α-Linolenic acid induces clearance of Tau seed via Actin-remodeling in Microglia

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Research

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Abstract

Background Tau seeds exhibit a detrimental role in the spread of disease in Alzheimer's disease. These species are found to be neurotoxic and activate microglia. However, the activation of microglia in pro-inflammatory response further elevates neurodegeneration. Omega-3 dietary fatty acids, on the other hand; exert an anti-inflammatory response by microglia. Along with the receptor expression, omega-3 fatty acids influence various important cellular functions. The role of omega-3 fatty acids on actin remodeling, which is the basis of cellular functions such as migration and phagocytosis is not known. Here in this study, we focus on effect of dietary supplement of ALA on extracellular Tau internalization and assisted actin polymerization for the process. ALA is found to induce membrane ruffling and phagocytic cup formation along with cytoskeletal rearrangement to induce lamellipodia and filopodia at the front end to move forward and assist the cell to identify the target. ALA is observed to promote the internalization of Tau and necessary actin remodeling for phagocytosis.

Methods α-Linolenic (ALA) acid has been used for the study. ALA was dissolved in 100% ethanol and solubilized at 50°C for 2 hours. The human Tau aggregates was prepared in vitro for the internalization study in microglia in presence of α-Linolenic acids (ALA) via fluorescence microscopy with Apotome. The studied the role α-Linolenic acids (ALA) actin remodeling in cellular processes in presence of Tau seed. The study of actin structures lamellipodia, filopodia, and membrane ruffling along with Iba-1 and Arp2/3 complex was observed on ALA exposure.

Results Extracellular Tau species are found to internalize more presence of ALA in microglia. The extensive polarization and migration was observed as indicated by extensive lamellipodia and filopodia formation. The formation of extensive actin branching in lamellipodia and membrane ruffling was studied with the help of ARP2/3 complex for nucleating actin network. The high density of ARP2/3 complex at the leading ends of migratory microglia confirmed the extensive branching of actin filaments on ALA exposure. Enhanced formation of lamellipodia and filopodia helps in migration and internalization of tau seed. The actin dynamics supports the phagocytosis process.

Conclusion Our approach provides the insights of beneficial role of ALA as anti-inflammatory dietary supplement to treat AD. ALA induces internalization of Tau and necessary actin remodeling for phagocytosis.

Background

Alzheimer's disease (AD) is a progressive neurodegenerative disease associated with misfolding and modification of two key proteins Amyloid precursor protein (APP) and microtubule-associated protein Tau (MAPT) followed by the production of amyloid plaques (Aβ) and Neurofibrillary tangles (NFTs) respectively. The physiological impairments associated with disease includes cognitive impairment, memory loss, loss of neuronal connections, loss of other mental abilities, *etc* [1]. Alzheimer's disease as a primary Tauopathy is characterized by excessive abnormal phosphorylation of Tau, which reduces its

affinity towards microtubules and leads to instability in microtubule functions and loss of synaptic neurons. The importance of Tau pathology in AD came into consideration after the failure of Aβ inhibiting drug in AD treatment and Tau being established as a new target for AD [1–3]. The spreading of Tau pathology in brain *via* prion-like manner contributes to the severity of disease. Various studies have been reported that the ability of Tau aggregates to spread in a prion-like manner in a neuronal culture to incorporate template-dependent aggregation in monomeric Tau [4, 5]. After being secreted in extracellular spaces; Tau monomer, oligomers, and aggregates are found to enter another cell through various cellular uptake mechanisms [6]. The extracellular species of Tau can exert toxicity in cultured hippocampal neurons *via* unbalanced calcium metabolism and causes cell death [7]. Polyunsaturated fatty acids (PUFAs), majorly omega-3 fatty acids have beneficial role on brain. Their dietary supplements are found to improve cognitive function and memory loss. PUFAs exist in the form of phospholipid content in cell membrane of brain cells. PUFAs abundance in cell membrane efficiently influences the membrane receptors expression, signaling cascade and effector function of cell. Presence of PUFAs as dietary supplement is found to enhance brain function, effectively improve cognitive functions and memory ability [8–10]. Omega-3 fatty acids are known to exert anti-inflammatory effect on brain cells. Especially DHA and EPA impart lipid changes in cell membrane composition and affect cell surface receptor expression. The cellular functions such as phagocytosis, migration are carried out with the assistance of actin remodeling [11, 12]. Various studies showed increased phagocytosis of Aβ since it reduces pro-inflammatory markers and induces anti-inflammatory cytokines in microglia cells [13]. For the process of phagocytosis to engulf extracellular pathogens, membrane extension induce by actin remodeling and phosphoinosites induced-signaling is imperative [14]. Omega-3 PUFAs significantly alters cytoskeleton associated gene expression including Rac1, CDC42, WASPL and ARP2/3 complex [11]. Microglia are resident immune cells of brain, which are myeloid in origin. The microglia in resting state inspects the brain environment with their long extensions. The communication with other glia cells and neuron take place *via* various cellular receptors, chemokine signals and soluble factor [15]. Activation of microglial receptors with inflammatory mediators turns resting microglia into motile effector cell, which exert an inflammatory reaction. After sensing the tissue injury, pathogen invasion or plaque deposition, microglia takes amoeboid form and migrate long distance towards concentration gradient of chemotactic signals. For the 2-dimensional migratory motion of microglia, the cells have been supported by protruding fan-shaped actin based structures (lamellipodia) on leading end and F-actin-rich thin extensions (filopodia) on rear end [16]. In this migratory state, microglia manifest presence of podosomes, which are actin-rich structures that mediates adhesion to substratum, migration and ECM degradation for invasion [17]. In phagocytosis after detecting target, the signaling cascades regulating actin cytoskeleton initiates. The membrane protrusion around targets and membrane ruffling to increase surface area initiates major actin remodeling [18]. The polarized state of microglia is maintained by the cytoskeletal network where actin provides directional sensing while microtubule dynamics is required for the mechanical strength to move cells forward direction [19]. The coordinated polymerization of actin filaments provides a protrusive force for the cell to move forward. ARP2/3 complex along with other actin-binding proteins plays important role in nucleating the branching of actin filament. The membrane protrusions and ruffling is initiated with ARP2/3 signaling cascade [20]. In the process of membrane ruffling, phagocytic cup formation along

with actin network Iba-1(ionized calcium adapted molecule-1) protein of microglia plays an pivotal role. Iba-1 is also reported to have a key role in the function of activated microglia [21, 22]. In AD, ability of microglia to undergo phagocytosis of extracellular Aβ plaques as well as aggregated Tau seeds is most affected along with the disrupted actin cytoskeleton [23]. The link between Aβ and disrupted actin cytoskeleton via dysregulation of phosphoinosites-mediated signaling can be considered as an affliction [24]. The extracellular Tau oligomer and aggregates has the potential to remodulate the actin cytoskeleton for active phagocytosis [25]. Since PUFAs induce anti-inflammatory response of microglia, the modulated membrane micro domains would induce changes in property of phagocytosis and related actin remodeling [11]. In this study, we aim to understand the effect of α-Linolenic acid as one precursor of DHA and EPA on actin remodeling-related phagocytosis and migration. In Tauopathies, extracellular Tau seeds tend to activate microglia to induce inflammatory activation. However, the study involves understanding the effect of ALA on phagocytosis and related actin-remodeling in microglia in the presence of extracellular Tau seeds.

