Brain transcriptome perturbations in the transferrin receptor 2 mutant mouse support the case for brain changes in iron loading disorders, including effects relating to long-term depression and long-term potentiation

Bulent Acikyol[1,2], Ross M. Graham[3,4,5,6], Debbie Trinder[3,4], Michael J. House[7], John K. Olynyk[6,8,10], Rodney J. Scott[1,2], Elizabeth A. Milward[1,2], Daniel M. Johnstone[1,2,9]

[1]School of Biomedical Sciences and Pharmacy, The University of Newcastle, Callaghan, NSW, Australia

[2]Hunter Medical Research Institute, New Lambton Heights, NSW, Australia

[3]School of Medicine & Pharmacology, Fremantle Hospital, University of Western Australia, Fremantle, WA, Australia

[4]Western Australian Institute for Medical Research, Perth, WA, Australia

[5]School of Biomedical Sciences and [6]Curtin Health Innovation Research Institute, Curtin University of Technology, Bentley, WA, Australia

[7]School of Physics, University of Western Australia, Perth, WA, Australia

[8]Department of Gastroenterology, Fremantle Hospital, Fremantle, WA, Australia

[9]Bosch Institute and Discipline of Physiology, University of Sydney, NSW, Australia

[10]Institute for Immunology and Infectious Diseases, Murdoch University, Murdoch, WA, Australia

Running Title: TFR2 mutant mice brain transcriptome changes

Corresponding author: Dr Dan Johnstone, Discipline of Physiology, F13 – Anderson Stuart, The University of Sydney, NSW 2006, Australia.

Tel.: +61 2 9351 5162; Fax: +61 2 9351 6470, Email: Daniel.Johnstone@sydney.edu.au

Abbreviations used: cDNA, complementary DNA; ICP-AES, inductively coupled plasma atomic emission spectroscopy; IRE/IRP, iron-responsive element/iron regulatory protein; LTD, long-term depression; LTP, long-term potentiation; TFR2, transferrin receptor 2

Abstract

Iron abnormalities within the brain are associated with several rare but severe neurodegenerative conditions. There is growing evidence that more common systemic iron loading disorders such as hemochromatosis can also have important effects on the brain. To identify features that are common across different forms of hemochromatosis, we used microarray and real-time RT-PCR to assess brain transcriptome profiles of transferrin receptor 2 mutant mice ($Tfr2^{mut}$), a model of a rare type of hereditary hemochromatosis, relative to wildtype control mice. The results were compared with our previous findings in dietary iron-supplemented wildtype mice and $Hfe^{-/-}$ mice, a model of a common type of hereditary hemochromatosis. For transcripts showing significant changes relative to controls across all three models, there was perfect (100%) directional concordance (i.e. transcripts were increased in all models or decreased in all models). Comparison of the two models of hereditary hemochromatosis, which showed more pronounced changes than the dietary iron-supplemented mice, revealed numerous common molecular effects. Pathway analyses highlighted changes for genes relating to long-term depression (6.8-fold enrichment, $p=5.4\times10^{-7}$) and, to a lesser extent, long-term potentiation (3.7-fold enrichment, $p=0.01$), with generalized reductions in transcription of key genes from these pathways, which are involved in modulating synaptic strength and efficacy and are essential for memory and learning. The agreement across the models suggests the findings are robust and strengthens previous evidence that iron loading disorders affect the brain. Perturbations of brain phenomena such as long-term depression and long-term potentiation might partly explain neurologic symptoms reported for some hemochromatosis patients.

1 Introduction

Iron has crucial roles in brain functions such as neurotransmitter synthesis and myelination but abnormally high brain iron levels have been proposed to be associated with neurodegenerative diseases such as Alzheimer's disease, Parkinson's disease, Huntington's disease and neurodegeneration with brain iron accumulation (reviewed (Johnstone and Milward, 2010b)). However debate surrounds the question of whether disorders of systemic iron accumulation, such as hereditary hemochromatosis, can also have detrimental effects on the brain.

