Ferroptosis and Brain Injury

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Abstract

Ferroptosis is a nonapoptotic form of cell death characterized by the iron-dependent accumulation of toxic lipid reactive oxygen species. Small-molecule screening and subsequent optimization have yielded potent and specific activators and inhibitors of this process. These compounds have been employed to dissect the lethal mechanism and implicate this process in pathological cell death events observed in many tissues, including the brain. Indeed, ferroptosis is emerging as an important mechanism of cell death during stroke, intracerebral hemorrhage, and other acute brain injuries, and may also play a role in certain degenerative brain disorders. Outstanding issues include the practical need to identify molecular markers of ferroptosis that can be used to detect and study this process in vivo, and the more basic problem of understanding the relationship between ferroptosis and other forms of cell death that can be triggered in the brain during injury.

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Introduction

During development and in adulthood, damaged or unwanted cells are eliminated through the activation of different regulated cell death pathways. In many devel-

opmental contexts, the caspase-dependent apoptosis pathway executes cell death [1]. However, in other contexts, especially those involving infection or trauma, cell death is executed by nonapoptotic cell death pathways, including necroptosis, pyroptosis, parthanatos, and ferroptosis [2–6]. Some of these pathways have been linked to pathological cell death in the nervous system. For example, necroptosis is associated with axonal degeneration in amyotrophic lateral sclerosis and frontotemporal dementia [7, 8], while parthanatos, i.e., poly-(ADP-ribose)-polymerase-1 (PARP1)-dependent cell death, is linked to neuronal cell death during glutamate-induced excitotoxicity and stroke [9–11]. In this review, we focus on ferroptosis, a nonapoptotic form of cell death first reported in 2012 [12, 13]. This process was initially characterized in vitro in cancer cells using synthetic small molecules, but it has since been linked to cell death events observed in vivo following acute injury to the brain and other tissues [14–20].

At the molecular level, ferroptosis is characterized by the iron-dependent accumulation of lipid reactive oxygen species (ROS), which are associated with irreparable lipid damage and membrane permeabilization. Recent studies have defined key enzymes and metabolites that either promote or prevent ferroptosis, and pinpointed chemical modulators of this process [13]. It is important to note that iron-dependent cell death is not a novel concept [21, 22]. The neurodegeneration and brain injury literature contains many descriptions of pathological cell death events linked to iron and/or oxidative stress [23–30].

Fig. 1. a Key metabolites and enzymes (bold text) involved in the ferroptosis pathway. **Insets** Upper: the regulation of cystine (Cys) import by glutamate (Glu) levels. Lower: additional detail on the regulation of lipid metabolism in ferroptosis. The purple-shaded box highlights the iron-catalyzed, free radical-mediated chain reaction of lipid oxidation. **b** The table lists key small-molecule modulators of ferroptosis and the associated mechanisms of action.

However, much of this work pre-dates the discovery that nonapoptotic cell death (i.e. necrosis) can be a highly regulated process [25]. It is therefore possible that many previous observations linking iron and ROS to brain injury could be explained by the induction of ferroptosis. A better understanding of the ferroptosis pathway and what makes this process unique could lead to new therapies that specifically block with this process in the brain and other tissues. Here, we review the discovery of ferroptosis, the relationship between this process and other forms of oxidative cell death that have been described previously, and recent examples of links between ferroptosis and brain injury.

The Mechanism of Ferroptosis

Ferroptosis is characterized by the iron-dependent accumulation of toxic lipid ROS (Fig. 1a). In particular, ferroptosis occurs when the oxidation of membrane polyun-

saturated fatty acids (PUFAs) is allowed to run out of control due to inactivation of the lipid hydroperoxidase glutathione peroxidase 4 (GPX4) [18, 31, 32]. It is presumed that unrestrained lipid oxidation and lipid ROS formation ultimately result in membrane damage and perforation [26], but the precise chemical details of how this occurs remain unclear. Unlike other forms of cell death, this process is not known to require the transcriptional upregulation or posttranslational modification of any specific cell death effector or pore-forming protein. Indeed, as far as is understood, there is no ferroptosis program latent in the cell and waiting to be activated. Rather, ferroptosis is triggered when disruption of the continuously operating endogenous antioxidant network of the cell results in lipid ROS accumulation to toxic levels [22]. Due to the central role of iron-dependent lipid peroxidation, this process can be inhibited by natural product and synthetic small-molecule iron chelators, such as deferox-

amine and ciclopirox [12, 27]. Lipophilic antioxidants, including vitamin E, trolox, ferrostatin-1, and liproxstatin-1 are also potent ferroptosis inhibitors (Fig. 1b).

Ferroptosis was initially identified and studied in vitro in human cancer cells treated with the synthetic small molecules erastin and 1S,3R-RSL3 (hereafter RSL3) [28, 29] (Fig. 1b). These molecules were discovered in phenotypic small-molecule screens based on their ability to selectively induce cell death in certain cancer cells. In sensitive cells, treatment with erastin and RSL3 does not cause caspase activation or lead to other biochemical or morphological features of apoptosis (e.g., chromosomal margination) [27, 28]. Bioenergetically, ferroptosis triggered by erastin occurs without the loss of intracellular adenosine triphosphate (ATP), which distinguishes this process from unregulated necrosis and PARP-dependent cell death [12]. Morphologically, the cell death phenotype induced by these molecules in human cancer cells is clearly distinct from apoptosis, unregulated necrosis (e.g., induced by H_2O_2), and autophagy, which can contribute to cell death in some contexts [12]. The only notable morphological phenotype of cells undergoing ferroptosis is an alteration in mitochondrial morphology, including smaller size and disorganized cristae [12, 18, 27]. The cause of these changes and whether they are functionally important to the execution of ferroptosis are important unresolved questions. Nonetheless, these changes may be sufficiently characteristic to identify ferroptotic cells in vivo [30, 31].