We have focused on the role of α-Linolenic acid on phagocytosis of extracellular Tau and cytoskeletal protein actin in microglial migration and membrane ruffling along with the microglial-activated protein Iba-1. The excessive branching of actin cytoskeleton in phagocytosis was studied with the help of ARP2/3 complex, which initiates branching of parental actin filaments. We have analyzed the enhancement of actin remodeling necessary for phagocytosis after ALA exposure by actin-mediated membrane ruffling.

Materials And Methods

Chemicals and Primary antibodies

Luria-Bertani broth (Himedia); Ampicillin, NaCl, Phenylmethylsulfonylfluoride (PMSF), MgCl2, APS, DMSO, Ethanol (Mol Bio grade), Isopropanol (Mol Bio grade) and methanol (Mol Bio grade) were purchased from MP biomedicals; IPTG and Dithiothreitol (DTT) from Calbiochem; MES, BES, SDS, α-Linolenic acid (ALA) (L2376) from Sigma; EGTA, Protease inhibitor cocktail, Tris base, 40% Acrylamide, TEMED from Invitrogen. For cell culture studies, the N9 microglial cell line no. is CVCL- 0452, Roswell Park Memorial Institute (RPMI), Fetal Bovine Serum (FBS), Horse serum, Phosphate buffer saline (PBS, cell biology grade), Trypsin-EDTA, Penicillin-streptomycin, RIPA buffer were also purchased from Invitrogen. MTT reagent and TritonX-100, Trypan -Blue were purchased from Sigma. The coverslip of 12 mm and 18 mm was purchased from Bluestar for immunofluorescence and copper-coated carbon grids for TEM analysis were purchased from Ted Pella, Inc. In immunofluorescence and western blot study we used the following antibodies: Beta-actin (Thermofisher cat no. MA515739), ARP2 MONO (Thermofisher cat no- 703394), Anti-Iba-1 (Thermo cat no-PA527436), anti-mouse secondary antibody conjugated with Alexa Fluor-488 (Invitrogen, cat no A-11001), Goat anti-Rabbit IgG (H+L) Cross-Adsorbed Secondary Antibody with Alexa Fluor 555 (A-21428), GOXMS ALEXA FLOUR 488 goat anti rabbit (Thermofisher- cat no A28175) DAPI (Invitrogen), Goat Anti Mouse secondary antibody Peroxidase conjugated (Thermo fisher 32430), Prolong Diamond antifade (Thermofisher cat no- P36961).

Protein expression and purification

Full-length wild type Tau protein (hTau40^{wt}) was expressed in BL21* cells with 100 µg/ml of ampicillin antibiotic selection and purified with two-step chromatography methods, cation-exchange chromatography and size-exclusion chromatography (Gorantla, MiMB, 2018). Induction was carried out with 0.5mM IPTG for 3 hrs at 37°. In brief the cell lysate was subjected to 90°C heating and supernatant was centrifugation at 40000 rpm for 45 minutes followed by dialysis overnight at 4° in 20 mM MES buffer and hTau40 purified by cation-exchange chromatography with Sepharose fast-flow column was used for chromatography. A fractions containing Tau proteins were collected after cation exchange chromatography, it was then concentrated and subjected to size-exclusion chromatography. Size-exclusion chromatography was carried out in the Superdex 75 Hi-load 16/600 column in 1X PBS supplemented with 2 mM DTT. A fractions containing Tau were collected, pooled, concentrated and the concentration of protein was determined with BCA (Bicinchoninic acid assay) assay.

Aggregation assay

Natively unfolded protein Tau undergo aggregation in presence of poly-anionic agent heparin or arachidonic acid to produce β- sheet structure [26]. Tau aggregation was induced by heparin (MW-17500 Da) in the ratio of 1:4 heparin to Tau along with other additives 20 mM BES buffer, 25 mM NaCl, 1 mM DTT, 0.01% NaN_3, PIC. Aggregation propensity of Tau was checked with ThS is a homogeneous mixture of methylation product of dehydrothiotoluidine in sulfonic acid, which can bind to β-sheet structure. Aggregation kinetics of Tau was studied with 2 µM of Tau and ThS in 1:4 ratios. The excitation wavelength for ThS is 440 nm and the emission wavelength is 521 nm, further analysis of data was done using Sigmaplot 10.0.

Transmission electron microscopy

Tau fibrils and ALA vesicles were studied by transmission electron microscopy (TEM) for morphological analysis. 2 µM Tau sample was incubated on 400 mesh, carbon-coated copper grid for observation and stained with 2% uranyl acetate for the contrast. For ALA vesicles working concentration of 40 µM was taken for grid preparation. The images were taken with TECNAI T20 120 KV.

Cell culture

N9 microglia cells were grown in RPMI media supplemented with 10% heat-inactivated serum, 1% penicillin-streptomycin antibiotic solution and glutamine and grown in T25 flask or 60mm dish to maintain the culture. Cells were passaged using 0.25% trypsin-EDTA solution after washing with PBS after attaining 90% confluency. For western blot experiment cells were seeded in 6 well plate. For α-Linolenic acid preparation, previously published protocol was followed [13]. Briefly, ALA was dissolved in 100% molecular biology grade ethanol and solubilized at 50°C in the stock concentration of 20 mM. ALA solution prepared fresh before every experiment. The working concentration of ALA 40µM was decided according to previous studies and final concentration of ethanol into culture was maintained below 0.5%.

Immunofluorescence analysis

For immunofluorescence 25,000 cells of N9 microglia were seeded on 12 mm coverslip (Bluestar) in 24 well plate supplemented with 10% FBS and 1% penicillin-streptomycin. During treatment of hTau40 and ALA the cells were supplemented with 0.5% serum-deprived RPMI media. The treatment was given for 24 hours. Cells were then fixed with chilled absolute distilled methanol for 20 minutes at -20°C then washed with 1X PBS thrice. Permeabilisation before staining was carried out using 0.2% Triton X-100 for 15 minutes followed by washing three times with 1X PBS and blocking with 2% serum in 1X PBS for 1 hour at room temperature. Primary antibody treatment was given to cells overnight at 4°C in 2% serum in 1X PBS in a moist chamber. The next day, cells were washed with PBS thrice. Then incubated with secondary antibody in 2% serum at 37°C for 1 hour. Further cells were washed with 1X PBS 3 times and counterstained with DAPI (300 nM). Mounting of coverslip was done in mounting media (80% glycerol). Images were observed under a 63x oil immersion lens in Axio observer 7.0 Apotome 2.0 Zeiss microscope.

