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# Does iron loading of oxygen-sensing prolyl hydroxylases rather than random Fenton-driven radical formation drive programmed ferroptosis and degeneration in neurological diseases?

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Cellular oxygen sensors are not only sensitive to oxygen but also to iron levels. Accordingly, they are ideally positioned to serve important roles not only in oxygen sensing but also in iron homeostasis and dyshomeostasis. Here, I review the literature which supports the notion that iron is essential for neurodegeneration including stroke, Alzheimer's disease, Parkinson's disease, and Neurodegenerations with Brain Iron Accumulation via its ability to optimize activities of oxygen-sensing, hypoxia inducible factor (HIF) prolyl hydroxylases. In this context, HIF PHDs act to drive prodeath transcription via the leucine zipper transcription factor ATF4, and not via decreases in HIF stability. I also discuss evidence which suggests that oxidative stress leads to cell death via a programmed pathway of ferroptosis that is unlikely to involve random, non-enzymatic, Fenton-mediated radical formation. Altogether, this evolving model broadens our view of how oxygen sensors and iron can regulate cell degenerations in neurological diseases under normoxic or hypoxic conditions.

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## Introduction: the origins of oxygen and iron homeostasis

Evolutionary changes in the earth's atmosphere and gas content of its oceans provided enormous pressure for organisms to utilize oxygen. The end result was the evolution of bacteria with the capacity to convert oxygen into energy via its role as a terminal electron acceptor. These bacteria eventually gave rise to mitochondria, the powerhouse of the eukaryotic cell. As oxygen became a critical energy source for unicellular and multi-cellular organisms, homeostatic mechanisms involving enzymes with oxygen sensing capacities evolved to allow these cells to adapt to local and global changes in oxygen tension [1]. The identity of these oxygen sensors, the HIF prolyl hydroxylases is now well established [2]. What is less appreciated, is the notion that these sensors might have evolved not only to sense oxygen but also to function as sensors of iron. The reason for this speculation is that metals were abundant in primordial oceans, and so as oxygen availability increased, mechanisms for leveraging the reactivity of oxygen with iron evolved, and similarly mechanisms for sensing and adapting to cellular iron overload conditions also likely developed. In this piece, I will discuss the evidence that suggests an exciting, novel dual role for oxygen sensing, HIF prolyl hydroxylases also as iron sensors under normoxia, and the implications of this sensing for regulation of a programmed formed of cell death in neurons, now known as ferroptosis.

## Iron homeostasis is disrupted in sporadic and genetic neurological conditions

Neuropathological studies in rodent models of neurological disease and in human brains have revealed disruptions in iron homeostasis in many conditions including stroke [3], traumatic brain injury [4], spinal cord injury [5], Alzheimer's disease [6], multiple sclerosis [7] and Parkinson's disease [8]. Moreover, a group of genetic disorders exist that are now referred to as Neurodegenerations with Brain Iron Accumulations and are characterized by genetic mutations leading to iron deposition in the basal ganglia, axonal spheroids, Parkinsonism, and intellectual deterioration [9]. The effectiveness of global chelators of iron in reducing pathology and improving functional outcomes in sporadic neurological conditions as well as those driven by genetic mutations suggests that changes in iron homeostasis following injury in these conditions are causally related to disease pathogenesis [10]. Despite this compelling and suggestive data supporting a role for iron in neurodegenerations, the precise mechanism of how iron catalyzes cell death in a host of conditions has remained unclear.

