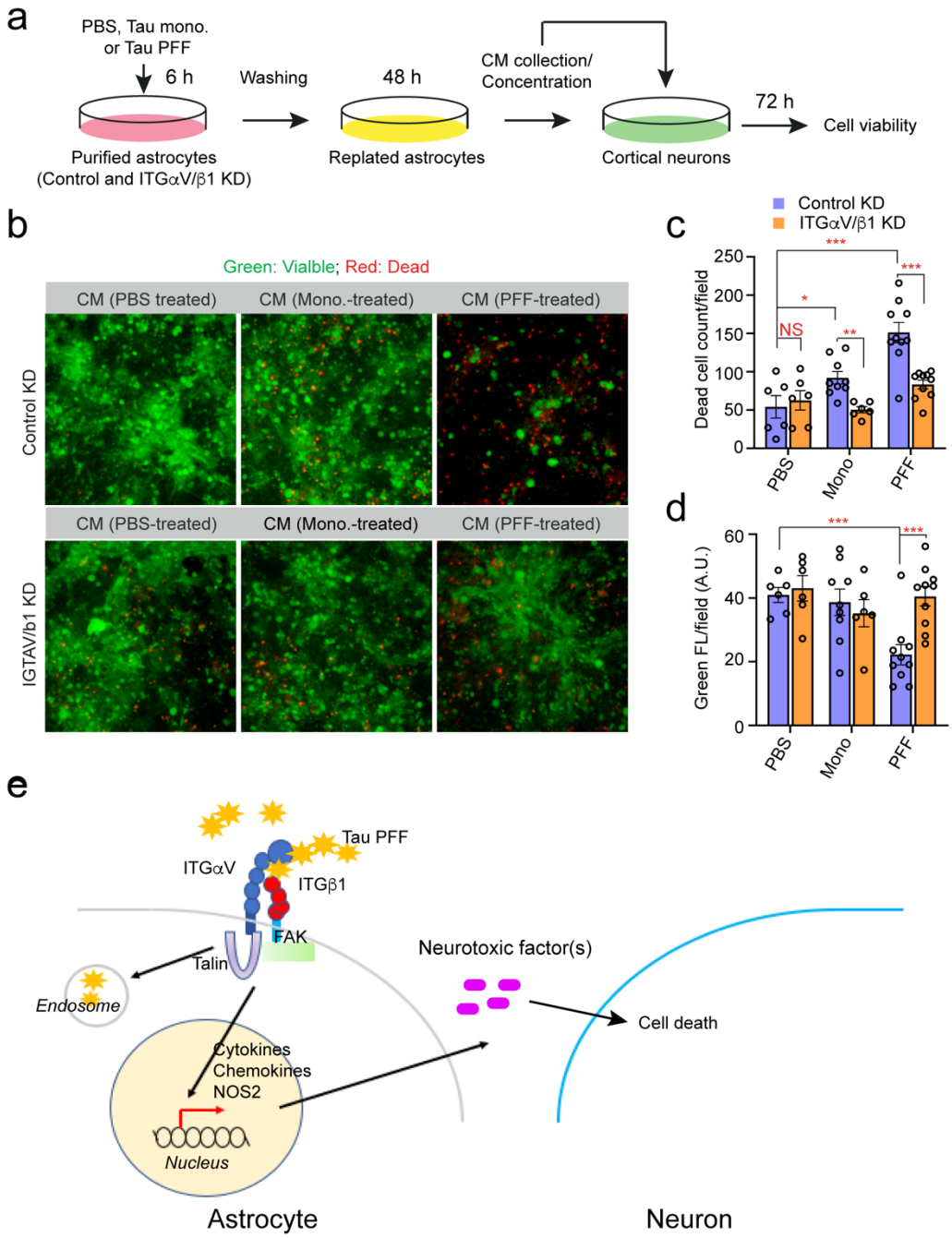

Figure 7. Tau PFF-treated astrocytes release a neurotoxic factor(s) via ITGαV/β1 activation.

(**a**) Scheme of the cell toxicity assay.

(**b**) Primary cortical neurons treated with the indicated condition medium (CM) were stained with a green-fluorescent dye calcein-AM to label viable cells and a red-fluorescent

dye ethidium homodimer-1 to label dead cells.
(**c**) The number of dead cells in randomly selected fields were quantified by Fiji. The green fluorescence intensity (FL) in randomly selected fields were quantified by Fiji.
(**d**) A working model showing the interplays between Tau-PFF, astrocytes and neurons.