Confocal- Super-resolution microscopy analysis

To study the actin structures associated with migration, phagocytosis in presence of ALA Zeiss LSM 980 with Airy scan 2 in super-resolution mode was used. The immunofluorescence staining for the previously described conditions were carried out with β-Actin (1:500) and Iba-1 (1:500) proteins to study the microglia activation and actin structures. The super-resolution mode helped to resolved and understand the minute cell structures such as lamellipodia, filopodia, membrane ruffling and polarization state of microglia. The image processing was carried out with Zeiss ZEN 2.3 software.

Western blot

For detection of protein levels in cells (3, 00, 000 cells/well) N9 Cells were seeded in 6 well plate. The desired treatment was given for 24 hours. Treatment exposure followed by washing with 1X PBS. Cell lysis was carried out using radioimmunoprecipitation (RIPA) assay buffer containing protease inhibitors for 20 min at 4°C. The cell lysate was centrifuged at 12000 rpm for 20 minutes. Protein concentration was checked by using Bradford's assay and equal amount of 75 µg total proteins for all the treatment groups were loaded on polyacrylamide gel electrophoresis of range 4-20% and the gel is electrophoretically transferred to polyvinylidene difluoride membrane and kept for primary antibody β-actin (1:5000), Iba-1 (1:1000) binding for overnight at 4°C. After the incubation blots washed three times with 1X PBST (0.1% Tween-20). The secondary antibody was incubated for 1 hour at RT. Then the membrane was developed using chemiluminiscence detection system. The relative quantification of protein was carried out with loading control β- Actin in each treatment group.

Statistical analysis

All the experiments have performed 3 times. The data is analyzed using SigmaPlot 10.0 and the statistical significance was calculated by student's *t*-test (ns- non-significant, * indicates $P \leq 0.05$, **

indicates P≤ 0.01, *** indicates P≤0.001). Measuring the absolute intensity of protein and the corresponding area of microglia with Zeiss ZEN 2.3 software for image processing carried out the quantification of levels of intracellular proteins in immunofluorescence experiments.

Results

Internalization of extracellular Tau induce by ALA

Microglia are professional phagocytes of brain, which assist to clear apoptotic cells, axonal and myelin debris, central infection of bacteria and viruses, misfolded aggregated protein-like Aβ and participate in neuronal connections by engulfing synapses [27]. Along with the Aβ plaques, microglia also recognizes extracellular Tau oligomers and aggregates and undergoes activation to engulf the Tau seeds. However, the increased production of IFN-γ and TNF-α by microglia hampers its phagocytic ability and are toxic to neuronal cells [23]. However, PUFAs treatment reduce the production of inflammatory cytokines while exerts anti-inflammatory cytokines production as well as improves phagocytosis [13]. We prepared hTau40 aggregates *in vitro* with the presence of heparin as polyanionic agent, which can induce Tau aggregation [28]. The hTau40 aggregates produced *in vitro* were characterized for the higher molecular weight aggregates by SDS-PAGE. Propensity of Tau aggregation was studied with ThS fluorophore that binds to Tau aggregates and gives the extent of aggregation. The fully formed mature Tau fibrils were assessed with Transmission electron microscopy (Fig. 1A). ALA was prepared by dissolving in 100% ethanol followed by heating at 50° C for 2 hours and characterized by TEM analysis (Fig. 1B). The scheme represents the experimental proceeding of the study as Tau internalization and the changes in actin cytoskeleton associated with the process (Fig. 1C). To study ability of ALA to cause anti-inflammatory effects in microglia, we observed the phagocytosis of extracellular Tau. We treated microglia with extracellular aggregated Tau species 1 μM Tau monomer and aggregates along with ALA 40 μM and respective controls were kept. Internalization of extracellular Tau in microglia was studied with fluorescence microscopy. Tau (red), Iba-1 (green) was detected in microglia cells after 24 hours exposure of Tau and ALA to N9 microglia culture. The internalization of Tau was studied with orthogonal view, which provides intracellular localization of internalized Tau in microglia (Fig. 1D). The internalization ability of microglia was found to be increased with ALA exposure. ALA induces anti-inflammatory properties to microglia assisting them to reduce the burden of extracellular Tau in Tauopathies.

Effect of ALA on actin dynamics and microglial activation

Actin cytoskeleton is involved in membrane-remodeling, downstream signal transduction and providing mechanical framework for phagosomes upon internalization. Actin cytoskeleton furnishes platform for receptors and other signaling cascades. Co-ordinated cycles of actin polymerization and depolymerization occurs at phagosomes for its internalization and maturation [29]. Membrane-ruffling and phagocytic cup formation is being assisted by actin cytoskeleton along with the Iba-1 protein in microglia. Iba-1 enhances membrane-ruffling and crosslinking of actin, which up-regulate the internalization events [21]. In phagocytosis, after interaction of target with phagocytic receptor the signal

transduction is initiated which involves actin cytoskeleton changes. The necessary remodeling with the membrane associated actin and introduction of more membrane protrusion enhance the phagocytosis [30]. The N9 microglial cells were treated with the different groups includes 1 μM hTau40 monomer, aggregates along with their respective treatment with 40 μM ALA and cell control (no treatment), ALA control (only ALA treatment) was kept for the comparison. We studied the role of actin (red) and Iba-1 (green) in phagocytosis in N9 cells on ALA exposure by immunofluorescence staining. In activated migratory microglia, the polymerization of actin at leading end is signified by the presence of fan shaped lamellipodia and thin filamentous filopodia-like structure whereas long extension is observed at the rear end (Fig. 2A). For the migratory cells such as immune cells, the front-end protrusion of lamellipodia formed by the forces of actin polymerization indicates the migratory state of microglia since they sense the extracellular Tau species. Whereas; the finger-like protrusion called as filopodia would cause the slow migration in absence of lamellipodia [31]. The immunofluorescence staining indicates enhanced colocalization between Iba-1 and actin at leading end of activates microglia cells in ALA treated cells[5] (Fig. 2A). These results indicates the involvement of actin and Iba-1 in phagocytic cup formation and membrane ruffling [21]. Intracellular intensity of Iba-1 was quantified and it was found to increase significantly in both monomer and aggregates in presence of ALA ($P<0.001$) (Fig. 2B). Iba-1 levels were also quantified by western blot, which showed that with respect to cell control (no treatment), Iba-1 levels do not increase significantly. The western blot quantification also suggests that no significant changes were observed as compared to cell control (Fig. 2C and D). The microscopic images showing all the panels are represented in supplementary figure (Fig. S1). In this study, we observe that, exposure of ALA and Tau species to microglia triggers the microglial activation and enhance actin remodeling which may induce processes such as migration and phagocytosis. In this process, actin and Iba-1 plays major role in microglial migration and activation, which assist the phagocytosis (Fig 2E). After detection of target, immune cells increase the membrane protrusion and membrane ruffling to increase the area in contact with the target to initiate the internalization process. Membrane ruffling enhanced by the actin polymerization and involvement of Iba-1 was shown with the super-resolution confocal microscopy images. The presence of ALA was found to enhance the membrane ruffling in both monomer and aggregates treated cells, which enhances the chances of internalization in microglia (Fig. 3). The overall view of the zoomed images with separate panel is shown in supplementary figure (Fig. S2)

