
Brain changes in iron loading disorders

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Abstract

Abnormal iron accumulation within the brain is associated with various neurodegenerative diseases; however, there is debate about whether milder disorders of systemic iron loading, such as haemochromatosis, affect the brain. Arguments on both sides of the debate are often based on some common assumptions that have not been rigorously tested by appropriate experimentation. Recent research from our lab has applied high-throughput molecular techniques such as microarray to models of dietary and genetic iron loading to identify subtle but important effects on molecular systems in the brain that may go undetected by other methods commonly used in the field. In this chapter, we review the existing research in animal models and human patients and discuss the strengths and limitations of the different approaches commonly used. Using our findings as an example, we argue that transcriptomic methods can provide unique insights into how systemic iron loading can affect the brain and suggest some basic guidelines for extracting the most robust and reliable information from microarray studies.

Keywords

Iron overload • Brain • Microarray • Mouse model • HFE

Introduction

As reviewed elsewhere, severe iron dyshomeostasis can impair cognition, movement and behaviour in rare conditions such as neurodegeneration with brain iron accumulation (NBIA) disease [1]. In this chapter, we will review the evidence that even relatively mild iron disorders give rise to brain changes of potential functional significance. Although iron deficiency will be considered briefly, we will focus primarily on iron loading disorders. We will first give an overview of some of the past work in this field. We will then go on to discuss some new approaches to studying this issue and some of the new data being generated by our

laboratory and others which challenge some common assumptions about the effects of iron overload on the brain.

This area is of current interest since patients with the common iron overload disorder haemochromatosis sometimes report neurological symptoms such as memory impairment, headaches or extreme fatigue, and it is not yet clear whether such symptoms are directly attributable to haemochromatosis or not. Haemochromatosis, most commonly caused by polymorphisms in the *HFE* gene [2, 3], is a disorder of iron metabolism in which there is excessive iron accumulation in a number of organs throughout the body, particularly in the liver and also in the pancreas, endocrine organs and heart [4–6]. Iron accumulation can cause liver fibrosis and cirrhosis, increasing the risk of hepatocellular carcinoma, as well as other disorders such as diabetes, cardiomyopathy and arthritis [4–6]; however, effects on the brain are still under debate.

Past animal models of iron overload have used iron supplementation and will be described in more detail below. More

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recently, new genetic models have been developed that may give more accurate pictures of human haemochromatosis. We have been studying brain gene expression changes in these models using high-throughput microarray technologies. Even relatively mild changes in (body) iron homeostasis appear to influence the expression of important genes within the brain, often in unexpected ways. In this chapter, we will discuss some common assumptions about iron and the brain and some of the key findings and limitations of past studies. We will go on to describe how high-throughput technologies such as microarrays and other methods of examining genome-wide gene expression can be used to expand our understanding of the effects of iron on brain and how the findings from such studies are starting to challenge many of the existing assumptions in this area.

A few common assumptions about iron and the brain

There have been relatively few experimental studies on the effects of iron perturbations in the CNS or CNS-derived models and these have often been performed under pharmacological conditions. For example, abnormally high levels of iron are often assumed to exert toxic effects primarily through the oxidative actions of free iron on neurons or other cells, but this is based primarily on studies using pharmacological iron doses. Surprisingly few studies provide *in vivo* evidence for iron-induced oxidative damage within the brain in conditions likely to be physiologically relevant.

Other common assumptions relate to the blood–brain barrier (BBB), which restricts the free passage of many substances between the brain and the rest of the body. Notably it is sometimes assumed that people with systemic iron overload conditions do not experience brain sequelae because the BBB limits the entry of iron into the brain. This in fact involves two assumptions, first that the BBB effectively protects the brain from iron loading in such conditions and second that if there is no iron loading in the brain, systemic iron loading in itself does not exert indirect effects on the brain.

With regard to these assumptions, as will be discussed in more detail further below, iron supplementation studies in rodents have demonstrated that brain iron levels can increase as a result of systemic iron increases, and that this can subsequently affect brain function [7–10]. This suggests that the BBB may not provide complete protection; however, these studies have generally used very high doses of iron and may not necessarily be relevant under more physiological conditions. Therefore, the validity of the first assumption remains uncertain.

Iron transport across the BBB is covered in detail in a chapter by Crichton and colleagues and will not be revisited in detail here. Another consideration that may be more

relevant in this context is that iron overload often arises as a result of genetic mutations, as further discussed below. This could lead to perturbations in brain systems influenced by the gene in question, independent of systemic iron status. These brain systems could be related to iron homeostasis within the brain or could be involved in other brain functions unrelated to iron. Therefore, genetic disorders of iron dyshomeostasis may affect brain systems even if the BBB is limiting entry of iron *per se* or even if there is no gross iron loading in the brain.

Also brain iron loading *per se* is not necessarily the only mechanism by which altered brain function may occur in response to systemic iron dyshomeostasis. There can be circumstances in which systemic iron loading can transduce indirect effects on the brain. For example, extreme systemic iron loading can cause severe liver damage, which can result in the accumulation of toxic substances such as ammonia in the blood. This, in turn, can lead to perturbation of brain functions through hepatic encephalopathy, a condition characterised by symptoms such as impairment of speech and movement and, in severe cases, seizures and coma [11]. A less extreme example may involve systemic inflammatory and immune changes. Iron overload in the liver can lead to low-grade inflammation [12] and alterations in circulating cytokines and other peripheral immune responses could act on the brain without brain iron levels being affected in any way.

Past animal studies

Iron supplementation studies in animal models have usually employed one of two broad approaches. One approach is to use acute models involving direct injection of one or more high doses of iron into the CNS of adult mice, in order to bypass the BBB [13–15]. Most such studies have involved highly artificial, often pharmacological, conditions, in large excess of usual exposure levels, and their relevance to human iron disorders remains to be established.