Hereditary hemochromatosis is a genetic disorder characterized by iron loading in peripheral tissues such as the liver (Pietrangelo, 2006; Ayonrinde et al., 2008). Evidence from case studies suggests that some hemochromatosis patients also have abnormal iron accumulation in the brain, particularly in the basal ganglia (Nielsen et al., 1995; Berg et al., 2000; Rutgers et al., 2007) and choroid plexus, pituitary, periventricular and perivascular regions (reviewed (Russo et al., 2004)). However there have been few, if any, studies of brain iron levels in large cohorts of hemochromatosis patients. While liver damage, arthritis and diabetes are the most well documented clinical consequences of hemochromatosis, some clinical and epidemiological studies have suggested possible associations with neurologic problems, including extreme fatigue (Whitlock et al., 2006; Allen et al., 2008; McLaren et al., 2008) and severe headaches (Hagen et al., 2002; Stovner et al., 2002).

Hemochromatosis is most commonly due to disease-causing polymorphisms in the *HFE* gene (Feder et al., 1996; Brissot et al., 1999; Olynyk et al., 1999), however loss-of-function mutations in the transferrin receptor 2 (*TFR2*) gene have also been demonstrated as causative (Camaschella et al., 2000; De Gobbi et al., 2001; Roetto et al., 2001). The *TFR2* gene is

transcribed in two main isoforms – the full-length form (α) and a shorter form (β) (Kawabata et al., 1999). In the liver, the primary site of iron storage and regulation, both HFE and TFR2-α (but not TFR2-β (Roetto et al., 2010)) are involved in regulating expression of the hormone peptide hepcidin, which in turn regulates dietary iron absorption (Nemeth et al., 2004). Loss-of-function mutations in either HFE or TFR2 lead to loss of hepcidin regulation and inappropriate iron absorption (Ajioka et al., 2002; Bridle et al., 2003; Nemeth et al., 2005; Drake et al., 2007).

Evidence from *in vitro* studies of hepatocytes has been interpreted to suggest that HFE and TFR2 form part of a common feedback mechanism that regulates hepcidin expression (Gao et al., 2009), however evidence from mice with combined deletion of *Hfe* and *Tfr2* genes suggest that the HFE and TFR2 proteins are involved in parallel pathways (Wallace et al., 2009a; Wallace et al., 2009b; Delima et al., 2012). It is not known whether HFE and TFR2 perform similar iron regulatory functions in other parts of the body, such as the brain, nor whether they are involved in common or parallel pathways in non-hepatic tissues.

We have previously investigated the effects of hemochromatosis on the brain by assessing the brain transcriptome in mouse models of dietary iron loading (Johnstone and Milward, 2010a) and HFE-related hemochromatosis (*Hfe*^{-/-} mice) (Johnstone et al., 2012a; Johnstone et al., 2012b). While these two models show comparable liver iron loading and no detectable brain iron loading, the brains of *Hfe*^{-/-} mice show substantially more changes at the transcript level than dietary iron-supplemented mice, suggesting hereditary hemochromatosis has more widespread effects on the brain than dietary iron loading. It is unclear whether this is due to the chronic nature of the iron accumulation peripherally, or due to disruption of internal brain systems which rely on HFE function.

To investigate this further, we have assessed the brain transcriptome of *Tfr2* mutant mice (*Tfr2*[mut]), another model of hereditary hemochromatosis that develops chronic iron loading, with the aim of delineating which brain effects are due to chronic systemic iron loading and which are due to gene-specific disruption within the brain. We also aimed to assess the replicability, in the *Tfr2*[mut] model, of important findings from the *Hfe*^{-/-} mouse study which could point to some of the underlying causes of neurologic sequelae reported for hemochromatosis patients.

2 Experimental Procedures

2.1 Animals

All animal work was conducted at the University of Western Australia and all protocols were approved by the institutional Animal Ethics Committee. The *Tfr2* mutant mouse model (*Tfr2*[mut]) has been described previously (Fleming et al., 2002; Drake et al., 2007; Chua et al., 2010). Mice are homozygous for the *Tfr2* Y245X nonsense mutation, orthologous to the *TFR2* Y250X mutation found in human patients (Camaschella et al., 2000). Wildtype and *Tfr2*[mut] mice were of the AKR strain, which has been shown to manifest a stronger iron loading phenotype than other mouse strains, with higher hepatic iron concentration and serum transferrin saturation (McLachlan et al., 2011). All mice were male and were fed standard mouse chow from weaning. At 10 weeks of age, mice were sacrificed under anesthesia (50 mg/kg ketamine, 10 mg/kg xylazine; Troy Laboratories Pty Ltd, Smithfield, NSW, Australia) and perfused transcardially with isotonic saline. Brains were removed, snap-frozen in liquid nitrogen and stored at -80^0C.