ALA enhance actin structure and dynamics

Immune cells are characterized by excessive motility to survey environment to find and destroy target pathogens. Actin polymerization provide necessary protrusion for the cells to move forward, which can be observed with the lamellipodia and filopodia to sense distant targets [20]. We observed the role of actin rich structures-lamellipodia and filopodia in migration and phagocytosis. The immunofluorescence staining of actin in microglia cells helped to study the minute fine structures (Fig. 4A). The percentages of lamellipodia and filopodia positive cells have increased with the ALA exposure in both monomer and aggregates treated cells ($P<0.001$) (Fig. 4B, C). The number of filopodia per cell has increased significantly with ALA exposure in monomer and aggregates treatment and the numbers of filopodia per cell is maximum in monomer with ALA treatment ($P<0.001$) (Fig. 4D). Actin plays major role in

phagocytosis and migration, which can be observed by, enhanced actin structures in the form of lamellipodia and filopodia (Fig. 4E).

ALA enhance actin branching network via ARP2/3 complex

In case of lamellipodia formation and protrusions to move forward, the continuous branching of mother actin filaments are important. The force required to move the plasma membrane forward is provided by actin filament polymerization. One of the actin binding protein ARP2/3 complex enhances the branching process. We have studied the presence of ARP2/3 complex in microglia after treatment of Tau monomer, aggregates and ALA. The 3D representation of images showing ARP2/3 (red), phalloidin (green) and DAPI indicates the presence of ARP2/3 at the leading ends of cells, inducing more branching of actin filaments for the protrusive force (Fig. 5A). Intensity of ARP2/3 complex protein was calculated from immunofluorescence images to check the protein levels. The intensity/μm^2 of ARP2/3 indicates no significant changes with incorporation of ALA with Tau species (Fig. 5B). The 2D representation of the immunofluorescence images with the entire panel is shown for the better understanding (Fig. S3).

Discussion

Extracellular deposits of Aβ and intracellular hyperphosphorylated neurofibrillary tangles of Tau depict the hallmark of AD. Accumulation of deposits of Aβ and aggregated Tau seeds in brain environment prompt cognitive decline and neuronal loss. Microglia as brain macrophages screens the brain environment by constantly extending the processes in resting stage. After encountering the site of injury or protein deposits, they extend the processes and migrate towards recognized site, ramified in shape and exert an immune response. In AD, microglia surrounds the Aβ plaques, activates and produce inflammatory response [32]. The gathered microglia towards senile plaques induce formation of Aβ deposits rather than clearance due to excessive inflammatory cytokines such as IFN-γ and TNF-α [23]. Iba-1 positive microglia also found to accumulate near NFT bearing neurons and induce inflammatory phase [33]. The Tau seeds have ability to propagate in a “prion-like” manner and efficiently induce microglia activation to fabricate inflammatory response [5, 34, 35]. In this condition, inducing anti-inflammatory phenotype of microglia would act as therapeutic strategy and omega-3 fatty acids speeds up the process [9]. Omega-3 PUFAs on dietary intake improves cognitive impairment, apoptosis of neurons and inflammatory phase by microglia [8]. DHA and EPA exert polarization of M2 phenotype of microglia along with the excessive expression of phagocytic receptors such as CD206 and anti-inflammatory cytokine IL-4, IL-10 etc. [13, 36]. The cytoskeleton changes needed for the phagocytosis by microglia is also induced via omega-3 PUFAs *via* various mechanism including phosphoinosites signaling [24]. Our results also suggest that necessary actin remodeling for migration and phagocytosis assists the phagocytosis of extracellular Tau species induced by ALA. Coordinated polymerization of branched network of actin filaments is a key phenomenon to generate pushing force for the migratory cells. Extensive motility of immune cells is necessary to locate a target to destroy. The identification of targets *via* receptors initiates a signaling cascade involving actin polymerization to produce membrane protrusions around the target to engulf. After engulfment, the target is enclosed in endocytic vesicle and

pinched out from cell membrane. In this process, actin remodeling plays a major role to induce changes in plasma membrane, membrane ruffling and protrusions that speed up phagocytosis [20, 37]. Membrane ruffling shows involvement of Iba-1 along with the actin, Iba-1 mutants hampers membrane ruffling and phagocytic cup formation [21]. The role of Iba-1 as an actin binding protein has been studied. The activated microglia after 24 hours of treatment of ALA and Tau showed high colocalization between actin and Iba-1 and enhanced membrane ruffling, lamellipodia and filopodia. The ALA was found to increase membrane ruffles to the great extent, which would be effective for increased phagocytosis. Previous studies have been reported for the physical binding of Iba-1 to F-actin and their involvement in membrane ruffling supports the increased colocalization of Iba-1 and actin in ALA treated cells. The results suggest importance of ALA to enhance microglial activation in presence of Tau species. After CNS injury, microglia has tendency to migrate towards injury site and this migration is supported by rearrangement of actin cytoskeleton [29]. The actin structures and dynamics supports migration, where lamellipodia structure produces forward protrusive forces and thin protrusion of filopodia inspect distant targets. The ALA treated cells showed greater polarized morphology with presence of dense lammellum at leading ends and uropods at rear ends. The lammellum and filopodia extensions were high in ALA treated cells, which suggests a high directional migration and supports protrusive and contractile migration. In the previous studies with the alternative activation of microglia by IL-4 treatment, lammellum with more membrane ruffles have been observed [19]. Similar morphology was observed with ALA treated cells. For the extensive branching required for the lamellipodia formation, ARP2/3 complex plays an important role. ARP2/3 introduce branching of mother actin filaments to produce dense branching network of the actin. The density of ARP2/3 in migratory cells is higher at leading end to produce dense actin network and fade out in other cell body [20, 37]. ALA found to enhance ARP2/3 at the leading ends, since it enhances lamellipodia formation. This depicts that ALA has tendency to remodulate actin network (Fig. 6).