It is sometimes assumed that the early phases of extreme pharmacological changes or changes in response to less extreme stimuli (acute or chronic) may be inferred from ‘scaling down’ observations in more extreme, pharmacological conditions. Yet often early changes in response to stimuli may involve completely different systems to those that become prominent under more extreme circumstances. One counter-example to this occurs for end-stage neurodegenerative disease. By the time patients die, the brain may be reduced in mass by as much as 30 % or more due to loss of particular cell populations. The original molecular profiles caused by the stimulus may have long since vanished, as cells move through early, possibly compensatory responses, through damage and repair profiles and into mechanistic profiles associated with cell death. In end-stage disease, the cells in which the first problems originally occurred may

have long since died, to be replaced by cells expressing different sets of genes or proteins. This needs to be considered when investigating phenomena that may lead to neuronal cell death, such as approaches that use pharmacologically high iron loading.

The other broad approach used by some researchers has been to look at the effects of iron supplementation in early post-natal rodents, with the goal of examining the effects of abnormally high iron exposures on the brain before the BBB is fully formed [8, 16]. While this kind of approach has been informative, one limitation is that it is still not yet fully clear how the transport of iron into and out of the brain alters through embryogenesis into infancy and adulthood. Also, while younger animals may have a more iron-permeable BBB, supplementing iron in young rodents can be difficult. Supplementation during embryogenesis or in pre-weanling neonatal mice has primarily used maternal dietary iron supplementation, making it difficult to achieve high levels of iron loading [17]. It may also be difficult to determine the actual exposure levels of individual neonates, although a few studies have used gavage (force-feeding) to deliver high known doses of iron [18, 19].

Issues relating to the sensitivity of methods used to measure iron also need to be kept in mind. The brain is a heterogeneous organ comprising various different anatomical regions and cell types which have different iron usages and contents. Measuring iron levels in whole brain homogenate tells only part of the story, as it provides no information on regional variation. Even when brain structure is preserved using histology, histochemical stains such as Perls' or Turnbull's stains are often not sensitive enough to pick up subtle changes in iron levels. Ideally, brain iron would be measured using a technique that combines the sensitivity of inductively coupled plasma-mass spectrometry (ICP-MS) or inductively coupled plasma-atomic emission spectroscopy (ICP-AES) with maintenance of brain morphology. Recent innovations and emerging technologies, for example laser ablation inductively coupled plasma mass spectrometry (LA-ICP-MS), may be the solution. Such methods can provide detailed and quantitative mapping of regional distributions of iron and other metals in brain sections [20].

Yet, despite these limitations, various studies using such approaches have successfully demonstrated alterations in brain iron levels and other brain changes in response to iron supplementation, as will now be reviewed in more detail.

Animal studies of how iron supplementation affects brain iron levels

Several studies have assessed changes in brain iron levels in response to iron administration at various ages. Pinero and colleagues investigated the effects of early systemic iron

loading on brain iron content using normal rat pups mothered by iron-supplemented dams [16]. This resulted in higher total iron content in the pups in most brain regions investigated. If weanling rats were instead given an iron-supplemented diet for two weeks only the pons and hippocampus showed increased total iron levels, suggesting that the brain may be less affected by increases in systemic iron at older ages. Yet, even at older ages, iron supplementation over longer periods can still accelerate accumulation of iron within the brain. Male weanling rats fed with a high iron diet for six or eight weeks showed significant increases of 15–30 % in total iron levels in the cortex, hippocampus, striatum and substantia nigra [21–23]. The degree of brain iron accumulation resulting from iron supplementation depends on the iron dose [7, 8, 18]. This supports the hypothesis that the BBB is capable of protecting the brain from excessive iron accumulation arising as a consequence of systemic iron overload, but only up to a certain threshold.

Effects of iron supplementation on brain expression of iron-related genes and proteins

1. Ferritin, transferrin receptor 1 and transferrin—Most studies of changes in brain gene and protein expression in response to systemic iron loading have focussed on specific molecules such as those controlling iron handling and homeostasis elsewhere in the body. In peripheral organs such as the liver, iron loading is often accompanied not only by increases in the iron storage protein ferritin but also by decreases in transferrin receptor 1 (TFR1), the main receptor for the iron transport protein transferrin. This has the effect of reducing the cellular uptake of iron, which is not only important in the liver but may be particularly important in organs such as the brain, which have not evolved the capacity to store large amounts of iron. The concerted inverse changes in ferritin and TFR1 occur in response to alterations in intracellular iron concentrations and this is coordinated at least in part by the iron-responsive element/iron regulatory protein (IRE/IRP) system [24].

Pinero and colleagues [16] observed decreased TFR1 protein levels in some brain regions in their iron-supplemented rat models, described in detail above, but no changes in ferritin levels in their model of early iron overload (rats mothered by dams iron-supplemented from postnatal day 10 to postnatal day 21). In contrast, their model of late iron overload (rats fed an iron-supplemented diet for two weeks following weaning) showed decreased TFR1 expression in different brain regions and increased ferritin expression in pons and cortex. There were no changes in transferrin levels in either model. Decreases in TFR1 and increases in ferritin are predicted to help maintain cellular iron homeostasis and

protect against iron toxicity by restricting cellular iron uptake and increasing sequestration within ferritin. For regions such as the pons, which also showed increased iron levels, the changes are consistent with post-transcriptional regulation through the IRE/IRP system.

