Disaggregation of Tau-induced by α-linolenic acid

By

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30EE21A26005

Major project submitted to AcSIR

For the award of the degree of

MASTER OF TECHNOLOGY

In

ENGINEERING

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May-2023

CERTIFICATE

This is to certify that the work incorporated in this master Thesis entitled, **"Disaggregation of Tau-induced by α-linolenic acid"** submitted by **Aashima Jain** to the Academy of Scientific and Innovative Research (AcSIR), in partial fulfillment of the requirements for the award of the Degree of MASTER OF TECHNOLOGY embodies original research work carried-out by the student. We, further certify that this work has not been submitted to any other University or Institution in part or full for the award of any degree or diploma. Research material(s) obtained from other source(s) and used in this research work has/have been duly acknowledged in the thesis. Image(s), illustration(s), figure(s), table(s) etc., used in the thesis from other source(s), have also been duly cited and acknowledged.

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STATEMENTS OF ACADEMIC INTERGRITY

I Aashima Jain, declares the integrity of the research conducted and is according to the ethical standards of scientific research. Throughout my journey, I have followed the path of morality, impartiality and transparency, including my data analysis, documentation and propagation.

I have looked through the results to ensure the authenticity and efficiency of the data collected and have reported without any bias or manipulation, acknowledging all information sources used in this thesis. I have not engaged in any form of plagiarism or academic misconduct.

I understand that to maintain the impact and credibility of research, scientific integrity is essential and I pledge to continue upholding these principles in my future research endeavors.

ACKNOWLEDGEMENT

I would like to express my sincere gratitude guide Dr. Mahesh Kulkarni, Principal Scientist and I would like to express my sincere gratitude to my Co-Advisor Dr. Subashchandrabose Chinnathambi, Principal Scientist, Biochemical Sciences Division, CSIR- National Chemical Laboratory, Pune, India for this exquisite opportunity to a part of his research lab and providing me all the resources to carry out my project. His invaluable guidance and support throughout the research process helped me to tune my skills and my knowledge. Their insights, expertise, and encouragement have been instrumental in shaping this thesis.

I am very grateful to thank Smita Eknath Desale, who has helped me in the project and guided me the through out my stay in the lab. I also thank all my lab members who generously gave their time and energy for helping me; Rashmi Das, Hari Hara Krishnan, Tazeen Qureshi, Mahima Kapoor and Ankita Namdeo. Their support are greatly appreciated and have been essential for me to compete my journey.

I am thankful to my Oral Examination Board (OEB) committee Dr. Shubhangi B Umbarkar, Dr. Rajesh G Gonnade and Dr H.V. Thulasiram for giving their precious time and evaluating me.

Finally, I would like to express my appreciation to my parents and friends for their unwavering support and encouragement throughout my academic journey. Their love and support have been the driving force behind my success.

Thank you all for your contributions to this thesis.

ABSTRACT

Intracellular Tau protein**,** resident at the axonal region of a neuron, undergoes post-translational modifications like phosphorylation, acetylation, ubiquitination etc. for its activated functioning. Microtubule associated Tau protein (MAPT) gene present at 17q21 chromosome and generates six different isoform of Tau, hTau40 being the largest isoform (4R Tau) with 441 Amino acids. The hexapeptide motifs present at R2 and R3 repeat domain of Tau is majorly responsible for prosperity for forming higher-order species. Hyperphosphorylation of Tau leads to destabilization of cytoskeleton, resulting in abnormal accumulation of Tau at various sites including at synaptic circuits, other healthy neurons etc. and is the major hallmark for Alzheimer's. The detachment of Tau from microtubules (MTs) leads to formation of heterogeneous oligomeric species and initiates aggregates formation. Alzheimer's is a chronic neurodenegrative disorder affecting millions of people worldwide above the age of 60 and is the major cause of dementia and cognitive impairment. In central nervous system, resident immune cells, Microglia and astrocytes contributes towards surveillance and removal of patho-proteins. Throughout their lifespan, microglia senses its micro-environment to maintain brain- homeostasis and under highly activated conditions, microglia retraces its extensions converting to amoeboid state, promoting pathogenesis. Due to high activation of glial cells, they polarizes towards M1 phenotype resulting in more production of toxic chemokines and cytokines like IFN- γ, affecting other healthy cells and instigating Aβ and Tau protein accumulation. To attenuate the higher-order species formation, α-linolenic acid (ALA) could be used as a therapeutic target, as it has a neuro-protective role and decreases the ROS conditions inside the brain. α-linolenic acid (ALA) with varied concentration was studied with higher-order species. The fluorometric assays showed the decreasing trend of higher order species upon ALA treatment, promoting its attenuation. Transmission electron microscopy (TEM) studies revealed that ALA restricts tau fibril formation; higher-order species with ALA with 80 µM resulted in shorter fibril formation. Alongside, Immunofluorescence studies also confirms, active modulation in cytoskeleton for migration and elevated internalization of extracellular Tau aggregates upon ALA treatment.

LIST OF ABBREVIATIONS

MAPT	**Microtubule-associated Tau protein**
A.D.	**Alzheimer's diseases**
MTs	**Microtubules**
IFN-γ	**Interferon gamma**
ALA	**α-linolenic acid**
TEM	**Transmission electron microscopy**
NFTs	**Neuro-fibrillary Tangles**
CNS	**Central nervous system**
Aβ	**Amyloid Beta**
IL-4	**Interleukin 4**
DHA	**Docosahexaenoic acid**
EPA	**Eicosapentaenoic acid**
PUFA	**Polyunsaturated Fatty acid**
NDs	**Neurodegenerative diseases**
PHFs	**Paired helical filaments**
SEC	**Size exclusion chromatography**
ThS	**Thioflavin S**
SDS-PAGE	**Sodium dodecyl sulphate- polyacrylamide gel electrophoresis**

LIST OF FIGURES

REVIEW OF LITERATURE:

Tau is a member of microtubule-associated protein (MAPT2) that are abundantly present in neurons and are less expressed in Oligodendrocytes and astrocytes [1]. They are considered an intrinsically disordered proteins, having repeat regions mediating electrostatic interactions with microtubule surfaces [2]. Under normal physiological conditions, Tau is localized primarily to the axonal region of neurons facilitating their assembly and disassembly [3]. They help in the stabilization of neuronal cytoskeleton, organization, and its development through the "repeat domain" in the C-terminal half [4]. MAPT (microtubule-associated protein Tau) gene present on chromosome 17q21 undergoes alternate splicing to generate six different isoforms of Tau. In the central nervous system, a total of 6 Tau isoforms are found ranging from 352 - 441 Amino acids. The major difference is the presence or absence of the R3 or R4 repeat domain. The structure of human Tau (hTau40) comprises of N-terminal (1-244 AA) comprising a proline-rich domain and

C-terminal (245-44 AA) including the Repeat domain [5]. The repeat region of Tau plays a vital role in aggregation [4], VQIVYK and VQIINK are the hexa peptide motifs present at the 2nd and 3rd repeat domain that contributes the highest for aggregation.[6]

The repeat region is responsible for the attachment of Tau with microtubules, conformational changes make it a target study. [5] However, Post-translational modifications like acetylation, phosphorylation, glycation, ubiquitination, truncation, etc. are realized to be necessary for physiological functions, whereas hyperphosphorylation is the pathological hallmark of Alzheimer's. In pathological conditions, Tau loses its affinity for microtubules, forming heterogeneous oligomers ultimately resulting in Neuro-fibrillary tangles (NFTs), one of the major causes of Dementia [7]. However, NFTs are considered to be the key pathological feature of several neurodegenerative diseases, including Alzheimer's disease (AD), frontotemporal lobar degeneration, and other Tauopathies.[8] The major factors responsible for A.D. could be disruption in clearance mechanism, abnormally folded protein and its accumulation, oxidative stress and inflammation, genetic factors, the imbalance between kinases and phosphatase activity, etc. leading to memory impairment and cognitive decline.[9] Additionally, they negatively impact normal CNS functions [3] . Progressive neurodegenerative diseases are affecting millions of people worldwide and it has been reported that AD affects over 10 Million people each year and would be reaching 78 million by 2030 [10]. Several organizations provide comprehensive and personalized training to patients so that they could be able to manage themselves. [11]

Microglia and astrocytes, both are resident immune cells, accounting for 10% and 61% of the Central nervous system respectively. They contribute towards the detection, maintenance of brain homeostasis, overall removes the foreign antigens and abnormal deposits present in the brain.[12]

Microglial cells, throughout their lifespan continuously communicate with the extracellular micro-environment to ensure the maintenance of cerebral homeostasis through various factors like TGF-β and CSF1R signaling and its extended finger-like projections. [12, 13] Microglial cells are stated to be existing in two states: "Ramified" and "activated". Normally, after every few hours, the brain undergoes surveillance to ensure the normal functioning of the CNS through its processes that scan the microenvironment for any lesions or injuries [12, 14] . But, upon histopathology (due to infections, attack of foreign antigens, deposition of proteins), the glial cells reflects abnormal activation and Microgliosis and astrogliosis becomes a common feature for Alzheimer's.

