Iron and glutathione at the crossroad of redox metabolism in neurons

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ABSTRACT

Neurons, as non-dividing cells, encounter a myriad of stressful conditions throughout their lifespan. In particular, there is increasing evidence that iron progressively accumulates in the brain with age and that iron-induced oxidative stress is the cause of several forms of neurodegeneration. Here, we review recent evidence that gives support to the following notions: 1) neuronal iron accumulation leads to oxidative stress and cell death; 2) neuronal survival to iron accumulation associates with decreased expression of the iron import transporter DMT1 and increased expression of the efflux transporter IREG1; and 3) the adaptive process of neurons towards iron-induced oxidative stress includes a marked increase in both the expression of the catalytic subunit of gamma glutamate-cysteine ligase and glutathione. These findings may help to understand aging-related neurodegeneration hallmarks: oxidative damage, functional impairment and cell death.

Key terms: neurons, iron metabolism, iron accumulation, oxidative stress, glutathione metabolism, neurodegeneration

The Janus face of iron in cell function: A link to neurodegeneration

Iron, in virtue of its ability to participate directly as a donor or acceptor in electron-transfer reactions, is an essential trace element for cell function. This property makes iron the most common cofactor within the oxygen handling biological machinery (4, 8). Additional roles for iron have been described in neurons, such as its participation in myelin synthesis (10) and neurotransmitter metabolism (30). However, the very property that enables iron to participate in oxygen metabolism explains its potential damaging effects: if not handled properly by the cell, iron interacts with molecular oxygen, generating reactive oxygen species (ROS) through Haber-Weiss and Fenton reactions (18). As reviewed elsewhere, uncontrolled ROS production leads to oxidative damage of cellular components, a condition termed ‘oxidative stress’ (14).

Recent evidence highlights the relevance of iron metabolism in neurodegeneration. Some genetic neurodegenerative diseases have been linked to mutations in the ferritin light chain gene (9) and the pantothenate kinase gene (20), demonstrating a direct relationship between mutations in iron handling proteins and neurodegenerative processes. Similarly, postmortem studies have shown increased iron accumulation in the substantia nigra pars compacta (SNpc) of Parkinson disease (PD) patients (16). With age, iron accumulates in redox-sensitive tissues such as substantia nigra even in normal individuals (42) (Fig. 1). Additionally, age-related increases in the iron content of different areas of the brain were demonstrated in rat and monkey (19, 36).

Mounting evidence indicates that neurons from Alzheimer disease (AD) brains are subjected to a high oxidative load (6). In addition, in PD models a link between iron deregulation and oxidative...
stress has been suggested (3). Data from our group indicate that iron accumulation leads to increase oxidative stress and cell death in cultured neuroblastoma cells and hippocampal neurons (2) (Fig. 2). All together, these findings suggest that iron accumulates and causes oxidative damage in selected brain areas. In the following sections, we review the available information about iron metabolism and iron-related oxidative stress in neuronal tissue. We also describe evidence obtained by our group suggesting a link between iron and glutathione metabolism.

Iron accumulation: Roles of the iron transporters DMT1 and Ireg1

The activities of the iron transporters DMT1 and Ireg1 (also termed ferroportin 1 and MTP1) determine iron movements into or out of cells. DMT1 mediates iron transport into cells by an electrogenic mechanism that involves the co-transport of Fe$^{2+}$ and H$^+$ (2). Although DMT1 mRNA expression in neuronal tissue is down regulated by an increase in cellular iron content (2, 3, 17, 39; reviewed in 25), there is no agreement about the specific mechanisms responsible for this regulation. DMT1 gene transcription produces four isoforms by alternative splicing of the 5'-end exons [exons 1A or 1B] and of the 3'-end exons [exons 16 (or +IRE) or 16A (or -IRE)] (21). Expression of the +1A/+IRE isoform is particularly sensitive to cell iron levels, but the 1A/-IRE isoform also responds to iron changes, whereas the 1B/+IRE and 1B/-IRE isoforms do not respond. Thus, it is possible that the regulation of DMT1 expression involves two regulatory processes, one mediated by exon 1A, probably of transcriptional origin, and another mediated by exon 16 (+IRE), probably of transductional origin.

