Ferroptosis Promotes Microtubule-Associated Protein Tau Aggregation via GSK-3 β Activation and Proteasome Inhibition

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Abstract

Ferroptosis is a form of regulated cell death resulting from iron accumulation and lipid peroxidation. In some particular brain regions, iron dyshomeostasis and peroxidation damage of neurons are closely related to a wide range of neurodegenerative diseases known as “tauopathies”, in which intracellular aggregation of microtubule-associated protein tau is the common neuropathological feature. However, the relationship between ferroptosis and tau aggregation is not well understood. The current study demonstrates that erastin-induced ferroptosis can promote tau hyperphosphorylation and aggregation in mouse neuroblastoma cells (N2a cells). Moreover, ferroptosis inhibitor ferrostatin-1 can alleviate tau aggregation effectively. In-depth mechanism research indicates that activated Glycogen synthase kinase-3 β (GSK-3 β) is responsible for abnormal hyperphosphorylation and accumulation. More importantly, proteasome inhibition can exacerbate the tau degradation obstacle and accelerate tau aggregation in the process of ferroptosis. Our results indicate that ferroptosis can lead to abnormal aggregation of tau protein and might be a promising therapeutic target of tauopathies.

1. Introduction

Ferroptosis is a recently discovered form of programmed cell death whose execution requires the accumulation of lipid peroxidation in an iron-dependent manner [1,2]. Ferroptosis is involved in diverse biological contexts and is genetically, biochemically, and morphologically distinct from apoptosis and necrosis. Interestingly, both the iron overload and lipid peroxidation in the brain are also closely related with the characteristic protein aggregation such as tau protein in Alzheimer’s disease (AD) and α -synuclein in Parkinson’s disease (PD), it highlights the associated research aiming at ferroptosis and neurodegenerative disease [3-6].

Tau protein, a highly soluble, microtubule-associated protein, whose primary function is to promote microtubule assembly and stabilization, as well as maintain axonal transport and neuron survival [7,8]. As a phosphoprotein, phosphorylation modification can negatively regulate its ability to stimulate microtubule assembly [9]. Moreover, hyperphosphorylation is assumed to be critical for the formation of tau aggregation [10] and alter its degradation through the proteasome or autophagy [11,12]. Besides the role in stabilizing neuronal microtubules [13], promoting axonal outgrowth [14], and regulating axonal transport [15], tau also functions to facilitate neuronal iron efflux. Lei *et al.* have reported that tau deficiency (i.e., the loss of soluble tau) can impair APP-mediated iron export then lead to neurotoxic iron accumulation and neurodegeneration [16,17].

Moreover, the aggregated tau protein in neurofibrillary tangles (NFTs) is associated with the induction of heme oxygenase-1 (HO-1) [18], which can oxidize cellular heme to generate biliverdin, free iron, and carbon monoxide (CO). The released ferrous iron subsequently catalyzes the Fenton reaction to generate high levels of endogenous reactive oxygen species (ROS), which perpetuate the intracellular oxidative stress of the brain [19,20]. These reports indicate that tau protein plays a crucial role in regulating cellular iron homeostasis, and tau abnormality is closely related to intracellular iron overload.

Iron is a crucial participant and regulator of neuronal physiological functions [21]. Iron is an indispensable cofactor for proteins and enzymes required to develop synapses, myelination, and neurotransmitter metabolism [22]. However, iron accumulates in the brain with age, associating with neurodegenerative disorders, including tauopathies such as AD [23]. Interestingly, it was also found that iron overload could induce tau hyperphosphorylation via the dysfunctional insulin signal in primary cultured neurons and mice [24]. Meanwhile, it has been extensively reported that iron overload generates redox-generated free radicals [25], which acts as a pro-oxidant to convert hydrogen peroxide into hydroxyl radicals through the Fenton reaction. The hydroxyl radical then attacks proteins or biological macromolecules [26], results in the formation of oligomeric tau via cysteine-cysteine binding [27] or via kinase pathways [28]. In addition, iron can also produce oligomeric tau by forming intermolecular coordination complexes mediated by phosphorylated amino acid residues [29,30].

All the above researches indicate that intracellular iron accumulation, lipid peroxidation, and tau aggregation are closely related, and a vicious cycle might be formed between iron overload and tau aggregation directly or indirectly by lipid peroxidation. It aroused our interest to unveil the relationship between ferroptosis, the iron-dependent form of regulated necrosis involved in lipid peroxidation, and tau aggregation. To address this directly, we treated N2a neuroblastoma (a neuron-like cell line) with erastin, which could inhibit the activity of cysteine-glutamate antiporter (system Xc⁻), leading to the depletion of the major cellular antioxidant glutathione (GSH), then decreased the activity of GPX and triggered ferroptosis [31,32]. The relationship between ferroptosis and tau aggregation was explored, with the associated mechanisms investigated in detail. Considering that intracellular iron overload and ROS accumulation play an essential role both in the process of ferroptotic cell death and in the pathogenesis of tau-related neurodegenerative diseases, our study indicates the possible new strategy for tauopathies therapy through the pharmacological interference with the ferroptosis processes of brain issues.

2. Methods And Materials

2.1 Antibodies and reagents

Rabbit polyclonal antibodies (pAb) against phospho-GSK-3 β at Ser9, tau phosphorylated at Ser396, and Thr231 were purchased from Bioworld Technology Inc. (Louis Park, MN, USA). Polyclonal antibodies against LC3 and total GSK-3 β were purchased from Cell Signaling Technology, Inc. (Beverly, MA, USA). pAb against β -actin was obtained from Proteintech Group Inc. (Wuhan, China) and pAb against Ubiquitin and Mouse polyclonal antibodies tau-5 and UCH-L1 were from Abcam (Cambridge, MA, USA). Vimentin, Goat Anti-Mouse IgG (H+L) Cy3, and Goat Anti-Rabbit IgG (H+L) Dylight 488 were purchased from Bioworld Technology Inc. (Louis Park, MN, USA). The specific ferroptosis inducer erastin and inhibitor ferrostatin-1 (Fer-1) were from Med Chem Express (Shanghai, China). Cell culture media were from Gibco Life Technologies (Grand Island, NY, USA).

2.2 Cell culture and drug treatments

Mouse neuroblastoma Neuro2a (N2a) cells were cultured in a 5% CO₂ humidified incubator at 37 °C with a medium consisting of 45% Dulbecco's modified Eagle's medium (DMEM), 45% Opti-MEM supplemented with 10% (v/v) fetal bovine serum. For drug treatment, 5, 10 and 20 µM erastin were used to treat cells for 6 and 12 hr to induce ferroptosis. During the incubation with 10 µM erastin, cells were either treated or not treated with 1, 2 and 4 µM ferroptosis inhibitor Ferrostatin-1 for 6 and 12 hr. The cells were treated with 0.2% dimethyl sulfoxide (DMSO) as a control.

2.3 Cell viability analysis

Cell Counting Kit-8 (Beyotime Institute of Biotechnology, Jiangsu, China) was used to determine cell viability. In brief, cells were seeded in 96-cell micro-culture plate at an initial density of 5000 cells per well and allowed to grow for 48 h in an incubator maintained at 37 °C. The cells were treated with corresponding drugs for 6 and 12 hr respectively, then 10 µL reagent was added to each well, and cells were incubated in the incubator for 2 hr. Finally, the plates were scanned with a microplate reader (Bio-Rad, Hercules, CA, USA) at 450 nm to measure the absorbance.