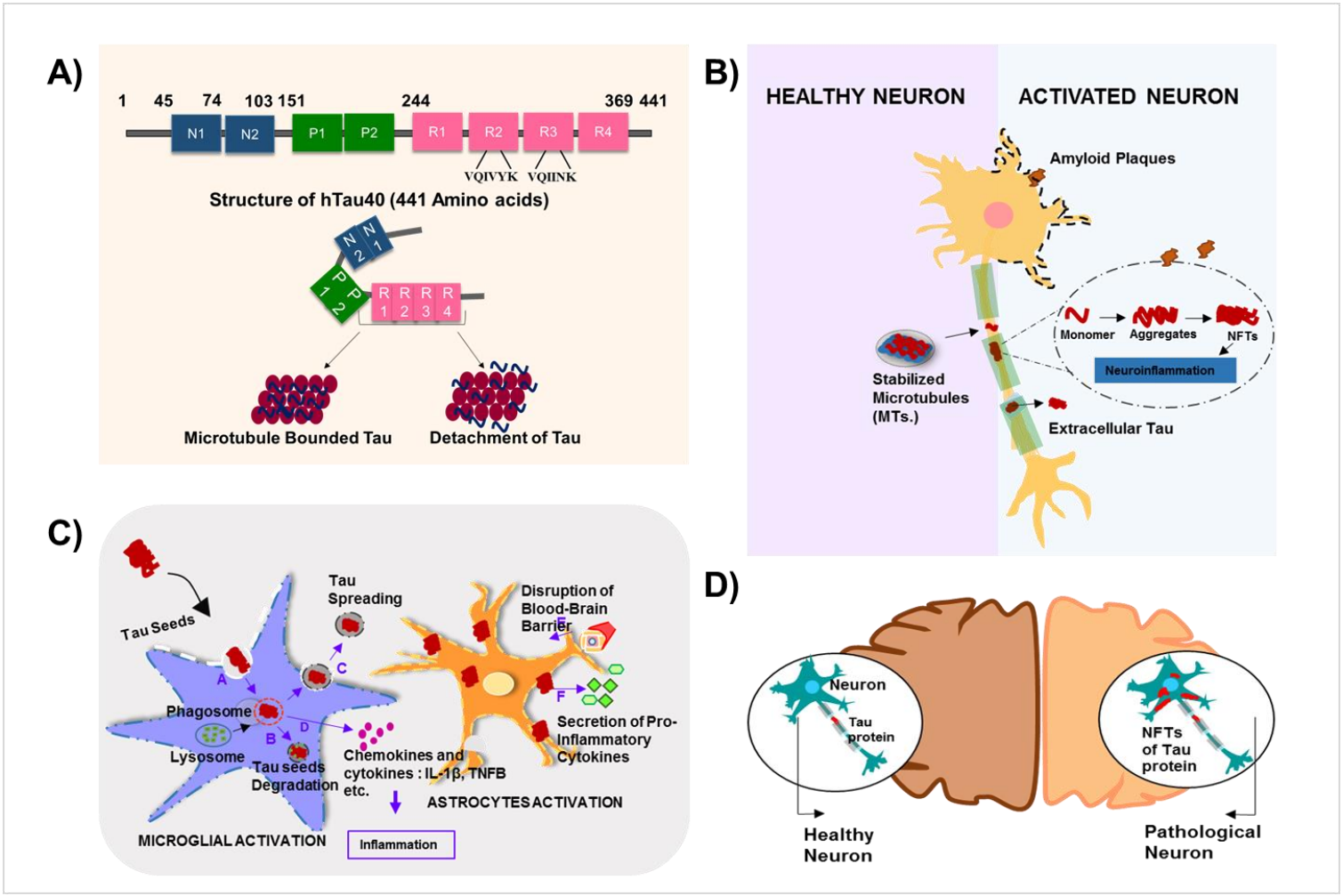


Figure 1. Structure of Tau-protein and its association with Alzheimer's disease.

A. Tau, a Microtubule-associated protein with molecular weight of approximately 45 KDa, carries a positive charge. Composed of several distinct regions, it is primarily found in the axonal region of neurons. Tau's N-terminal region has two inserts, whereas its proline-rich domain is situated in the middle of the protein. Under normal physiological conditions, stability and interaction with the cytoskeleton are provided by a four-repeat sequence located in the C-terminal region. Modulating Tau's behavior and allowing it to form higher-order species are important functions of the two hexapeptide motifs (VQIVYK and VQIINK) in the repeat domain. Tau plays a crucial part in preserving the structural stability of microtubules in neurons.

B. Tau interacts with microtubules via electrostatic interactions in a healthy neuron. Tau can form higher-order species when it becomes detached from microtubules under certain conditions, such as hyperphosphorylation. The loss of normal function and accumulation of abnormal Tau species, resulting from hyperphosphorylation of Tau, can ultimately lead to the formation of neurofibrillary tangles and other neurodegenerative diseases, including Alzheimer's disease.

C. Neighboring microglia and astrocytes, can take up Tau released from dying neurons, which can trigger a cascade of events ultimately resulting in neuroinflammation. Activated glial cells release various toxic chemokines and cytokines, which can activate other healthy cells in the central nervous system and lead to a cycle of inflammation and toxicity. In addition, the incomplete degradation process can lead to the generation of Tau fragments that act as seeds, causing the formation of abnormal Tau species in neighboring cells. This, in turn, can contribute to the progression of neurodegenerative diseases like Alzheimer's.

D. Neuro-fibrillary tangles of Tau are a pathological hallmark in an Alzheimer's patient, while in a normal healthy individual, Tau protein exists as monomeric form.

Additionally, cells undergo morphological modifications in association with inflammatory responses generated due to changes in the microenvironment. On the immune challenge, microglial cells are characterized by: M1 and M2 phenotypes. The classical pathway (M1 phenotype) is initialized through the first line of defense i.e. to destroy the invaded pathogen and secretes pro-inflammatory responses like IFN-γ exhibiting antigen presentation and its clearance. The alternate pathway (M2 phenotype), also referred to as would healing state where microglial cells promote the healing process and are associated with the secretion of anti-inflammatory cytokines and chemokines like IL-4-mediated responses. [12, 15] between these two phenotypes of microglia, there is a very thin line of differentiation, so the particular activated phenotype is critical for the brain environment. Under AD conditions, the M1 phenotype dominates over the M2 phenotype leading to impairment of the healing process and leading to an increase in inflammation and neurotoxicity. Hyper activation of immune cells over the site of lesions induces inflammatory cytokines-chemokines production and ultimately kills other healthy neurons along 00with hyperphosphorylation of proteins. [15]

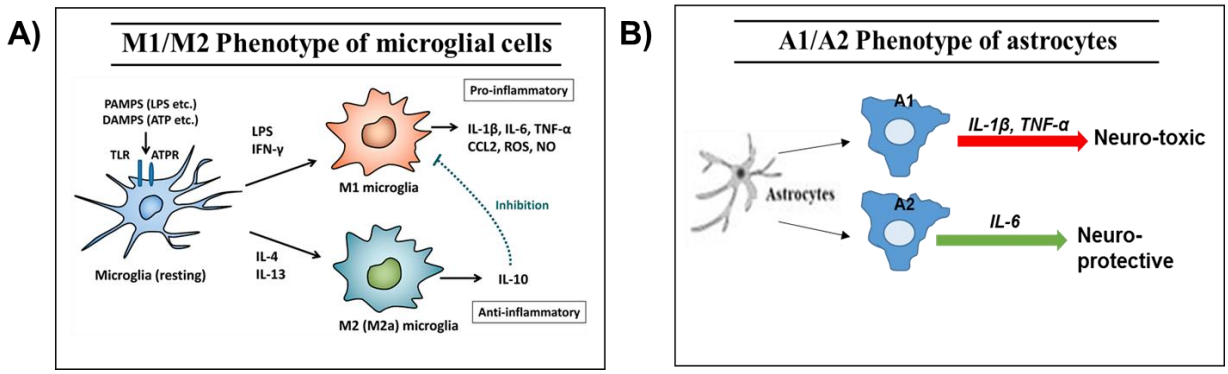


Figure 2. Polarization of microglia and astrocytes from M1/M2 and A1/A2 respectively.

A. Activation of Microglial cells can be done by various stimuli, including pathogen-associated molecular patterns (PAMPs), which are recognized by Toll-like receptors (TLRs) or by ATP receptors, present on the surface of microglial cells. M1 and M2 are the two polarized state of activated microglial cells. The M1 phenotype, pro-inflammatory cytokines production phenotype, which can be toxic to neurons and contribute to neuroinflammation. In contrast, the M2 phenotype, responsible for generation of anti-inflammatory responses. For proper brain functionality and homeostasis, balance between M1 and M2 phenotype is crucial, and dysregulation of this balance can contribute to the progression of various neurological diseases.

B. In central nervous system, Astrocytes are the most abundant glial cells, under activated condition can differentiate into A1 and A2 phenotype. The A1 phenotype, characterized by its neurotoxicity and ability to produce pro-inflammatory cytokines and chemokines whereas, in contrast A2 phenotype, anti-inflammatory responses are generated through secretion of cytokines and chemokines like IL-6, promoting tissue repair and regeneration.

Despite all extensive research, there is no effective cure or treatment available that decreases the progression of this destructive disease [7, 16]. To date, the treatments available in nature (memantine, galantamine, etc.) are only symptomatic, trying to counterbalance the neurotransmission disturbance, not deaccelerating nor preventing the progression of the disease. However, available therapies are benefitting cognition, and functionality but are self-effacing. Nowadays, research is being conducted focusing largely on the Attenuation of Tau aggregation and targeting its signaling pathways. [7]One promising therapeutic approach that can be used for targeting the accumulated Tau protein is the use of dietary interventions, as they can modulate Tau phosphorylation and its accumulation [17]. Alpha-linolenic acid (ALA) is a polyunsaturated fatty acid found in several foods, including flaxseed oil, chia seeds, and walnuts. [18] It has been shown to ALA has neuroprotective effects in animal models of AD, Huntington's disease, and other neurodegenerative diseases through ontogenesis.