The protein targets of erastin, RSL3, and related molecules act to restrain ferroptosis. Erastin is a potent and specific inhibitor of the system x_c^- cystine/glutamate antiporter, a heterodimer composed of the SLC3A2 (4H2hc, CD98) regulatory subunit and the SLC7A11 (xCT) 12-pass transmembrane protein [12, 32, 33]. System x_c^- imports extracellular cystine in for exchange for intracellular glutamate. This transport cycle is ATP-independent, being driven by the high concentration of intracellular glutamate. This renders system x_c^- sensitive to inhibition by high concentrations of extracellular glutamate, as can occur in a variety of brain injury contexts [34] (Fig. 1a). Once transported into the cell, cystine is reduced to cysteine, which is used to synthesize proteins and the nonribosomal tripeptide antioxidant (reduced) glutathione (GSH). Of the 3 amino acids found in GSH, cysteine is typically the least abundant, and therefore considered rate-limiting for de novo GSH synthesis. Cysteine can be synthesized in some cells from methionine, via the transsulfuration pathway. However, in many other cells, cystine import appears to

be essential to maintain cysteine and GSH levels and prevent ferroptosis, at least in vitro.

RSL3 and related molecules covalently bind to and inhibit the function of the GSH-dependent selenoenzyme GPX4 [35, 36]. Loss of GPX4 function either directly (e.g., due to covalent inhibition) or indirectly (e.g., due to GSH depletion) is currently thought to be the key event leading to the onset of ferroptosis. This is due to the central role of GPX4 in the prevention of lethal lipid peroxidation. Relative to saturated fatty acids (SFAs) and monounsaturated fatty acids (MUFAs), the *bis*-allylic carbons of PUFAs are highly susceptible to oxidation. GPX4 uses GSH as a cofactor to reduce PUFA lipid peroxides (L-OOH) to lipid alcohols (L-OH) (Fig. 1a). The catalytic cycle of GPX4 consumes 2 GSH molecules which are converted to GSSG. GSSG can be recycled back to GSH by GSH reductase in an NADPH-dependent manner. Normally, a balance is maintained between L-OOH formation and L-OOH reduction to L-OH by GPX4. Lipid hydroperoxides (L-OOH) can be oxidized by Fe^{2+} to yield highly reactive alkoxy radicals (L-O $\bullet$). These radicals can directly damage adjacent PUFAs through free radical-mediated chain reactions (Fig. 1a, purple-shaded area) [37]. When GPX4 is absent or inactivated, L-OOH can accumulate to higher levels than normal, resulting in a greater production of L-O $\bullet$, presumably leading to catastrophic membrane damage. As predicted from this model, lipidomic studies show that cells treated with erastin are depleted for various PUFAs, whereas the levels of these species are maintained in cells cotreated with erastin and the radical-trapping antioxidant ferrostatin-1, despite the effective inhibition of system x_c^- and the depletion of intracellular GSH [38]. These findings place lipid oxidation downstream of GSH depletion and/or GPX4 inactivation in the ferroptotic cascade.

Various PUFA-containing phospholipids (PLs) are oxygenated during ferroptosis, with PUFA-containing phosphatidylethanolamines (PEs) being highly oxidized following GPX4 inactivation in mouse fibroblasts [39, 40]. The PE C20:4 (arachidonoyl) and C22:5 (adrenoyl) fatty acyl chains, in particular, are doubly or triply oxygenated following *Gpx4* inactivation [39]. The incorporation of these PUFAs into PLs, and thus sensitivity to ferroptosis, is attenuated by the disruption of specific lipid-metabolic enzymes required for the metabolism of PUFAs within the cell. To date, genetic screens in cancer cells and mouse embryonic fibroblasts (MEFs) have identified 2 lipid-metabolic enzymes that are required to execute ferroptosis, at least in certain nonneuronal cells [39–42]. Acyl-CoA synthetase long-chain family member 4 (ACSL4)

preferentially activates PUFA free fatty acids to PUFA-CoAs, and this activation is required for their incorporation into PLs. Lysophosphatidylcholine acyltransferase 3 (LPCAT3) is an enzyme that inserts acyl-CoA molecules into lysophosphatidylcholine to generate phosphatidylcholine. LPCAT3 preferentially acts on PUFA-CoAs, including arachidonoyl-CoA. Together, these 2 enzymes help define a lipid metabolic pathway essential for PUFA insertion into membrane PLs and sensitivity to ferroptosis. Recent evidence suggests that it is the PUFA-containing PLs found at the plasma membrane whose oxidation is necessary for ferroptosis, and that the relative ratio of plasma membrane PLs acylated with MUFAs (e.g., oleic acid) versus PUFAs (e.g., arachidonic acid) helps determine cellular ferroptosis sensitivity (Magtanong et al. [128]). It is possible that cells dynamically modulate the “oxidizability” of the plasma membrane by manipulating the relative levels of PUFAs versus MUFAs that are incorporated into this structure.