2.4 Intracellular glutathione peroxidase (GPX), reactive oxygen species (ROS), and iron content detection

Following drug treatment, cells were washed once with cold PBS. They were lysed directly in an Eppendorf tube with lysis buffer (50 mM Tris-HCl, 150 mM NaCl, 0.1% sodium dodecyl sulfate(SDS), 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.02% NaN₃, 100 µg/ml phenylmethylsulfonyl fluoride, 2 µg/ml Aprotinin) for 30 min and centrifugation for 15 min at 12000 ×g. The supernatant was removed, and protein concentration was determined. The relative indicators were then measured using commercial assay kits. In brief, the ROS levels were measured by adding 100 µL of protein supernatant and 100 µL of 2',7'-dichlorofluorescein diacetate (DCFH-DA) to a 96-well plate, which was incubated for 10 min at 37 °C in the dark. Fluorescence detection was then carried out using an excitation wavelength of 488 nm and an emission wavelength of 525 nm. GPX and iron levels were assessed following the manufacturer's instruction (Nanjing Jiancheng Bioengineering Institute, Nanjing, China), and the samples were incubated for 30 min at room temperature. The absorbance was measured at 340 nm to calculate the GPX level and calculate the iron content at 590 nm.

2.5 Cell morphology observation

N2a cells were seeded in the 24-well plate. When cells grew to 80% abundance, they were treated with erastin, erastin plus ferrostatin-1 for 6 and 12 hr, respectively. The morphology of the N2a cells with different treatments was observed using a phase-contrast microscope as in our previous study [33]. The obtained images were processed with image processing software, Image J, where neurites were manually traced with a Wacom tablet to measure the length. Average lengths of the neurites were then obtained by dividing the total length by the number of neurons measured.

2.6 Western Blot

Cells were seeded in six-well plates and treated as described above. The protein concentration was determined using the BCA protein assay. For Western blot analysis, twenty micrograms of total protein were separated using 10% SDS polyacrylamide gels and then transferred by electroblotting onto polyvinylidene difluoride membranes. After the membranes were blocked with 5% nonfat milk for 1 hr, washing the membranes three times with PBS (containing 0.1% Tween-20) for 10 min each time. Membranes were probed overnight at 4 °C with antibodies. The immunoblots were washed and treated with the appropriate species of horseradish peroxidase (HRP)-conjugated secondary antibody at 37 °C for 1 hr and then wash the membranes three times with PBS. The immunoreactive bands were quantified using an Infrared Imaging System.

2.7 Immunofluorescence cytochemistry

N2a cells were mounted on coverslips in a 24-well plate. After drug treatment, cells were fixed with Precooled methanol for 10 min at -20 °C. After washing with 0.2% Triton-PBS and 0.5% Triton-PBS, respectively for three times, cells were incubated for 1hr in blocking buffer (5% BSA), followed by incubation for 48 hours with primary antibodies at 4 °C (tau-5, 1:100; vimentin, 1:100). The coverslips were then washed three times with 0.1% Triton-PBS and incubated in the second antibody for 1 hr at 37 °C. The coverslips were counterstained with Hoechst and washed three times with Triton-PBS. Finally, cells on the coverslips were observed and imaged using a fluorescence microscope.

2.8 Measurement of 20S Proteasome Activity

According to the manufacturer's instructions and our previous literature [34], the 20S proteasome catalytic core chymotrypsin-like activity kit was measured using the 20S proteasome activity assay kit (Boston Biochem, Cambridge, MA, USA). In brief, following the above drug treatment, the cells were collected, resuspended with the 20S proteasome assay buffer, then centrifuged at 8000 ×g for 8 min, and the precipitation was collected. The precipitation was treated with the 20S proteasome lysis buffer for 30 min at room temperature. The cytosolic extract was subsequently collected as the supernatant following centrifugation for 12 min at 12000 ×g. For the proteasome activity assay, the cell lysates were incubated with chymotrypsin fluorogenic substrate Suc-Leu-Leu-Val-Tyr-AMC. The chymotrypsin-like activity was determined to increase of the fluorescence intensity in the reaction products by using the Synergy 2 Multi-Mode Microplate Reader (Bio Tek, Winooski, VT, USA) at 360 nm excitation and 480 nm emission wavelengths [35].

2.9 Immunoprecipitation

Cells were seeded in six-well plates and treated as described above. After one wash with cold PBS, the cells were lysed directly with RIPA lysis buffer (50 mM Tris-HCL, 150 mM NaCl, 5 mM EDTA, 1% Triton-X, 1% DOC, 0.1% SDS, 0.2 mM TPCK, 1 mM PMSF, 2 mM TLCK) for 30 min on ice, and centrifuged at 12000 ×g for 20 min subsequently. Add protein G to the supernatant at a ratio of 100:1 between the supernatant and protein G, and shake it slowly on a shaker at 4 °C for 30 min to remove non-specific protein binding. After centrifugation at 12000 ×g for 15 min, an anti-tau-5 antibody was added to the sample according to

the antibody ratio to sample at 1:100 and incubated overnight on a shaker at 4 °C. After incubating the primary antibody overnight, add protein G at a ratio of 5:1 between the supernatant and protein G, slowly incubate in a low-temperature studio for 2 hr and resuspend the pellet in loading buffer. After boiling for 10 min and centrifuging at 12000 ×g for 1 min, the protein concentration was determined using the BCA protein assay.

2.10 Statistics Analysis

All experiments were performed in triplicate. Data were expressed as mean±SD and analyzed using SPSS 27.0 statistic software. The One-Way ANOVA procedure followed by LSD's post hoc tests was used to determine the different means between groups ($p<0.05$).

3. Results

3.1 Erastin decreases the viability of N2a cells

To determine the effect of the ferroptosis inducer erastin on the viability of N2a cells, we treated N2a cells with different concentrations of erastin for 6 and 12 hr. It was shown that the cell viability decreased in a concentration and time-dependent manner following erastin treatment. Compared to the control group, 5, 10 and 20 µM erastin treatment for 6 hr decreased the relative cellular viability by 7.35%, 12.72% and 12.24%, respectively. What's more, the relative cell viability decreased by 17.21%, 24.53% and 24.77%, respectively, when cells were treated with 5, 10 and 20 µM erastin for 12 hr (Fig. 1A). As there was no significant difference in the activity inhibition between the concentrations of 10 µM and 20 µM, we selected 10 µM erastin as the optimal treatment concentration in the subsequent experiments. We also found that 2 or 4 µM ferrostatin-1 (Fer-1) treatment, an effective inhibitor of ferroptosis, which can inhibit the accumulation of ROS then inhibit oxidative lipid damage and cell death [36], can effectively reverse erastin induced cell viability inhibition (Fig. 1B).

3.2 Erastin treatment induces intracellular ROS accumulation and iron overload in N2a cells

Erastin can induce system Xc^- inhibition and cysteine deprivation, then lead to GSH deletion and GPX inactivation, finally initiating ferroptosis in many cell types. To confirm the sensitivity of N2a cells to erastin-induced ferroptosis, we assayed the intracellular GPX activity, ROS level and iron content after erastin treatment.

The results showed that compared with the control group, N2a cells treated with 10 µM erastin resulted in a significant decrease of intracellular GPX activity after 6 (Fig. 2A) and 12 hr (Fig. 2B). Meanwhile, the ROS content (Fig. 2C, 2D) and iron levels increased significantly (Fig. 2E, 2F). The results also demonstrated that 2 µM ferrostatin-1 treatment could significantly reverse erastin induced GPX, ROS and iron imbalance. These results confirmed that erastin could induce ferroptotic cell death in N2a cells.

3.2 Erastin treatment caused morphological abnormality of N2a cells

The unique ability of neurons is to compute and allocate massive information, which relies on their polarized morphology. Damage factors such as peroxidation can cause microtubule depolymerization and neuron death [37]. Therefore, we observed the morphology changes of N2a cells following erastin exposure by using phase-contrast microscopy.