Conclusions

ALA effectively promotes actin-remodeling indicated with membrane ruffling, lamellipodia, and filopodia formation necessary to migrate towards the target and undergo phagocytosis. The enhanced actin remodeling contributes to microglia activation, migration and also phagocytosis. This indicated beneficial role of dietary supplement of ALA has potential to target microglia to clear Tau seeding.

Abbreviations

ALA- α- Linolenic acid

CNS- central nervous system

AD- Alzheimer's disease

PUFAs- polyunsaturated fatty acids

DHA- Docosahexaenoic acid

EPA- Eicosapentaenoic acid

NFTs- Neurofibrillary tangles

ARP2/3- Actin related proteins

Ethics Declarations

Ethics approval and consent to participate

Not applicable

Consent for publication

Not applicable

Availability of data and materials

All data generated or analyzed during this study are included in this published article and its supplementary information files.

Competing interests

The authors declare that they have no competing interests.

Additional information

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Contributions

SD and SC performed the experiments and prepared the initial draft. SC conceived, designed, supervised, initial draft, review editing and wrote the paper. All authors read and approved the final paper

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Figures

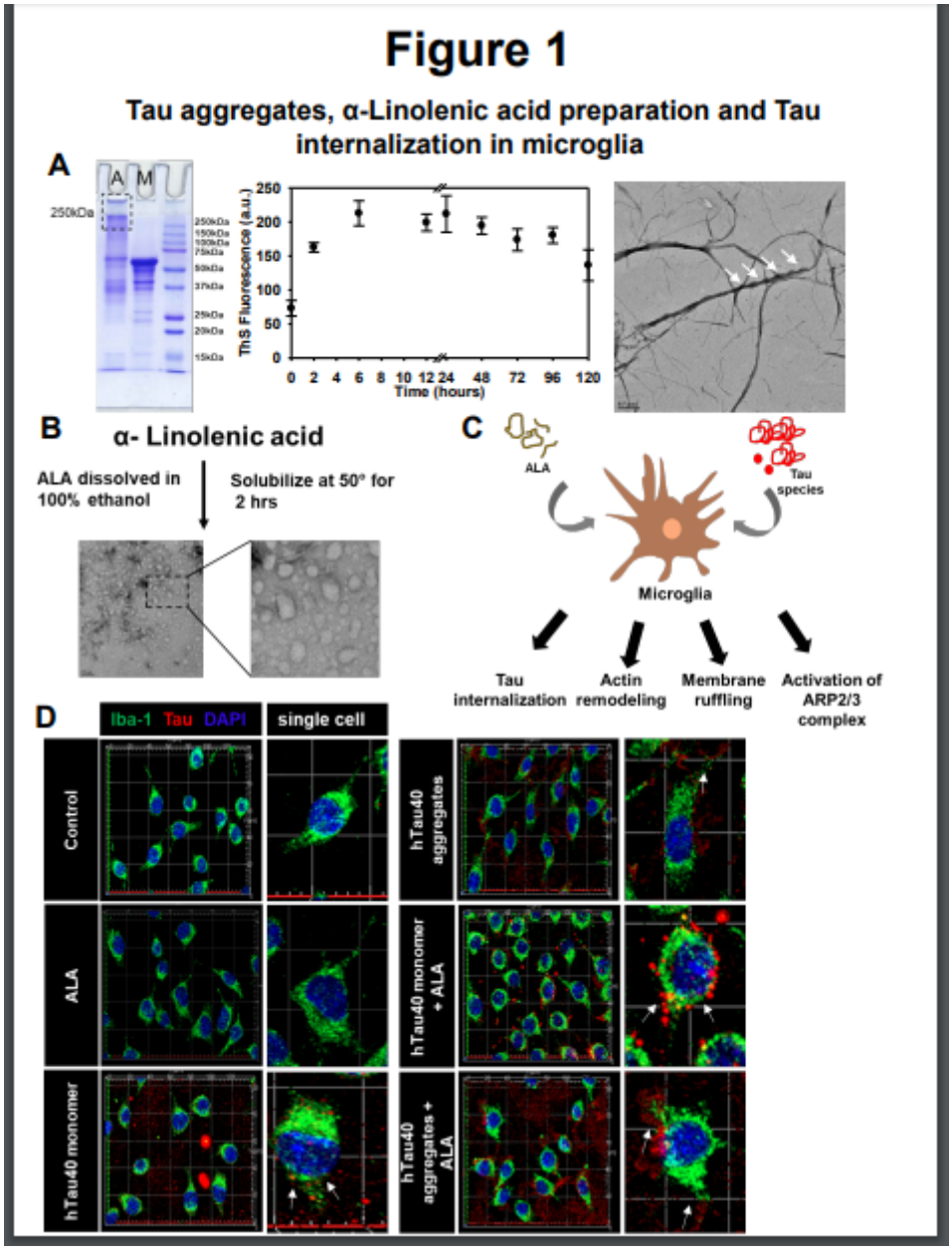

Figure 1

Internalization of extracellular Tau influence by ALA. A) Human Tau40 aggregates were prepared as a starting material for internalization studies. Heparin was used as polyanionic reagent and incubated with Tau for 120 hours. The biophysical characterization was carried out with SDS PAGE, ThS fluorescence, TEM analysis. B) ALA was prepared as a starting material in presence of 100% ethanol and solubilized at 50° for 2 hours. C) Schematic representation of experimental proceeding depicts the role of ALA in phagocytosis of extracellular Tau and necessary actin remodeling for the process. D) Study of internalization of extracellular Tau monomer, aggregates in presence of ALA. The treatment was given for 24 hours and studied with fluorescence microscopy to understand the internalization of Tau (red) in Iba-1 (green) positive cells. Figure showing 3D image of fluorescence microscope image, single cell zoom with arrow marks indicates internalized Tau. Axio observer 7.0 Apotome 2.0 Zeiss microscope were used to the study of internalization extracellular Tau.