2. Divalent metal transporter 1—Another protein important in cellular and organelle iron uptake is divalent metal transporter 1 (DMT1). Ke and colleagues assessed DMT1 protein and mRNA in different brain regions by Western and Northern blotting in male weanling rats fed a high-iron or normal diet for eight weeks. As described in the preceding section, all brain regions investigated showed increases in brain iron content of 15–30 %. Yet there was no difference in brain DMT1 mRNA or protein levels relative to rats fed a normal diet, despite one DMT1 transcript variant containing an IRE. So, at least in adulthood, DMT1 expression may not be regulated by iron in the particular brain regions examined [22].
3. The ferroxidases hephaestin and caeruloplasmin—Movement of iron between different cells in the brain is influenced by ferroxidases in the brain interstitium which oxidise ferrous iron exported from neural cells or transported across the BBB. This facilitates binding to transferrin, which binds ferric iron with greater affinity than ferrous iron.

One ferroxidase essential for normal brain iron homeostasis is caeruloplasmin—patients with acaeruloplasminaemia due to loss-of-function mutations in the caeruloplasmin gene or with low levels of caeruloplasmin in Wilson's disease show brain MRI abnormalities suggestive of basal ganglia iron accumulation [25–27] and often display symptoms such as cerebellar ataxia and cognitive impairment [28–30].

In iron-supplemented rat models similar to those used in the Ke DMT1 study above, with increased brain iron content in all regions investigated, hephaestin protein is increased in cortex and hippocampus but decreased in striatum and substantia nigra [23], while caeruloplasmin protein is increased in the substantia nigra only [21]. This suggests that the ferroxidases hephaestin and caeruloplasmin are not regulated in the same way in response to alterations in iron status and that regulation of these proteins differs between different brain regions. This may relate to differences in iron usage between brain regions with different functions.

Recently the Alzheimer's disease amyloid precursor protein (APP) has been reported to have ferroxidase activity and proposed to be 'the neuronal ferroxidase' [31]. This protein has both membrane-bound and secreted forms and contains a region with sequence similarity to the ferroxidase domain of ferritin heavy chain [31]. Furthermore, the corresponding mRNA transcript contains an iron-responsive element in the 5' untranslated region [32]. The contributions of APP to brain ferroxidase activity remain to be determined but are likely to be limited insofar as APP appears unable to protect adequately

against caeruloplasmin deficiency and accompanying iron deposition in patients with acaeruloplasminaemia or Wilson's disease.

Iron supplementation and the brain microvasculature

Studies of changes in protein levels in brain microvasculature in response to iron supplementation have found no evidence for changes in ferritin, TFR1, transferrin or DMT1 [33]. This suggests any changes which occur in expression of these proteins in response to iron supplementation do not take place in brain capillary endothelial cells, influencing uptake of blood iron into the brain, but instead occur in neurons or glia and influence iron trafficking and distribution within neural tissue after uptake across the BBB. However, it is unclear what changes occur in brain microvasculature in response to more extreme circumstances of iron release, for example during cerebrovascular haemorrhage.

Functional changes

On the whole, the weight of current evidence suggests that iron supplementation affects the expression within the brain of genes and proteins important in iron homeostasis. Several investigations suggest there may also be functional phenotypic changes in response to iron supplementation, including effects on movement and behaviour. Fredriksson and colleagues found that oral administration of iron to mice (37 mg/kg body weight) at 10–12 days of age, a critical period for brain development, resulted in significantly increased total iron content (60 %) in the basal ganglia at three months of age [8], as assessed by atomic absorption spectroscopy. This was accompanied by signs of neurobehavioural dysfunction, including altered spontaneous motor behaviour, poor performance in radial arm maze learning tests and deficits in habituation.

Similarly, Sobotka and colleagues found that supplementing the diet of male weanling rats with a high iron dose (20,000 parts per million carbonyl iron) for 12 weeks caused increased brain non-haem iron levels in association with neurobehavioural dysfunctions. These included deficits in conditioned active avoidance response, which indicates changes in associative processes, impaired startle response, which indicates decreased ability to respond to environmental stimuli, and decreased motor activity [7].

Using an iron-supplementation protocol sufficient to cause regional brain iron increases, as described above [16], Pinero and colleagues showed evidence of altered motor functions in iron-supplemented rats, including

decreased activity and stereotypic behaviour [9]. In addition, studies by Maaroufi and colleagues demonstrated that intra-peritoneal injection of ferrous sulphate (3 mg/kg) in adult male rats for five days or more, not only increases iron levels in various brain regions (see above), but causes impaired emotional behaviour and spatial learning [10].

Some of these effects may be due to altered neurotransmitter levels in certain brain regions as a result of iron supplementation. Kaur and colleagues administered a dose of iron (120 mg/kg body weight) that was 40 times the usual daily iron intake to neonatal mice at 10–17 days of age, resulting in increased total iron levels in the substantia nigra by two months of age, as assessed by inductively coupled plasma mass spectrometry. This was accompanied by depletion of striatal dopamine levels and increased levels of oxidative stress markers [18]. Whether pharmacological effects have more generalised validity and applicability is always open to question but another study also found depletion of dopamine levels in rats fed an iron-supplemented diet (0.3 % w/w ferrous sulphate) for 10 weeks, in addition to decreased brain serotonin levels [34].

Neither of the two studies just described investigated higher level brain function such as movement, behaviour or cognition. Even so, taken together, the body of findings in the various animal models subjected to iron supplementation suggests that systemic iron levels can influence some brain functions, in addition to brain gene and protein expression.

However, most of these studies have used pharmacological doses of iron that may not accurately reproduce what occurs in most human iron overload conditions. Supplementation models are also constrained by the acute, short-term nature of typical experimental iron-supplementation protocols as well as the partial restriction of brain iron entry by the blood–brain barrier and the blood–cerebrospinal fluid (CSF) barrier. All these factors may limit the usefulness of such models in addressing how brain iron abnormalities cause neurologic dysfunction. Further insights can be gained by studying the effects on the brain of longstanding perturbations in brain iron homeostasis due to inherent genetic mutations. In addition, such mutations also have the potential to disrupt the brain's internal homeostasis by mechanisms which are partly or fully independent of systemic iron status.

