Abstract

Ferroptosis is a regulated form of cell death which is considered an oxidative iron-dependent process. Although neurodegeneration seems to be a multifactorial process, it is now commonly accepted that iron and free radicals are considered to cause lipid peroxidation as well as the oxidation of proteins and nucleic acids, leading to increased membrane and enzymatic dysfunction, and finally contributing to cell death. The lipid hydroperoxidase glutathione peroxidase 4 (GPX4) prevents the iron (Fe\(^{2+}\))-dependent formation of toxic lipid reactive oxygen species (ROS). While emerging evidence indicates that inhibition of GPX4 as a hallmark of ferroptosis in many cancer cell lines, the involvement of this biochemical pathway in neuronal death remains largely unclear. To test this, erastin (0.5 µM bis 0.8 µM) was applied to hippocampal HT22 neurons for 16h. The optimal experimental conditions occurred after incubation with 0.5 µM erastin. In addition, cells were cultured with the autophagy inhibitor, 3-methyladenin (3-MA at 10 mM) and/or ferroptosis inhibitors, ferrostatin 1 (Fer-1 at 10 µM to 20 µM) or deferoxamine (DFO at 100 to 200 µM) before exposure to erastin. In this study, we demonstrated by immunofluorescence and western blot analysis, that erastin downregulates dramatically the expression of GPX4, the sodium-independent cystine-glutamate antiporter (x-CT) and nuclear receptor coactivator 4 (NCOA4). The protein levels of ferritin (FTH) and mitochondrial ferritin (mtFT) in HT22 hippocampal neurons did not remarkably change following erastin treatment. In addition, we demonstrated that not only the ferroptosis inhibitor, ferrostatin/deferoxamine abrogated the ferroptotic cell death induced by erastin in hippocampal HT22 neurons, but also the potent autophagy inhibitor, 3-MA. We conclude that (1) in contrast to cancer cells, erastin-induced ferroptosis in hippocampal HT22 neurons, despite reduced NCOA4-levels, (2) that either NCOA4-mediated ferritinophagy does not occur or is of secondary importance in this model, (3) that ferroptosis seems to share some features of the autophagic cell death process.

Keywords: Erastin; Ferroptosis; Ferritin; Ferritinophagy; GPX-4; NCOA4; HT22 Neurons
Introduction

Ferroptosis is an iron-dependent and oxidative damage-related form of regulated cell death, which is morphologically, biochemically, and genetically different from other forms of cell death [1]. It has been reported that cellular iron overload leads to increased reactive oxygen species (ROS) and accumulation of lipid peroxidation in many cell types [2-4]. In addition, ferroptosis can be prevented by glutathione peroxidase 4 (GPX4) and ferroptosis inhibitors, such as ferrostatin 1 (Fer1) and the iron chelator deferoxamine [3,1,5-8]. Ferroptosis can be induced chemically by erastin, which inhibits the glutamate/cystine antiporter, and subsequently suppresses cellular cystine uptake and depletes glutathione (GSH). GSH is fundamental in maintaining the redox balance and defending against oxidative stress, including reactive oxygen species [9]. Erastin has been widely found to induce ferroptosis in several types of cancer cells [10,11]. However, the involvement of these erastin-related biochemical pathways in neuronal death remains largely unclear.

Under oxidative stress conditions, cells must actively regulate their iron-carrier proteins such as transferrin, lactotransferrin [12]. Transferrin is an iron carrier protein that can be transported into the cell via receptor-mediated endocytosis [13]. Lactotransferrin is known as an iron-binding protein. Mechanisms controlling iron-loading of transferrin and or lactotransferrin may impact ferroptosis. On the other hand, cells must buffer their intracellular iron by using cytosolic iron storage proteins such as NCOA4 and ferritins. Mitochondrial ferritin (mtFt) and H-chain ferritin (FTH) are the most known iron storage proteins in the brain. Accumulating evidence indicates the involvement of mtFt in some neurodegenerative diseases, especially Alzheimer’s disease and Parkinson’s disease [14,15]. Mitochondrial ferritin seems to have inhibitory effects on oxidative stress-dependent neuronal damage and acts as a neuroprotective factor to maintain normal neuronal function [16,17]. Previous studies suggested that overexpression of mtFt may prevent cytosolic iron accumulation and protect neuroblastoma cells from oxidative stress [18,19]. In addition, it has been shown that ferritin is a source of iron and protection from iron-induced toxicities [20,21]. Mouse brain deficient in ferritin exhibited increased evidence of oxidative stress [22] and oligodendrocytes provide antioxidant functions for neurons by secreting ferritin [23]. The ferritin turnover is checked by an autophagic cargo receptor, NCOA4, which facilitates the delivery of ferritin for lysosomal degradation via a selective autophagy process, called ferritinophagy [24,25]. It has been reported that ferritinophagy is involved in ferroptosis and that the genetic deletion of NCOA4 inhibits ferritinophagy and blocks lipid peroxidation and ferroptosis by decreasing the levels of bioavailable intracellular iron [12].