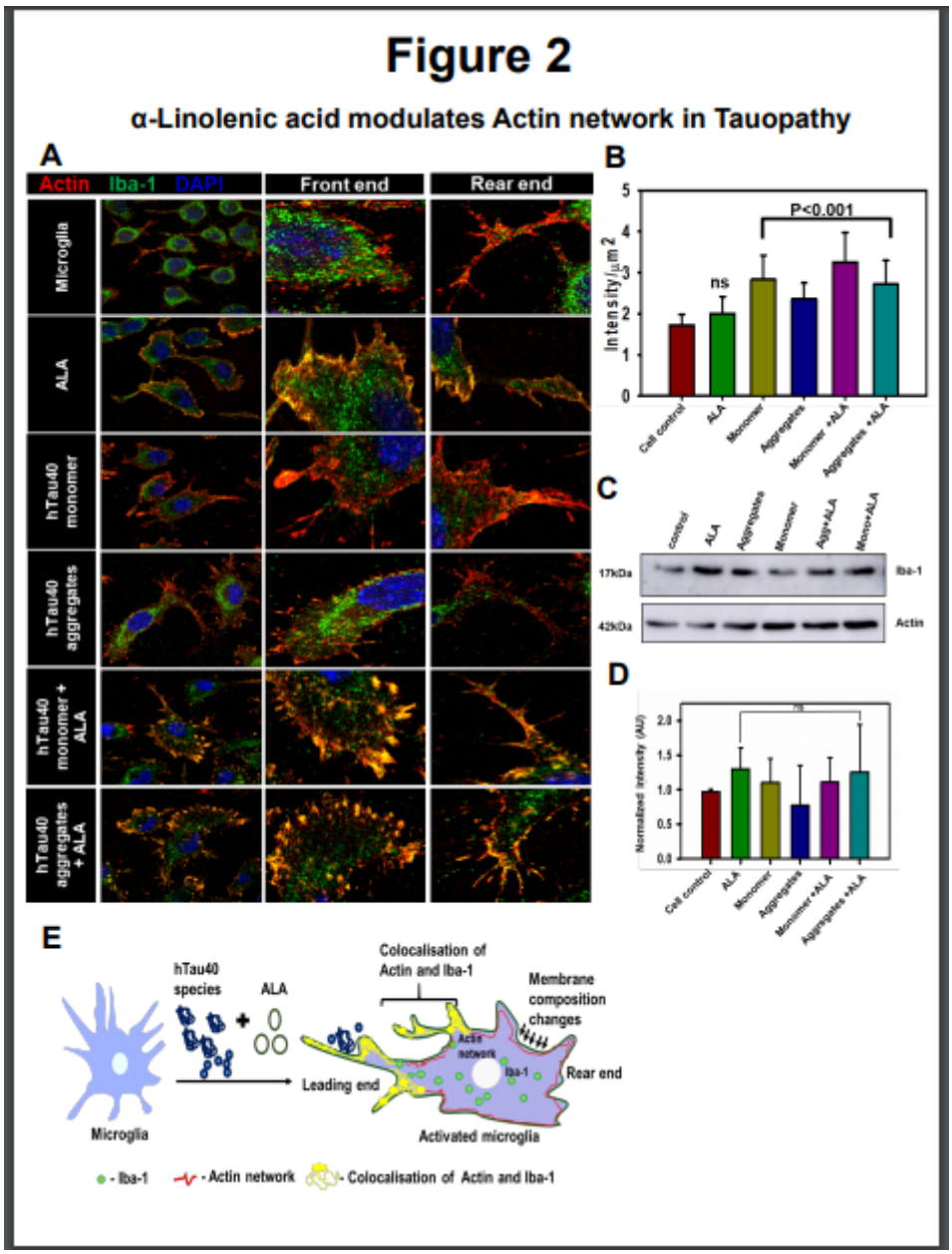

Figure 2

Polarization of Microglia under influence of α-Linolenic acid. Role of actin-based migration and Iba-1 in phagocytosis. A) N9 microglia cells were treated with hTau40 aggregates species, hTau40 monomer species with α-Linolenic acid for 24 hours. After fixation cells were stained with α-actin (red) and anti-Iba-1 antibody (green). Colocalization of actin and Iba-1 was studied by Super-resolution confocal microscopy, scale bar is 20 µm. The migratory microglia are shown with the front; leading end and rear end and the colocalization of actin and Iba-1 in the ends assisting the microglia for membrane ruffling and phagocytosis. B) The quantification of Iba-1 in cell indicating its activation status and its levels on increased phagocytosis, significance is P<0.001.C) The protein expression of Iba-1 was measured by western blot analysis in all the treated groups after 24 hours of treatment and stained with anti-Iba-1 and β-actin antibody for loading control. D) Quantification of intensity of protein bands from western blot normalized with the β-actin loading control E) Schematic overview of actin remodeling at leading ends of microglia and involvement of Iba-1 in the process of phagocytosis after ALA treatment to cells.

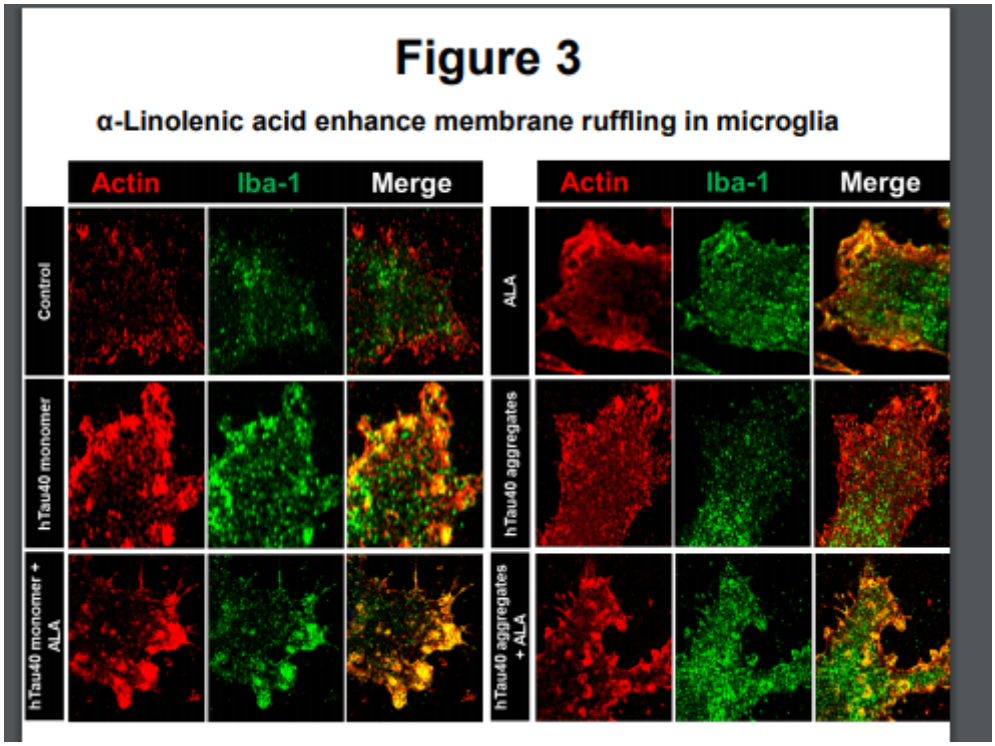

Figure 3

Formation of membrane ruffles induced by ALA. Role of actin and Iba-1 in formation of membrane ruffling was studied upon exposure of ALA. The effect of ALA in membrane ruffling was observed with the super-resolution confocal microscopy. A) N9 cells were studied for the membrane ruffling after 24 hours of exposure of hTau40 monomer, aggregates and ALA and stained with actin (red) and Iba-1 (green). The zoom images are indicated with the colocalization of actin and Iba-1 showing membrane ruffling in images.