2.2 Iron measurements

Non-heme iron levels in brain and liver homogenates were assessed using the method of Kaldor (Kaldor, 1954), as described previously (Johnstone and Milward, 2010a; Johnstone et al., 2012a).

Total brain iron levels were assessed by inductively coupled plasma atomic emission spectroscopy (ICP-AES). Approximately 150 mg of homogenized brain tissue from wildtype controls (n=7) or $Tfr2^{mut}$ mice (n=5) was digested with 10 ml concentrated nitric acid (69%) at 60ºC. Following complete solubilization of tissue, samples were heated to 95ºC until volume was reduced to ~1 ml. Upon cooling, samples were diluted to ~8 ml in 1% nitric acid. Samples were sent to the Marine and Freshwater Research Laboratory, Murdoch University, Australia for measurement of iron and other metals (cadmium, copper, manganese, lead, selenium, zinc) by ICP-AES.

2.3 Western immunoblotting

To isolate protein, whole brain tissue homogenates from wildtype mice (n=7) and $Tfr2^{mut}$ mice (n=5) were treated with a lysis buffer (0.5% Triton X-100, 25 mM Tris-HCl, 150 nM NaCl) containing 1 tablet of CompleteMini protease inhibitor cocktail (Roche Diagnostics GmbH, Mannheim, Germany) per 10 ml buffer. Protein concentration was quantified by BCA assay (Pierce, Rockford, IL, USA). Proteins (60 μg/lane) were separated by 12% sodium dodecyl sulfate polyacrylamide gel electrophoresis and Western immunoblotting and visualization performed as described previously (Chua et al., 2008). Ferritin was detected using rabbit anti-human ferritin primary antibody (1:2000; Dako, Carpinteria, CA, USA), which recognizes both heavy and light chains, and goat anti-rabbit IgG-HRP secondary

antibody (1:2000; Santa Cruz Biotechnology, Santa Cruz, CA, USA). Actin, used as a reference protein, was detected using a mouse anti-human primary antibody (1:1000; Millipore, Billerica, MA, USA) followed by goat anti-mouse IgG-HRP secondary antibody (1:2000; Santa Cruz Biotechnology). Images were obtained using a Versadoc imaging system and densitometry performed using the QuantityOne software package (Bio-Rad Laboratories, Hercules, CA, USA). Ferritin protein levels were quantified relative to levels of actin.

2.4 Microarray

Whole brains homogenates from biological replicates of wildtype mice (n=4) and $Tfr2^{mut}$ mice (n=4) were subjected to RNA isolation using TRI Reagent (Ambion, Austin, TX, USA), according to the manufacturer's instructions. Following isolation, total RNA was purified and concentrated using the RNeasy MinElute Kit (QIAGEN, Hilden, Germany). Total RNA (500 ng) was prepared for microarray using the Illumina TotalPrep RNA Amplification Kit (Ambion).

The brain transcriptome was assessed using Illumina Sentrix MouseRef-8 (v1.1) BeadChip microarrays, which contain ~24,000 specific oligonucleotide probes. Arrays were scanned using the Illumina BeadArray Reader and BeadScan software. Data were analysed using BeadStudio v3 (Illumina, San Diego, CA, USA), with normalization by Cubic Spline and differential expression analysis using the Illumina Custom algorithm. This generated a list of probes with significant ($p<0.05$) differences in signal intensity between $Tfr2^{mut}$ mice and wildtype controls. This probe list was then filtered by detection p-value in order to eliminate probes detecting non-specific signals. Probes assigned a detection p-value ≥ 0.01 in both control and $Tfr2^{mut}$ groups were removed from further consideration. Probe annotations from the microarray manifest file were updated using the SOURCE database

(http://smd.stanford.edu/cgi-bin/source/sourceSearch), using the listed NCBI transcript accession numbers as the search terms. Where the accession number was no longer listed in the database, annotations were updated by aligning the probe sequence against the mouse transcriptome using BLAST (http://blast.ncbi.nlm.nih.gov/Blast.cgi).