Functional effects in haemochromatosis patients and animal models

There are several case reports [35–37] of movement disorders in patients with the iron loading disorder haemochromatosis, described earlier in this chapter. However, it has been argued that the movement impairment in these patients is coincidental and not attributable to haemochromatosis [38, 39]. There is

a dearth of studies investigating *HFE* genotype in patients with movement disorders other than Parkinson's disease, but one case study does report a female with severe *HFE* haemochromatosis and cerebellar ataxia. This patient showed evidence of brain iron accumulation and symptoms of ataxia were stabilised following phlebotomy [37], suggesting that iron may have had a causative role.

There are virtually no papers on brain effects in animal models of *HFE* dysfunction, but Golub and colleagues investigated motor function in male *Hfe*^{-/-} knockout mice at three months of age [40]. The *Hfe*^{-/-} mice experienced more falls from a rotarod, in addition to having a wider forelimb landing footsplay, greater variability in stride length and hypersensitivity to proximal stimulation. These features are consistent with a motor co-ordination deficit, which did not appear to be attributable to deficits in motor learning or strength or generalised debility.

Interestingly, histochemical staining revealed no obvious accumulation of iron in the *Hfe*^{-/-} brain [40], suggesting that movement disorders can occur in the absence of gross brain iron accumulation. As noted earlier, lack of gross brain iron accumulation does not rule out the possibility that systemic iron overload causes other brain changes. At least three different animal models—a rat model of dietary iron overload [7], *Hfe*^{-/-} mice [40] and *Irf2* knockout mice, deficient for iron regulatory protein 2 [41, 42]—all show behavioural or motor abnormalities without apparent increases in brain iron. Such findings raise the possibility that some iron disorders, such as those due to *HFE* gene mutations, may disrupt brain functions by mechanisms that are at least partially independent of brain iron accumulation as well as through effects directly due to changes in brain iron homeostasis.

In summary, there is now considerable evidence that both iron deficiency and iron overload can cause changes in gene expression and protein levels in the brain. To date, most studies have focussed on changes in iron-related genes and proteins but we believe there may also be expression changes for important genes and proteins relating to key brain functions, although as yet few studies have investigated this.

Exploratory studies of brain gene expression changes in response to iron status

As exemplified by most of the studies discussed above, most research relating to the effects of iron on the brain has been restricted to investigations of a small number of molecules of interest. So far there have been very few studies of changes at the level of molecular systems, yet some studies have now begun to use exploratory, discovery driven approaches rather than focussing solely on select molecules.

Liu and colleagues measured gene expression changes in response to iron supplementation or deprivation in rat

hippocampal cells by suppression subtractive hybridisation [43]. Expression changes for select genes were then confirmed *in vivo* by performing Northern blot analysis on hippocampal tissue isolated from rats fed either an iron-supplemented or iron-deficient diet for two months following weaning. This is one of the earliest examples in the brain iron field of combining both discovery-driven and hypothesis-based approaches to find new genes of interest. In total, 31 genes were identified by SSH as differentially expressed in comparisons of iron-supplemented and iron-deprived hippocampal cultures. Quantitative analysis of ten of these by Northern blot analysis revealed only modest expression changes (less than twofold). The genes that were identified as having higher expression in response to iron supplementation were endothelial-derived gene 1, ribophorin II, ubiquitin-specific protease 14, *etoile* Sam68-like protein and the partial coding sequence Ir22. These genes are generally poorly characterised and had few known functions.

Four of the genes with higher expression in response to iron deprivation (BS69, Pdcd5, Anapc8, Ruvbl2) related to cell cycle regulation and may encode proteins with functions in DNA damage and repair and the transition to apoptosis [43]. This suggests that iron deficiency in the hippocampus may be associated with cell damage or apoptosis.

This illustrates a common problem confronting researchers working with emerging high-throughput technologies. What can be made of a bevy of small changes in poorly characterised proteins? Such changes may simply be artefacts of the model or the technology. Yet even small changes may be of real relevance, not only if acting in concert but even when acting in opposing directions if this indicates systems under stress which are exhibiting both potentially pathogenic and compensatory changes.

When performed rigorously, gene expression microarray studies can provide unprecedented insights into the behaviour of molecular systems. However, some facets of rigorous array data analysis are still not well understood by many researchers. Array data often show a mass of changes, many involving molecules whose functions are unknown. Out of the sea of changes, often of relatively small magnitude even in grossly abnormal samples such as cancers, how do we decide, first, which are real and, second, which of these are important? Resources need to be focused on effects of real importance. Yet the volume of information generated is often so large that it is far beyond the capacity of individual groups to follow up and validate all important effects. The following sections address these issues in more detail.

High-throughput advantages and concerns

The emergence of microarray technology has facilitated high-throughput gene expression studies unimaginable only years ago, allowing differential gene expression to be examined

across the entire genome in a single experiment. Microarray technology has several advantages over approaches traditionally used to assess gene expression. Researchers often studied molecules artificially isolated from their natural environment, making it difficult to assess how molecular phenomena were affected by interactions with other molecules within cells and tissues. Methods such as RT-PCR or Northern blot analysis require the user to select a small number of specific genes for investigation of expression. In contrast, the microarray technique measures the expression of thousands of genes in a single experiment, providing a cost-effective and time-efficient way of acquiring information on genome-wide gene expression. This allows researchers to explore biomolecular systems and the orchestrated behaviour of large groups of molecules in far greater depth than ever before. Microarray experiments can provide valuable insights into molecular mechanisms or pathways or ontologies that are perturbed due to a given experimental manipulation, as opposed to simply identifying changes in the expression of select individual genes.