To the DMSO treated cells, the cell bodies showed tapered or with clear edges, and the neurites were stretched. However, after erastin treatment for 6 and 12 hr, the cell bodies became round, and the neurites retracted obviously (Fig. 3A). Compared with the erastin treatment group, the cell bodies of the erastin plus ferrostatin-1 co-treatment group recovered the polarity gradually. Most of the cell bodies were tapered, and the length of the neurites partially increased (Fig. 3B). The above results showed that erastin exposure could dramatically destroy the microtubule structure and inhibit the neurite outgrowth in N2a cells.

3.4 Erastin induced abnormal aggregation of tau protein

The microtubule is a major cytoskeletal component of neurites, and the regulation of microtubule stability is essential for neurite morphogenesis. Microtubule-associated protein tau plays a crucial role in establishing cell polarity and maintaining microtubule stability [38]. The dissociation of tau from microtubules would impede the microtubule assembly and disrupt the stability of the microtubule network, then destroy the process of axonal transport and synaptic plasticity. Meanwhile, the dissociated tau would self-aggregate and form intracellular inclusions. Based on the result that erastin treatment destroyed cytoskeleton structure significantly, to clarify whether the abnormality of neuronal morphology is related to tau protein, we double-stained the cells with antibody tau-5 (identified total tau) and vimentin (a known marker of aggregate-forming protein).

After N2a cells were treated with erastin for 6 and 12 hr, tau protein aggregated obviously around the nucleus (Fig. 4A and 4B. e, red), the strongly positive staining of vimentin (Fig. 4A and 4B. f, green) indicated that erastin could lead to the formation of intracellular protein aggregates. Furthermore, the co-localization of tau protein (red) and vimentin (green) around the nucleus suggested that the aggregates contain tau protein (Fig. 4A and 4B. h, orange). It was also shown that the aggregated tau protein disappeared when cells were treated with erastin plus ferrostatin-1 (Fig. 4A and 4B. i, orange). The above results demonstrate that erastin treatment would induce abnormal aggregation of tau protein in the cytoplasm.

3.5 Erastin remarkably increases the content of total tau and phosphorylated tau

Tau function and affinity for the microtubules mainly depend on its phosphorylation status. Hyperphosphorylation of tau protein at some key sites such as Ser396 and Thr231 can decrease the affinity between tau and microtubules, then cause the dissociation of tau from microtubules and the formation of intracellular tau aggregates [39,40]. In the present study, we detect the content of total tau and phosphorylated tau at Ser396 and Thr231 sites by Western blot (Fig. 5). It was shown that after erastin treatment for 6 and 12 hr, the intracellular total tau increased significantly. Meanwhile, the relative

level of phosphorylated tau at Ser396 and Thr231 sites also upregulated dramatically, especially in the 6 hr erastin treatment group.

Interestingly, 2 μ M ferrostatin-1 could effectively reverse erastin induced increase of total tau and phosphorylated tau. Statistical analysis revealed a positive correlation between the ROS content and the iron level ($r=0.65$), and we also found ROS induced a highly positive correlation with tau phosphorylation at Ser396 ($r=0.82$) and Thr231 sites ($r=0.76$) (Fig. 6I). These results indicate that during neuronal ferroptosis, tau was hyperphosphorylated at Ser396 and Thr231 sites.

3.6 Erastin activated GSK-3 β in N2a cells

Most studies on tau hyperphosphorylation explored the kinase-phosphatase imbalance and regarded GSK-3 β as the significant kinase involved in tau pathology [41]. GSK-3 β mediated tau phosphorylation is observed in areas that are in close proximity to the microtubule-binding domains and their amino acid residues [42]. Therefore, the hyperphosphorylated tau induced by GSK-3 β is prone to dissociate from microtubules and self-aggregate in the cytoplasm [43]. Given that GSK-3 β is one of the most important kinases that regulate tau phosphorylation at Ser396 and Thr231 sites [44], to clarify the underlying mechanisms of tau hyperphosphorylation in the process of erastin-induced ferroptosis, we detected the level of total GSK-3 β and Ser9-phosphorylated GSK-3 β (negative related to GSK-3 β activity). As shown in Figures 6A and 6C, after 6 and 12 hr erastin exposure, the total level of GSK-3 β showed no significant change, while the relative level of Ser9-phosphorylated GSK-3 β (p-GSK-3 β) decreased with time. Compared to the control group, the relative phosphorylated GSK-3 β at Ser9 decreased to $\sim 40\%$ and 30% , respectively. Meanwhile, 2 μ M ferrostatin-1 treatment effectively rescued erastin induced GSK-3 β activity upregulation. These results suggest that GSK-3 β activation is involved in the erastin induced tau hyperphosphorylation in N2a cells.

3.7 Effect of erastin on the content of Poly-Ub and Mono-Ub

Based on the above results that erastin treatment produces tau aggregates (Fig. 4) and elevates the total tau level (Fig. 5), we wonder whether degradation impairment contributes to erastin induced tau accumulation. Intracellular misfolded proteins that fail to be rescued by molecular chaperones are sent for degradation by proteasome and autophagy. The recognition signal for degradation of aggregated proteins by both the proteasome and autophagy is driven by polyubiquitin chain labeling of substrates [45] which depends on the ubiquitin pool homeostasis. To determine whether the metabolic abnormality of tau protein is associated with the ubiquitin cycle, we first detected the content of polyubiquitin (Poly-Ub) and monomer ubiquitin (Mono-Ub) in N2a cells. It was shown that after the erastin treatment for 6 and 12 hr, the intracellular poly-Ub level increased significantly, while the level of mono-Ub decreased obviously (Fig. 7A, 7D). We also observed that ferrostatin-1 treatment could successfully reverse erastin induced ubiquitin cycle impairment. The above results indicate that during the process of erastin induced ferroptotic cell death, the degradation of polyubiquitin-labeled substrates was inhibited.

3.8 Erastin induced abnormal ubiquitination modification of tau protein

As pathologically modified proteins such as hyperphosphorylated tau can be degraded by ubiquitin-proteasome system [46] and autophagy-lysosome pathway [47], and polyubiquitin label is the prerequisite of proteins degradation by these two systems. In view of the significant down-regulation of monomer ubiquitin and apparent up-regulation of poly-ubiquitin after erastin treatment, we further tested the effect of erastin on the ubiquitination of tau by immunoprecipitation. Following incubating with the tau-5 antibody (analyzed total tau proteins), the samples from different treatment groups were used for immunoblot analysis with tau-5 and anti-ubiquitin antibody (probed ubiquitination-conjugated proteins). It was shown that compared to the control group, the ubiquitinated tau increased significantly, most so with 6 hr erastin treatment (Fig. 8). Moreover, ferrostatin-1 could rescue erastin induced association abnormality between ubiquitin and tau protein. The above results indicate that erastin can cause polyubiquitinated tau protein degradation impairment, which may be involved in the abnormal aggregation of tau.

3.9 Erastin treatment inhibited 20S proteasome activity in N2a cells

The proteasome is the main route for misfolded proteins degradation, and the 20S proteasome is a complex containing activities similar to trypsin, chymotrypsin, and caspase. As chymotrypsin-like activity inhibition can cause the protein degradation efficiency of the proteasome to be significantly reduced [48], we focused on the chymotrypsin-like activity alteration of the proteasome after erastin treatment. Figure 9 shown that chymotrypsin activity decreased significantly in a time-dependent manner after 6 and 12 hr erastin treatments, and 2 μ M ferrostatin-1 dramatically mitigated erastin induced proteasome activity down-regulation. The above results indicate that the activity of the proteasome system is inhibited during ferroptosis and might be contributing the tau aggregation.