2.5 Pathway classification and enrichment analysis

Pathway enrichment analyses were performed using the Database for Annotation, Visualization and Integrated Discovery (DAVID; http://david.abcc.ncifcrf.gov/) (Huang da et al., 2009b, a). Pathway classification within DAVID used the Kyoto Encyclopedia of Genes and Genomes database (KEGG; http://www.genome.jp/kegg/pathway.html). We assessed enrichment among the list of transcripts with significantly altered levels between wildtype and *Tfr2*^{mut} groups, and also further subdivided these lists into up-regulated and down-regulated transcripts. Enrichment statistics were adjusted by Benjamini correction.

2.6 Real-time RT-PCR

Levels of select transcripts of interest were measured by real-time RT-PCR in additional wildtype mice (*n*=8) and *Tfr2*^{mut} mice (*n*=9). Reverse transcription of 1 μg total RNA to complementary DNA (cDNA) was performed using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Carlsbad, CA, USA). Real-time PCR was performed on the ABI 7500 Real Time PCR System (Applied Biosystems). Each reaction was performed in triplicate and contained Power SYBR Green PCR Master Mix (Applied Biosystems), 200 nM forward and reverse primers (Table 1) and 4.5 μl cDNA. Transcript levels of interest were quantified relative to transcript levels for two reference genes (*Rpl13a*, *Gapdh*) using the geometric mean of threshold cycle values as the reference. Student *t*-testing was used for statistical comparisons of wildtype control and *Tfr2*^{mut} group means.

3 Results

3.1 Iron measures

Animals used in this study were aged 10 weeks, an age at which $Tfr2^{mut}$ mice have been reported to manifest liver iron loading comparable to that in mild human hemochromatosis but no gross damage to the liver or other tissues (Drake et al., 2007). This age is the same as that at which changes have been observed in our previous studies of other mouse models of iron loading (Johnstone and Milward, 2010a; Johnstone et al., 2012a) and facilitates assessment of early changes occurring in response to TFR2 dysfunction, uncomplicated by subsequent secondary effects.

As expected, liver non-heme iron levels were significantly higher (>3-fold) in $Tfr2^{mut}$ mice than wildtype controls (955±73 μg Fe/g tissue vs. 307±28 μg Fe/g tissue; $p<0.05$). In contrast, $Tfr2^{mut}$ and control mice showed no difference in brain total iron levels measured by ICP-AES (13.2±0.2 μg Fe/g tissue vs. 13.1±0.6 μg Fe/g tissue). Similarly, there was no difference in brain non-heme iron levels between the two groups ($p>0.05$). Measurement of other metals by ICP-AES revealed no difference in levels of copper, zinc and manganese, while levels of cadmium, lead and selenium were below the detection limits of the assay (data not shown).

3.2 Summary of microarray results

Levels of numerous mRNA transcripts differed significantly ($p<0.05$) between brains from $Tfr2^{mut}$ mice and wildtype controls. In summary, of the 24,611 probes on the array, 10,835 detected signals significantly above background levels. Of these, 742 probes, targeting 712

different transcripts, detected significantly altered expression in group comparisons of $Tfr2^{mut}$ and wildtype brain, however most of these changes (~95%) were less than 2-fold in magnitude.

3.3 Expression of iron-related genes

The $Tfr2^{mut}$ mice showed few expression changes for genes relating to iron metabolism or regulation. Transcripts for TFR2 were detectable in wildtype brain and significantly reduced in $Tfr2^{mut}$ brain (2.2-fold decrease, p=0.007), presumably due to nonsense-mediated decay. Other transcripts with key roles in iron metabolism or regulation, including hepcidin, showed either unaltered levels or were below microarray detection limits (data not shown). One notable exception was a significant increase in transcripts by microarray (1.3-fold increase, p=0.033) for ferritin light chain 1 (*Ftl1*), encoding an essential component of the iron storage protein ferritin, however this transcript level change was not validated by real-time RT-PCR (Fig. 1). Western immunoblotting of whole brain homogenate showed increased levels of total ferritin protein in $Tfr2^{mut}$ mouse brain relative to wildtype controls (1.5-fold increase, p=0.01; Fig. 2), however we were unable to resolve light and heavy chain subunits.