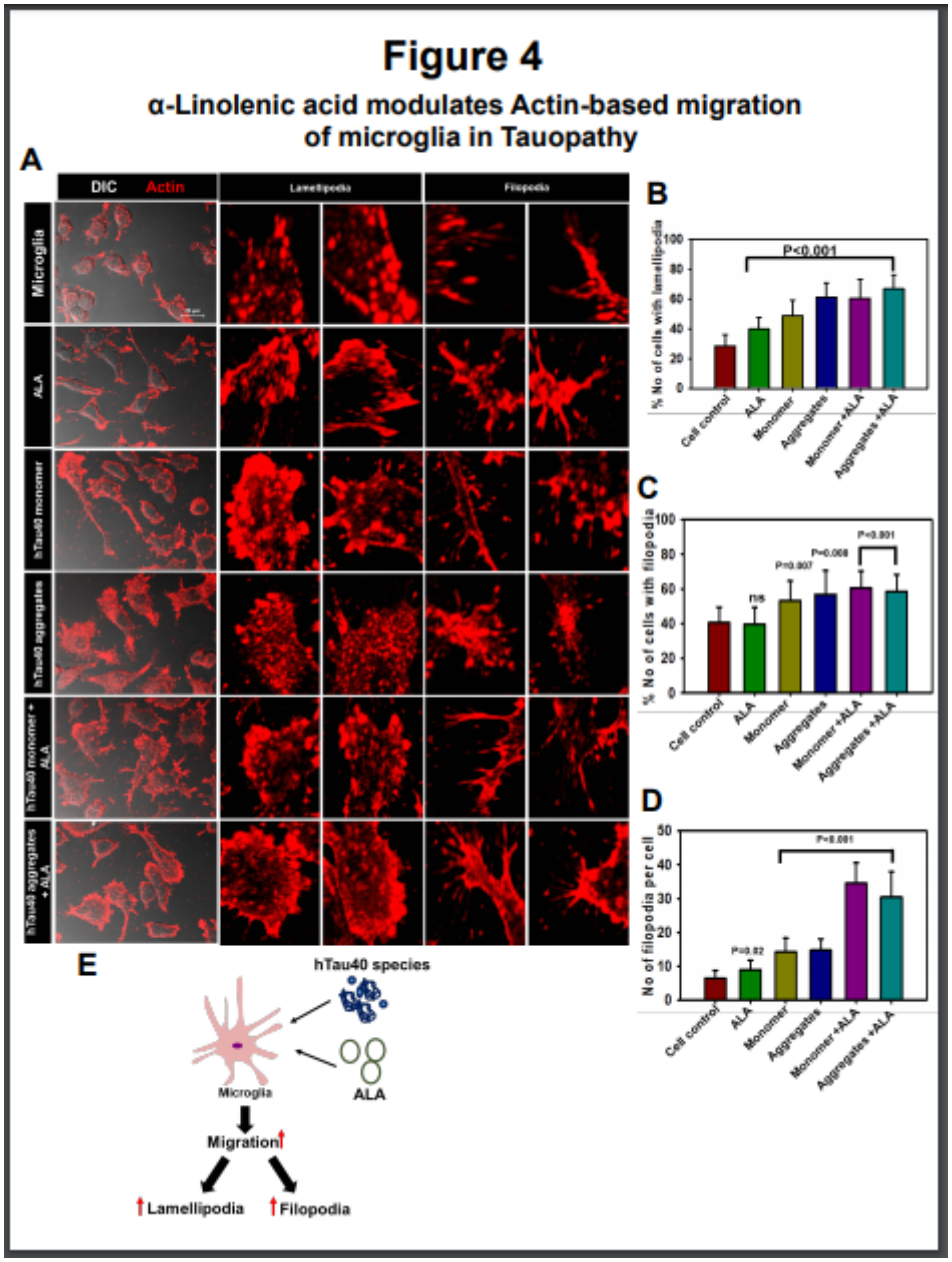

Figure 4

Enhancement of actin-rich structures for migration in microglia. Actin plays significant role in migration of microglia by providing the mechanical strength as well as direction support. A) Actin based structures lamellipodia, filopodia were observed after 24 hours exposure of hTau40 monomer, aggregates and ALA to microglia cells by fluorescence microscopy. Cells were fixed, stained with β- actin antibody and observed for fluorescence imaging. The panels suggest merge image with actin and DIC and zoomed images indicates actin-based lamellipodia, filopodia structures, scale bar is 20 μM. B) The graph represents quantification of percentage of lamellipodia positive cells per 10 fields of different treatment groups, P<0.001 as compared to cell control not activated cells. C) The graphical representation of percentage of filopodia positive cells per 10 fields of different treatment groups, monomer and aggregates treated groups showed significance of p<0.05; whereas monomer, aggregates with ALA showed high significance of p<0.001 D) Quantification of number of filopodia extensions present per cell

per 10 fields of different groups, significance is p<0.001. E) Migration of microglia is important aspect in phagocytosis process; the ALA in presence absence of hTau40 monomer and aggregates enhances the process by promoting actin rich structure lamellipodia and filopodia, which assist the phagocytosis.