Based on the link between NCOA4 and ferritin in regulating intracellular iron levels, we intend to investigate, first whether the ferroptosis key players are involved in the neuronal cell death induced by erastin HT22 neurons. HT22 neuronal cell line is a suitable model for studying oxidative stress due to deficiency of N-methyl-D-aspartate (NMDA) receptor. The second objective was to address neuron response to erastin, with a special focus on ferritin and NCOA4-mediated ferritinophagy.

Materials and Methods

HT22-Hippocampal cells were kindly provided by Dr. David Schubert (The Salk Institute for Biological Studies, La Jolla, CA, USA). HT-22 is an immortalized mouse hippocampal cell line subcloned from the HT-4 cell line. The parental HT-4 cell line was derived from the immortalization of mouse neuronal tissues with a temperature-sensitive SV40 T-antigen.

Drugs Treatment

The ferroptosis inducer erastin, ferrostatin 1, and 3-MA (10 mM) were dissolved in DMSO (Sigma Aldrich). Deferoxamine (Sigma) was dissolved in deionized water. Cells were seeded in 96-well plates for viability assay, 24-well plates for immunofluorescence staining, and western blot assay. To assess the optimal concentration of erastin, hippocampal HT22 neurons were exposed to the drug at 0.3, 0.4, 0.5, 0.6, 0.7, and 0.8 µM for 16 hrs.
Cell Viability Detection

The cell viability was performed using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay (Jiang et al., 2014b). The results were determined using a microplate reader (Spectra Max M2e, Sunnyvale, CA, USA) at 490 nm.

Immunofluorescence

Fixed cells were incubated overnight at 4°C with primary antibodies. The following antibodies have been used: GPX4 (Rabbit mAb; #52455, Cell signaling, Germany), x-CT (Rabbit mAb; #12691, Cell signaling, Germany), FTH (Rabbit mAb; #4393 Cell signaling), MtFt (Rabbit mAb, #31718, Abcam, Germany), NCOA4 (Rabbit mAb; #66849, Cell Signaling, Germany), ACSL4 (Rabbit polyclonal #PA5-27137). Briefly, cells (miniscule) were pre-incubated for 1 h at room temperature in PBS, 5% NGS (Sigma), and primary antibodies were applied at 4°C for 24 h in PBS plus 5% normal goat serum. Alexa Fluor 488 and Alexa 568 goat anti-mouse secondary antibodies were used at a 1:200 dilution (Molecular Probes, Göttingen, Germany). Adjacent wells that were not treated with the primary antibody were run for each animal in parallel. After rinsing the cells with 0.1 M PBS, they were mounted in Dako fluorescent mounting medium (Dako, Hamburg, Germany). Fluorescent images were acquired using an Axio-Cam digital camera mounted on a Zeiss microscope (Carl Zeiss, Jena, Germany).

For semiquantitative densitometric analyses of the immunoreactions, images were digitized with an Axiocam system (Zeiss, Germany; 1,030 x 1,030 pixels, 8-bit color depth), using NIH ImageJ software (Image Processing and Analysis in Java, developer Wayne Rasband), as described previously. Six to 8 ROIs were selected individually, and the relative optical density (rel. O.D.) of background staining was measured within the selected areas.

Western Blotting

Homogenates were prepared in ten volumes of homogenization buffer (150 mM NaCl, 20 mM Tris pH 7.5, 1 mM EDTA, 0.5% Sodium Deoxycholate, 0.1% SDS, 1% Nonidet P-40), and polytron homogenization for 10 s. Aliquots were stored at -70°C and 30 µg of total protein was used per lane. Samples were resuspended to contain 30 µg of total protein in loading buffer, heated for 5 min at 95°C, and separated using a MINI-PROTEAN II electrophoresis system from Bio-Rad on either a 10% Tris-glycine gel, or a 15% Tris-glycine gel with 4% stacking gels. Gels were run at 180 V for 1 h. The matched gel was soaked for 15 min in transfer buffer (25 mM Tris, 190 mM glycine, 20% methanol) and then transferred to a PVDF membrane for 30 min at 100 mA constant current using a Bio-Rad Semi-Dry Blotting apparatus. Blots were blocked with 10% rehydrated nonfat dry milk for 1 h at room temperature. Primary antibodies against GPX4 (Rabbit mAb; #52455, Cell signaling), mtFt (Rabbit pAb, #66111, Abcam), FTH (Rabbit mAb; #4393 Cell signaling), NCOA4 (Rabbit mAb; #6684, Cell Signaling) x-CT (Rabbit mAb; #12691, Cell signaling) and actin (mAb, #A5316, Sigma) were used.