3.4 Pathway enrichment analysis

To determine whether particular pathways were over-represented in the lists of differentially-expressed genes, we used the online tool DAVID to classify the list of significantly altered transcripts into KEGG pathways and assess pathway enrichment. This list was further divided into two sets comprising transcripts that were i) up-regulated or ii) down-regulated, and enrichment assessed within these subsets. While there were no significantly enriched pathways among the list of up-regulated transcripts, several pathways were identified as significantly enriched among the list of down-regulated transcripts (Table 2). The most

highly enriched pathway, which remained significant after adjustment for multiple hypothesis testing using the conservative Benjamini correction, related to long-term depression (LTD), with the related pathway of long-term potentiation (LTP) also identified as significantly enriched. These processes relate to a reduction or enhancement, respectively, in synaptic strength and efficacy and are key to the process of synaptic plasticity. The list of down-regulated genes classified in these pathways is given in Table 3. Notably, the long-term depression pathway was also identified as down-regulated in our previous study of $Hfe^{-/-}$ mouse brain (Johnstone et al., 2012b).

There was also significant enrichment, among the list of down-regulated transcripts, of various pathways relating to cancer, most notably glioma (Table 2). Most of the genes identified in this pathway also form part of the LTD or LTP pathways (Table 3), including the important oncogenes v-raf-leukemia ($Raf1$) and its upstream effector Kirsten rat sarcoma ($Kras$). A similar result was observed in the $Hfe^{-/-}$ mice for $Raf1$ (1.5-fold decrease in transcripts relative to wildtype controls, $p=0.028$) but not $Kras$ (no significant change, $p>0.05$).

3.5 Comparison of transcriptome profiles of $Tfr2^{mut}$, $Hfe^{-/-}$ and dietary iron-supplemented mice

As noted above, we have previously published studies of the brain transcriptome in mouse models of dietary iron loading (Johnstone and Milward, 2010a) and HFE hemochromatosis (Johnstone et al., 2012a; Johnstone et al., 2012b), also aged 10 weeks. Comparison of differentially-expressed gene lists from the three different models of iron loading revealed 34 transcripts common to all lists (Fig. 3). Strikingly, for these 34 transcripts (shown in Table 4), across the three models there was 100% concordance in the direction of change relative to

wildtype (i.e. transcripts were increased in all models or decreased in all models), much higher than the 25% that would be expected by chance alone, suggesting that the majority of these findings are not false positives. Of interest, all models showed significantly increased transcripts for ferritin light chain 1 and spastin, which is genetically linked to spastic paraplegia, and decreased transcripts for the metal binding protein metallothionein 3 (Table 4).

When comparing just the two models of hereditary hemochromatosis, $Tfr2^{mut}$ and $Hfe^{-/-}$ mice, there was substantial overlap in the list of differentially expressed transcripts, with two-thirds of the transcripts identified in $Tfr2^{mut}$ mice also significantly altered in $Hfe^{-/-}$ mice. Again the direction of change relative to wildtype was highly consistent, with 96% concordance. We focused our validation studies for the $Tfr2^{mut}$ mice on various key transcripts previously validated in the $Hfe^{-/-}$ model, including transcripts relating to the LTD or LTP pathways, as the consistency observed across different models suggests these are likely to be the most robust findings.

We have previously reported increased transcripts by both microarray and real-time RT-PCR for the immediate early gene Fos, as well as other genes encoding transcription factors, in $Hfe^{-/-}$ mice (Johnstone et al., 2012a). We also observed a large increase in Fos transcripts in $Tfr2^{mut}$ mice by microarray (Table 5) and this change was also validated with statistical significance by real-time RT-PCR (Fig. 1).