3.10 Erastin treatment activated the autophagy pathway

Considering that the autophagy-lysosomal system can also degrade abnormally aggregated tau protein and a lipidated form of LC3, LC3-II, is an autophagosomal marker in mammals, we further analyzed the relative ratio of LC3-II/LC3 in N2a cells after erastin treatment. It was shown that the intracellular LC3-II/LC3 ratio increased about 10% at 6 hr and 20% at 12 hr than the controls and showed clear time-dependence (Fig. 10). The above results indicated that in the first 12 hours of erastin treatment, proteasome activity is inhibited. However, the autophagy-lysosome system is compensatively activated to maintain intracellular protein balance. But, obviously, the activation of the autophagy-lysosome system is insufficient to degrade the abnormally aggregated tau protein.

4. Discussion

Abnormal deposition of aggregated microtubule-associated protein tau is a common final pathway of tau-related neurodegenerative diseases named “tauopathies”, including Alzheimer’s disease(AD), progressive supranuclear palsy (PSP), Huntington disease (HD), frontotemporal dementia with

parkinsonism-17 (FTDP-17), and so on. It clarified that the factors that promote tau aggregation are important to elucidate the pathogenesis and acquire a good curative effect of these diseases. Iron has been shown to promote aggregation of some neurodegenerative diseases characteristic aberrant proteins, such as tau [49,50] and β -amyloid [51]. Further support for the involvement of iron in the pathogenesis of tauopathies is provided by the recent discovery of a new form of cell death, ferroptosis. Arising from iron-dependent lipid peroxidation, ferroptosis is augmented in conditions of cysteine depletion and glutathione peroxidase (GPX) inactivation [20,52,53,26]. What's more, it has been widely observed that many neurodegenerative diseases such as AD are pathologically characterized by the accumulation of iron in the brain and peroxidation damage of neurons [54]. However, the detailed relation between ferroptosis and abnormal tau aggregation is still unclear.

In the present study, ferroptotic cell death was constructed by treating N2a cells with ferroptosis inducer erastin for 6 hr and 12 hr. It was shown that erastin exposure significantly inhibited the viability of N2a cells, prominently decreased the intracellular GPX activity (a key marker of ferroptosis), elevated ROS and iron content. What's more, 2 μ M ferroptosis inhibitor ferrostatin-1 can effectively rescue the cytotoxic effect caused by erastin. The above results confirm that erastin can induce ferroptosis in N2a cells. Meanwhile, it was also found that erastin treatment caused noticeable morphology change of N2a cells, especially significantly shortened neurites, which means the neuronal skeletal system was impaired during ferroptosis. As tau protein is one of the most important microtubule-related proteins in neurons and plays a key role in maintaining the stability of the neuronal skeletal system [2, 55,56], the dissociation of tau from microtubules can induce not only the collapse of cytoskeletal structure but also the formation of intracellular tau aggregates. Naturally, the obvious neurites retraction aroused our interest to assay the status of tau protein during ferroptosis. Interestingly, the immunofluorescence cytochemistry results showed clear tau-positive aggregates in the cytoplasm after erastin treatment, and it directly demonstrated the causal relationship between ferroptosis and abnormal tau aggregation.

Based on the above results, we further explored the mechanisms involved in tau aggregation during ferroptosis. It was accepted widely that several post-translational modifications were proposed to play important roles in tau aggregation [57]. Among them, phosphorylation modification can negatively regulate the ability of tau to stimulate microtubule assembly [9]. It was reported that phosphorylation of tau at the Thr231 site could lead to a trans-to-cis conformational change trigger the detachment of tau from microtubules [58]. Likewise, phosphorylation of tau at Ser396 reduces its affinity for MTs and its ability to stabilize MTs against depolymerization [59]. Thus, we assayed the phosphorylation profile of Thr231 and Ser396 sites. It was found that compared to the control group, the phosphorylation modification levels of tau protein at Ser396 and Thr231 sites increased significantly after erastin treatment for 6 and 12 hr. Considering the equilibrium disruption between tau kinase and phosphatase activities is the major reason responsible for the hyperphosphorylation of tau protein, we further measured the content and activity of GSK-3 β , a crucial tau kinase corresponding to 70% of tau pathological phosphorylation sites including Ser396 and Thr231 sites [60]. We found that the level of GSK-3 β was not significantly changed after erastin treatment, but the reduction of phosphorylated GSK-3 β at Ser9 suggested that erastin could induce GSK-3 β activation.

Interestingly, Wu *et al.* also found that GSK-3 β silence can block erastin-induced ferroptosis with less production of ROS, and GSK-3 β overexpression can exacerbate erastin induced ferroptosis in breast cancer cells [45]. These results clearly demonstrate the relationship between ferroptosis and GSK-3 β activation. Considering that GSK-3 β dysregulation almost influences all the major hallmarks of tauopathies, including tau hyperphosphorylation and aggregation [42,61,62], synaptic dysfunction, and memory impairment [63], clarifying its contribution to the ferroptosis will be the central objectives of further study and may provide a viable therapeutic approach to prevent or halt tauopathies.

Erastin induced tau aggregation strongly indicated a concomitant loss of proteostasis, which is maintained by several systems in the cell, including the ubiquitin proteasome system (UPS) and autophagy-lysosome system. Given the two protein degradation pathways both required ubiquitin label of substrates [64-67], we first assayed the content change of poly-ubiquitin and mono-Ub after erastin treatment and found that intracellular poly-ubiquitin obviously increased, but the level of mono-Ub decreased significantly. Moreover, erastin treatment increased the amount of ubiquitinated tau protein significantly. These results suggested that ferroptosis would lead to tau degradation impairment.

As the ubiquitin-proteasome system is the principal route for the degradation of intracellular misfolded or damaged proteins [68], including tau protein [69], we further explored the mechanistic research and found that chymotrypsin-like activity of 20S proteasome, which directly affects the degradation efficiency of the proteasome [48], decreased in a time-dependent manner after erastin treatment. As proteasome cannot adequately handle misfolded protein aggregates [70], what's more, aggregates of some pathogenic proteins can directly inhibit proteasome activity [71]. These data together suggested that in the process of ferroptosis, hyperphosphorylated tau can't be effectively degraded by the proteasome system and even can induce proteasome activity inhibition.

In addition to the proteasome, autophagy also plays an important role in removing aggregated proteins [72,73], including tau proteins [74]. But whether autophagy is involved in tau clearance during erastin-induced ferroptosis is not demonstrated. LC3-I and LC3-II are two important biomarkers in autophagy, the conversion of LC3-I to LC3-II indicates autophagy activation [75]. In this study, the increased LC3-II/LC3-I ratio suggested an enhanced autophagic flux following erastin administration. The finding is consistent with previous studies that erastin induced LC3 conversion and autophagy activation [76]. Since there is a contradiction between autophagy activation and tau accumulation in the process of erastin-induced ferroptosis, we consider that proteasome inhibition might play a more critical part than autophagy in the pathological aggregation of tau protein.

In conclusion, our data suggest that in the process of erastin-induced ferroptotic cell death, microtubule-associated protein tau undergoes hyperphosphorylation and aggregation. The pathological changes of tau are related to the activation of GSK-3 β and the inhibition of the proteasome system (Fig. 11). It provides a clear clue about the relationship between ferroptosis and tau aggregation, opens new therapeutic targets and strategies for tauopathies.

Declarations

Conflict of interest statement

No potential conflicts of interest relevant to this article were reported.

Ethics approval

This experiment was approved by the Ethics Committee of Central China Normal University.

Consent to participate

Not applicable

Consent for publication

Not applicable.

Availability of data and materials

The data are available upon reasonable request from the author for correspondence.

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Authors' contributions

XM-L conceived the experiments and supervised the project. YJ and YB-L performed the experiments. SH-W collected the data, made the figures and wrote the manuscript. MJ-M did the analysis. HW-S contributed essential reagents or tools. All authors read and approved the final manuscript.