The requirement for iron is a defining feature of ferroptosis. Both ferric and ferrous iron chelators (deferrioxamine and ciclopirox, respectively), but not chelators of other divalent metals, are effective inhibitors of ferroptotic cell death [13]. Iron is carried in the bloodstream by the glycoprotein transferrin. Depletion of transferrin from cell culture medium can block ferroptotic cell death of MEFs, and resupplementation with holotransferrin (i.e., iron-loaded) but not apotransferrin (i.e., iron-free) restores ferroptosis in this system [15]. In cancer cells, genetic silencing of key iron metabolic genes including *IREB2* and *TFRC* (encoding the transferrin receptor) reduce iron uptake and ferroptosis sensitivity, while perturbation of mitochondrial iron/sulfur cluster biogenesis can increase ferroptosis sensitivity [12, 15, 29, 43]. How iron impacts lipid oxidation to promote ferroptosis is not yet completely understood. Intracellular Fe^{2+} can promote the formation of hydroxyl and alkoxyl radicals that can initiate or help propagate lipid ROS production. Additionally, the function of the lipid-oxidizing lipoxygenase (LOX) family of enzymes is iron-dependent, and genetic silencing of LOX enzyme expression can block ferroptosis in some models, as anticipated from early studies of glutamate toxicity [44, 45]. In some contexts, LOX function and the oxidation of specific PUFA-containing PEs is orchestrated by 15-LOX binding to phosphatidylethanolamine-binding protein 1 (PEBP1) (Fig. 1a). PEBP1 binds LOX and orients PE-PUFAs at the membrane, facilitating lipid oxidation [20]. Whether there is a universal requirement for LOX activity, however, remains unclear. Joint deletion or silencing of *Alox15* and

Alox5 does not impair ferroptosis induced by *Gpx4* deletion in mice [18], and in at least 1 animal model of brain injury, the *Alox5* enzyme appears most important [46]. Moreover, some cultured cells completely lacking LOX enzyme expression execute ferroptosis normally [47]. Thus, the role of individual LOX enzymes in ferroptosis may be species- and/or tissue-specific. Importantly, many commonly employed putative LOX inhibitors (e.g., nordihydroguaiaretic acid [NDGA] and zileuton) can act as direct radical-trapping antioxidants [47, 48], which could account for their ability to inhibit ferroptosis and related forms of oxidative cell death, apart from LOX inhibition.

Ferroptosis, Oxidative Glutamate Toxicity, and Oxytosis: A Common Process?

Given the mechanistic details elucidated above, it is likely that ferroptosis is related or identical to oxidative cell death phenotypes first observed as early as 1977 in fibroblasts and cultured brain cell lines deprived of cystine or exposed to high levels of extracellular glutamate [45, 49–55]. This process was termed oxidative glutamate toxicity, and subsequently oxytosis, to distinguish it from apoptosis [45, 56]. The central role of system x_c^- inhibition, GSH depletion, and iron-dependent lipid ROS accumulation is broadly consistent in studies of ferroptosis and oxytosis, and it may represent the core of 1 common lethal process [57–59]. However, glutamate-induced oxytosis in neuronal-like cells displays certain differences when compared to ferroptosis in cancer cells, so the actual degree of overlap between oxytosis and ferroptosis still remains somewhat murky [57, 58]. Reconciling these differences has not been straightforward, as most mechanistic studies on ferroptosis have been carried out in cancer cells and fibroblasts, while most studies on oxytosis have been carried out in neurons or neuronal-like cells, especially the HT-22 cell line.

In human cancer cells, the accumulation of lipid ROS appears necessary and sufficient for membrane permeabilization and the induction of ferroptosis. Moreover, Ca^{2+} influx and mitochondrial function are not necessary for the execution of ferroptosis [12, 59], although they may contribute to the process (e.g., [81, 82]). By contrast, in neuronal cell models (e.g., the HT-22 cell line), additional molecular events are required downstream of lipid ROS accumulation for the execution of cell death, including Ca^{2+} influx into the cell via ORAI calcium release-activated calcium modulator 1 (ORAI1), BH3-interacting domain death agonist (BID) translocation to the mitochondria, mitochondrial ROS production, and the release of apoptosis-inducing factor (AIF) from mitochondria

[50, 60–66]. Whether the execution of oxytosis requires the recently identified AIF-binding partner, the nuclease macrophage migration inhibitory factor (MIF), is unclear [11]. It is possible that these downstream events dependent on Ca^{2+} , mitochondria, and AIF are specific to neurons, and represent a module that is “bolted on” to the core GSH-GPX4-lipid-ROS ferroptotic pathway in these cells. Indeed, AIF appears dispensable for cell death in fibroblasts, T cells, and B cells, suggesting that the role of this enzyme could be tissue-specific [67]. From a therapeutic standpoint, it might not matter. Given that lipid ROS accumulation lies in the shared portion of the pathway common to both mechanisms, targeting this molecular event should be sufficient to block ferroptosis/oxytosis in all cases.