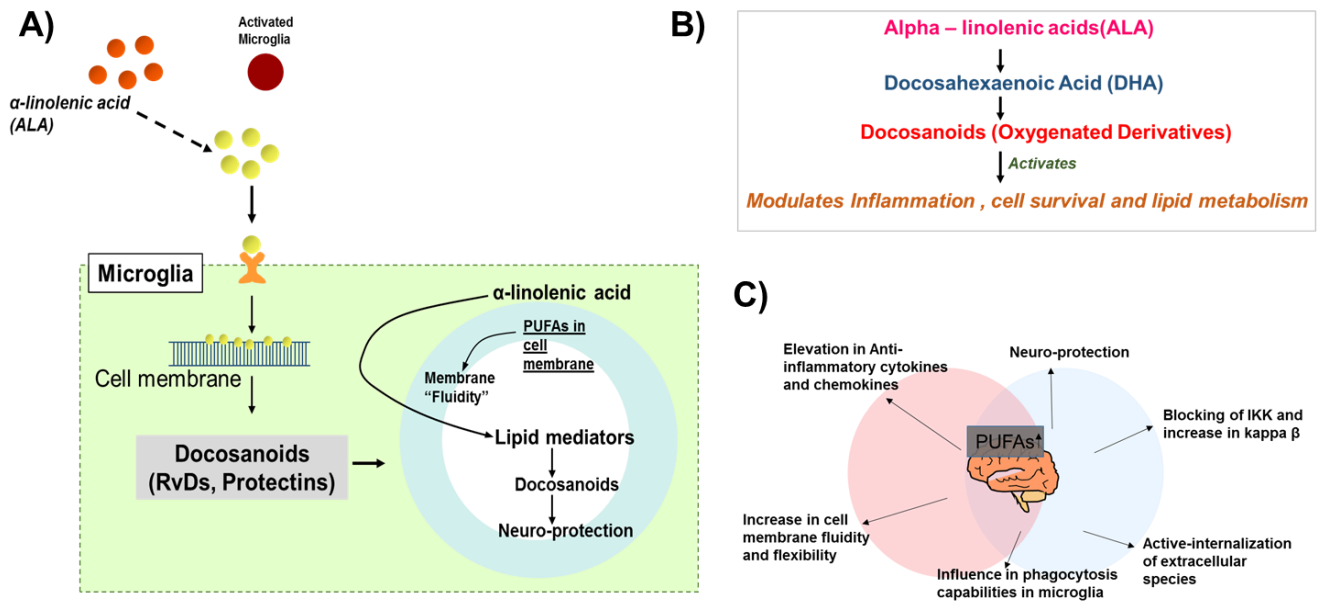


Figure 3. Metabolism of ALA and its neuro-protective effects in CNS.

A. The dietary fatty acid, α-linolenic acid has been found to provide neuroprotection and increases plasma membrane fluidity, which can be beneficial in Alzheimer's disease. ALA was seen have anti-inflammatory benefits towards Tauopathies, where neuroinflammation is a major hallmark. ALA is metabolized to produce lipid mediators that bind to microglial cells and influences their phagocytotic capabilities, leading to the production of anti-inflammatory cytokines, such as IL-10, as well as specialized pre-resolving mediators (SPMs) like Resolvins and Protectins resolves inflammation and was found to be neuroprotective in various neurological disease.

B. Metabolic pathway of α-linolenic acid.

C. PUFAs, such as Omega-3 fatty acid have shown to provide various benefits to the brain such as increased anti-inflammatory cytokine production, tissue repair and regeneration, blockage of pro-inflammatory response, modulation of microglial cells,

Additionally, ALA has been shown to modulate the activity of microglial cells towards M2 phenotype i.e. anti-inflammatory instead of M1 phenotype - pro-inflammatory [19] . α-linolenic acid (ALA) n-3 or ω- 3 polyunsaturated fatty acid also participates in modulating AD pathology by protecting against Aβ - neurotoxicity, reduction in nitric-oxide production, reduction in neuro-inflammation, protection against neuronal damage, etc. Eicosapentaenoic acids (EPA; 20: 5n-5) and Docosahexaenoic acid (DHA; 22: 6n-3), are the important long-chain n-3 fatty acids metabolized from α-linolenic acid (ALA), having a vital role in brain development and inflammatory response. [20]

Studies suggest that through dietary interventions i.e. when Omega-3 fatty acids are incorporated into the human body through diet, they reside in membrane bilayer in almost all cell types and have the potency to modulate lipid mediators pathways as well as cell signaling cascades. [17, 21]. EPA and DHA synthesize bioactive lipid mediators – Docosanoids and Elovanoids that have beneficial properties and also escalate the fluidity of the cellular membranes. They are incorporated into lipid molecules (Diacylglycerol) at the sn-2 position facilitating a positive response in CNS. Such observations suggest a decrease in Tauopathies, an increase in synaptic plasticity, attenuation of over-activated glial cells, and a decrease in the secretion of inflammatory cytokines: IFN- gamma, IL-6, IL-1β, C1q, etc. [17, 22] Additionally, ALA has been shown to modulate the activity of several kinases and phosphatases involved in Tau phosphorylation, suggesting

that it may directly affect the accumulation of Tauopathies. Hence, there is a necessity to study α-linolenic acid and screen it against Tauopathies. [15]

In this present study, we have focused on the role of α-linolenic acid over Tau species that get accumulated in extracellular spaces in Alzheimer's disease. ALA is an important PUFA with a large number of biological activity that includes, anti-inflammatory, and modulation of microglial cell from the M1 phenotype to the M2 phenotype. ALA was prepared by heat dissolution in 100% ethanol at 50 °C for 2 hours and was screened for its therapeutic properties. The beneficial role of ALA is known, so the present study focuses on the effect of ALA against extracellular Tau species through fluorescence assays and morphological analysis of disaggregates followed by cell biological assays. The current results suggest that α-linolenic acid can be used as a dietary- fatty acid that inhibits Tau to form higher-order species and increases microglial phagocytotic capabilities. The In-vitro attenuation of Tau aggregation proposes ALA as a promising fatty acid in the reduction of Tauopathies.

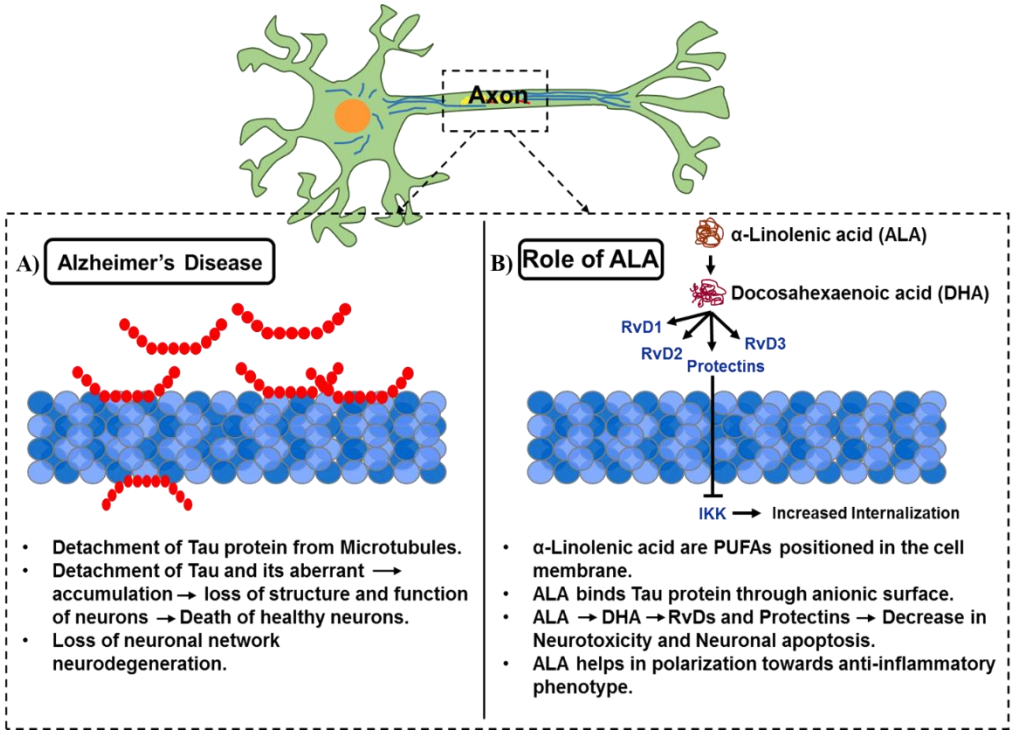


Figure 4. The fate and structure of axonal region in a) Alzheimer's disease and b) ALA-treated.

A. The formation of neurofibrillary tangles (NFTs) in neurons is caused by the abnormal accumulation and hyperphosphorylation of the Tau protein, which characterizes Alzheimer's disease. The cognitive decline and neurodegeneration associated with Alzheimer's disease are believed to be caused by NFTs, which are a characteristic pathological feature of the disease. Although NFTs have been recognized as a significant therapeutic objective for Alzheimer's disease, there is presently no efficient cure accessible to halt its advancement.

B. Alpha-linolenic acid, an essential omega-3 fatty acid, have shown as a therapeutic agent for higher-order species. Conversion of ALA to EPA and DHA leads to the production of specialized pro-resolving mediators (SPMs), such as Resolvins and protectins, which blocks pro-inflammatory responses and promoting tissue regeneration through transporting these molecules to cell membrane facilitated by a MFSd2a transporter. Together, these beneficial properties makes ALA a therapeutic agent for neurodegenerative diseases.

MATERIALS AND METHODS

Lists of chemicals and cell Culture materials

Serial Number	Chemicals	Company/Supplier
1.	4'-6' diamidino-2-phenlyindole (DAPI)	Thermo Fisher Scientific
2.	Thioflavin-S (ThS)	Sigma
3.	8-Anillinonapthalene-1-sulfonic acid (ANS)	Sigma
4.	Acrylamide	Invitrogen
5.	Ammonium Acetate	MP Biomedicals
6.	Ammonium per sulfate (APS)	MP Biomedicals
7.	Ampicillin	MP Biomedicals
8.	N,N-bis(2-hydroxyethyl)-2-aminoethanesulfonic acid (BES)	Sigma
9.	Bicinchconinic acid (BCA)	Sigma
10.	Bis-Acrylamide	Invitrogen
11.	Bovine Serum Albumin std (BSA)	Sigma
12.	Bradford reagent	Biorad
13.	Bromophenol blue	MP Biomedicals
14.	Coomassie brilliant blue R-250	MP Biomedicals
15.	Copper sulfate (II)	Sigma
16.	Dimethyl sulfoxide (DMSO)	Life Tech / MP Biomedicals
17.	Dithiothretol	Calbiochem
18.	Ethanol	MP Biomedicals
19.	Ethylene glycol tetra acetate (EGTA)	MP Biomedicals
20.	Fetal Bovine serum (FBS)	Thermo Fisher
21.	Formaldehyde	MP Biomedicals
22.	6X Gel loading dye	New England Biolabs
23.	Glacial acetic acid	MP Biomedicals
24.	Glycerol	MP Biomedicals
25.	Glycine	Invitrogen
26.	Heparin (MW – 17500)	MP Biomedicals
27.	Horse Serum	Invitrogen