Author information

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References

1. Dixon SJ, Lemberg KM, Lamprecht MR, Skouta R, Zaitsev EM, Gleason CE, Patel DN, Bauer AJ, Cantley AM, Yang WS, Morrison B 3, Stockwell BR (2012) Ferroptosis: an iron-dependent form of nonapoptotic cell death. *Cell* 149(5):1060–1072. <https://doi.org/10.1016/j.cell.2012.03.042>
2. Yang WS, Stockwell BR (2016) Ferroptosis: Death by Lipid Peroxidation. *Trends Cell Biol* 26(3):165–176. <https://doi.org/10.1016/j.tcb.2015.10.014>
3. Jellinger KA (1999) The role of iron in neurodegeneration: prospects for pharmacotherapy of Parkinson's disease. *Drugs Aging* 14(2):115–140. <https://doi.org/10.2165/00002512-199914020-00004>
4. Zhang GM, Zhang YR, Shen YX, Wang YC, Zhao M, Sun L (2021) The Potential Role of Ferroptosis in Alzheimer's Disease. *J Alzheimers Dis* 80(3):907–925. <https://doi.org/10.3233/jad-201369>
5. Logroscino G, Marder K, Graziano J, Freyer G, Slavkovich V, Lolocono N, Cote L, Mayeux R (1997) Altered systemic iron metabolism in Parkinson's disease. *Neurology* 49(3):714–717. <https://doi.org/10.1212/wnl.49.3.714>
6. Chiueh CC (2001) Iron overload, oxidative stress, and axonal dystrophy in brain disorders. *Pediatr Neurol* 25(2):138–147. [https://doi.org/10.1016/s0887-8994\(01\)00266-1](https://doi.org/10.1016/s0887-8994(01)00266-1)
7. Fulga TA, Elson-Schwab I, Khurana V, Steinhilb ML, Spires TL, Hyman BT, Feany MB (2007) Abnormal bundling and accumulation of F-actin mediates tau-induced neuronal degeneration in vivo. *Nat Cell Biol* 9(2):139–U117. <https://doi.org/10.1038/ncb1528>
8. Johnson GV, Hartigan JA (1999) Tau protein in normal and Alzheimer's disease brain: an update. *J Alzheimers Dis* 1(4–5):329–351. <https://doi.org/10.3233/jad-1999-14-512>
9. Lindwall G, Cole RD (1984) Phosphorylation affects the ability of tau protein to promote microtubule assembly. *J Biol Chem* 259(8):5301–5305
10. Mandelkow EM, Mandelkow E (2012) Biochemistry and cell biology of tau protein in neurofibrillary degeneration. *Cold Spring Harb Perspect Med* 2(7):a006247. <https://doi.org/10.1101/cshperspect.a006247>
11. Guillozet-Bongaarts AL, Cahill ME, Cryns VL, Reynolds MR, Berry RW, Binder LI (2006) Pseudophosphorylation of tau at serine 422 inhibits caspase cleavage: in vitro evidence and implications for tangle formation in vivo. *J Neurochem* 97(4):1005–1014. <https://doi.org/10.1111/j.1471-4159.2006.03784.x>
12. Dickey CA, Kamal A, Lundgren K, Klosak N, Bailey RM, Dunmore J, Ash P, Shoraka S, Zlatkovic J, Eckman CB, Patterson C, Dickson DW, Nahman NS Jr, Hutton M, Burrows F, Petrucelli L (2007) The high-affinity HSP90-CHIP complex recognizes and selectively degrades phosphorylated tau client proteins. *J Clin Invest* 117(3):648–658. <https://doi.org/10.1172/jci29715>
13. Feinstein SC, Wilson L (2005) Inability of tau to properly regulate neuronal microtubule dynamics: a loss-of-function mechanism by which tau might mediate neuronal cell death. *Biochim Biophys Acta* 1739(2–3):268–279. <https://doi.org/10.1016/j.bbadis.2004.07.002>
14. Caceres A, Kosik KS (1990) Inhibition of neurite polarity by tau antisense oligonucleotides in primary cerebellar neurons. *Nature* 343(6257):461–463. <https://doi.org/10.1038/343461a0>

15. Magnani E, Fan J, Gasparini L, Golding M, Williams M, Schiavo G, Goedert M, Amos LA, Spillantini MG (2007) Interaction of tau protein with the dynactin complex. *Embo j* 26(21):4546–4554. <https://doi.org/10.1038/sj.emboj.7601878>
16. Lei P, Ayton S, Appukuttan AT, Moon S, Duce JA, Volitakis I, Cherny R, Wood SJ, Greenough M, Berger G, Pantelis C, McGorry P, Yung A, Finkelstein DI, Bush AI (2017) Lithium suppression of tau induces brain iron accumulation and neurodegeneration. *Mol Psychiatry* 22(3):396–406. <https://doi.org/10.1038/mp.2016.96>
17. Lei P, Ayton S, Finkelstein DI, Spoerri L, Ciccotosto GD, Wright DK, Wong BX, Adlard PA, Cherny RA, Lam LQ, Roberts BR, Volitakis I, Egan GF, McLean CA, Cappai R, Duce JA, Bush AI (2012) Tau deficiency induces parkinsonism with dementia by impairing APP-mediated iron export. *Nat Med* 18(2):291–295. <https://doi.org/10.1038/nm.2613>
18. Perry G, Nunomura A, Hirai K, Zhu X, Prez M, Avila J, Castellani RJ, Atwood CS, Aliev G, Sayre LM, Takeda A, Smith MA (2002) Is oxidative damage the fundamental pathogenic mechanism of Alzheimer's and other neurodegenerative diseases? *Free Radical Bio Med* 33(11):1475–1479. [https://doi.org/https://doi.org/10.1016/S0891-5849\(02\)01113-9](https://doi.org/https://doi.org/10.1016/S0891-5849(02)01113-9)
19. Zukor H, Song W, Liberman A, Mui J, Vali H, Fillebeen C, Pantopoulos K, Wu TD, Guerquin-Kern JL, Schipper HM (2009) HO-1-mediated macroautophagy: a mechanism for unregulated iron deposition in aging and degenerating neural tissues. *J Neurochem* 109(3):776–791. <https://doi.org/10.1111/j.1471-4159.2009.06007.x>
20. Ward RJ, Zucca FA, Duyn JH, Crichton RR, Zecca L (2014) The role of iron in brain ageing and neurodegenerative disorders. *Lancet Neurol* 13(10):1045–1060. [https://doi.org/10.1016/s1474-4422\(14\)70117-6](https://doi.org/10.1016/s1474-4422(14)70117-6)
21. Salvador GA (2010) Iron in neuronal function and dysfunction. *BioFactors* 36(2):103–110. <https://doi.org/10.1002/biof.80>
22. Carpenter KLH, Li W, Wei H, Wu B, Xiao X, Liu C, Worley G, Egger HL (2016) Magnetic susceptibility of brain iron is associated with childhood spatial IQ. *NeuroImage* 132:167–174. <https://doi.org/10.1016/j.neuroimage.2016.02.028>
23. Zhang G, Zhang Y, Shen Y, Wang Y, Zhao M, Sun L (2021) The Potential Role of Ferroptosis in Alzheimer's Disease. *J Alzheimers Dis* 80(3):907–925. <https://doi.org/10.3233/jad-201369>
24. Wan W, Cao L, Kalionis B, Murthi P, Xia S, Guan Y (2019) Iron Deposition Leads to Hyperphosphorylation of Tau and Disruption of Insulin Signaling. *Front Neurol* 10:607. <https://doi.org/10.3389/fneur.2019.00607>
25. Smith MA, Harris PL, Sayre LM, Perry G (1997) Iron accumulation in Alzheimer disease is a source of redox-generated free radicals. *Proc Natl Acad Sci U S A* 94(18):9866–9868. <https://doi.org/10.1073/pnas.94.18.9866>
26. Huang X, Atwood CS, Hartshorn MA, Multhaup G, Goldstein LE, Scarpa RC, Cuajungco MP, Gray DN, Lim J, Moir RD, Tanzi RE, Bush AI (1999) The A beta peptide of Alzheimer's disease directly produces