Ferroptosis and Development

Whether ferroptosis is engaged during development to eliminate unwanted cells in a programmed manner is unknown. With a few notable exceptions (e.g., cornification of skin epithelia), nonapoptotic cell death pathways do not appear to play a primary role in most developmental contexts, although they can serve an important backup function [3, 68]. It is therefore perhaps not surprising that, to date, there is little evidence that ferroptosis is required for normal development. In the mouse autopod, a decrease in Gpx4 activity correlates with increased interdigital cell death, a process that can occur in a caspase-independent manner, but whether this is a primary means of eliminating these cells or a backup mechanism is not clear [60, 61]. In mice lacking the E3 ligase gene, mouse double minute 2 (*Mdm2*), and where the endogenous *Trp53* gene has been mutated to encode a variant with 3 lysines in place of arginine, treatment with the ferroptosis inhibitor ferrostatin-1 can improve embryonic development, consistent with the possibility that p53 may promote cell death in the developing embryo, in part via ferroptosis [62]. However, these studies do not suggest a primary role for ferroptosis in the elimination of a defined cell population. Rather, these findings, and those described below, are more consistent with the model that GPX4 and other enzymes in the ferroptosis pathway are simply essential for cell viability.

GPX4 is expressed in the brain of developing animals. In rats, *Gpx4* mRNA and protein are detected at various stages of cerebral development, with the hippocampus being one structure that maintains high Gpx4 expression into adulthood [63]. In the adult rat brain, dentate granule cells and hippocampal cornu ammonis pyramidal neurons express Gpx4. The pyramidal neurons in the

frontal and entorhinal cortex exhibit strong Gpx4 expression [63]. In an in vitro model of embryogenesis, 2 of the 3 Gpx4 isoforms (mitochondrial, m-Gpx4; and nuclear, n-Gpx4) were shown to promote normal development. Injection of small-interfering (si)RNA against n-*Gpx4* led to a defect in development of the left atrium of the heart [64]. Injection of m-*Gpx4* siRNA resulted in drastic defects of rhombomeres 5 and 6 of the developing hindbrain, and the neuroepithelium between these 2 rhombomeres was smaller than normal [64]. The importance of Gpx4 activity during embryonic brain development was also demonstrated by studying the posttranscriptional regulation of m-*Gpx4* mRNA. Guanine-rich sequence-binding factor 1 (Grsf1) is an RNA-binding protein that binds to a 14-base pair G-rich element sequence [65]. Grsf1 binds to a specific region in the 5' end of m-*Gpx4* mRNA, and mRNA of both genes was detected in the embryonic brain [66]. siRNA silencing of *Grsf1* results in decreased m-*Gpx4* expression, higher levels of isoprostanes (indicative of increased lipid peroxidation) and cell death, and defects in midbrain and hindbrain development [66]. These findings are corroborated by the results of mouse germline knockout studies. *Gpx4*^{-/-} embryos die at embryonic day 7.5 (E7.5), exhibiting failed gastrulation and very little germ layer differentiation [69]. Whole-body deletion of *Gpx4* deletion in adults is also lethal due to kidney failure, while adult tissue-specific deletion of this gene can result in the loss of neuronal and skin cell viability [18, 70, 71]. Intriguingly, introduction of a mutation causing replacement of the Gpx4 active site selenocysteine with a less-reactive cysteine residue results in enzyme inactivation due to irreversible hyperoxidation; however, this is compatible with normal embryonic development, and animals ultimately die only around postnatal day 15 due to the loss of parvalbumin-positive GABAergic interneurons that leads to fatal seizures [19]. Thus, only minimal Gpx4 activity appears to be required for normal embryonic development, and this enzyme plays a more important role in postnatal cell survival.

Unlike GPX4, deletion of the system x_c^- transport subunit *Slc7a11* in mice does not impair development and has only mild effects on homeostasis in adults [72–74]. One possibility is that the requirement for system x_c^- function to prevent ferroptosis only manifests under tissue culture conditions (e.g., high oxygen) [72]. Another possibility is that deletion of *Slc7a11* results in metabolic compensation that enables these mutant cells to maintain intracellular cysteine and GSH levels by alternative means. For example, loss of system x_c^- function could

lead to upregulation of the transsulfuration pathway, which generates cysteine from methionine. This possibility would be consistent with recent knockout studies that have confirmed extensive plasticity between redox pathways in development [75]. The expression of antioxidant enzymes including GPX1 and superoxide dismutase 1 (SOD1) can vary over time during development, altering sensitivity to brain injury [76, 77]. Whether the expression levels of GPX4, SLC7A11, and other key regulators of ferroptosis per se change over time during development and aging, or in response to slow-onset neurological diseases (e.g., [78]), in a manner that influences ferroptosis sensitivity in different tissues, requires further investigation.