Western blots were performed with horseradish peroxidase conjugated anti-rabbit IgG (DAKO, Germany) using enhanced chemiluminescence Western blotting detection reagents (Amersham International). To ensure equivalent protein loading, the stripped membranes were rehybridized with an actin antibody as an internal control.

Expression of proteins was examined by Western blot. For each group to evaluate the results of the Western blot study, we scanned the film with an imaging densitometer (ChemiDoc XRS) and quantified the optical density using a gel analysis software QuantiScan program from Biosoft. Three blots from each group have been examined. Rectangular regions are identified within the image and background subtracted. The center of mass within the region and the net and gross total volume are calculated. Volume is calculated as the summed value of all pixels within the region. Data are reported as means ± SD of n experiments. Significant differences between means were statistically assessed by ANOVA. Significant differences between means at each time point were assessed by paired Student’s t-test and *p<0.05 was considered statistically significant.
Results

Ferroptosis is involved in erastin-induced toxicity in HT-22 cells

Dose-response tests evaluated the effect of treatment of HT-22 cells with different concentrations of erastin and showed decreased survival of neurons over time (MTT assay). Erastin decreased significantly the cell viability of hippocampal HT22 neurons. The cell survival rate was near its lowest in HT-22 cells treated with 0.6 µM erastin for 16 hours. For our study, we selected the 0.5 µM dose of erastin (Figure 1a).

Ferrostatin-1 (Fer-1) or Deferoxamine (DFO) were preadministered to determine if ferroptosis is involved in erastin-induced toxicity. The results indicated that Fer-1 or DFO reduced the cell death induced by erastin (Fig.1b). In addition, 3-methyladenine (3-MA) as an inhibitor of autophagy was used to determine if autophagy is involved in erastin-induced ferroptosis. The autophagy inhibitor 3-MA protected hippocampal HT22 neurons from ferroptotic cell death induced by erastin (Figure 1c).

Figure 1: a) Erastin induces cell death in hippocampal HT22 neurons. HT22 neurons treated with different concentrations of erastin (0.3, 0.4, 0.5, 0.6, 0.7, and 0.8 µM) for 16 hours and examined by light microscopy. Neurons were severely damaged after treatment with erastin at 0.5; 0.6; 0.7 and 0.8 µM. MTT assay for the viability of HT22 neurons after treatment with erastin shows a significant decrease of viability. Significant differences between means were statistically assessed by ANOVA (n = 6). The samples were detected in six replicates, and each experiment was performed in triplicate. *p < 0.05; **p < 0.01; ***p < 0.001

Figure 1: b) DFO/Fer1 inhibit erastin-induced cell death in HT22 neurons: Fer1 and DFO (50 µM) were added to wells 12 hours before erastin treatment. The samples were detected in six replicates. Data are shown as the mean ± SEM (ANOVA). Each experiment was performed in triplicate. **P < 0.01 different from controls, ## P < 0.01 different from erastin.
Figure 1: c) 3-MA protects HT22 cells from erastin-induced cell death: 3-MA (10 mM) was dissolved in DMSO and added to wells 12h before erastin treatment. The samples were detected in six replicates. Data are shown as the mean ± SEM (ANOVA). Each experiment was performed in triplicate. **P < 0.01 different from controls, ## P < 0.01 different from erastin

Erastin Reduced GPX4- and x-CT-Expression

As biomarkers of ferroptosis, the expression of x-CT (a sodium-independent cystine-glutamate antiporter) and GPX4 were analysed. We determined the expression of GPX4 after treating the HT22 cells with the GPX4 inhibitor and ferroptosis inducer, erastin. The densitometric analysis of GPX4-immunoreactivity and GPX4-immunoblots revealed a statistically significant decrease upon erastin treatment as early as 6h post-incubation as shown in (Figure 2). The x-CT-immunoreactivity as well as the density of immunoblots was more intense in controls than in erastin-treated cells. The x-CT levels were significantly decreased at all-time points examined in erastin-treated cells (Figure 2 & Figure 3a).