28.	IPTG	MP Biomedicals
29.	Isopropanol	MP Biomedicals
30.	LB Broth	Invitrogen / Himedia
31.	MES hydrate	Sigma
32.	Methanol	MP Biomedicals
33.	Penicillin-Streptomycin	Invitrogen
34.	Polysorbate 20	MP Biomedicals
35.	Polyvinylidene fluoride membrane	Merck Millipore
36.	Potassium acetate	MP Biomedicals
37.	Potassium chloride	MP Biomedicals
38.	Protease Inhibitor Cocktail (PIC)	Roche
39.	Precision Plus ProteinTM Dual Color Standards	Biorad
40.	RIPA buffer	Thermo Scientific
41.	Sodium acetate trihydrate	MP Biomedicals
42.	Sodium Azide	MP Biomedicals
43.	Sodium bicarbonate	MP Biomedicals
44.	Sodium chloride	MP Biomedicals
45.	Sodium dodecyl sulfate (SDS)	Sigma
46.	Sodium hydroxide	MP Biomedicals
47.	SuperSignal TM West Atto Chemiluminescent Substrate	Thermo Scientific
48.	Tetramethylethylenediamine	Invitrogen
49.	Tris base	Biorad
50.	Tris HCl	Invitrogen
51.	Triton X 100	Sigma
52.	Trypan Blue	Invitrogen
53.	Trypsin-EDTA	Invitrogen

Lists of Antibodies

Serial Number	**Antibodies**	**Company/ Supplier**
1.	K9JA (A0024)	DAKO
2.	Beta -Tubulin Polyclonal Antibody (Loading control) (PA1-86602)	Thermo Fisher Scientific
3.	Beta - Actin Polyclonal Antibody (Loading control) (PA5-16914)	Thermo Fisher Scientific

4.	Ionized binding adaptor molecule 1(Iba1) (PA5-27436)	Thermo Fisher Scientific
5.	Lysosomal associated membrane protein (LAMP1) (MA1-164)	Thermo Fisher Scientific
6.	Alexa Fluor 488 Phalloidin (A12379)	Thermo Fisher Scientific

Laboratory Instruments and Equipment used

Serial Number	Instrument/Equipment	Company
1.	AKTA Pure FPLC system	GE Healthcare
2.	AKTA Start FPLC system	GE Healthcare
3.	Amersham Imager 600	GE Healthcare
4.	Analytical weighing balances	Mettler Toledo
5.	Autoclave	Spire
6.	Avanti JXN26 High speed centrifuge	Beckman Coulter
7.	BioSafety cabinet/Clean Bench	Thermo Fisher Scientific
8.	CO2 Incubator	Thermo Fisher Scientific
9.	Dry bath	Genei
10.	Gel rocker	Benchmark
11.	Heraeus Incubator	Thermo Fisher Scientific
12.	High speed Centrifuge 5804R	Eppendorf
13.	Homogenizer	Constant System Ltd.
14.	Laminar air flow	Microfit
15.	Magnetic stirrer	Genei
16.	Microcentrifuge 5418 R	Eppendorf
17.	Microplate reader Infinite 200 PRO	Tecan
18.	MilliQ unit Direct 16	Millipore
19.	Mini-PROTEAN electrophoresis system	Biorad
20.	MiniSpin Plus Table top Centrifuge	Eppendorf
21.	Optima XPN10 Ultracentrifuge	Beckman Coulter
22.	pH meter Five Easyplus	Mettler Toledo
23.	Shaker Incubator (H1010-MR)	Benchmark Scientific

24.	Shaker Incubator Multitron Standard	Infors HT
25.	Semi Dry Blotting apparatus	GE Healthcare
26.	T20 Transmission Electron Microscope	Tecnai
27.	Vacuum Pump	Millipore
28.	Vortexer mixer	Genei
29.	Water Bath	Genei
30.	Zeiss Axio observer 7 microscope with Apotome 2.0	Zeiss

hTau40 Protein Isolation and Purification:

The Primary culture was prepared by inoculation of the BL-21 Bacterial strain in LB Media, strain was taken from glycerol stock and was augmented with Ampicillin (100 µg/ml). The culture flask was incubated at 37°C in the Benchmark Incu-shakerTM 10 L, providing proper shaking overnight. The next day, Augmentation with ampicillin was given and the Secondary culture was inoculated from the primary culture and was incubated in a rotatory shaker at 37°C till the culture reaches its exponential phase (where O.D. at 600 nms reaches 0.5). Once optical density was achieved, induced with 0.5 mM IPTG in the rotatory shaker at 37°C. After 4 hours, the culture was pelleted down by centrifugation in an Avanti JXN26 High-speed centrifuge at 4,500 rpm for 10 minutes. The pellets were separated and later re-suspended in lysis buffer and prepared for lysis. Once the pellets become slurry, they are subjected to Constant cell disruption system Ltd (mechanical disruption system). Pelleted cells were disrupted in a constant homogenizer at 15,000 psi, leading to cell lysis. The lysed cells were collected in a glass bottle kept on ice and the process was repeated 1-2 times for complete lysis. The lysate was heated for 20 minutes at 90°C in presence of 0.5 M NaCl and 5 mM DTT, collecting supernatant once the process was complete. As supernatant comes to RT, was subjected to centrifugation in Optima XPN10 Ultracentrifuge at 40,000 rpm for 45 minutes at 4°C. Later the supernatant was kept for overnight Dialysis in presence of a buffer containing 50 mM NaCl. The dialyzed protein was centrifuged at 40,000 rpm for 45 minutes at 4°C and was subjected to two-step chromatography. hTau40 protein is positively charged with a pH of 8.8, in consequence, it was subjected to cation-exchange chromatography. The Buffer A was used for the calibration of the Sepharose Fast-flow column and the sample was loaded using super-loop, elution of Tau protein was done in presence of gradient 1M NaCl. The eluted fractions were picked out through SDS-PAGE, concentrated, and processed to the second step of chromatography – Size exclusion. In a Superdex 200 pg High Load 16/600 chromatography using 1X PBS and 2 mM DTT sample was loaded using a loop of 2 ml. The eluted protein fractions were collected and identified on SDS-PAGE. The selected fractions were concentrated and Bicinchoninic Acid assay (BCA) was used to estimate the concentration of protein in presence of BSA (1 mg/ml) as standard.

Evaluation of protein concentration:

The Bicinchoninic acid assay is a method to quantify protein concentration. BCA is based on the principle that the protein of interest present in the sample leads to the reduction of Cupric ion (Cu +2) to cuprous ion (Cu +) in presence of Bicinchoninic acid. Protein chelates the Copper leading to the formation of a blue color complex, to which Bicinchoninic acid binds at 562 nms, generating a purple-colored complex. As the

concentration of protein in the sample increases the intensity of the purple complex increases and vice-versa.

In –vitro hTau40 Oligomer preparation:

hTau40 Oligomerization is induced by a polyanionic reagent, Heparin (17500 Da) which acts as a co-factor responsible for charge compensation of the monomeric protein. Tau protein was RT incubated for 12 hours in presence of a reaction mixture containing heparin in the ratio of 4:1 (4 parts of Tau and 1 part of heparin), along with 2 mM DTT, 1X PBS, 25 mM NaCl, with protease inhibitor cocktail and 0.01% NaN3. Fixating with 4% Formaldehyde and concentrating. The characterization is done by SDS-PAGE, ThS, ANS, and HR-TEM.

In –vitro hTau40 Aggregation preparation:

hTau40 protein is an intrinsically disordered protein with a positive charge and a pH of 8.8. Tau protein upon inaccurate Post-Translational modification undergoes speeding of the aggregation process. For in-vitro aggregates formation, Heparin (17500 Da) was used as an inducer. It is a polyanionic agent that binds to the repeat motifs of Tau. The ratio of Tau to heparin (5 µM) is 4:1 and was added in along with 2 mM DTT, holding up the formation of the aggregates. The aggregation mixture carried 20 Mm BES, along with 25 mM NaCl, 0.01% NaN3, and a Protease inhibitor cocktail.

The mixture was kept at 37 °C for 120 hours and was characterized by SDS- PAGE, ThS, ANS, and HR-TEM. Pre-formed aggregates (20 µM) were incorporated with 20, 40, and 80 µM of ALA to study the kinetics of disaggregation.

Fluorometric assay:

Unfolded Tau is a random coil-structured protein with overall hydrophilic in nature but upon aggregation it has protruding β- sheet structure with elevated hydrophobicity. The charge compensation and conformational change at repeat motifs lead to the formation of the secondary structure of Tau. The modulation towards 2° structure and elevated hydrophobicity is characterized through Thioflavin-S at Ex/Em 440/451 nms and ANS at Ex/Em 390/475 nms, incubating for 10 minutes. The measurements were done in Triplicates in Eppendorf 384 well plate and plots were prepared in SigmaPlot 10.0.

Sodium-dodecyl sulfate – PAGE analysis of hTau40 aggregates:

The SDS-PAGE is an analytical technique that separates proteins according to their molecular weight. The SDS is a detergent that masks the protein with a uniform negative charge. Tau aggregates were characterized on SDS- PAGE, since aggregates are high molecular species they tend to remain at the top. In the BIO-RAD Mini-PROTEAN electrophoresis system, 5 µl of the reaction mixture (aggregates) was loaded on 10 % Resolving gel and once the process was complete the gel was stained and visualized.

Analysis of aggregates through Transmission electron Microscopy:

hTau40 tangles were observed through High Resolution -TEM (TECNAI T20 120 KV). Firstly, a reaction mixture of 2 µM was placed over Para film and the carbon-coated copper grid was RT incubated for 3 minutes. Two water washes for 30 seconds were given to remove the excess sample and the grid was negatively stained with 2 % uranyl acetate for 5 minutes, observed at 1,000,000 X magnification. the α-linolenic acid sample was prepared from 40 µM concentration.