But these powerful tools bring new challenges with regard to interpreting the mass of data now pouring out. Some deeply rooted assumptions about how best to progress bioscientific research may need to be re-assessed in the light of the new perspectives now starting to emerge. As just one obvious example, the high-throughput 'omics' approaches now being applied have already begun to raise awareness of the validity of conducting both discovery-driven research and hypothesis-driven research and indeed of the value of viewing these as complementary approaches in order to maximise yields of useful information.

Explanation of arrays

Microarrays are manufactured on small chips or slides, with an individual gene expression array containing many different oligonucleotide or cDNA probes, each targeting a specific gene transcript. Each probe is present in millions of copies, allowing transcript levels to be assessed quantitatively. With advances in current technology, microarrays are now commercially available that, in theory, contain a sufficient number of different probes to measure every transcript encoded by the genome, although the comprehensiveness and effectiveness of such investigations are reliant on probe quality.

One important barrier to uptake of any high-throughput technology, whether array-based or sequencing-based, is concerns about the validity of the output data. The difficulty lies in extracting reliable, relevant information from the mass of molecular data generated by high-throughput studies. This raises some important issues in analysing and interpreting array and other high-throughput datasets, particularly in circumstances where effect sizes are relatively small. These

issues include assessment of background thresholds, minimising effects of technical variation, assessing whether or not an observed signal difference is likely to be real and, if this is done statistically, deciding whether or not it is appropriate to correct for multiple testing [44–46].

In addition, the cost of microarrays, although small in terms of the amount of data obtained, is still a barrier for many labs. In our experience, sample pooling, while superficially appealing on economic grounds, is problematic, since even a single outlier sample can make an entire experimental run useless, over-riding any potential cost efficiencies of pooling. We therefore recommend avoiding pooling if at all possible. But this does restrict the number of variables that can be examined effectively. For example, it is difficult to examine the effects of iron on gene transcript levels over a wide range of different ages, dietary regimens or brain regions. However, initial microarray results can guide validation studies over expanded experimental conditions using cheaper technologies such as PCR, Western analysis of protein or other more traditional approaches. Issues relating to the accuracy of high-throughput data are addressed in more detail below.

Normalisation and analysis issues when investigating small magnitude expression changes

Microarray intensity values are usually normalised by one or more transforming functions. Reasons for normalising can include forcing a normal data distribution or increasing comparability between probes, samples, chips, machines or platforms [47–49]. Even small technical variations (e.g. cRNA loading on arrays, scanning and hybridisation inconsistency) can sometimes cause considerable differences in signal intensities. The overarching aim of normalisation is to reduce differences due to technical variation (false positives) while conserving true biological effects (i.e. maximising true positives and minimising false negatives).

The particular normalisation methods which are most appropriate will vary depending on the type of experiments, the array platform and type of array (e.g. RNA or cDNA, one-colour or two-colour). It is often advisable to apply more than one normalisation approach to identify robust effects that are not artefacts of a particular normalisation method. These issues are particularly relevant for datasets where expression changes are expected to be modest but similar considerations also apply even for datasets where most expression changes are large, since these will also contain some genes of biological interest with small expression changes.

The challenge for any analytical approach lies in reducing false positives while avoiding false negatives. A statistical

p-value approach allows estimation of false positive error probability, which can be considerable when conducting large numbers of comparisons, as occurs in arrays. However, the methods currently used to adjust for multiple comparisons [50] are often conservative, missing real changes. Such adjustments may be most useful for identifying restricted groups of target genes (e.g. class prediction aimed at identifying biomarkers for diagnosis or prognosis). For studies aimed at identifying complete sets of target genes (e.g. class comparison or class discovery aimed at understanding biological mechanisms), accepting non-informative false positives may be less problematic than omitting informative genes. Minimising false negatives by not applying a multiple testing correction has been recommended for such studies [51, 52].

One drawback of using statistical metrics is that important expression changes can be missed if there is high replicate variation due to chance outliers. To avoid this, fold-change metrics have been advocated by various groups including the Microarray Quality Control (MAQC) consortium, which has recommended cut-offs based on fold-change in combination with a relaxed *p*-value cut-off [53]. However, other groups disagree [46, 54]. Chen and colleagues note that if there is no treatment effect, the strategy of a fold-change cut-off with a non-stringent *p*-value cut-off will result in 100 % false-positive error selection [46]. Other problems may occur when analysing datasets with low fold-changes, where small but biologically significant alterations may be missed by fold-change filters (false negatives).

We recommend routinely using multiple normalisation and analytical approaches to decrease the likelihood of false-positive results due to artefacts of the analytical methodology. In addition, we suggest focusing most attention on molecular systems containing several genes with altered expression, as co-ordinated expression changes in multiple related genes may reflect perturbations of pathways or functions and may be more likely to be real findings of biological relevance than changes in individual genes with no common functions. This is achieved in part through the use of pathway and ontology tools and other bioinformatics approaches, as well as by more traditional investigative strategies.

Microarray studies of brain gene expression in models of iron disorders

Despite the potential opportunities for discovery using microarray, few researchers have utilised the technology to investigate brain molecular systems perturbed as a result of iron disorders. Clardy and colleagues used microarray to investigate brain expression of ~8,000 genes in a rat model of developmental iron deficiency [55]. There were 334 genes

with altered expression, including decreased transferrin and increased transferrin receptor 1 gene expression. Down-regulation of myelin-related genes was also seen and may contribute to the hypomyelination that occurs in states of developmental iron deficiency [56–58]. Iron repletion following weaning corrected most of the alterations, with only five genes showing alterations in iron replete rats [55]. This suggests that the effects of iron deficiency early in development may be partially or even fully reversible, at least when deficiency is corrected at a relatively young age.

There are few other array studies examining genome-wide brain gene expression. However, recently our group has performed microarray and real-time RT-PCR analyses of brain gene expression in mouse models of iron loading. We believe these data may be painting a far richer picture of what iron is doing in the brain than has ever been captured before.