Both $Tfr2^{mut}$ and $Hfe^{-/-}$ mice showed significantly decreased transcripts for calcium/calmodulin-dependent protein kinase II alpha ($Camk2a$; Table 5), which has key roles in synaptic plasticity and learning and memory and is part of the LTP pathway

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(Yamauchi, 2005; Lee and Silva, 2009). This was validated by real-time RT-PCR for the *Tfr2*[mut] model (Fig. 1), having been validated previously in the *Hfe*^{-/-} mice (Johnstone et al., 2012a). There were also decreased transcripts in both models for insulin-like growth factor 1 receptor (*Igf1r*), involved in LTD and other important brain functions (Bondy and Cheng, 2004). Again this decrease was validated by real-time RT-PCR in both *Tfr2*[mut] mice (Fig. 1) and *Hfe*^{-/-} mice (Johnstone et al., 2012a).

Furthermore, both models show reduced transcripts for the calcium channel gene *Cacna1a* (Table 5), another component of the LTD pathway, and this was also validated by real-time RT-PCR (Fig. 1). In humans, the *CACNA1A* gene is causatively linked to familial hemiplegic migraine (Ducros et al., 1999; Hans et al., 1999; Pietrobon, 2002) and may provide an intriguing explanation for the increased prevalence of severe headaches reported for people with hemochromatosis or brain iron abnormalities (Hagen et al., 2002; Stovner et al., 2002; Boes et al., 2006; Gaul et al., 2007).

3.6 Key transcripts identified as altered in *Hfe*^{-/-} mice but not *Tfr2*[mut] mice

As illustrated by Fig. 3, there were substantially fewer transcripts with altered levels in *Tfr2*[mut] mice than *Hfe*^{-/-} mice. We have previously reported altered transcript levels in *Hfe*^{-/-} mice for several key genes relating to Alzheimer's disease (amyloid precursor protein *App*, presenilin 1 *Psen1* and other γ-secretase complex components) and Notch signaling (e.g. hairy and enhancer of split 1 and 5 *Hes1*, *Hes5*) (Johnstone et al., 2012b) but these transcripts did not show significantly altered levels by microarray in the *Tfr2*[mut] brain (data not shown). To confirm that microarray findings of unaltered expression of these two genes in the *Tfr2*[mut] mice, we assessed transcript levels of *App* and *Psen1* in additional wildtype and *Tfr2*[mut] mice by real-time RT-PCR. While PCR confirmed that *App* transcript levels were unchanged,

Psen1 transcript levels showed a small but significant decrease in *Tfr2*^{mut} mice relative to wildtype controls (Fig. 1), consistent with the finding in *Hfe*^{-/-} mice (Johnstone et al., 2012b).

4 Discussion

This study of the brain transcriptome in the *Tfr2*^{mut} mouse model of hereditary hemochromatosis, together with our previous studies in age-matched dietary iron-supplemented (Johnstone and Milward, 2010a) and *Hfe*^{-/-} mice (Johnstone et al., 2012a; Johnstone et al., 2012b), provides further evidence that conditions of systemic iron loading have detectable influences on brain gene expression. While microarray findings are sometimes dismissed because of perceived high potential for false positive findings, the observation of common gene expression changes across various models of the same disorder, the high degree of concordance in the direction of these changes and the validation of changes by real-time RT-PCR strongly supports the assertion that real changes are indeed occurring within the brain under conditions of systemic iron loading.

The *Tfr2*^{mut} mouse model display a similar iron phenotype to the *Hfe*^{-/-} and dietary iron-supplemented mice we have reported previously, with significantly increased liver iron levels relative to control mice but no change in brain iron levels. This was confirmed using two different methods (non-heme iron assay, ICP-AES). Interestingly, despite the lack of detectable brain iron changes, there were increased levels of ferritin protein. Ferritin is regulated post-transcriptionally by intracellular iron levels through the iron-responsive element/iron regulatory protein (IRE/IRP) system – an increase in ferritin protein in the absence of an increase in transcripts is generally indicative of an increase in intracellular 'free' iron (Hentze and Kuhn, 1996; Muckenthaler et al., 2008). The findings may therefore

indicate a redistribution of iron within the *Tfr2*^{mut} mouse brain to favour more iron moving into a stored form, either through redistribution between extracellular and intracellular compartments or redistribution between subcellular compartments. While there were no detectable alterations in transcripts for iron transporters such as ferroportin or divalent metal transporter 1 that might mediate these kinds of redistribution, translation of these transcripts is also regulated by the IRE/IRP system (Muckenthaler et al., 2008), so changes may only be evident at the protein level.