- hydrogen peroxide through metal ion reduction. *Biochemistry* 38(24):7609–7616.
<https://doi.org/10.1021/bi990438f>
27. Soeda Y, Yoshikawa M, Almeida OF, Sumioka A, Maeda S, Osada H, Kondoh Y, Saito A, Miyasaka T, Kimura T, Suzuki M, Koyama H, Yoshiike Y, Sugimoto H, Ihara Y, Takashima A (2015) Toxic tau oligomer formation blocked by capping of cysteine residues with 1,2-dihydroxybenzene groups. *Nat Commun* 6:10216. <https://doi.org/10.1038/ncomms10216>
 28. Uranga RM, Giusto NM, Salvador GA (2009) Iron-induced oxidative injury differentially regulates PI3K/Akt/GSK3 β pathway in synaptic endings from adult and aged rats. *Toxicol Sci* 111(2):331–344. <https://doi.org/10.1093/toxsci/kfp152>
 29. Bader B, Nübling G, Mehle A, Nobile S, Kretzschmar H, Giese A (2011) Single particle analysis of tau oligomer formation induced by metal ions and organic solvents. *Biochem Biophys Res Commun* 411(1):190–196. <https://doi.org/10.1016/j.bbrc.2011.06.135>
 30. Nübling G, Bader B, Levin J, Hildebrandt J, Kretzschmar H, Giese A (2012) Synergistic influence of phosphorylation and metal ions on tau oligomer formation and coaggregation with α -synuclein at the single molecule level. *Mol Neurodegener* 7:35. <https://doi.org/10.1186/1750-1326-7-35>
 31. Perry G, Nunomura A, Hirai K, Zhu X, Pérez M, Avila J, Castellani RJ, Atwood CS, Aliev G, Sayre LM, Takeda A, Smith MA (2002) Is oxidative damage the fundamental pathogenic mechanism of Alzheimer's and other neurodegenerative diseases? *Free Radic Biol Med* 33(11):1475–1479. [https://doi.org/10.1016/s0891-5849\(02\)01113-9](https://doi.org/10.1016/s0891-5849(02)01113-9)
 32. Schipper HM, Bennett DA, Liberman A, Bienias JL, Schneider JA, Kelly J, Arvanitakis Z (2006) Glial heme oxygenase-1 expression in Alzheimer disease and mild cognitive impairment. *Neurobiol Aging* 27(2):252–261. <https://doi.org/10.1016/j.neurobiolaging.2005.01.016>
 33. Wang S, Zhang P, Liu R, Li Y, Liu C, Liao X (2017) A DEHP plasticizer alters synaptic proteins via peroxidation. *Toxicol Res (Camb)* 6(1):89–97. <https://doi.org/10.1039/c6tx00361c>
 34. Xie M, Li Y, Wang SH, Yu QT, Meng X, Liao XM (2017) The Involvement of NR2B and tau Protein in MG132-Induced CREB Dephosphorylation. *J Mol Neurosci* 62(2):154–162. <https://doi.org/10.1007/s12031-017-0919-8>
 35. Cai Y, Yu SS, He Y, Bi XY, Gao S, Yan TD, Zheng GD, Chen TT, Ye JT, Liu PQ (2021) EGCG inhibits pressure overload-induced cardiac hypertrophy via the PSMB5/Nmnat2/SIRT6-dependent signalling pathways. *Acta Physiol (Oxf)* 231(4):e13602. <https://doi.org/10.1111/apha.13602>
 36. Skouta R, Dixon SJ, Wang J, Dunn DE, Orman M, Shimada K, Rosenberg PA, Lo DC, Weinberg JM, Linkermann A, Stockwell BR (2014) Ferrostatins inhibit oxidative lipid damage and cell death in diverse disease models. *J Am Chem Soc* 136(12):4551–4556. <https://doi.org/10.1021/ja411006a>
 37. Choi WS, Palmiter RD, Xia Z (2011) Loss of mitochondrial complex I activity potentiates dopamine neuron death induced by microtubule dysfunction in a Parkinson's disease model. *J Cell Biol* 192(5):873–882. <https://doi.org/10.1083/jcb.201009132>
 38. Mandelkow E-M, Mandelkow E (1998) Tau in Alzheimer's disease. *Trends Cell Biol* 8(11):425–427. [https://doi.org/https://doi.org/10.1016/S0962-8924\(98\)01368-3](https://doi.org/https://doi.org/10.1016/S0962-8924(98)01368-3)

39. Ding H, Matthews TA, Johnson GV (2006) Site-specific phosphorylation and caspase cleavage differentially impact tau-microtubule interactions and tau aggregation. *J Biol Chem* 281(28):19107–19114. <https://doi.org/10.1074/jbc.M511697200>
40. Neddens J, Temmel M, Flunkert S, Kerschbaumer B, Hoeller C, Loeffler T, Niederkofler V, Daum G, Attems J, Hutter-Paier B (2018) Phosphorylation of different tau sites during progression of Alzheimer's disease. *Acta Neuropathol Commun* 6(1):52. <https://doi.org/10.1186/s40478-018-0557-6>
41. Ly PT, Wu Y, Zou H, Wang R, Zhou W, Kinoshita A, Zhang M, Yang Y, Cai F, Woodgett J, Song W (2013) Inhibition of GSK3 β -mediated BACE1 expression reduces Alzheimer-associated phenotypes. *J Clin Invest* 123(1):224–235. <https://doi.org/10.1172/jci64516>
42. Jackson GR, Wiedau-Pazos M, Sang TK, Wagle N, Brown CA, Massachi S, Geschwind DH (2002) Human wild-type tau interacts with wingless pathway components and produces neurofibrillary pathology in *Drosophila*. *Neuron* 34(4):509–519. [https://doi.org/10.1016/s0896-6273\(02\)00706-7](https://doi.org/10.1016/s0896-6273(02)00706-7)
43. Hernández F, Gómez de Barreda E, Fuster-Matanzo A, Lucas JJ, Avila J (2010) GSK3: a possible link between beta amyloid peptide and tau protein. *Exp Neurol* 223(2):322–325. <https://doi.org/10.1016/j.expneurol.2009.09.011>
44. Ferrer I, Gomez-Isla T, Puig B, Freixes M, Ribé E, Dalfó E, Avila J (2005) Current advances on different kinases involved in tau phosphorylation, and implications in Alzheimer's disease and tauopathies. *Curr Alzheimer Res* 2(1):3–18. <https://doi.org/10.2174/1567205052772713>
45. Dikic I (2017) Proteasomal and Autophagic Degradation Systems. *Annu Rev Biochem* 86:193–224. <https://doi.org/10.1146/annurev-biochem-061516-044908>
46. Wang P, Joberty G, Buist A, Vanoosthuyse A, Stancu IC, Vasconcelos B, Pierrot N, Faeltsh-Savitski M, Kienlen-Campard P, Octave JN, Bantscheff M, Drewes G, Moechars D, Dewachter I (2017) Tau interactome mapping based identification of Otub1 as Tau deubiquitinase involved in accumulation of pathological Tau forms in vitro and in vivo. *Acta Neuropathol* 133(5):731–749. <https://doi.org/10.1007/s00401-016-1663-9>
47. Vaz-Silva J, Gomes P, Jin Q, Zhu M, Zhuravleva V, Quintremil S, Meira T, Silva J, Dioli C, Soares-Cunha C, Daskalakis NP, Sousa N, Sotiropoulos I, Waites CL (2018) Endolysosomal degradation of Tau and its role in glucocorticoid-driven hippocampal malfunction. *Embo j* 37(20). <https://doi.org/10.15252/embj.201899084>
48. Jung T, Catalgol B, Grune T (2009) The proteasomal system. *Mol Aspects Med* 30(4):191–296. <https://doi.org/10.1016/j.mam.2009.04.001>
49. Spotorno N, Acosta-Cabronero J, Stomrud E, Lampinen B, Strandberg OT, van Westen D, Hansson O (2020) Relationship between cortical iron and tau aggregation in Alzheimer's disease. *Brain* 143(5):1341–1349. <https://doi.org/10.1093/brain/awaa089>
50. Sayre LM, Perry G, Harris PL, Liu Y, Schubert KA, Smith MA (2000) In situ oxidative catalysis by neurofibrillary tangles and senile plaques in Alzheimer's disease: a central role for bound transition metals. *J Neurochem* 74(1):270–279. <https://doi.org/10.1046/j.1471-4159.2000.0740270.x>