We first investigated genome-wide gene expression changes in a mouse model of dietary iron overload. In this model, male mice were maintained on normal chow from weaning until 7 weeks of age and then transferred to a high-iron diet comprising normal chow supplemented with 2 % carbonyl iron for 3 weeks. The control group consisted of mice maintained on normal chow from weaning until 10 weeks of age. These experiments utilised mice of the AKR strain, which exhibit a strong iron loading phenotype [59]. The high iron diet has previously been shown to increase serum iron indices and liver iron loading [59, 60], and this has been confirmed specifically in the AKR strain under the experimental conditions used in our study [61].

Yet, despite the peripheral iron loading phenotype seen in these mice and confirmed in our study, there was no increase in the amount of non-haem iron in the brain of mice on the high iron diet, as shown in Fig. 1. Therefore, in this model utilising short-term dietary iron loading, the brain appears to be at least partly protected from the increased iron present throughout the rest of the body, whether by the BBB or by other mechanisms.

However, even though there was no apparent increase in brain non-haem iron levels, we observed various other changes. Notably, small but significant increases in brain transcripts for ferritin light chain of approximately 20 % were detected by microarray and validated by real-time RT-PCR. Consistent with this, brain transcripts for iron regulatory protein 1, which negatively regulates ferritin translation, decreased 30 %.

These changes suggest brain gene expression can be influenced even by relatively mild systemic iron loading insufficient to cause significant gross increases in brain iron levels. However, the extent of the observed changes was small and levels of transcripts for the transferrin receptor and various other important iron-related proteins were unaltered. This is consistent with previous reports that, in general, average brain levels of transferrin, transferrin receptor 1 and

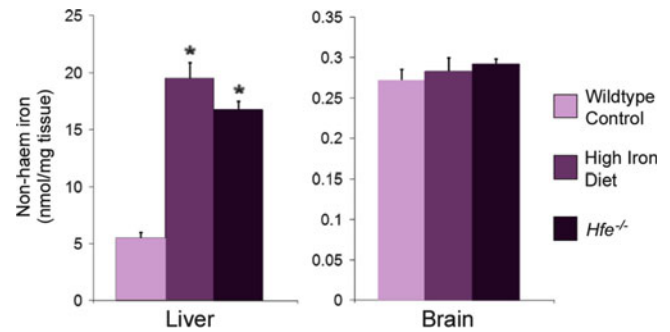


Fig. 1 Comparison between non-haem iron levels in the liver and the brain of mouse models of iron loading. All mice were male and of the AKR background strain. The levels of non-haem iron in the liver increased by similar amounts in both models of iron loading, while the levels of non-haem iron in the brain remained unchanged. * $p < 0.05$, $n \geq 4$ per group

divalent metal transporter 1 proteins often do not change substantially in response to high systemic iron [62, 63].

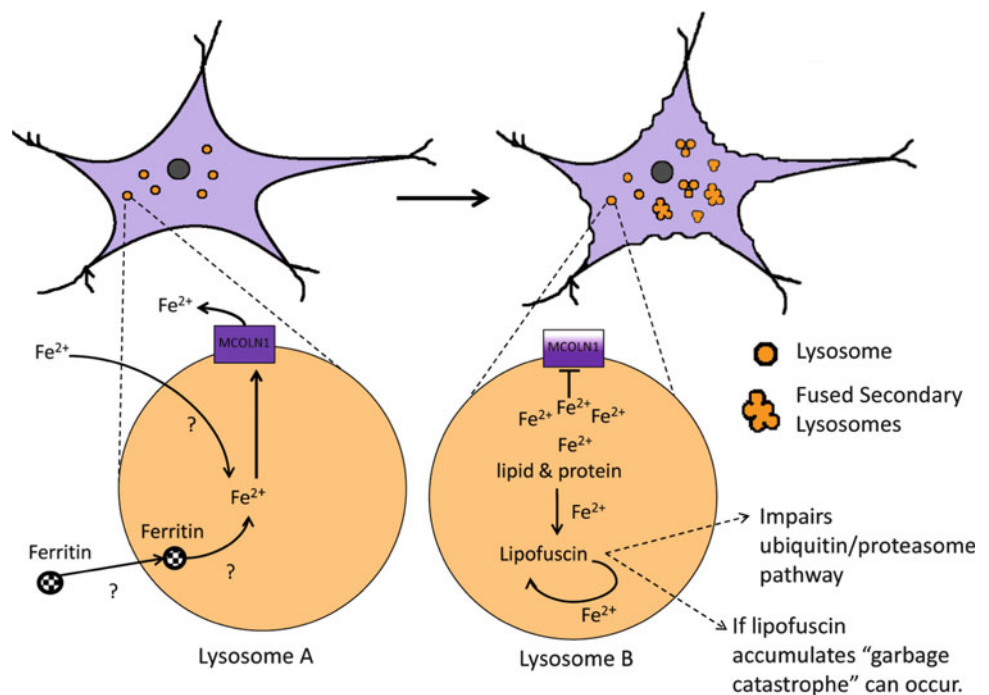
So the most likely explanation for our findings is probably that on the one hand the BBB is indeed partly protecting the brain against the effects of peripheral iron loading in the short-term dietary model. On the other hand, the level of systemic iron loading is apparently sufficiently high to partially over-ride the protective effects enough to perturb brain iron homeostasis at the subcellular level and trigger early compensatory responses. These appear to involve initial increases in ferritin gene transcription, as well as transcriptional and probably also post-transcriptional changes involving the IRE/IRP system. However, these effects do not appear to be sufficient to lay down significantly detectable extra iron stores within ferritin or to cause gross brain iron loading.