Ferroptosis and Brain Injury

The first report describing ferroptosis linked this process to acute brain injury, establishing the concept that these 2 processes may be related [12]. Exposure of dissected rat organotypic hippocampal slices to a high concentration of extracellular glutamate induced cell death that could be partially (around 50%) suppressed by cotreatment with ferrostatin-1 or cilopirox, consistent with the induction of ferroptosis. Subsequently, improved ferrostatin-1 analogs were tested in an *in vitro* model of periventricular leukomalacia. Previous studies showed that periventricular white matter injury was associated with elevated levels of lipid peroxidation, and that cell death was exacerbated by iron supplementation or *de novo* GSH synthesis inhibition and suppressed by N-acetylcysteine [79–81]; this appeared reminiscent of ferroptosis. Indeed, ferrostatin-1 and its improved analogs could block cell death in this model of oligodendrocyte death [38]. Moreover, the lethality of extracellular glutamate towards cultured oligodendrocytes is suppressed by ferrostatin-1, liproxstatin-1, iron chelators, and other small molecules known to inhibit ferroptosis [82]. Cell death under these conditions is not blocked by the pancaspase inhibitor Z-VAD-fmk or the necroptosis inhibitor necrostatin-1s, and it does not lead to the formation of necrosome complex, further ruling out the involvement of apoptosis and necroptosis, respectively, in this process.

Emerging evidence implicates ferroptosis as a *bona fide* mechanism of acute pathological brain cell death *in vivo*. This was investigated by Tuo et al. [83] in the context of unilateral transient middle cerebral artery occlusion (MCAO). These investigators focused specifically

on the role of tau, amyloid precursor protein (App), and the ferrous iron exporter ferroportin (Fpn) in iron-handling, and how this impacted tissue injury and behavior. In rat and mouse models, MCAO resulted in elevated iron levels in the lesioned hemisphere that correlated with a reduction in iron export. It was hypothesized that this iron accumulation could sensitize to, or perhaps induce, ferroptosis. Consistent with such a model, liproxstatin-1 or ferrostatin-1 treatment limited the infarct size and reduced the behavioral losses occasioned by MCAO, even when administered 6 h after reperfusion. The authors proposed that, through an unknown mechanism, ischemia results in reduced tau protein. This, in turn, leads to the inhibition of App-regulated, Fpn-dependent iron export. Loss of iron export results in intracellular iron accumulation and increased susceptibility to and/or the induction of ferroptosis. Another means of increasing the local concentration of iron at the site of injury could involve an influx of holotransferrin from leaking blood vessels near the damaged area [84]. Interestingly, increasing the levels of apotransferrin in the bloodstream (to lower the percentage transferrin saturation) decreased brain injury and improve neurological outcomes, likely associated with reduced neuronal lipid peroxidation and cell death. Other studies confirm the efficacy of ferroptosis inhibitors in the inhibition of MCAO-induced brain damage but suggest that additional nonapoptotic processes could also be active here [85].

Another process in which ferroptosis has been implicated is brain injury following intracerebral hemorrhage, which results from blood vessel rupture and the leakage of blood into the brain. During this process, iron from hemoglobin found in the blood can be released and cause neuronal damage by enhancing ROS formation [30]. Ferrostatin-1 or liproxstatin-1 can inhibit cell death in the hippocampal region of brain-slice cultures treated directly with hemoglobin or free iron. Mechanistically, exposure to hemoglobin-bound iron leads to GSH depletion and GPX4 inactivation in these hippocampal cells. In an *in vivo* model of collagenase-induced vessel damage, direct injection of ferroptosis inhibitors at or distal to the site of injury reduced the number of damaged cells and the size of the injury, improving the subsequent neurological function of the animals [30]. Protection against intracerebral hemorrhage-associated cell death is also conferred by the cell-permeable cysteine analog, N-acetyl-cysteine (NAC) [46]. Intracellular cysteine can contribute to the production of GSH, and these results therefore indicate that ferroptosis following intracerebral

hemorrhage results from a defect in GSH synthesis (e.g., a loss of cystine uptake).

In a rat model of intracerebral hemorrhage, partial protection against cell death is also conferred by overexpression of Gpx4, whose levels in the brain drop in the damaged area in the immediate aftermath of the injury [86]. Brain injuries involve a complex interplay between different cell types, and, interestingly, bone marrow-derived polymorphonuclear neutrophils (PMNs) that infiltrate the site of damage following intracerebral hemorrhage can be stimulated by interleukin 27 to release lactotransferrin, an iron-binding molecule that can clear free iron from the damaged area, leading to decreased levels of 4-hydroxynonenal (4-HNE), a lipid peroxidation breakdown product, and improved neurological outcomes [87]. Speculatively, but in line with other recent results, this beneficial effect could involve a reduction in the degree of ferroptosis within the damaged area.

Ferroptosis has also been linked to traumatic brain injury. In a rat controlled cortical-impact model, elevated levels of oxidized PE lipid species could be detected 1 h after injury, consistent with the loss of GPX4 function [20]. In a mouse controlled cortical-impact model of traumatic brain injury, a loss of iron-positive cells and morphological evidence consistent with ferroptosis (i.e., shrunken mitochondria) was observed at or near the injury site [31]. Moreover, cell death and the associated behavioral changes were reduced by cotreatment with ferrostatin-1, administered at the time of injury directly into the cerebral ventricles. Other studies of brain compression injury demonstrate an increase in ROS accumulation and cell death that can, likewise, be partially suppressed by direct administration of reduced GSH [88]. This is consistent with other *ex vivo* data that ferroptosis and other cell death processes may act in parallel [12], especially following traumatic brain injury. Indeed, it is possible that previous studies linking brain injury to both apoptosis and nonapoptotic (i.e., necrotic) processes can be rationalized by assuming that each different process (e.g., apoptosis, parthanatos, and ferroptosis) contributes to the death of different cells, depending on the type of stress experienced by a particular region of the brain [89–91].