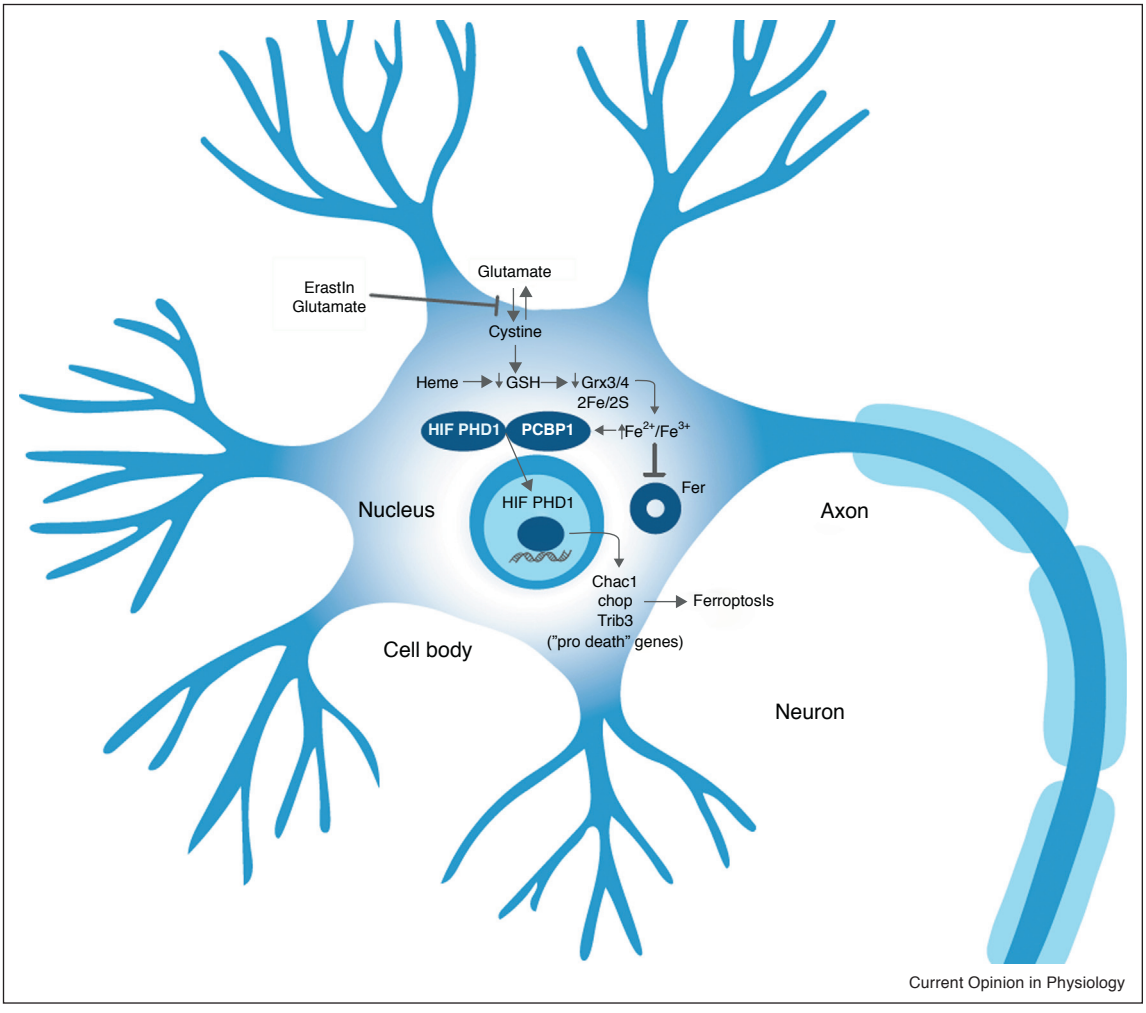
## Fenton Chemistry mediated neuronal damage: dogma without data?

The dominant theory for how excess and presumably free iron leads to neuronal dysfunction and degeneration involves Fenton Chemistry [11]. In classical Fenton reaction schemes purported to mediate damage, cytosolic

peroxide or lipid peroxides interact with reduced iron to produce highly reactive hydroxyl radicals, peroxyl or alkoxyl radicals [12••]. There are at least two conceptual problems with hydroxyl, peroxyl and alkoxyl radicals as pervasive toxins. First, the concentrations of exogenously added hydrogen peroxide required to induce cell death in neuronal systems are often in the high micromolar to millimolar range. To achieve these levels of peroxide would require almost all of cellular oxygen to be converted to peroxide. There is little evidence to support the notion that all oxygen is fed into the production of peroxide in neuronal cell death paradigms. Indeed, the best estimates of cellular peroxide levels based on classical and more recent methods run everywhere from the nanomolar to the low micromolar range [13], while concentrations of lipid peroxides that exist in cultured neurons or those in the brain following injuries have not been measured dynamically in living cells or in tissue, the precise physiological concentrations that would be required to trigger death by reactive lipids are unclear [14] (Figure 1).