A number of key genes identified in our previously published transcriptomic studies of the *Hfe*^{-/-} mouse model of hemochromatosis (Johnstone et al., 2012a; Johnstone et al., 2012b) also showed expression changes in the *Tfr2*^{mut} mouse model, validating our previous findings and providing support for the assertion that disruption of iron homeostasis can lead to important changes in the brain. These include effects on genes involved in long-term depression, a molecular pathway that was significantly enriched among down-regulated transcripts in the two models.

Long-term depression, characterized by a long-lasting decrease in synaptic strength, is a key process in neural plasticity and therefore in learning and memory. Findings from animal models suggest that brain region-specific disruption of LTD results in a diminished capacity for specific types of learning and memory, as reviewed elsewhere (Collingridge et al., 2010). As described in more detail by Collingridge and colleagues, LTD is proposed to have roles in a range of cognitive functions, including hippocampus-dependent learning and memory (Brigman et al., 2010), fear conditioning in the amygdala (Migues et al., 2010), recognition memory in the perirhinal cortex (Griffiths et al., 2008) and cerebellar learning (Massey and Bashir, 2007).

It is therefore conceivable that the reduced expression of key genes relating to LTD observed in our mouse models of hereditary hemochromatosis will have a detrimental impact on various facets of learning and memory. Behavioural studies of these models, together with electrophysiological studies of brain slices to determine net effects on LTD/LTP, will help clarify this issue. In addition, it would be valuable to thoroughly assess learning and memory in large cohorts of patients with clinical hemochromatosis (i.e. not just individuals with pre-disposing mutations) and to further investigate suggestions of detrimental effects of high hippocampal iron on verbal memory and high basal ganglia iron on working memory (Bartzokis et al., 2011).

As far as we are aware, there have been no studies of the effects of iron loading on glioma susceptibility, however high expression of TFR2 in glioblastoma biopsies is positively correlated with patient survival (Calzolari et al., 2007; Calzolari et al., 2010). It was therefore interesting to observe reduced expression of various genes in the 'glioma' pathway in $Tfr2^{mut}$ brain, including the important and closely related oncogenes $Kras$ and $Raf1$. Although activating mutations in $KRAS$ and $RAF1$ are required for oncogenicity or in Noonan Syndrome (a common developmental disorder), deletion of either gene in mice causes embryonic lethality (Johnson et al., 1997; Huser et al., 2001; Mikula et al., 2001), indicating that both genes are essential for normal function. The consequences of reduced expression of these genes in the brain is unclear but suggest potentially important effects on the RAS-MAPK signal transduction pathway, which could have far-reaching effects on the intracellular cascades that control the cell cycle.

While there were similarities between the brain transcriptome profiles of $Hfe^{-/-}$ and $Tfr2^{mut}$ mice, there were also a number of differences, with $Tfr2^{mut}$ mice showing far fewer changes relative to wildtype controls than $Hfe^{-/-}$ mice. The reasons for this are unclear but may relate to differences in the expression patterns of HFE and TFR2 in the brain. Although there are few relevant studies to date, HFE transcripts appear to be widely expressed across different brain regions, albeit at low levels (Hanninen et al., 2009). In addition, immunohistochemical studies have provided preliminary evidence that HFE protein is expressed by brain capillary endothelial cells, choroid plexus and ependymal cells (Connor et al., 2001), in addition to scattered cells, possibly astrocytes, throughout the cortex and cerebellum (Bastin et al., 1998).