![Figure 1](image-url)
Figure 2. Iron-induced oxidative stress and cell death. A: iron accumulation induces ROS. SH-SY5Y cells were grown in glass cover slips for 8 days in standard 5 µM Fe media after which they were challenged for 2 days with 1.5, 5, 10, 40 or 80 µM iron. The levels of ROS were determined by measuring 2’,7’-dichlorofluorescein (DCF) fluorescence (34). Stabilization in cell ROS was apparent at high iron concentrations, evidencing an adaptive response (adapted from reference 31, with permission from the publisher). B: iron-induced cell death. SH-SY5Y cells were grown in 96-well plates for 8 days in standard 5 µM Fe media as described in the Methods, after which they were challenged with varied concentrations of iron and the culture continued for 2 days. Cell viability at the end of the culture period was determined by the MTT method. Each individual point was done in triplicates. Shown is one of three similar experiments. Decay in cell viability was apparent with both increased iron concentration and with time of incubation (adapted from reference 31).
Ireg1 is the only member of the SLC40 family of transporters and the first reported protein that mediates the exit of iron from cells. Structural modeling indicates an Ireg1 membrane topology of nine transmembrane domains with a cytoplasmic N-terminal domain and an extracellular C-terminal region (reviewed in 26). This protein is expressed mainly in enterocytes and macrophages, but the presence of Ireg1 also has been described in neurons, glioma cells and astrocytes (5, 13, 22). In enterocytes, Ireg1 is responsible for iron efflux during the process of intestinal iron absorption, while in Kupffer cells Ireg1 mediates iron export for reutilization by the bone marrow (11). The mechanism underlying regulation of Ireg1 expression is unknown, but it is clearly cell-specific; in enterocytes, iron deficiency induces Ireg1 expression (27), whereas in macrophages iron deficiency decreases Ireg1 expression (7). In a recent study, we reported that SH-SY5Y neuroblastoma that survived conditions of iron overload display an adaptive response consisting of decreased synthesis of DMT1 and increased synthesis of Ireg1 (2) (Fig. 3). Ireg1 expression correlates with iron content in SH-SY5Y and hippocampal cells; similarly, there is a correlation between Ireg1 expression and the rate of iron efflux (2). Thus, one of the clues of neuronal survival to iron overload seems to be the coordinated regulation of both influx and efflux iron transporters.

Glutathione: The main antioxidant molecule in neurons

Neuronal antioxidant defenses rely mainly on the cellular levels of reduced glutathione (GSH) (15, 28). Elevated GSH levels in hippocampus and midbrain were reported in AD (1), an indication that AD neurons may be over-reacting to an oxidative load. Similarly, decreased activity of antioxidant enzymes occurs in AD brains (32), an indication that the normal handling of GSH may be altered in these cells. A 30-40% decrease in GSH concentrations, without a corresponding increase in the levels of oxidized GSH (GSSG), was reported in PD brains (33). In addition, GSH levels in PD are specifically decreased in SNpc without a concomitant increase in the levels of GSSG (37), and the decrease in GSH content correlates with the severity of neurodegeneration (34). Thus, substantial evidence points to profound changes in GSH metabolism in neurodegenerative processes.

Figure 3. Regulation of iron transporters DMT1 and Ireg1 by iron accumulation. DMT1 and Ireg1 from SH-SY5Y cells cultured for 2 days with different iron concentrations were determined by Western blot. A decrease in DMT1 expression and an increase in Ireg1 expression was observed in the 1.5-80 µM Fe range. In Ireg1 determination, bands of 65.3 and 122.1 KDa, corresponding to putative monomers and dimers, were evident (adapted from reference 3).
Progressive iron accumulation induces a biphasic change in GSH levels

SH-SY5Y cells cultured in 1.5 μM iron present basal GSH levels, but increasing iron from 1.5 to 5 μM results in a marked increase in GSH levels (Fig. 4). Increased GSH levels are due to increased synthesis, as determined by the inhibitory effect of the GSH synthesis inhibitor L-buthionine sulfoximine (31). Further increases in iron concentration lead to significant reductions in GSH content. Thus, iron accumulation sequentially induces an increase and then a decrease in GSH content. Since treatment of cells with iron in the 40-80 μM range induces significant cell death (31), the adaptation of cells surviving a high iron offer was further studied. To that end, cells that survived the initial onslaught caused by high iron offer were kept in culture for two additional days prior to GSH evaluation. Under these conditions, the cells presented important increases in GSH levels at 40 and 80 μM Fe in the culture media (Fig. 4). This increase in GSH levels in surviving cells can be viewed as an adaptive response to iron-induced oxidative stress and underlines the particular relevance of GSH in the antioxidant response.