Preparation of ALA:

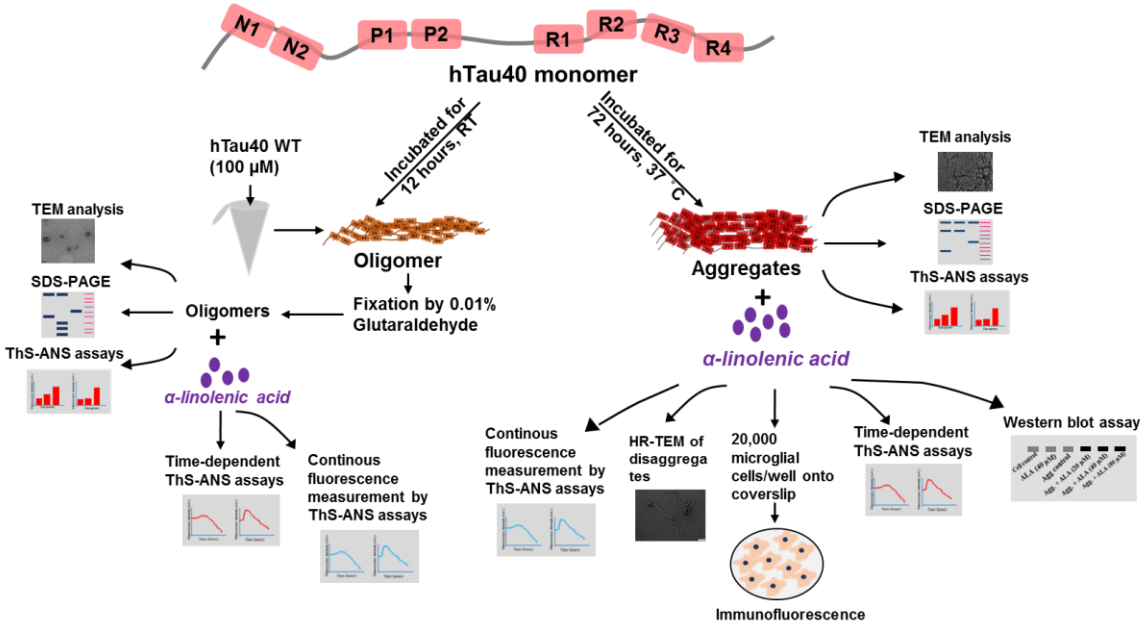
ALA working volume was prepared by dissolving it in 100% ethanol and the Eppendorf was sealed with a Para-film. Kept it in a water bath at 50° C for 1 hour.

Western Blotting Assay:

Western blotting is an analytical technique used to detect and quantify the expression of a specific protein. N9 cells with a density of 3 * 10^6 / well were seeded to study the expression of Iba1 and LAMP1 markers on microglial cells and their activation. The experiment consists of six treatment groups- Untreated cell control, 40 µM of α-linolenic acid treatment, Aggregate control (only aggregates) treatment, Aggregates with 20 µM ALA treatment, Aggregates with 40 µM ALA treatment, and Aggregates with 80 µM ALA treatment. After treatment cells were incubated for 24 hours and later pelleted down. Pelleted cells were lysed with RIPA buffer and sedimented at 14,500 rpm at 4°C for 20 minutes. Bradford assay was used to analyze the protein concentration and 75 µg was loaded on SDS-PAGE gel. After the completion of SDS-PAGE, the gel was transferred to a methanol-activated PVDF membrane, and the blot was blocked with 10% skimmed milk at RT for 1 hour. Then blot was overnight incubated with 1° antibody at 4°C, given three 1X PBST washes, and the blot was incubated with 2° antibody for 1 hour. After the secondary, the blot was given three 1X PBST washes 10 minutes each and was developed using SuperSignal TM West Atto Chemiluminescent Substrate solution, and Amersham AI600 chemiluminescent imager was used to image the blot.

Immunofluorescence Assay:

Immunofluorescence is a technique that allows the visualization of target molecules/antigens through their conjugation with fluorescent dyes in fluorescence microscopy.N9 cells with density of 25 * 105 / well were seeded on a 12 mm coverslip. After treatment, cells were incubated for 24 hours at 37 °C, visualized, and fixed with 4% PFA. Cells were permeabilized with Triton-X 100, followed by blocking by 5% horse serum. Permeabilization and Blocking were done together with 5% horse serum and 0.2% Triton-X 100, RT incubating for 1 hour. Then coverslips were given 3 1 X PBXT washes 10 minutes each, overnight incubating with 1° antibody in a humid condition. After overnight incubation, coverslips were washed with 1X PBST thrice for 10 minutes each. Later coverslips were RT incubated with Alexa fluoro tagged 2 ° antibody for 1 hour. Coverslips were washed thrice with 1X PBS for 10 minutes and were 10 minutes incubated with 300mM of DAPI. The coverslips were mounted over the clean glass slide with 5 µl of mounting media.



Scheme 1. The schematic representation of experiments performed.

RESULTS:

Preparation of Tau species and its Characterization

hTau40 is a natively unfolded protein with 441 amino acids, a microtubule-associated protein present at the axonal region of neurons and less expressed in oligodendrocytes and astrocytes. Tau Oligomer is an unstable, short-lived, toxic intermediate species, leading to aggregation of Tau. In the AD brain, Tau oligomers tend to accelerate and form neurofibrillary tangles leading to neurotoxicity and neurodegeneration. Stable hTau40 WT oligomers were prepared by 12 hours RT incubation of Heparin with repeat motifs of monomer in presence of 1X PBS (pH7.4). The wild-type oligomers were stabilized by 0.01% glutaraldehyde and were prepared for SEC. Collection of the eluted protein fractions and identification on SDS-PAGE. Fractions were selected (indicating the presence of higher-order oligomers), concentrated and concentration was estimated through Bicinchoninic Acid assay (BCA) in presence of BSA (1 mg/ml) as standard. In-vitro aggregation of hTau40 WT can be induced by RNA, Lipids, Heparin, etc. In this study, for aggregation induction, hTau40 monomer was incubated with Heparin for 72 hours and was characterized by SDS-PAGE analysis.

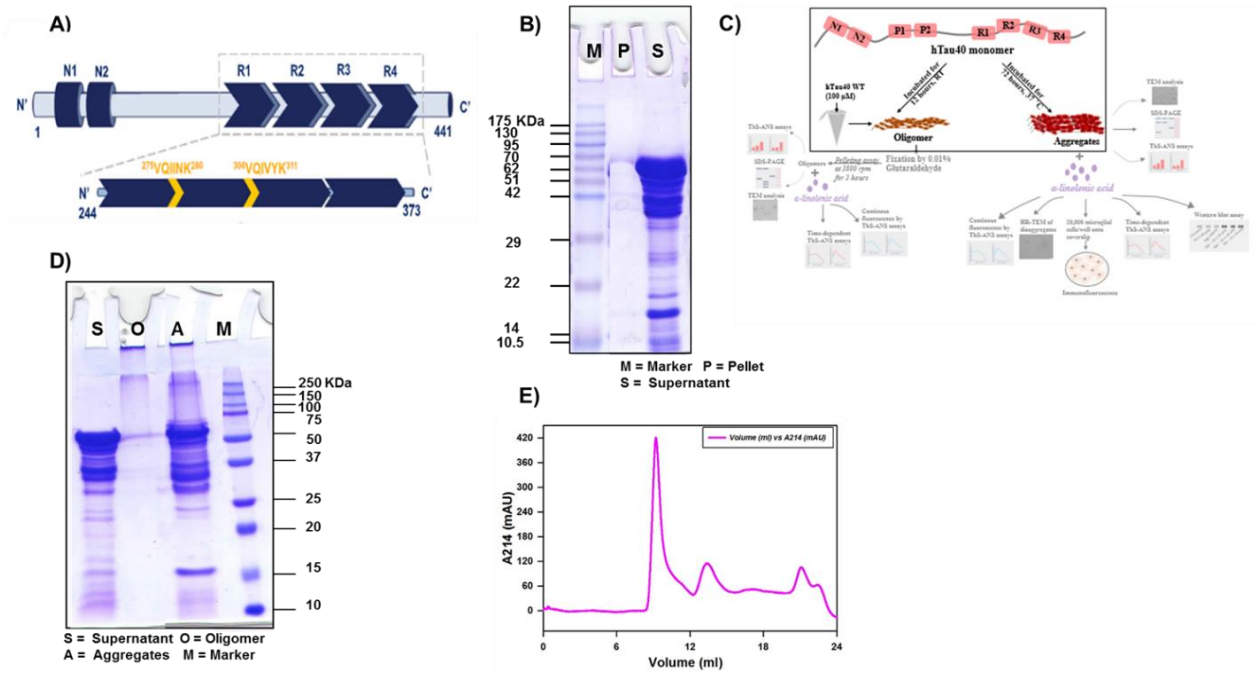


Figure 5. Preparation of Tau Oligomers and aggregates and its characterization.

A. The full-length structure of the hTau40 monomer protein comprises an N-terminal with two inserts, a C-terminal with four-repeat domains, and a flanking region, where the repeat domain plays a crucial role in attaching the protein to the cytoskeleton via the assembly domain under physiological conditions.

B. The hTau40 WT protein, weighing 45 KDa, was centrifuged at 60,000 rpm for 60 minutes, leading to the separation of the supernatant and pellet, which were subsequently characterized on Sodium-dodecyl sulphate –PAGE with a marker as a reference.

C. Schematic representation of experimental procedure performed depicting the preparation of hTau40 Oligomeric and aggregates species.

D. Prepared Tau species, including oligomers and aggregates, were characterized by 10% resolving gel through SDS-PAGE analysis and higher-order oligomer bands were observed after incubation with Heparin in presence of 1X PBS buffer and a trail of protein was observed in aggregates after incubation in BES buffer.

E. Tau oligomers were fixed by 0.01% glutaraldehyde and were separated on Size-exclusion chromatography (SEC) and the elution volume was plotted against the absorbance at 214 nm.

hTau40 species confirmed by fluorometric analysis and TEM

Thioflavin-S, a fluorescent dye interacts with cross- β structure formed at the gist of Tau oligomers as well as aggregates. Thereby, ThS can be used as a fluorophore to study aggregation modulations. Due to transient and unstable nature of oligomeric species, there is no significant increase as compared to hTau40 ^{WT} monomer (1:4 M ratio, 1 part of Tau and 4 parts of ThS).Oligomers have slight increase in ANS fluorescence (1:20 M ratio, 1 part of Tau and 20 parts of ANS), dye that interacts with the exposed hydrophobic groups on Tau. Altogether illustrating oligomers have hydrophobic groups and lesser extent to form β-sheet structure. Similarly, hTau40 ^{WT} aggregates have higher exposed hydrophobicity and β-sheet structure.

hTau40^{WT} oligomer and aggregates were characterized by High Resolution-TEM. Oligomeric species were observed as small globular structures (5-60 nms) whereas elongated mature fibrils were seen in aggregates TEM grids.