51. Rottkamp CA, Raina AK, Zhu X, Gaier E, Bush AI, Atwood CS, Chevion M, Perry G, Smith MA (2001) Redox-active iron mediates amyloid-beta toxicity. *Free Radic Biol Med* 30(4):447–450. [https://doi.org/10.1016/s0891-5849\(00\)00494-9](https://doi.org/10.1016/s0891-5849(00)00494-9)
52. Kim AC, Lim S, Kim YK (2018) Metal Ion Effects on A β and Tau Aggregation. *Int J Mol Sci* 19(1). doi:10.3390/ijms19010128
53. Lee S, Viqar F, Zimmerman ME, Narkhede A, Tosto G, Benzinger TL, Marcus DS, Fagan AM, Goate A, Fox NC, Cairns NJ, Holtzman DM, Buckles V, Ghetti B, McDade E, Martins RN, Saykin AJ, Masters CL, Ringman JM, Ryan NS, Förster S, Laske C, Schofield PR, Sperling RA, Salloway S, Correia S, Jack C Jr, Weiner M, Bateman RJ, Morris JC, Mayeux R, Brickman AM (2016) White matter hyperintensities are a core feature of Alzheimer's disease: Evidence from the dominantly inherited Alzheimer network. *Ann Neurol* 79(6):929–939. <https://doi.org/10.1002/ana.24647>
54. Dziewulska D, Rafałowska J (2005) Proteinaceous intracellular inclusions in neurodegenerative disorders. *Folia Neuropathol* 43(2):51–63
55. Yang WS, Stockwell BR (2008) Synthetic lethal screening identifies compounds activating iron-dependent, nonapoptotic cell death in oncogenic-RAS-harboring cancer cells. *Chem Biol* 15(3):234–245. <https://doi.org/10.1016/j.chembiol.2008.02.010>
56. Friedmann Angeli JP, Schneider M, Proneth B, Tyurina YY, Tyurin VA, Hammond VJ, Herbach N, Aichler M, Walch A, Eggenhofer E, Basavarajappa D, Rådmark O, Kobayashi S, Seibt T, Beck H, Neff F, Esposito I, Wanke R, Förster H, Yefremova O, Heinrichmeyer M, Bornkamm GW, Geissler EK, Thomas SB, Stockwell BR, O'Donnell VB, Kagan VE, Schick JA, Conrad M (2014) Inactivation of the ferroptosis regulator Gpx4 triggers acute renal failure in mice. *Nat Cell Biol* 16(12):1180–1191. <https://doi.org/10.1038/ncb3064>
57. Martin L, Latypova X, Terro F (2011) Post-translational modifications of tau protein: implications for Alzheimer's disease. *Neurochem Int* 58(4):458–471. <https://doi.org/10.1016/j.neuint.2010.12.023>
58. Lu PJ, Wulf G, Zhou XZ, Davies P, Lu KP (1999) The prolyl isomerase Pin1 restores the function of Alzheimer-associated phosphorylated tau protein. *Nature* 399(6738):784–788. <https://doi.org/10.1038/21650>
59. Bramblett GT, Goedert M, Jakes R, Merrick SE, Trojanowski JQ, Lee VM (1993) Abnormal tau phosphorylation at Ser396 in Alzheimer's disease recapitulates development and contributes to reduced microtubule binding. *Neuron* 10(6):1089–1099. [https://doi.org/10.1016/0896-6273\(93\)90057-x](https://doi.org/10.1016/0896-6273(93)90057-x)
60. Martin L, Latypova X, Wilson CM, Magnaudeix A, Perrin ML, Yardin C, Terro F (2013) Tau protein kinases: involvement in Alzheimer's disease. *Ageing Res Rev* 12(1):289–309. <https://doi.org/10.1016/j.arr.2012.06.003>
61. Pérez M, Hernández F, Lim F, Díaz-Nido J, Avila J (2003) Chronic lithium treatment decreases mutant tau protein aggregation in a transgenic mouse model. *J Alzheimers Dis* 5(4):301–308. <https://doi.org/10.3233/jad-2003-5405>

62. Uemura K, Kuzuya A, Shimozone Y, Aoyagi N, Ando K, Shimohama S, Kinoshita A (2007) GSK3 β activity modifies the localization and function of presenilin 1. *J Biol Chem* 282(21):15823–15832. <https://doi.org/10.1074/jbc.M610708200>
63. Giese KP (2009) GSK-3: a key player in neurodegeneration and memory. *IUBMB Life* 61(5):516–521. <https://doi.org/10.1002/iub.187>
64. Nedelsky NB, Todd PK, Taylor JP (2008) Autophagy and the ubiquitin-proteasome system: collaborators in neuroprotection. *Biochim Biophys Acta* 1782(12):691–699. <https://doi.org/10.1016/j.bbadis.2008.10.002>
65. Ciechanover A, Kwon YT (2017) Protein Quality Control by Molecular Chaperones in Neurodegeneration. *Front Neurosci* 11:185. <https://doi.org/10.3389/fnins.2017.00185>
66. Deng Z, Purtell K, Lachance V, Wold MS, Chen S, Yue Z (2017) Autophagy Receptors and Neurodegenerative Diseases. *Trends Cell Biol* 27(7):491–504. <https://doi.org/10.1016/j.tcb.2017.01.001>
67. Clague MJ, Urbé S (2010) Ubiquitin: same molecule, different degradation pathways. *Cell* 143(5):682–685. <https://doi.org/10.1016/j.cell.2010.11.012>
68. Glickman MH, Ciechanover A (2002) The ubiquitin-proteasome proteolytic pathway: destruction for the sake of construction. *Physiol Rev* 82(2):373–428. <https://doi.org/10.1152/physrev.00027.2001>
69. Xie M, Shi R, Pan Y, Zeng T, Chen Q, Wang S, Liao X (2014) Proteasome inhibition-induced downregulation of Akt/GSK-3 β pathway contributes to abnormality of tau in hippocampal slice. *Mol Neurobiol* 50(3):888–895. <https://doi.org/10.1007/s12035-014-8702-0>
70. Galves M, Rathi R, Prag G, Ashkenazi A (2019) Ubiquitin Signaling and Degradation of Aggregate-Prone Proteins. *Trends Biochem Sci* 44(10):872–884. <https://doi.org/10.1016/j.tibs.2019.04.007>
71. Thibautaud TA, Anderson RT, Smith DM (2018) A common mechanism of proteasome impairment by neurodegenerative disease-associated oligomers. *Nat Commun* 9(1):1097. <https://doi.org/10.1038/s41467-018-03509-0>
72. Berger Z, Ravikumar B, Menzies FM, Oroz LG, Underwood BR, Pangalos MN, Schmitt I, Wullner U, Evert BO, O'Kane CJ, Rubinsztein DC (2006) Rapamycin alleviates toxicity of different aggregate-prone proteins. *Hum Mol Genet* 15(3):433–442. <https://doi.org/10.1093/hmg/ddi458>
73. Ravikumar B, Vacher C, Berger Z, Davies JE, Luo S, Oroz LG, Scaravilli F, Easton DF, Duden R, O'Kane CJ, Rubinsztein DC (2004) Inhibition of mTOR induces autophagy and reduces toxicity of polyglutamine expansions in fly and mouse models of Huntington disease. *Nat Genet* 36(6):585–595. <https://doi.org/10.1038/ng1362>
74. Wang Y, Krüger U, Mandelkow E, Mandelkow EM (2010) Generation of tau aggregates and clearance by autophagy in an inducible cell model of tauopathy. *Neurodegener Dis* 7(1–3):103–107. <https://doi.org/10.1159/000285516>
75. Lin WM, Li ZG (2015) Blockage of cisplatin-induced autophagy sensitizes cervical cancer cells to cisplatin. *Genet Mol Res* 14(4):16905–16912. <https://doi.org/10.4238/2015.December.14.18>