A second problem with a model in which iron generates indiscriminately reactive electrophiles emerges from our recent understanding of an iron-dependent form of programmed cell death called ferroptosis. The term ferroptosis was coined by Dixon *et al.* as result of their experiments to evaluate the cytotoxic effects of a small molecule called erastin [15]. Their elegant work revealed that erastin induces tumor cell death by inhibiting a plasma membrane antiporter which exchanges glutamate in return for cystine (the Xc-transporter). Inhibition of the transporter by erastin or glutamate leads to depletion of cellular glutathione and oxidative stress [16]. The resulting cell death from erastin or glutamate is caspase-independent and therefore not classical apoptosis. However, chemical biology studies revealed that erastin-induced death in KRAS mutant cancer cells was abrogated by

**Figure 1**




Ferroptosis.

agents that increased glutathione, small molecules that bound iron, inhibitors of 12/15 lipoxygenase and selective inhibitors of the ERK signaling pathway [15]. Indeed, ERK inhibitors completely rescued death arguing that under conditions of depletion of the major cellular antioxidant glutathione, unopposed oxidants trigger programmed cell death which is completely dependent on aberrant signaling [17••,18]. Depletion of glutathione in neurons, labeled incorrectly in the early 90s by the author of this review as apoptosis [19], had been previously shown to be inhibited by small molecules that bind iron [10], as well as dependent on ERK signaling [17••]. More relevant to the arguments against Fenton Chemistry, glutathione depletion-induced ferroptosis in neurons was shown to be inhibited by global inhibitors of transcription [19]. Subsequent studies identified the leucine zipper transcription factor ATF4 as the major driver of greater than 90% of the genes induced by glutathione depletion and necessary for ferroptosis in neurons [20]. More recent unpublished studies from our lab indicate that biomarkers of oxidative damage (e.g. protein carbonylation) occur transiently at the time of cell death commitment or well past cell death commitment (e.g. oxidative DNA damage). Accordingly, it is difficult to imagine a scheme in which iron, acting non-specifically to generate hydroxyl radicals could trigger a methodically executed, controlled cell death process involving the prodeath transcription factor ATF4 and the upregulation of prodeath genes including Chop (CEBp homologues protein, linked to ER-stress induced death), Trib3 (Tribbles 3, a pseudokinase inhibitor of Akt), and Chac1 (an enzyme that degrades glutathione) [20].

## An alternate model for ferroptotic death in neurons: iron loading of prodeath oxygen sensors not non-enzymatic Fenton Chemistry