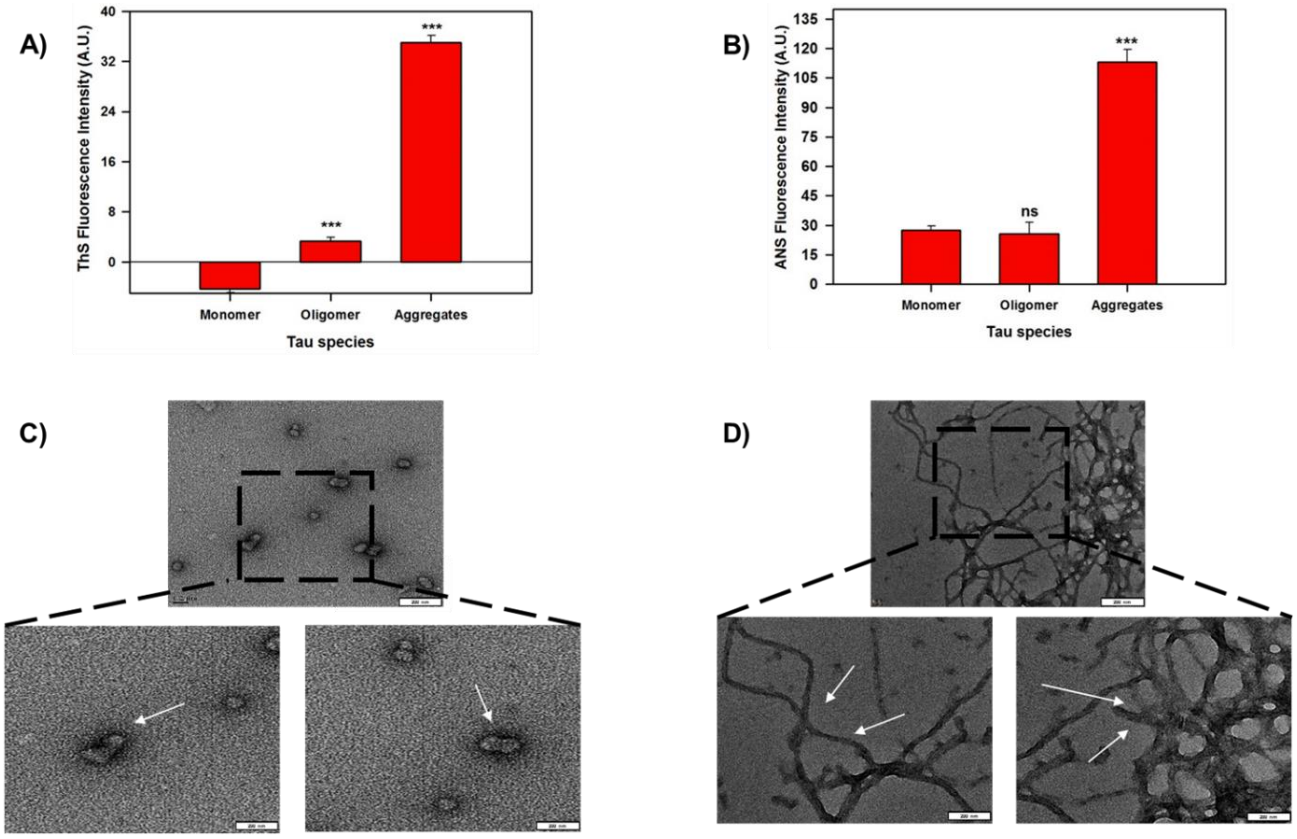


Figure 6. Characterization of higher-order species by fluorometric analysis and HR-TEM.

A. B. Fluorescence analysis confirmed β-sheet structure and changes in hydrophobicity in hTau40 oligomers and aggregates, with ThS and ANS showing significant increases in fluorescence intensity compared to monomers - 3-fold and 5-fold for ThS and 0.5-fold and 4-fold for ANS, respectively.

C. D. hTau40 Oligomers were observed by Transmission electron microscopy, they formed heterogonous globular structure with size ranging from 5-50 nms as indicated by white arrows, whereas hTau40 aggregates were observed to form long-fibrillar structures and white arrows indicates presence of intact thread-like structure of Tau aggregates.

α-Linolenic acid repressed Tau mediated aggregation in-vitro

hTau40^{WT} monomeric protein have an unfolded structure with a flanking proline-rich domain, responsible for binding with MTs and its assembly. Tau has an aggregation prone region ranging from Repeat 1-Repeat 4 domain. Docosahexaenoic acid, a metabolite of α-linolenic acid (ALA) is a potent dietary fatty acid having protection against cardiovascular, neurological and tissue diseases. Through Transmission electron microscopy, ALA was characterized and was observed small globular vesicles. To study the effect of ALA on Tau, Tau oligomer (20 µM) and aggregates (20 µM) was incubated with 20 µM, 40 µM and 80 µM of ALA in a reaction mixture respectively. ALA stock volume was prepared by dissolution in 100% ethanol, at 50° C for 2 hours. The schematic hypothesis depicts the domain organization of Tau oligomers and aggregates and its interaction with ALA. For the assay, the reaction mixture of Tau species with ALA was prepared and Aggregates (20 µM) without ALA was kept as a control to study the kinetics. The kinetics was measured through ThS and ANS fluorometric assays at different time intervals (0-24 hours) and the results suggested that ALA widely inhibited the propensity of aggregation.

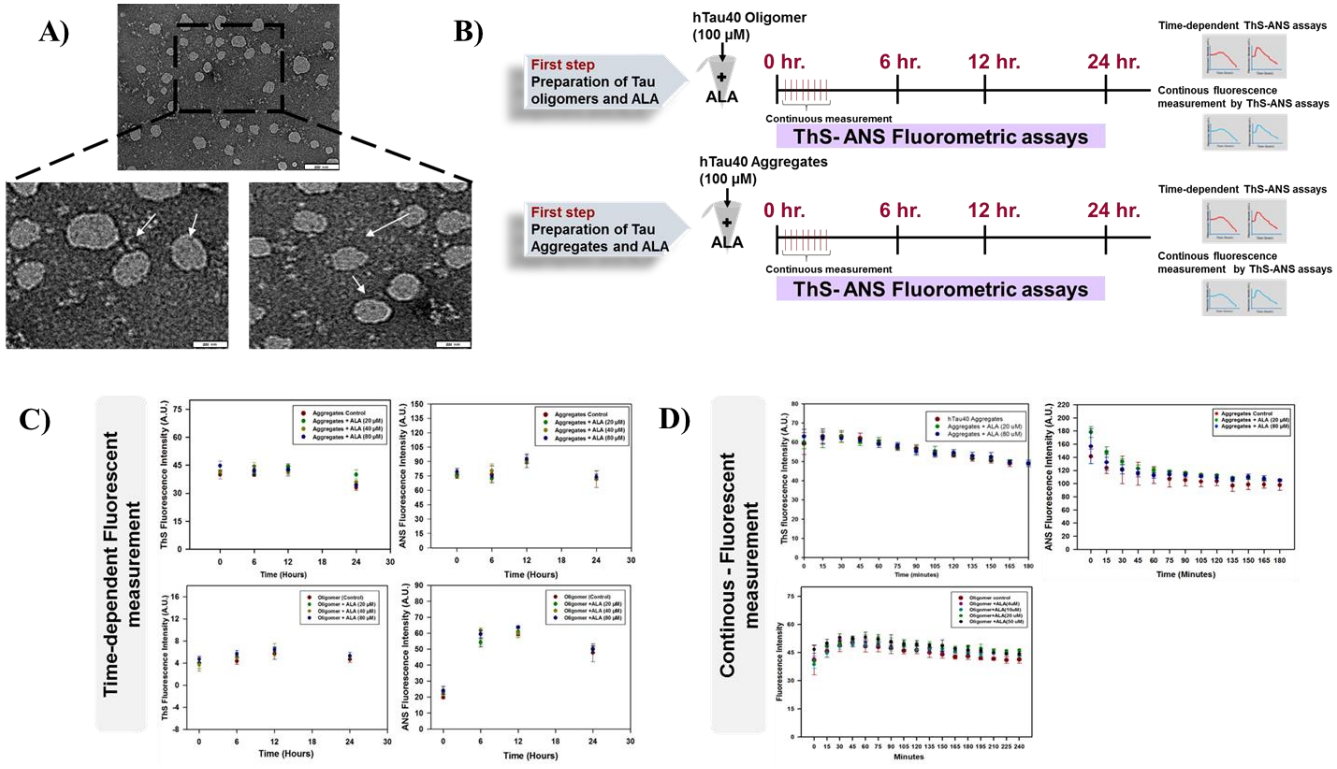


Figure 7. Disaggregation of Tau higher-order species by ALA

A. α-linolenic acid (40 µM) were characterized by Transmission electron microscopy and were observed as small vesicular structures ranging from 15-100 nms as indicated by white arrows.

B. The experimental procedure involved mixing prepared Tau species with varying concentrations of ALA to study the in-vitro disaggregation, followed by time-dependent analysis and continuous measurement of fluorescence.

C. D. The fluorescence kinetics of hTau40 ^{WT} Species was measured at different time intervals using ThS and ANS assays, and the results showed that ALA disaggregates and prevents aggregation-prone sites, while continuous incubation for 180 minutes also revealed a decrease in fluorescence intensity in the presence of ALA compared to the control group.

The fluorescence intensity tends to decrease as the incubation period increase with ALA increases for ALA treated groups, illustrating inefficacy of Tau to aggregate further. ThS (Ex/ Em at 440/521 nms) binds to higher order species through cross-β structure and ANS (Ex/ Em at 390/475 nms) interacts with hydrophobic regions, evaluating the degree of aggregation. Moreover, the continous fluorescence was studied by ThS-ANS fluorometric assay, the reaction mixture was prepared by mixing hTau40 Oligomers and Aggregates with ALA (20 µM, 40 µM and 80 µM) along with Aggregate control (carries 0% ALA). The sample was poured onto 384 well plate and fluorescence was measured continuously for 180 minutes. We estimated that with increase in incubation time the trend for fluorescence was getting decreased in presence of ALA. Furthermost, it could be concluded that ALA is inhibiting aggregation through blocking the aggregation-prone domains on repeat regions.