One might therefore conclude that the BBB is essentially fully effective at protecting the brain against the effects of dietary iron overload in this short-term model. However, in addition to the changes observed for ferritin gene transcription, around 300 other genes showed significant expression changes.

Most of these changes were small in magnitude (less than twofold) and unable to be readily validated by real-time RT-PCR or other methods, suggesting short-term increases in dietary iron intake produce only subtle effects on brain gene expression. However, the alterations included sets of expression changes for groups of genes relating to particular pathways or functions already known to involve iron and likely to be biologically relevant.

We have observed similar effects in a model of genetic haemochromatosis, the *Hfe*^{-/-} deletion mutant mouse model. This mouse was generated using a targeting vector to introduce a 1.7-kb PGKneo cassette to replace a 360-bp fragment of the *Hfe* gene encompassing a portion of exon 4 and intron 3 [64]. This produces a truncated dysfunctional protein. Two strains of mice commonly used as backgrounds

Fig. 2 The role of iron in the formation of cellular lipofuscin. Lysosome A shows the normal movement of iron through lysosomes in a healthy cell where MCOLN1 transports iron across the membrane of the lysosome. Lysosome B comes from a cell in which MCOLN1 function is impaired and iron is building up within the lysosomes. This iron acts as a catalyst in the formation of lipofuscin from degraded lipids and proteins. Some lysosomes are able to fuse together



in *Hfe*^{-/-} deletion mutant mice studies are C57BL/6J and AKR [59]. Both models replicate the human disease with increased liver iron content (a gauge of total body content) [59, 64]; however, liver iron concentrations differ markedly between the two strains, with the AKR strain showing higher levels of hepatic iron [59]. We found that *Hfe*^{-/-} deletion mutant mice on the AKR background, like the short-term dietary iron supplementation model, again showed relatively few changes in key genes associated systemic iron regulation yet still displayed many gene expression changes, across numerous important brain systems.

Overall, the dietary and genetic models of iron loading we have examined to date have shown very little evidence for changes in oxidative stress-related systems. There is virtually no evidence for inflammation (in strong contrast to other preliminary brain microarray studies we have analysed in adult mouse models of systemic infection, where there are numerous increases in expression of genes encoding cytokines and other inflammatory molecules, even after clearance of the systemic infection). We have also so far observed little if any evidence of changes relating to angiogenesis or vasculogenesis and few changes related to reactive oxygen species, although in the dietary iron model in particular we did observe some changes that may relate to nitric oxide signalling, which is dependent on iron, and nitrosative stress [65]. We have also seen few if any signs of gene expression changes clearly related to apoptosis or other forms of cell death in these models.

However, we do see effects on a range of other molecular systems. Notably expression changes were observed for genes causatively linked to neuronal ceroid lipofuscinosis and mucopolipidosis. These diseases involve intralysosomal lipofuscin build-up that may reflect lysosomal iron

accumulation (Fig. 2). The *Hfe*^{-/-} brain showed most total changes and had expression changes for several genes causatively linked to neuronal ceroid lipofuscinosis [66]. However expression changes for genes causatively linked to lipofuscinosis diseases were also seen even in the short-term dietary iron model, which had fewer total changes [65].

Intriguingly, these included decreased levels of transcripts for the mucolipin 1 gene (MCOLN1), an iron channel protein and calcium transporter thought to play a role in iron release from late endosomes and lysosomes [67]. Mutations in MCOLN1 can impair the iron permeability of lysosomal membranes, preventing iron release and causing the disease Type IV mucopolipidosis [67].

Lipofuscin is an intralysosomal substance that cannot be exocytosed from the cell or degraded by lysosomes [68]. It is hydrophobic and 'yellow-brownish' in colour [69]. It is considered to be a telltale sign of the ageing process because its rate of accumulation is proportional to the age of an individual [68].

The prevailing hypothesis for lipofuscin formation is that hydrogen peroxide produced as a by-product by mitochondria and other organelles permeates into the lumen of secondary lysosomes [70]. (Newly formed lysosomes are termed 'primary' lysosomes, becoming 'secondary' lysosomes once they acquire and start to degrade exogenous materials such as pathogens or membrane fragments.) Lysosomal degradation of iron-containing proteins such as ferritin and cytochromes releases iron, which can then catalyse the Fenton reaction with hydrogen peroxide [68]. The Fenton reaction produces hydroxyl radicals which cause protein and lipid peroxidation, which is followed by intermolecular cross-linking and lipofuscin formation [70].

Secondary lysosomes can fuse with other secondary lysosomes or with primary lysosomes [71]. When this occurs, materials within the lysosomes aggregate, so over time lipofuscin accumulates [71]. The buildup of lipofuscin can have strong effects on cellular functioning as it can impair the ubiquitin/proteasome pathway. Accumulation of lipofuscin has been proposed to lead to a phenomenon dubbed the ‘garbage catastrophe’, the cumulative effect of cellular waste not being properly eliminated due to concurrent inhibition of both proteasomes and lysosomes [72]. These changes are potentially pathogenic and therefore have the potential to affect brain function unless compensated.

We have also observed changes in transcripts of genes tied more directly to brain function. These included expression changes for genes involved in important brain functions such as neurotransmission. One prominent example of a gene showing consistent brain expression changes across both dietary and genetic models of iron overload is the gene encoding calcium/calmodulin-dependent protein kinase II α (Camk2a), a protein of considerable prominence in learning, memory and other cognitive processes. This gene shows reproducible decreases in transcripts across the models, in conjunction with a variety of other changes potentially affecting cognition [66].

As far as we are aware, there have not been any other reports examining changes in genome-wide brain gene expression in the whole brain in response to iron supplementation. The study by Liu and colleagues described above identified 31 genes with altered expression in the hippocampus of rats fed an iron-supplemented diet for 2 months after weaning [43]. None of these genes were identified as having altered expression in whole brains from iron-supplemented mice in our study.