76. Park E, Chung SW (2019) ROS-mediated autophagy increases intracellular iron levels and ferroptosis by ferritin and transferrin receptor regulation. *Cell Death Dis* 10(11):822.
<https://doi.org/10.1038/s41419-019-2064-5>

Figures

Figure 1

Effects of erastin and ferrostatin-1 on N2a cell viability. A, the relative viability of N2a cells treated with 5,10 and 20 μM erastin for 6 and 12 hr respectively. B, the relative viability of N2a cells co-treated with 10 μM erastin and 1, 2 and 4 μM ferrostatin-1 (Fer-1) for 6 and 12 hr respectively. Data are represented as means± SD (n= 3). *p<0.05, **p<0.01, ***p<0.001 as compared with the control group; #p<0.05, as compared with erastin treatment group.

Figure 2

Erastin treatment induces GPX activity, ROS and iron levels imbalance in N2a cells. A-B, changes of intracellular GPX activity in N2a cells treated with 10 μ M erastin or 10 μ M erastin plus 2 μ M ferrostatin-1 for 6 and 12 hr respectively. C-D, changes of intracellular ROS content in N2a cells treated with 10 μ M erastin or 10 μ M erastin plus 2 μ M ferrostatin-1 for 6 and 12 hr, respectively. E-F, Changes of intracellular iron levels in N2a cells treated with 10 μ M erastin or 10 μ M erastin plus 2 μ M ferrostatin-1 for 6 and 12 hr,

respectively. $**p<0.01$, as compared with the control group; $\#p<0.05$, $\#\#p<0.01$, compared with the erastin treatment group.

Figure 3

The effects of erastin and ferrostatin-1 on the morphology of N2a cells. A, the morphology of N2a cells treated with DMSO, 10 μM erastin, and 10 μM erastin plus 2 μM ferrostatin-1 for 6 hr (a-c) and 12 hr (d-f), respectively. B, the quantification of neurite length of 6 hr group and C, the quantification of neurite length

of 12 hr group. *** $p < 0.001$, as compared with the control group; ## $p < 0.01$, as compared with the erastin treatment group. Scale bar = 50 μm .

Figure 4

Erastin induced tau aggregation in N2a cells. The staining of tau protein (red) and vimentin (green) in N2a cells treated with erastin (10 μM) or erastin plus 2 μM ferrostatin-1 for 6 hr (A) and 12 hr (B). Red fluorescence represents tau protein labeled with tau-5 antibody (a, e, i); green fluorescence represents the vimentin (b, f, j); blue fluorescence represents the nucleus of DAPI labeled cells (c, g, k), arrows represent tau protein aggregates. Scale bar = 20 μm .

Figure 5

Erastin increased the level of total tau and phosphorylated tau in N2a cells. A and E, representative blots of total tau and phosphorylated tau-Ser396 and Thr231 after cells were treated with 10 μ M erastin for 6 hr (A) and 12 hr (E) respectively. B-D, Quantitative analysis of the relative levels of tau-5, p-tau at Ser396, and p-tau at Thr231 after treating for 6 hr. F-H, Quantitative analysis of the relative levels of tau-5, p-tau at Ser396, and p-tau at Thr231 after treating for 12 hr. I, the correlation coefficients between ROS content,

iron levels, and tau phosphorylation at Ser396 and Thr231 sites. Data were expressed as the means $\pm$ SD (n=3). *p<0.05, **p<0.01, as compared with the control group; #p<0.05, ##p<0.01 as compared with erastin treatment group.

Figure 6

Erastin activated GSK-3β in N2a cells. The level of intracellular GSK-3β and Ser9-phosphorylated GSK-3β in N2a cells following erastin (10 μ M), and erastin plus ferrostatin-1 (2 μ M) treatment for 6 hr (A, B) and 12 hr (C, D), respectively. The protein levels were expressed as fold changes over control cells and are

represented as mean $\pm$ SD. ** $p < 0.01$, as compared with the control group; ## $p < 0.01$ as compared with the erastin treatment group.

Figure 7

The effects of erastin on ubiquitin levels in N2a cells. The intracellular Poly-Ub and Mono-Ub content changes were detected by Western blot after erastin, or erastin plus ferrostatin-1 treatment for 6 hr and 12 hr, respectively. A, B, and C, the relative levels of Poly-Ub and Mono-Ub after 6 hr. D, E, and F, the relative levels of Poly-Ub and Mono-Ub after 12 hr. The values were expressed as fold changes over control and are represented as mean $\pm$ SD ($n=3$). ** $p < 0.01$, as compared with the control group; ## $p < 0.01$ as compared with the erastin treatment group.

Figure 8

Erastin treatment increased ubiquitinated tau in N2a cells. Extracts from cells were immunoprecipitated with anti-IgG control and anti-total tau antibody (tau-5). The immunoprecipitation was detected with an anti-ubiquitin antibody and tau-5. A and C, the input panel shows the presence of the studied proteins prior to immunoprecipitation in the extracts. B and D, quantification of the ratios of immunoprecipitated tau versus total input tau protein from three independent experiments. Values were normalized to those of the control group and represented the mean $\pm$ SD (n=3). * p<0.05, **p<0.01, as compared with the control group; #p<0.05, ##p<0.01 as compared with the erastin treatment group.

Figure 9

Erastin treatment decreased the chymotrypsin-like activity of the proteasome in N2a cells. The changes of intracellular chymotrypsin-like activity in N2a cells after 6 hr (A) and 12 hr (B) erastin or erastin plus ferrostatin-1 treatment, respectively. ** $p < 0.01$, as compared with the control group; # $p < 0.05$, ## $p < 0.01$ as compared with the erastin treatment group.

Figure 10

Erastin treatment activated autophagy in N2a cells. The changes of intracellular LC3-II/I ratio in N2a cells were detected by Western blot after the treatment with erastin (10 μ M) or erastin plus ferrostatin-1 for 6 hr (A and B) and 12 hr (C and D). Data were expressed as the means $\pm$ SD (n=3). **p<0.01, as compared with the control group; ##p<0.01 as compared with the erastin treatment group.

Figure 11

The potential mechanism underlying tau aggregation induced by erastin. Erastin inhibits cystine-glutamate antiporter (system Xc⁻) activity, leads to the GPX activity inhibition and ROS increasing, then triggers the ferroptosis. Ferroptosis can improve the activity of GSK-3β, which induces the hyperphosphorylation of tau proteins. Meanwhile, the proteasome system is impaired, which attenuates the degradation efficiency of hyperphosphorylated tau. So the activation of GSK-3β and proteasome impairment might co-contribute to the tau aggregation.