Disaggregation of hTau40 species by ALA

High-order Tau species were characterized by SDS-PAGE and HR-TEM analysis. hTau40 ^{WT} oligomer and aggregates were incubated with ALA (20 µM, 40 µM and 80 µM) for 24 hours, keeping Aggregate (carries 0% ALA) as a Control indicated in red. Tau higher-order species were absent in ALA (20 µM, 40 µM and 80 µM) treated group. As the incubation period increases, ALA treated group leads to imperceptive higher-order species.

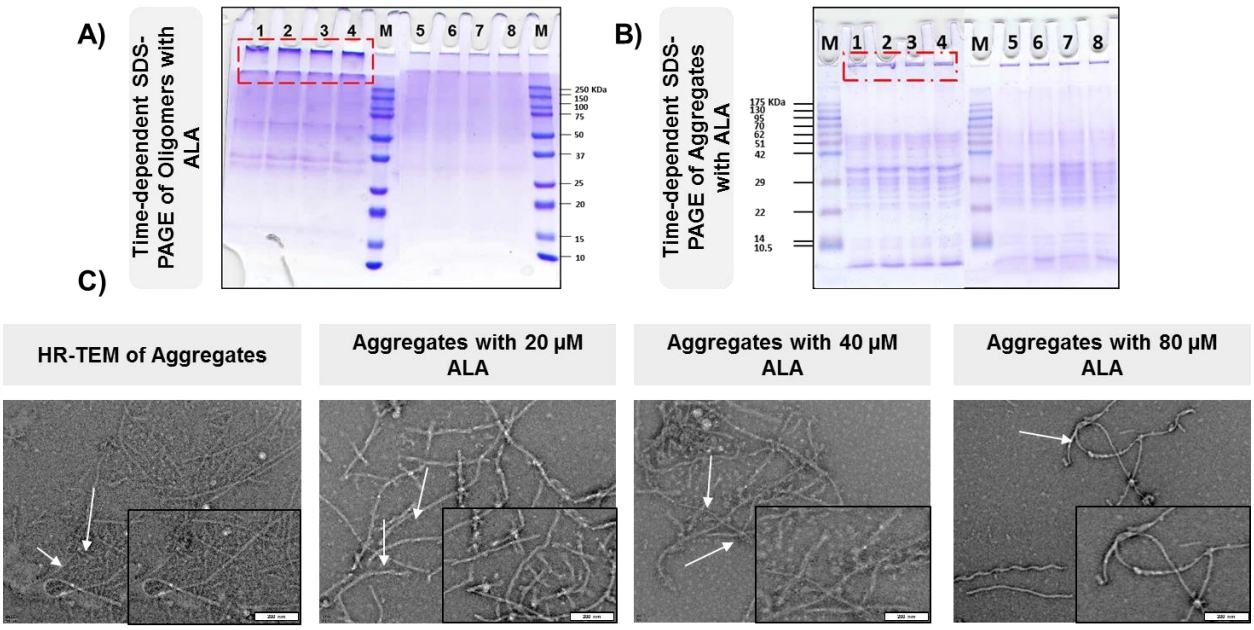


Figure 8. Higher-order Propensity was vented by ALA

A. B. Oligomers and aggregates were treated with varying concentrations of ALA for up to 24 hours, with pre-formed aggregates serving as a control. In the presence of ALA, the absence of higher-order bands on a 10% resolving gel was observed for Tau species, and as incubation time increased, ALA led to a decrease in the perception of higher-order bands.

C. HR-TEM characterization revealed mature bundles of Tau fibrils in the aggregate control group, while groups treated with ALA (20 µM, 40 µM, and 80 µM) showed broken fibrils, indicating successful disaggregation.

Tau oligomers and aggregates in presence of ALA with 80 µM showed maximum confiscation of bands at top of the 10% Resolving gel. Moreover, to study the disaggregation of Tau aggregates with ALA, Tau (20 µM) was incubated for 24 hours with increased conc. of ALA (20 µM, 40 µM and 80 µM) in a reaction

mixture, keeping aggregate (0% ALA) as control. Characterization was done through HR-TEM, mature bundles of Tau fibrils were observed in case of aggregate control (0% ALA). White arrows and zoomed area illustrates presence of fibrils. Whereas with ALA (20 µM, 40 µM and 80 µM) treated groups showed broken fibrils, depicting disaggregation in presence of ALA.

α-linolenic acid have the potency to increase internalization of hTau40 aggregates

N_9 microglial cells were exposed to extracellular Tau in presence of ALA with varied concentration. Cells were treated with 40 µM ALA for 24 hours and were analyzed for internalization through Immunostaining. N_9 cells were treated with 1 µM Aggregate control (0% ALA), 1 µM Aggregate + ALA (20 µM), 1 µM Aggregate + ALA (40 µM) and 1 µM Aggregate + ALA (80 µM) for 24 hours at 37°C. The schematic representation explains the property of ALA in activating N_9 cells for increased internalization. Immunofluorescence staining was performed to study internalization of extracellular Tau (pink) in presence of ALA through Iba-1 (green)-positive cells, Iba1 is microglial marker and increased intensity of Iba1 indicates increase in phagocytosis and internalization. The 2-D images indicates increased internalization of extracellular Tau upon ALA treatment. Zen 2.3 software was used to calculate the mean intensity of Tau and Iba1 inside microglial cells .The enlarged images indicates the specific area of co-localization between extracellular Tau and Iba1 in microglial cells. The mean intensity of Tau was increased in aggregates with 80 µM of ALA as compared to other treatment groups. The fluorescence intensity of aggregates with 80 µM ALA was increased by 5 fold as compared to cell control. Increased co-localization of Iba1 indicates activation of microglial cells for membrane ruffle formation and phagocytosis. The mean intensity of Iba1 was increased in ALA treated group as compared to cell control. ALA helps in activation of microglial cells for active-internalization through increased phagocytosis as well as retraction of ends were observed for forward movement. The increased levels of Iba1for aggregates with ALA treated groups indicates microglial motility and internalization of aggregates. These results suggests that αlinolenic acid increases membrane fluidity which corresponds in polarization of microglia to be activated for higher internalization. The expression of Iba1 was also analyzed through western blot and results illustrated increased levels of Tau aggregates with ALA treated groups.

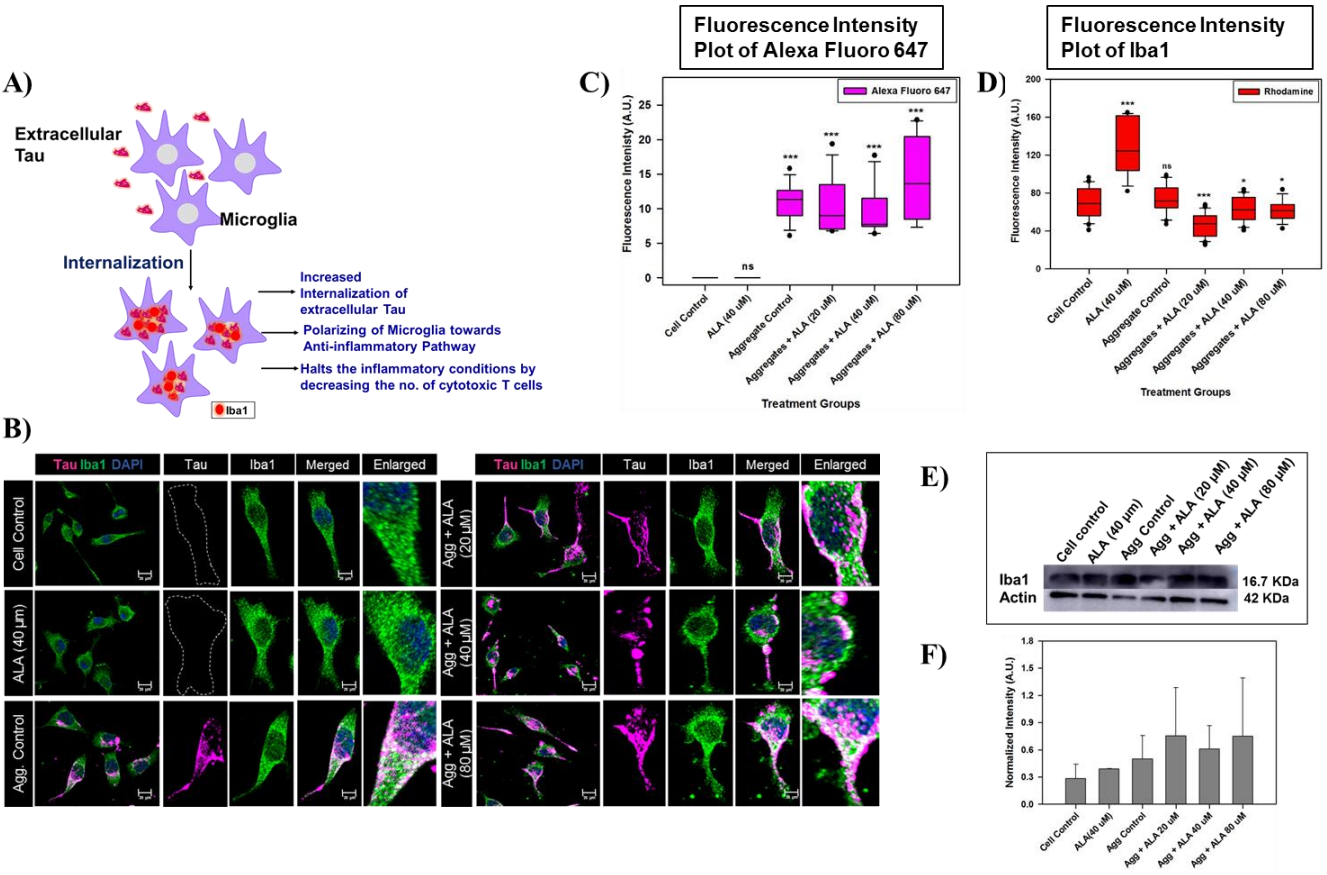


Figure 10. Internalization of hTau40 aggregates and activation of microglia.