Neuronal oxidative balance must be regulated; therefore, compensatory mechanisms are expected to surge upon oxidative stress. In the case of AD, this is not entirely clear since both a decrease (24) and an increase (7) in GSH content have been reported. Since under our conditions increased GSH was a consequence of increased cellular iron, we hypothesize that, compared to normal neurons, AD neurons have undergone increased iron accumulation that results in excessive GSH synthesis. Indeed, increased GSH synthesis may correspond to late steps in AD where major compensatory changes in the reduction state of affected neurons have been reported (35, 43).

Figure 4. Iron induction of GSH synthesis. A. SH-SY5Y cells were cultured for 8 days in standard culture medium and then for 2 or 4 days in media with 1.5, 5, 10, 20, 40 or 80 μM iron. Cell extracts were prepared and GSH levels were determined as described (34). Note the inverse relationship between iron and GSH levels in the range 20-80 μM Fe at 2 days of culture. When compared with the 2-day values, a significant increase in GSH content (*: p<0.001) was observed at 4 days for the 40 and 80 μM Fe conditions. Data represent means of 4 (2 days) or 3 (4 days) independent determinations.
The GSH content increase correlates with GCL expression

Glutamate cysteine ligase (GCL) is the rate-limiting enzyme for GSH synthesis (12). GCL is a heterodimer composed by a catalytic or heavy subunit (GCLC) and a modulatory or light subunit (GCLM), which are encoded by different genes. Both genes display upstream regions with antioxidant response elements, AREs, also known as electrophilic response elements (9, 38, 40). Both Nrf2 and c-Jun transcription factors are involved through their AREs in the up-regulation of the GCLC gene in human hepatoblastoma cells (23). The possibility that iron-induced oxidative stress might up-regulate GCLC or GCLM expression in SH-SY5Y cells was explored (Fig. 5). After two days of challenging SH-SY5Y cells with 1.5, 7 or 80 µM iron, two- and three-fold increases in the expression of the catalytic GCLC subunit mRNA were apparent at 7 and 80 µM iron. On the contrary, no significant changes in expression of the GCLM modulatory subunit were found. It was recently shown that hemin treatment of SH-SY5Y cells leads to Nrf2 nuclear translocation and to up-regulation of ARE-dependent oxidative-stress related enzymes (29). Therefore, it is likely that the oxidative stress induced by iron overload caused activation of Nrf2 and increased expression of the GCLC subunit.

![Figure 5. RT-PCR analysis of GCLC and GCLM expression.](image)

**Figure 5. RT-PCR analysis of GCLC and GCLM expression.** mRNA expression of the catalytic (GCLC) and regulatory (GCLM) sub-units of γ-glutamate cysteine ligase were determined by RT-PCR of total RNA obtained from cells cultured with varied concentrations of iron for 2 days. GAPDH mRNA expression was used as an internal loading standard. The lower panel shows densitometry quantification of the RT-PCR bands normalized to GADPH expression. Mean ± SD of 2 determinations. Iron promoted a 2.9-fold increase in the mRNA of the GCSC subunit, while no significant change in the GCSM subunit was apparent.
Concluding remarks: A model for the time course changes of neuronal iron and GSH levels during a lifespan

During the last few years, mounting evidence has been obtained indicating a strong relationship between iron accumulation, oxidative stress and neurodegeneration. A hypothetical time progression chart, describing changes in iron, GSH, DMT1 and Ireg1 levels in neurodegeneration-prone neurons during the human lifetime, is depicted in figure 6. Iron increases markedly during the initial 25 years of age. As a response to the initial iron accumulation, expression of DMT1 decreases and expression of Ireg1 increases. During this period, GSH levels present a biphasic response: an initial increase due to increased synthesis is followed by a decrease due to increased consumption. The decrease in GSH levels accompanied by the increased iron content result in oxidative conditions leading to cell death. Cells that adapt to the redox challenge enter a metastable condition characterized by a persistent pattern of iron accumulation accompanied by increasing levels of GSH.

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