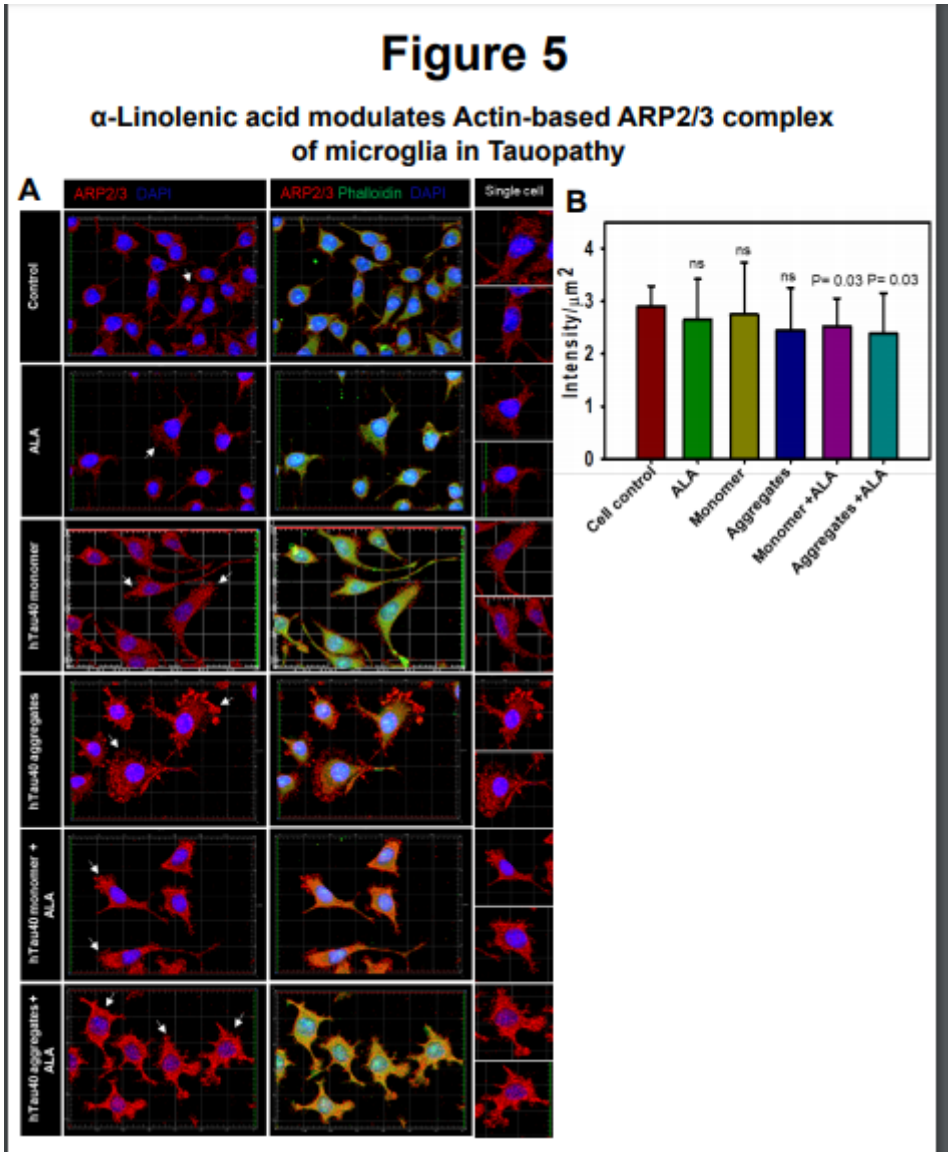

Figure 5

Enhancement of lamellipodia and ARP2/3 complex in presence of ALA in microglia. ARP2/3 complex plays important role in formation of lamellipodia and provide pushing force for cell to move forward. After exposure ofhTau40 monomer, aggregates and ALA the abundance of ARP2/3 in microglia was studied by fluorescence microscopy. A) Immunofluorescence images showing 3D view of microglia cells stained with ARP2/3 (red), phalloidin (green), DAPI. Single cell panel showing enlarged view of single cell indicating abundance of ARP2/3 in cell. B) Intracellular intensity of ARP2/3 in cell was quantified from immunofluorescence images and plotted as intensity per sq. area. P< 0.05 for ALA treated groups as compared to cell control.

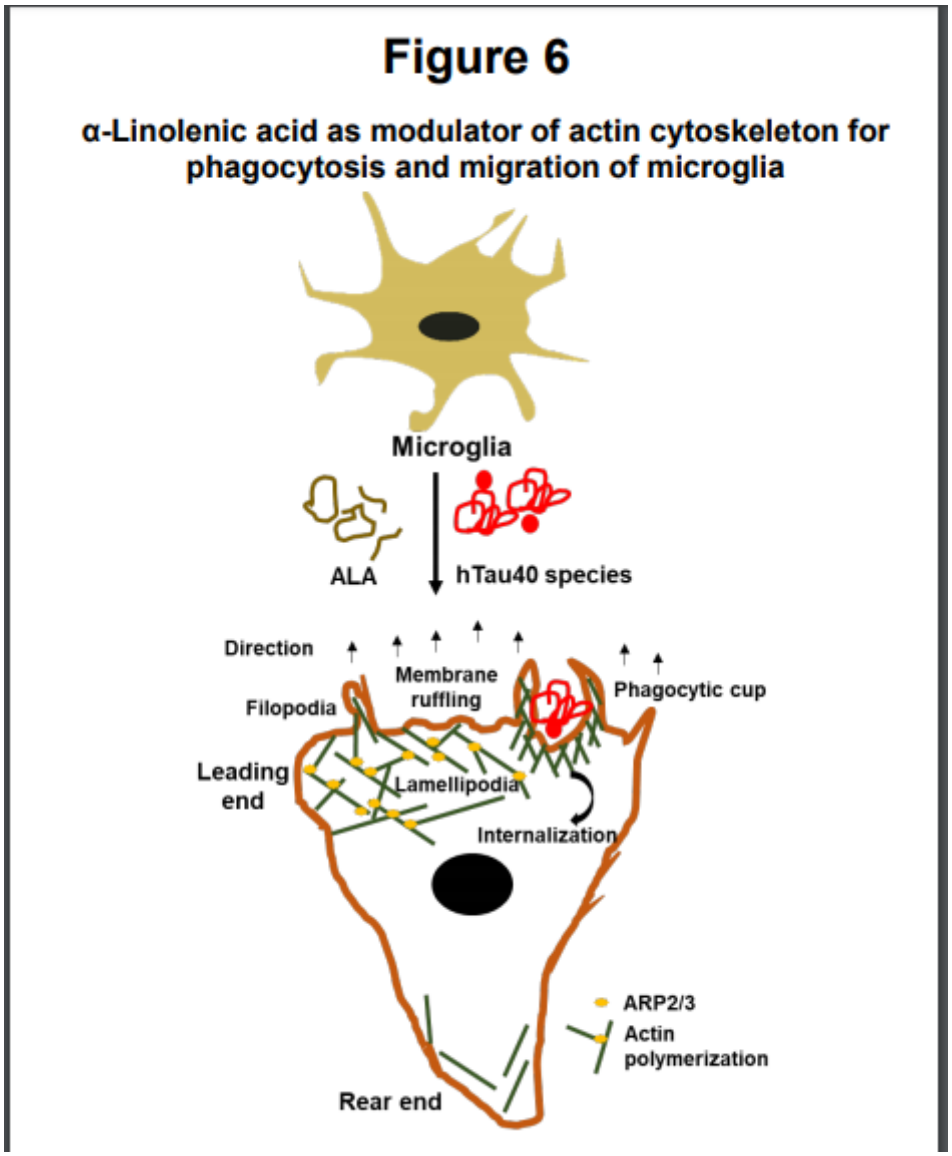

Figure 6

α-Linolenic acid as modulator of actin cytoskeleton for phagocytosis and migration of microglia. The picture representation suggests effects of ALA after exposure to N9 cells in enhancing extracellular Tau phagocytosis and associated actin-remodeling. ALA enhances phagocytosis of microglia with increased actin dynamics, the actin structure lamellipodia, and filopodia for the migration and promotes membrane ruffling to support the process of phagocytosis.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- supplement5.pdf