In this review, we propose an alternate model for how excess iron is sensed and it drives ferroptosis: loading of iron-dependent oxygen sensors, the HIF prolyl hydroxylases. The origins of the model come not from studies of oxygen sensing *per se*, but rather an interest, more than 25 years ago, to understand how chelators of iron protect neurons. As mentioned, exposure of cultured immature neurons to glutamate (before they develop functional glutamate receptors) leads to inhibition of the cystine–glutamate transport, depletion of intracellular cyst(e)ine, and a 50% drop in cellular concentrations of glutathione. Glutathione, a tripeptide composed of glycine, cysteine, and glutamate, is believed to be a versatile antioxidant, in that, it can act with glutathione reductase to maintain protein thiols in their reduced state; it can act with glutathione peroxidases to reduce lipid and hydroperoxides; it can also act with glutathione transferases to detoxify xenobiotics and DNA hydroperoxides. Accordingly, immature neurons exposed to glutamate leading to glutathione depletion operationally represents an *in vitro* model of oxidative stress. Oxidative stress is classically defined as an imbalance of cellular oxidants and cellular antioxidant defenses in favor of oxidants. The model holds a significant advantage, in that, oxidative stress occurs from 6 to 16 hours after the addition of an inhibitor of cystine uptake, but the cells can be completely rescued, allowing events that are causal for death to be distinguished from those that are a consequence. We used this model to understand how iron chelation abrogates oxidative stress. Not unexpectedly, we found that chelators of iron, deferoxamine and the plant amino acid, mimosine, while structurally diverse, could both abrogate ferroptotic death [10]. As expected, loading of deferoxamine or mimosine with iron reversed their protective effect. Throughout these studies we measured cell death by monitoring the level of LDH released into the medium as an index of whether the plasma membrane was breached much like what is done when cardiac enzymes are measured to detect myocardial infarction. What was entirely unexpected is that despite being protected as measured by use of a live and dead cell stain, cells treated with iron chelators had a 400% increase in their LDH activity levels. As a result of the collaborative help of Gregg Semenza, we were able to subsequently demonstrate the protective effects of iron chelators against ferroptosis were correlated with activation of the transcriptional activator hypoxia inducible factor-1 (HIF1) and the induction of LDH (a glycolytic enzyme), as well as other glycolytic enzymes, erythropoietin, and VEGF [10]. Subsequent studies that followed the identification by the Kaelin and Ratcliffe laboratories of the HIF prolyl hydroxylases as iron, 2-oxoglutarate, and oxygen dependent oxygen sensors [2], showed that HIF PHDs were the target for protection from ferroptosis by iron chelation in neurons [21]. Indeed, small molecules that did not bind iron, but that inhibited the HIF PHDs also blocked oxidative death; moreover, molecular deletion of HIF PHDs *in vivo* abrogated cell death and behavioral dysfunction related to brain hemorrhage (which leads to ferroptotic death) [22]. While we originally assumed that protection was related to activation of HIF-dependent gene expression, subsequent, unbiased transcriptomic studies showed the protective effects of a selective inhibitor of the HIF PHDs, adaptaquin, were correlated with suppression of prodeath transcription via ATF4, not prosurvival signaling via HIF1. Indeed, higher concentration of adaptaquin were able to induce HIF target genes but those did not correlate highly with neuroprotection. In this scheme, HIF PHDs act as a transcriptional 'coactivator' and bind ATF4 in the nucleus to hydroxylate its prolines and drive prodeath transcription. Indeed, mutation of five conserved prolines in ATF4 abrogated hydroxylation of ATF4's prodeath capacities in neurons. As mutating five prolines represents a significant structural change to any protein, more precise and nuanced mutations are required to fully evaluate this model [22].

## HIF PHDs as iron sensors that drive ferroptotic death

A common feature of ferroptotic stimuli is their ability to inhibit cystine transport leading to glutathione depletion and oxidative stress. A critical but as yet, unanswered question is whether glutathione depletion leads to an increase or redistribution of iron to enzymes like the HIF PHDs or alternatively, it simply requires the iron-dependent HIF PHDs to mediate death in the absence of any changes in iron levels.

Several lines of investigation favor that notion that glutathione depletion drives a change in iron homeostasis during ferroptosis, and this change could be sensed as increased activity of the HIF PHDs. First, canonical iron overload models in which cells in culture are exposed to heme (iron complexed with the tetrapyrrole, protoporhyrin IX) lead to increases in iron and ferroptotic death of neurons [23]; preliminary studies from our lab indicate that heme exposure augments HIF PHD activity and diminishes the activity of these enzymes to convert decreases in oxygen into HIF stability. These observations suggest that the HIF PHDs exist in two forms, an 'apo' form devoid of iron but ready to sense and be loaded with iron, and an iron-replete form that is ready to sense oxygen.