The study by Clardy and colleagues described above, which investigated changes in 21 day-old pups of female rats fed an iron-deficient diet through gestation and lactation, identified 334 genes with altered expression [55]. Only one, stathmin-like 4, showed altered expression in our study of brains from mice following short-term (3 weeks) dietary iron supplementation [65]. The stathmin-like 4 gene is believed to be involved in microtubular organisation but is not well characterised and expression was decreased both in our study of iron supplementation and in the Clardy study of developmental iron deficiency, making it hard to interpret the findings. One possibility is that iron abnormalities of any kind, high or low, can perturb microtubular organisation.

None of the gene clusters highlighted in the Clardy iron deficiency study were found to be enriched in our study of short-term dietary iron supplementation, including myelin-related genes, although we did observe expression changes for two genes (Gdap1, Litaf) causatively linked to demyelinating forms of Charcot–Marie–Tooth disease [73]. Taken together, the above considerations suggest that gene

expression changes in systemic iron loading in adults are not simply the opposite of changes in developmental iron deficiency.

Validation and the limitations of PCR

Any experimental technology can give rise to artefacts, making it important that experimental replication is carried out wherever possible using alternative approaches. Validation of RNA microarray data is often performed by real-time RT-PCR. This raises the question of phenotype and effect size. For genes with strong genetically dominant disease associations, an abnormality of one allele is sufficient to cause clinical disease. Yet this may represent a change of 50 % or less in gene function. For genes of this kind, capable of strong effects with relatively small changes, it is unreasonable to expect to observe big changes in experimental models that accurately represent the disease if a 50 % reduction or less in function causes a severe disease phenotype. Yet smaller changes are often below the limits of detection of relevant technologies such as real-time RT-PCR.

Although people often think of PCR as having molecular scale sensitivity, with the ability to amplify and detect a single molecule of DNA, sensitivity is often lower in practice, in particular for reverse transcription PCR, where RNA samples must first be reverse transcribed to complementary DNA (cDNA) before PCR. The exponential nature of PCR also imposes sensitivity limits in detecting differential expression when the relative gene expression of the test and control differs by less than twofold. This is because a twofold difference represents one cycle of a PCR reaction. While it may be possible to detect smaller fold changes that are statistically significant, basic principles of measurement suggest that it is not possible to determine effects of less than 0.5 cycles with certainty.

This is not a problem for genome-wide technologies such as microarrays, since the expression of genes of interest can be normalised relative to ‘averaged’ expression of large sets of genes, and typically the full background gene set (global gene expression). For changes of twofold or less microarrays may be more sensitive than real-time RT-PCR. This raises the question of the utility of using a less sensitive technique to validate the results from a more sensitive technology, at least for small changes, and suggests new approaches may be required for more accurate validation.

Other emerging technologies

New technologies now emerging include next-generation and real-time sequencing technologies. Although hybridisation-based approaches such as microarray can already provide

fast and cost-effective large-scale transcriptomics for many applications, there are some applications for which sequence-based approaches are better suited [74–76]. These include determination of exact transcription boundaries as well as detection of transcripts that do not map to a known genomic sequence or are not specifically targeted by microarray probes. In addition, RNA-sequencing techniques may avoid certain problems that can lead to errors in microarray data. These include non-specific background signal or signal saturation for genes with very low or very high expression, as well as probe binding anomalies due to affinity differences, the existence of different transcript spliceforms or other factors [74–77].

As yet, these technologies are rarely utilised in clinical practice, drug discovery or pharmacogenomics. More detailed molecular studies will be facilitated by new technologies such as real-time DNA and RNA sequencing. This will allow the tracking of a range of molecular changes over time to determine the mechanisms underlying disease progression and to help distinguish primary causative changes from secondary effects. These sensitive techniques may also overcome some of the limitations of currently available methods such as real-time RT-PCR, which has been demonstrated as not sufficiently sensitive to validate gene expression changes detected by microarray with a fold-change of less than approximately 1.4 [78], most likely due to the exponential nature of the amplification protocol.

Focussed studies of particular brain regions of interest could utilise cutting-edge RNA sequencing techniques, which provide information on novel transcripts and splice variants not specifically targeted by the arrays. This technology has already been used to determine region-specific gene expression changes in post-mortem brains from patients with Alzheimer's disease [79], although accurate quantification of transcript levels using these methods is currently limited due to issues relating to normalisation [76].

It will be valuable to clarify which gene expression changes result from effects dependent on systemic iron overload and which result from effects of deletion of *Hfe* or other iron-related genes independent of systemic iron overload. This could be investigated by performing regular venesection or iron chelation on *Hfe*^{-/-} mice to maintain body iron levels within normal ranges or by using conditional knockout mice with brain-specific disruption of the *Hfe* gene. Rodent or human neural cell culture models may help further delineate which changes arise due to iron and which are due to dysfunction of HFE or other gene targets. This could be achieved by assessing genome-wide gene expression after either treating cells with iron or using RNA interference to knock down HFE expression in iron-replete or iron-deficient cells.

Although there is clearly still a long way to go in understanding how iron acts, we believe the new high-throughput

and other technologies now emerging will reveal that iron is not only a key player in assorted brain functions but that even relatively small, short-term changes in systemic iron levels may be having far more effect on everyday brain activities than is presently realised. This is particularly true for conditions such as haemochromatosis which involve effects accumulating over long time periods of decades or more, even in the juvenile forms of the disease. We therefore believe that it is important to report on systems undergoing large numbers of small changes in the literature, with the appropriate caveats on the limitations of this kind of data. Ultimately confirmation or negation will come from the cumulative weight of independent studies from different groups using a variety of different approaches. In the interim, we believe that the data now coming from high-throughput technologies is providing valuable signposts for guiding future research.

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