Erastin does not alter significantly the FTH- and mtFt-levels

We determined the expression levels of FTH and mtFt after treating the HT22 cells with the GPX4 inhibitor and ferroptosis inducer, erastin. FTH- and mtft levels were measured in control and erastin-treated cultures at each time point, i.e., 6, 16, and 24h post-incubation. Western blot and immunofluorescence analysis revealed no remarkable alterations in FTH/mtft expression upon erastin-treatment (Figure 2 & Figure 3b), except for a slight transient increase in FTH-levels at 6h after incubation with erastin.

Erastin induced alterations in NCOA4- and ACSL-4-levels

NCOA4 was recently identified as a key player in ferritinophagy, serving as an autophagic cargo receptor to facilitate the delivery of ferritin for lysosomal degradation. We followed the time course of the expression of NCOA4 by immunofluorescence- and WB-analysis after erastin treatment. We found a gradual decrease in NCOA4 levels at 6, 16, and 24h post-incubation (Figure 2 & Figure 3c).

Acyl-CoA synthetase long-chain family member 4 (ACSL4) is one of the main enzymes which promote ferroptosis [20]. Cell lines with deleted ACSL4 gene showed less susceptibility to ferroptosis inducers such as erastin and RSL-3. In addition, in some cell lines, the abundance of ACSL4 could be regarded as a biomarker of ferroptosis sensitivity. We followed the time course of the expression of ACSL4 by immunofluorescence- and WB-analysis after treatment of HT22 cells with erastin. We found a gradual decrease in ACSL4 levels at 6, 16, and 24h post-incubation (Figure 2 & Figure 3d).
Figure 2: Western blot assay and densitometric analysis of immunoblots of GPX4, xCT protein, NCOA4, ACSL4 and FTH (6, 16 and 24 h after 0.5 μM erastin). Each experiment was performed in triplicate. Significant differences between means were statistically assessed by ANOVA. *p < 0.05, **p < 0.01 were considered statistically significant.
Figure 3a-e: shows the GPX4-, x-CT-, NCOA4-, ACSL4-, mtFt and FTH-immunoreactivities in HT 22 neurons collected from controls and erastin-treated cells at 6h, 16h and 24h after incubation with erastin. Significant differences between means were statistically assessed by ANOVA. *p< 0.05 and **p< 0.01 were considered statistically significant.
Discussion

Although neuronal death seems to be a multifactorial process, there are now indications that neuronal iron overload and release of stored intracellular iron are critical events leading to irreversible neuronal damage. On the one hand, iron homeostasis is indispensable for neuronal cell function, however, a deregulation of cellular iron is potentially harmful due to its reactive nature [2,27,29]. Deregulation of cellular iron leads to increased reactive oxygen species (ROS). Increased ROS leads in turn to impairment of the cellular antioxidant system, which may exacerbate the susceptibility of neurons to iron. Under stress conditions, cells must actively buffer their intracellular iron by using cytosolic iron storage proteins such as ferritin [29,30].

Our initial goal was to determine whether there were alterations in the expression levels of ferroptosis key players in HT22 neurons following erastin treatment. We first addressed GPX4-protein, which is a strong antioxidant enzyme. Accordingly, we demonstrated that erastin treatment downregulates dramatically the GPX-4 expression in HT22 neurons. This supports the hypothesis that the cell death induced by erastin in HT22 neurons is ferroptotic and indicates that neurons are specifically sensitive to GPX4-depletion. In this context, GPX4-deletion triggers degeneration of spinal motor neurons [31] and that GPX4-ablation in adult mice results in a lethal phenotype accompanied by neuronal loss in brain [32]. GPX4-knockout mice exhibit a selective degeneration of CA1 pyramidal hippocampal neurons and interneurons [33-35].

The x-CT levels were also reduced significantly in HT22 neurons after erastin treatment. In general, a decrease in x-CT-levels should stabilise glutamate homeostasis and prevent neuronal damage. However, it is not the case in our system, cell damage occurred even with a decrease in x-CT-levels. This lets assume that the capacity of this process seems to be limited and not powerful enough to rescue neurons from the damaging effects of erastin.

Seeing that ACSL4 expression is well correlated with ferroptosis sensitivity in various cancer cells and is considered a biomarker of apoptosis [30], this is not the case in hippocampal HT22 neurons. We found in our study that erastin reduced the ACSL4-levels in hippocampal HT22 neurons. Comparing HT22 neurons to cancer cell lines, the reduction of ACSL4-levels in erastin treated HT22 cells should mean consequently an attenuation of ferroptosis. On the contrary, we found in our study that ferroptosis occurred despite reduced ACSL4 levels in HT22 neurons. We can assume that neuronal- and cancer cells responses to ferroptosis inducers are quite different.