Ferroptosis and Acute Brain Injury: Reinterpretation of Early Studies

With our new understanding of ferroptosis, it is possible to revisit results obtained previously that further support the notion of ferroptosis as a bona fide mecha-

nism of neuronal cell death occurring after acute brain injury in both the developing and adult brain. For example, free iron accumulates in the ipsilateral hypoxic-ischemic neonatal rat cortex as early as 4 h postinjury [92], and the iron chelator deferoxamine protects from neonatal brain damage sustained after hypoxia-ischemia [93, 94]. Children diagnosed with traumatic brain injury have increased levels of F₂-isoprostane, a biomarker of lipid peroxidation, and decreased levels of the antiapoptotic metabolite GSH [95]. These features are highly suggestive of ferroptosis, but the involvement of this process *per se* remains to be confirmed in this and other models that use highly potent and specific inhibitors such as ferrostatin-1 or liproxstatin-1.

New understanding of small molecule inhibitor effects may also provide evidence for the importance of ferroptosis in brain injury. One of the first small-molecule MEK1/2 inhibitors, U0126, reduces neuronal cell death and brain atrophy in gerbils following MCAO-induced injury [96], as well as glutamate-induced toxicity in HT-22 cells *in vitro* [97]. These effects were attributed to the ability of this compound to block MEK1/2 activity and MAPK pathway activity. Later, U0126 was identified as a potent inhibitor of ferroptosis in cancer cell-based models, and likewise attributed to the inhibition of MEK1/2 activity [27]. However, it is now clear that U0126 can act as a free radical scavenger and that its ability to block ferroptosis is not necessarily linked to the inhibition of MEK1/2 activity [15]. By inference, some of the protective effects of U0126 observed previously in brain injury studies may be attributable to the inhibition of ferroptosis via an off-target antioxidant mechanism.

A similar example is provided by necrostatin-1, a potent inhibitor of RIPK1 kinase activity and the RIPK1-dependent nonapoptotic process of necroptosis. Necrostatin-1 was shown to reduce infarct size in the brain in an MCAO model of stroke in mice [25]. Problematically, at high concentrations necrostatin-1 can inhibit ferroptosis through an unknown mechanism that is independent of RIPK1 [18]. It has been observed that necrostatin-1 potently suppresses glutamate-induced ROS accumulation and cell death in HT-22 cells, but only when used at concentrations far above what is needed to inhibit RIPK1 activity [98, 99]. Moreover, studies that have directly compared necrostatin-1 to an improved analog, necrostatin-1s, have shown that only the parent compound possesses the ability to suppress cell death under ferroptosis-inducing conditions in brain cells [82]. Thus, it is possible that some of the pro-

tective effects of the original parent compound observed previously (e.g., [25, 100]) may be attributed to the inhibition of ferroptosis. Likewise, as noted above, many putative small-molecule lipoxygenase inhibitors prevent ferroptosis by acting directly as radical-trapping antioxidants, rather than through effects on LOX enzyme function, at least in vitro [47]. The above considerations do not invalidate the previous results; MAPK pathway activity, RIPK1-dependent necroptosis, and LOX enzyme activity could all contribute to neuronal injury in vivo. However, the potential contribution of these processes to ferroptosis must also be considered in the light of new information about how these inhibitors can function as antioxidants.

Ferroptosis and Degenerative Brain Injury

In addition to acute brain injury, ferroptosis is being linked to the cell death observed in a number of degenerative conditions. There are well-established links between iron and neurodegeneration [101, 102], which likely make these conditions fertile ground in the search for the occurrence of ferroptosis in vivo. For example, overexpression of a pathogenic Huntington (Htt) protein fragment in cultured rat corticostriatal brain slices can trigger cell death that is prevented by cotreatment with ferrostatin-1 [38]. Likewise, dopaminergic cell death in vitro and in vivo caused by exposure to the neurotoxin, 1-methyl-4-phenylpyridinium (MPP+), which produces a Parkinson's-like phenotype, is blocked by ferrostatin-1 and the iron chelator deferiprone [78, 103]. Links between ferroptosis and Alzheimer's disease may also exist, given the known associations between this disease and increased iron levels, and the protective effects of certain molecules that can act as antioxidants (e.g., lipoic acid) against disease progression [104, 105]. However, definitive evidence linking neurodegeneration to ferroptosis in long-term animal model studies is currently lacking.