A. The schematic representation explains the property of ALA in activating N_9 cells for increased internalization.

B. After incubating microglial cells with Extracellular Tau and ALA, a control group with pre-formed aggregates was maintained. Immunostaining revealed a significant increase in accretion of Iba1 in microglial cells activated by ALA treatment compared to control group with 0% ALA.

C. D. Quantification of intensities indicated an increase in Fluorescence intensity of Tau (Alexa fluoro 647) in ALA treatment group, with a corresponding increase in intensities of Iba1 for ALA treated group.

E. F. Western blot analysis revealed elevated expression levels of Iba1 protein in ALA treated group, and is confirmed by Normalized intensity plot of Iba1.

DISCUSSION

Tau, a positively charged protein with a pH of 8.8, is a natively disordered protein, highly hydrophilic in nature. The transition of monomeric Tau protein to higher-order species are associated with the pathology of Alzheimer's disease. [23] Due to disturbance in the distribution of the protein along the cytoskeleton, Tau forms heterogeneous oligomers followed by aggregates. Thus, deposition at several neuronal junctions, decreasing synaptic plasticity, increasing ROS conditions etc. leads to neuro-inflammation and neuro-toxicity in the brain [24]. There are several factors that contribute in instigation of aggregation: anionic charge compensation at the repeat domain, PTMs leading to hyperphosphorylation of Tau, detachment of MTs from the repeat region of Tao protein etc. Alzheimer's disease is characterized as most prevalent chronic neurodegenerative disease affecting millions of people worldwide above the age of 60, making the meshwork still unresolved. Till date, diagnosis and therapies considered for Alzheimer's are focusing on reduction of damage caused by the deposition of patho-proteins and there is no effective treatment found out until now.[24, 25] Researchers studied fatty acids for its beneficial properties towards the pathology of Tau aggregation and contribution of neuroinflammation in elevation of neurodegenerative diseases [26, 27] PUFAs are making upto 20% dry weight of the brain, contributing in improvement of neurotransmission, increasing lipid bi-layer fluidity and optimizing protein channel function.[26] There is a study associated with administration of α-linolenic acid against synaptic dysfunction and alleviation in patho-protein based neuroinflammation. Basically, Alzheimer's mouse model showed control of various inflammatory cytokines and chemokines like p65, TLR4 etc.[27, 28]

Previous studies suggested that PUFAs are responsible for speeding up the process of therapeutics. ALA to have anti-inflammatory properties, neuroprotection activity, maintenance of BBB, improving cognitive impairment in microglial cells. ALA sparges immune cells when extracellular Tau was given as treatment through remodeling of cytoskeleton through their active migration.[29] Exposure of α-linolenic acid enhanced the phagocytotic capabilities of cells through receptor-mediated phagocytosis, as well as antigen was readily cleared through lysosomal-degradative pathway. Increased Colocalization of endosomal marker with extracellular Tau suggested beneficial effects of ALA over the increased internalization and its clearances.[30]

Our study suggests the α-linolenic acid assists in ceasing the higher-order species formation. The relationship of α-linolenic acid and Tau higher-order species were investigated in-vitro. The pre - formed higher – order species were mixed in a reaction mixture, containing varied concentration of α-linolenic acid (20 µM, 40 µM and 80 µM). An omega-3 PUFAs have neuroprotective properties inside the brain. The fluorometric analysis was done using ThS - ANS fluorescence assay. The higher-order species have modulation towards 2 ° structure with β-sheet formation and higher hydrophobicity, detected by ThS and ANS respectively, which in case of ALA with 20 µM, 40 µM and 80 µM was decreased. The aggregation propensity is induced within 6 hours of in-vitro treatment with poly-anionic agent, but the presence of ALA attenuated after 24 hours. Both ThS and ANS showed in attenuation of higher-order species formation of both oligomeric and aggregates species upon ALA treatment as compared to control. The inability of formation of higher-order aggregates was also confirmed through SDS-PAGE, which indicated the termination after 12 hours in presence of ALA. Higher-order SDS stable band at 250 KDa was seen at 0^{th} hour but at 24^{th} hour fainted higher-order bands were seen. However, pre-formed aggregate was taken as control to study the reaction kinetics. This suggests that ALA contributes in attenuation of Tau higher - order species formation. TEM studies after 24 hours of incubation of pre-formed aggregates with ALA showed short fibrils from aggregates mixed with 80 µM ALA as compared to the control group. The continuous fluorescence measurement was done for both oligomeric as well as aggregates mixed with ALA

was done using ThS and ANS respectively. The trend to fluorescence intensity tend to decrease with ALA treated group as compared to control group.

Immunofluorescence results suggested that phagocytosis of extracellular Tau species is induced by α-linolenic acid (ALA). The extracellular targets *via* membrane receptors are the key for pushing towards the generation of phagocytotic cup and its internalization. Due to phagocytosis of higher-order aggregates, microglial cells tend to get highly activated after 24 hours of treatment. Plasma membrane undergoes modification generating membrane ruffles and phagocytotic cup formation.[30] Iba-1, an activated microglial marker, was studied for its involvement as an actin -binding protein. ALA was found to increase the internalization of extracellular Tau species as increased co-localization of Iba-1 with ALA treated group was seen. The western blot analysis confirmed the active-internalization of Tau species in presence of ALA, as normalized intensity plot were increased with ALA treated groups. Targets are subjected to get degraded through lysosomal degradative pathway, as lysosomal compartment has low pH and is responsible for clearance of debris. Results suggests that, in presence of ALA microglial cells greatly follows lysosomal biogenesis. LAMP1-1, an integral lysosomal protein responsible for binding to lysosomal compartment and the end result illustrated increased co-localization of LAMP1 with ALA

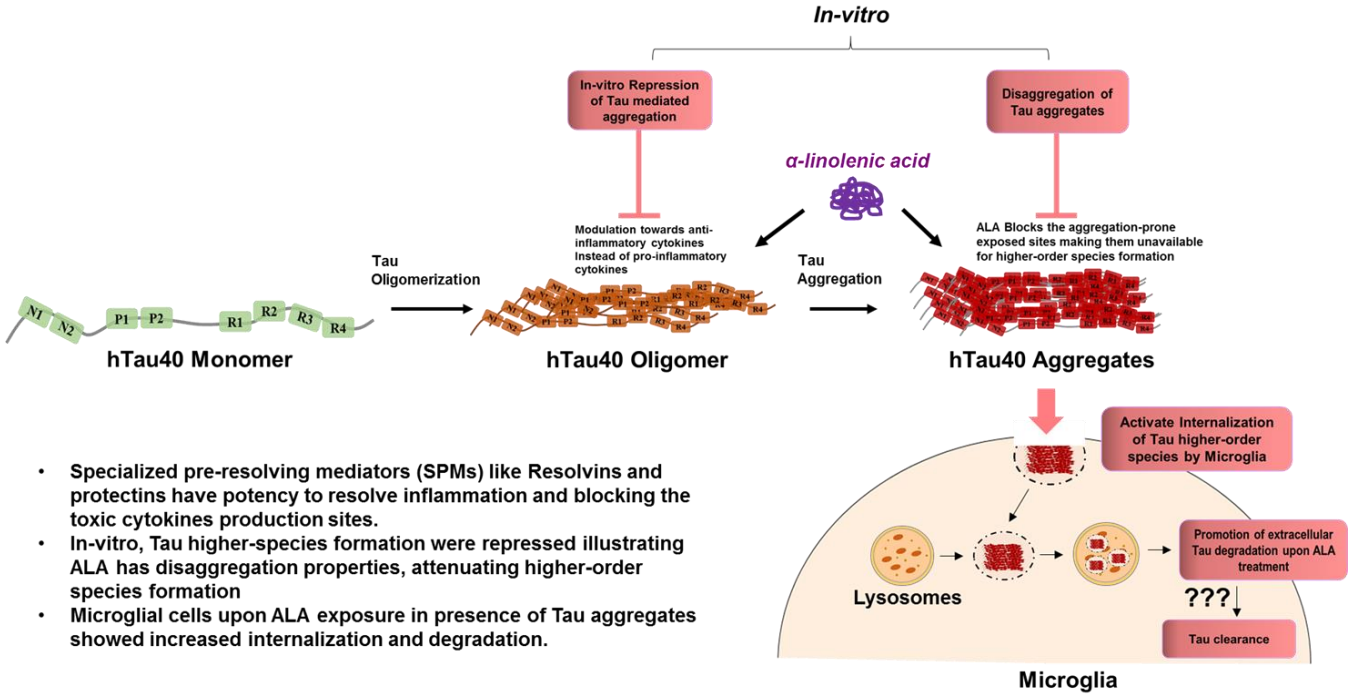


Figure 12. Summary: Disaggregation of higher-order species in presence of ALA

The positively charged hTau40 protein plays a crucial role in cytoskeleton development, but abnormal PTMs can cause it to form higher-order species through charge compensation, leading to oligomerization and toxicity. However, ALA, an ω-3 fatty acid, has been found to have neuroprotective effects by regulating glial cell membrane lipids, improving cognitive decline and increasing plasma membrane's fluidity Our results suggests that formation of higher-order species was attenuated upon ALA treatment, also modulating the phenotype from pro-inflammatory to anti-inflammatory, thus increasing the internalization of extracellular species

treated group. This was verified with western blot analysis, where the ALA treated group showed increased normalized intensity plots of LAMP1. Polymerization of actin at the leading-edge of a cell, a vital feature for its cytoskeleton modulation. We studies the targets for readily remodels the actin cytoskeleton in presence of ALA.Suggesting, the role of ALA in activating microglial cells in presence of extracellular Tau. It has been seen that ALA enhances the membrane ruffles to a great extent and increased the co-localization of Iba1-LAMP1 in ALA treated cells. All these findings suggest, the vital role of ALA towards the enhancement of microglial cells in presence of extracellular Tau species. Microglial cells after sensing the micro-environment tend to clear the debris through degradative pathway and ALA treated groups supports degradation. These reports the neuro-protective nature of ALA inside the brain.

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