On the other hand, elevated cytosolic iron levels triggered the expression of neuronal ferritin, presumably to bind and buffer iron. It is likely that ferritin is one of the metalloproteins which plays an active, protective role within neuronal cells by diminishing the availability of reactive iron upon accumulation, and readily providing iron for utilization under physiological conditions. The ferritin homeostasis is checked by NCOA4, which regulates the lysosomal degradation of ferritin via a selective form of autophagy called ferritinophagy [19,37,38]. Accordingly, we next determined whether a change in NCOA4 expression occurs during erastin-induced ferroptosis in HT22 neurons. We observed that the protein level of NCOA4 was remarkably reduced following the treatment of HT22 neurons with erastin. Comparing to cancer cells, this should mean consequently a reduction of NCOA4-mediated ferritinophagy in HT22 neurons and attenuation of ferroptosis. On the contrary, we found in our study that ferroptosis occurred despite reduced ACSL4 levels in HT22 neurons. We can assume that neuronal- and cancer cells responses to ferroptosis inducers are quite different.

These discordant results may be a consequence of a number of differences between tumor-derived cell lines and neurons, including the relative dependence on NCOA4-mediated ferritinophagy for iron balance in cell culture models and differences in iron availability between cells. However, our results are in accordance with the studies of [3], who have shown that erastin reduced NCOA4-levels in mouse embryonic fibroblasts and this was accompanied by NCOA4 autophagic degradation.

Ferritinophagy is the process of autophagic degradation of the iron storage protein ferritin, which is critical for the regulation of cellular iron levels. Accordingly, we next determined whether a change in ferritin expression (FTH and mtFt) occurs during erastin-induced ferroptosis in HT22 neurons. FTH/mtFt levels were not remarkably altered in erastin-treated HT22 cells, which may indicate that ferritinophagy does not occur or it is of secondary importance in this experimental model. In contrast, recent data support the protective effect of ferritin accumulation, because the lack or deficiency of mitochondrial ferritin exacerbates neurological deficits and oxidative stress in a traumatic brain injury model [39,40]. In addition, it was shown that FTH upregulation in neuroblastoma...
was an important neuroprotective response to the oxidative insult caused by the neurotoxic agent rotenone [41]. A protective role of ferritin was also observed in dopamine neurons of the substantia nigra after treatment with the proteasome inhibitor lactacystin [42]. To further confirm the eventual involvement of autophagy in ferroptosis, we tested the autophagy inhibitor, 3-MA and demonstrated that 3-MA ameliorates significantly the cell viability in erastin-treated hippocampal HT22 neurons. Consistently, inhibition of autophagy in erastin-treated hippocampal neurons abrogated ferroptotic cell death. Therefore, ferroptosis seems to share some features of the autophagic cell death process, and the so-called NCOA4-mediated ferritinophagy does not support ferroptosis by controlling cellular iron homeostasis in hippocampal HT22 neurons.

Basically, it has been reported that NCOA4-levels were enhanced in multiple cancer cell lines and that NCOA4-dependent ferritinophagy can promote ferroptosis through releasing free iron from ferritin [43]. However, to date there is no direct evidence associating ferritinophagy and ferroptotic cell death in neurons.

**Conclusion**

In summary, the present study provides important evidence that hippocampal HT22 neurons respond differently from cancer cell lines to the ferroptosis inducer erastin. In addition, autophagy seems to promote erastin-induced ferroptosis (Figure 4). Besides ferritinophagy, a further understanding of the functional characterization of different pathways leading to ferroptosis, NCOA4-degradation, and its contribution to intracellular and systemic iron homeostasis, will be important.

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**Figure 4:** (1) Erastin induced iron accumulation, reduction of glutathione peroxidase 4-levels and ferroptosis in hippocampal HT22 neurons, despite reduced NCOA4-levels. Decreased levels of NCOA4 lead to stabilization of FTH/mtFt by decreased ferritinophagy flux. Ferroptosis inhibitors, ferrostatin1/deferoxamine as well as the potent autophagy inhibitor, 3-MA abrogated the ferroptotic cell death induced by erastin in hippocampal HT22 neurons. (2) Ferritinophagy does either not occur or is of secondary importance in this model, (3) ferroptosis seems to share some features of the autophagic cell death process.
Declarations

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Data Availability: the datasets generated during and/or analysed during the current study are available from the corresponding author on reasonable request.

Ethics approval: “This study was performed by using immortalized hippocampal cultures”
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