Frontiers in the Investigation of Ferroptosis and Brain Injury

The vast majority of studies characterizing the biochemical mechanisms of ferroptosis have been performed in cultured cancer cells or MEFs. Even here, basic questions concerning the biochemical regulation of ferroptosis remain. For example, in addition to the key role of GPX4, a second endogenous antioxidant, the metabolite coenzyme Q₁₀, is now thought to be required to suppress

lipid ROS accumulation and the onset of ferroptosis [16, 36]. Does coenzyme Q₁₀ act in parallel to GPX4, or are these functions linked in some way? Intriguingly, administration of coenzyme Q₁₀ markedly improved outcomes in a rat MCAO model of stroke [106]. This protective effect was attributed to the inhibition of apoptosis, but it could be worth considering whether this metabolite prevents brain injury by inhibiting ferroptosis. Two related areas where greater mechanistic clarity is required concern the role of different LOX enzymes in promoting ferroptosis and the specific oxidized lipid species that are generated by LOX enzymes and other processes during ferroptosis. There is evidence from cancer cell models that not all ferroptotic triggers cause the same type of oxidative lipid damage [44], and it might be that different cells respond to the same proferroptotic stimulus with unique patterns of lipid oxidation. How different endogenous (e.g., GPX4 and coenzyme Q₁₀) and exogenous (e.g., ferrostatin-1 and liproxstatin-1) inhibitors interfere with the production and composition of different oxidized lipid species in brain tissues is also unknown. For all studies conducted using cultured cells, it will also be important to consider whether the composition of the growth medium itself can alter ferroptosis sensitivity [107].

Animal studies investigating the link between ferroptosis and brain injury typically employ inhibitors (e.g., ferrostatins) to block cell death, but have not yet elucidated the specific signals within the complex environment of the brain that actually cause the induction of ferroptosis; in many cases, even the specific class of brain cells that is spared by ferroptosis inhibitors remains unknown. Thus, a fundamental area of future investigation concerns the biochemical regulation of the ferroptotic process in brain cells. It is possible that ferroptosis will exhibit greater regulatory complexity in brain cells or interact with other brain-specific cell death pathways in a way that would not be anticipated from results obtained in cancer cell studies of this process. Studies of the related process of oxytosis already suggest that, in neurons, ferroptosis may exhibit greater regulatory complexity (as described above). However, other complexities are likely to exist due to the unique structure and function of brain cells. NMDA receptor stimulation can induce a molecular cascade that leads to increased neuronal iron uptake via the transferrin receptor and the divalent metal transporter 1 (DMT1), and iron chelation attenuates NMDA-induced excitotoxic neuronal death [108–110]. Could NMDA receptor stimulation-dependent iron uptake sensitize neurons to ferroptosis, or could an increased iron

uptake itself even be sufficient to trigger ferroptosis in parallel to classic Ca^{2+} -mediated excitotoxicity? If so, this could provoke a re-examination of existing studies of NMDA-receptor mediated neuronal damage, to ascertain whether this involves the induction of ferroptosis. Similarly, links between oxytosis and PARP1-mediated parthanatos may also exist. The execution of both oxytosis and parthanatos is reported to involve the translocation of AIF from mitochondria to the nucleus to trigger DNA degradation and cell death. This implies that, at least in certain brain cells, ferroptosis/oxytosis and parthanatos may converge at the level of AIF, leading to the induction of cell death through a common terminal effector. If so, therapies that target this particular step (i.e., AIF translocation or function) may prove especially useful at blocking pathological brain cell death in cases where both processes are implicated, such as stroke [9–11]. Whether ferroptosis and parthanatos may act in parallel, or in different cell types in response to different configurations of gene expression and ischemia-reperfusion exposure, is an interesting question for future studies.

Another area where progress is required is in the identification of useful molecular markers of ferroptosis that can be employed in vivo or in ex vivo samples. Studies of apoptosis have long benefitted from the ability to detect cleaved caspase-3 or cleaved PARP as biochemical markers of this process in tissue sections or lysates from damaged brain [111]. Likewise, detection of phosphorylated mixed-lineage kinase domain-like (MLKL) can be used as a molecular marker for execution of necroptosis in various pathological samples [7, 112]. Analogous protein-based molecular markers do not exist for ferroptosis. Fundamentally, this may reflect the fact that the execution of ferroptosis does not appear to require the upregulation or posttranslational modification of any one particular protein that could be the target of a specific antibody, or the surface exposure of a new antigen that could be detected by a specific reagent (e.g., Annexin V), although further investigation is required. To date, the best available markers of ferroptosis are those based on altered gene expressions that occur as a result of ferroptosis. Cysteine deprivation downstream of system x_c^- inhibition is sensed by the canonical GCN2/ATF4 pathway, leading to transcriptional upregulation of a battery of ATF4 target genes, including *CHAC1* [32]. However, *CHAC1* mRNA upregulation is not universal during ferroptosis (e.g., it is not upregulated by direct GPX4 inhibition). Moreover, *CHAC1* upregulation is not linked to the execution of ferroptosis in the same way that PARP cleavage or MLKL phos-

phorylation are uniquely and mechanistically linked to the execution of apoptosis and necroptosis, respectively. Indeed, *CHAC1* levels are increased by diverse ER stresses that cause ATF4 activation, including the inhibition of glycosylation, membrane vesicle transport, and intracellular calcium handling [113, 114]. An alternative gene expression marker of ferroptosis, *PTGES2*, may be a broader indicator of the induction of ferroptosis whose expression is increased downstream of either system x_c^- blockade or direct GPX4 inhibition [35]. However, it is unclear whether *PTGES2* is specific for ferroptosis or whether this marker would be useful in the context of brain injury.