A second line of investigation that supports the notion that HIF PHDs could function as iron sensors is the discovery by the Philpott and Bruick labs in 2011 of the iron chaperone of the HIF PHDs, poly (rC) binding protein 1 (PCBP1) [24]. Until these important studies were published, little information was available as to how iron is transferred from ferritin, the cytosolic depot for iron, to distinct metalloenzymes. They showed that under conditions of Iron deficiency, loss of PCBP1 leads demetallation of HIF PHD2, diminished hydroxylation of HIF1 and augmentation of HIF dependent genes. By contrast, under conditions of iron deficiency, incubation of iron loaded PCBP1 to lysates restored HIF PHD activity. Together, these data suggest that PCBP1 transduces changes in iron availability into changes in HIF PHD activity. A question that remains unanswered is given the known molecular similarity in protein motifs of ferritin or HIF PHD2 that receive iron from PCBP1, how does the cell decide whether to load ferritin or to load the HIF PHDs? Also, the studies by the Philpott team focused on the interactions between PCBP1 and HIF PHD2. HIF PHD2 can be cytosplasmic or nuclear, but what about HIF PHD1, which is primarily nuclear and which we have shown can regulate cell death and ATF4 activity [25,22]. Does HIF PHD1 use PCBP1 as well as an iron chaperone? Despite these unanswered questions, the model suggests that under conditions of iron overload or iron redistribution that the cells ability to synthesize new ferritin is overwhelmed, and so free iron is targeted to PCBP1 where it loads the HIF PHDs. Increased activity of HIF PHD1 leads to increased hydroxylation of ATF4 and enhanced transcription of prodeath genes [22].

A third line of investigation that supports the notion that ferroptosis associated with GSH depletion leads to disruptions in iron homeostasis; and that changes in iron that lead to death are independent of iron-dependent production of oxidants (Fenton Chemistry) are from the labs of Toledano, Muhlenhoff, Lillig and Lill [26••,27••]. They elegantly showed using yeast mutant strains that GSH participates with Glutaredoxin 3 and 4 (Grx 3/4) to maintain an iron sulfur cluster motif (2Fe-2S) in Grx 3 or Grx 4 which is critical for delivery of iron into a host of iron-dependent enzymes (e.g. iron sulfur cluster proteins and heme). The absence of reduced glutathione or glutaredoxin3/4 leads to decreased incorporation of iron to target proteins and a significant increase in cellular iron and cell death. Cell death cannot be attributed in this circumstance to oxidants as removing oxygen has no effect on viability; second yeast with reduced Grx3 and Grx4 have no classical signs of oxidative stress. For example, Grx3 and 4 deficiency leads to an accumulation of reduced not oxidized glutathione. According to this scheme, depletion of GSH leads to decreased activity of monothiol Grx3 and 4 activity because the iron sulfur cluster formed by bringing two GRX 3 or 4 monomers together cannot be assembled. Diminished Grx3 or GRx4 2Fe-2S formation leads to decreased iron incorporation in these cells and an increase in cytosolic iron [28]. In our as yet untested model, this increased cytosolic iron would be incorporated into PCBP1 and delivered to HIF PHD1. Enhanced Nuclear HIF PHD1 activity would then result in hydroxylation of ATF4 to drive prodeath transcription.

## Conclusion

Iron dyshomeostasis has been linked to almost all sporadic and some inherited neurological conditions (e.g. Neurodegeneration with Brain Iron Accumulation). The ability of broad and non-selective chelators of iron to reduce cell death and improve behavioral recovery in rodent models of these conditions supports the notion that iron could be causally related to cell death. Most models of iron-mediated injury to neurons invoke Fenton-mediated generation of highly reactive hydroxyl or alkoxyl radicals. Here, we offer a different model based on converging lines of inquiry from a number of groups. In this model, depletion of reduced glutathione leads to decreased activity of monothiol glutaredoxins and diminished incorporation of iron into target metalloproteins, which are abundant in the cell. The increased 'available' iron is diverted to the iron chaperone PCBP1 to load existing 'apo' forms of oxygen-sensing, HIF PHDs in the cytoplasm and nucleus. Increased HIF PHD1 activity leads to enhanced transcription of ATF4 dependent prodeath genes and induction of ferroptotic death.

## Conflict of interest statement

RRR serves on the SAB for Neuronasal, Inc., a biotech company that has licensed intellectual property from Cornell developed in the Ratan Laboratory related to Ferroptosis inhibition and hypoxia inducible factor prolyl hydroxylase inhibition.

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