The optimal molecular marker of ferroptosis in vivo would be linked to the unique features of this cell death program. The best candidate in this connection may be increased levels of PUFA oxidation and membrane lipid ROS. PL oxidation can be detected in brain samples following injury [115], and methods have been developed to perform similar oxylipidomic analyses of cultured cells and animal tissues undergoing ferroptosis due to Gpx4 inactivation or in response to certain injuries (e.g., controlled cortical impact) [20, 39]. It is possible that these methods could be applied to samples obtained from the brain under other pathological conditions and examined for patterns of PL oxidation that are known to be characteristic of ferroptosis. Whether ferroptosis results in a unique signature of PL oxidation has not been determined. Furthermore, associating the pattern of lipid oxidation to a specific cell type is currently beyond the technical reach of this method. A final practical consideration is that oxylipidomic methods themselves are highly technical and likely not practical for most investigators to implement independently or routinely.

Another approach is to examine the accumulation of lipid ROS directly. This could potentially be performed in live samples using the probe C11 BODIPY 581/591 (e.g., using a 2-photon microscope or intravital microscopy), or via the administration of a less specific ROS-reactive probe such as Amplex Red [88, 116]. In fixed tissues or lysates, it may be possible to detect 4-HNE or other lipid breakdown products that can modify intracellular proteins to generate a suitable epitope [37, 54, 117, 118], but it is not known if these products are formed during ferroptosis in vivo, and, even if they are, whether they are specific for this process. New chemical probes that report on oxidative damage and are suitable for use in fixed tissues are urgently needed. Currently, functional inhibitor studies demonstrating that a cell death event is blocked by cotreatment with a

ferroptosis-specific inhibitor are the sole route to confirming the existence of a ferroptotic phenotype *in vivo* [119].

A final area where progress is required concerns the development of small-molecule ferroptosis inhibitors suitable for advancement to human clinical trials. Ferrostatin-1 is not considered ideal for most *in vivo* and *ex vivo* studies due to its poor stability. This has not prevented some investigators from having success with this molecule, both in *ex vivo* slices and when injecting directly at or relatively near the site of brain damage (e.g., intranasally) [12, 30, 38, 83]. Nonetheless, improved analogs of ferrostatin-1 that exhibit better metabolic stability *in vivo* have demonstrated utility in blocking pathological cell death in animal models of kidney disease, when administered systemically [14, 38]. Whether these molecules or other derivatives of the ferrostatin core scaffold effectively penetrate the blood-brain barrier and/or will be suitable for development into true clinical candidates for use in humans, is presently unclear, despite the promising results of animal studies [14, 120]. A second distinct class of radical-trapping antioxidant inhibitor is liproxstatin-1. Like improved ferrostatin analogs, liproxstatin-1 has demonstrated antiferroptotic activity *in vivo* in several animal models [18, 30] and, like ferrostatin-1, it is widely available commercially. Again, however, like the ferrostatin series, it is not clear whether liproxstatin-1 can or will be developed further for human use.

A more speculative therapeutic direction could be to directly modulate lipid metabolism, with the aim of preventing lipid ROS accumulation in the first place. Small molecules capable of inhibiting ACSL4, an enzyme required for the activation of PUFAs that are subsequently oxidized during ferroptosis, include the natural product triacsin C and synthetic thiazolidinediones such as rosiglitazone [121]. It is predicted that these would reduce the PUFA load and therefore the “oxidizability” of membrane PLs. Additional inhibitors of ACSL4 could be sought. Alternatively, a potentially distinct approach was established by experiments showing that certain dietary MUFAs such as oleic acid can suppress ferroptosis, at least in cancer cell models *in vitro* (Magtanong et al. [128]). Given that time would be required for exogenous MUFAs to alter membrane lipid composition, it is unclear whether MUFA treatment would ever be useful in the setting of acute injury. Intriguingly, however, increased MUFA intake may reduce the risk of hemorrhagic but not ischemic stroke [122].

In this review, we have outlined our understanding of the ferroptosis pathway, derived mostly from studies con-

ducted on nonneuronal model systems, and describe the contexts in which these may be most applicable to brain injury. Ferroptosis may also contribute to pathological cell death in neurodegenerative processes such as Huntington’s disease, Parkinson’s disease, and, potentially, degenerative brain conditions characterized by elevated levels of iron [38, 78, 123, 124]. How ferroptosis is triggered at the molecular level in different acute and chronic neurodegenerative disorders, and whether the regulation of ferroptosis might differ according to brain cell type, age, or other factors, remains to be elucidated. To answer this question, it will be essential to develop ways to identify the individual cells that undergo ferroptosis in the brain.

Given the successes observed in animal models of brain injury, major questions also now lie in the realm of immediate clinical application. Should new clinical trials be initiated to test the concept of ferroptosis inhibitors as key modulators of pathological cell death during brain injury? Could earlier trials be reinterpreted in the light of our new mechanistic understanding of this pathway? There is a long history of developing iron chelators and antioxidant small molecules as therapies for brain injury with varied success (reviewed in [117, 118, 125–127]). New approaches, incorporating our past knowledge of antioxidant function into our growing mechanistic knowledge of ferroptosis, may aid in the identification or development of compounds that lead to better ways of specifically blocking iron-dependent ROS accumulation *in vivo*. While many hurdles remain, the study of this process has the potential to lead to a new understanding of brain injury and new routes to effective treatment.

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Statement of Ethics

The authors have no ethical conflicts to disclose.

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