In contrast, while there has been even less investigation of TFR2 in the brain, some evidence suggests that TFR2 expression is confined to the cerebellum in humans (Hanninen et al., 2009) and that transcripts for TFR2-α are below RT-PCR assay detection limits when averaged across the whole mouse brain (Kawabata et al., 1999). The roles of TFR2-α in the brain may therefore be restricted to particular regions, notably the cerebellum, while disruption of HFE may have the potential to affect systems across a larger number of brain regions.

It should be noted, however, that while transcripts for TFR2-α are present at low levels, transcripts for TFR2-β are strongly expressed in mouse brain (Kawabata et al., 1999). This isoform of TFR2 is not involved in hepcidin regulation but is proposed to be involved in transcriptional regulation of the cellular iron export protein ferroportin in the spleen (Roetto et al., 2010) and, while its functions in the brain are not known, disruption of the $Tfr2$ gene

and consequently the TFR2-β isoform could potentially have far-reaching effects within the brain.

While this study has illuminated several important effects of iron loading disorders on the brain, some specific limitations warrant discussion. First, both the evaluations of iron levels and the transcriptomic studies focused on whole brain rather than specific regions. Due to differences in the distribution and functions of iron across different brain regions and cell types, the downstream effects of perturbed iron homeostasis are likely to vary between different brain compartments. It will be informative in future studies to investigate transcriptomic profiles of particular brain regions to determine if changes are global or restricted to certain regions, specifically regions with high concentrations of iron such as the basal ganglia (Hallgren and Sourander, 1958; Hill and Switzer, 1984; Bartzokis et al., 1997), as well as validating array results at the protein level using immunohistochemistry. In addition, it will also be valuable to further characterize the different forms of TFR2 and investigate their expression and functions in different brain regions.

Second, *Tfr2*[mut] mice showed no net brain iron loading relative to wildtype mice. It is therefore important to note that this is not a model for studying the effects of gross brain iron accumulation – rather it provides a model for investigating the effects of both systemic iron loading in general and TFR2-related iron loading in particular on the brain transcriptome. The observed effects may be due to disruption of internal brain systems that normally rely on TFR2 or due to peripheral effects of iron loading being transduced to the brain. We plan in future studies to investigate models of more severe hemochromatosis, which we expect to have detectable brain iron accumulation.

In summary, the transcriptome perturbations observed in the brains of $Tfr2^{mut}$ mice add to the growing body of evidence that disorders of systemic iron loading have important effects on the brain. The findings highlight changes to some specific molecular systems, such as long-term depression, that might partly explain sporadic reports of neurologic sequelae in some hemochromatosis patients.

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Figure Legends

Fig. 1. Real-time RT-PCR validation of transcript levels for select genes in *Tfr2*^{mut} mice.
Levels of *Ftl1*, *Camk2a*, *Igf1r*, *Cacna1a*, *Fos*, *App* and *Psen1* transcripts were determined by real-time RT-PCR and expressed relative to the levels of two reference genes (*Gapdh*, *Rpl13a*). Results are presented as mean ± SEM (n≥8 per group). * $p<0.05$ relative to wildtype mice.

Fig. 2. Ferritin protein levels in *Tfr2*^{mut} mouse brain. A) Representative Western blot showing anti-ferritin immunoreactivity in the wildtype (W) and *Tfr2*^{mut} (T) mouse brain. Actin was used as a reference protein. B) Ferritin levels were quantified by densitometry and are expressed relative to actin. Results are presented as mean ± SEM (wildtype n=7, *Tfr2*^{mut} n=5). * *p*=0.01 relative to wildtype mice.

Fig. 3. Venn diagram of probes detecting differentially-expressed transcripts in each model.

Tables

Table 1. Primer sequences for real-time RT-PCR

Gene name and symbol	Forward Primer	Reverse Primer
Amyloid precursor protein *App*	CCGTTGCCTAGTTG GTGAGTTT	CGACGGTGTGCCA GTGAA
Calcium/calmodulin-dependent protein kinase II alpha *Camk2a*	AGTGACAGAGCAG CTGATCGAA	GTTCCGGGACCAC AGGTTTT
Calcium channel, voltage-dependent, P/Q type, alpha 1A subunit	CTGGGCCTCGTCTT GCAT	GGTGTTAATGTCC TTTCCTTTGCT
FBJ osteosarcoma oncogene *Fos*	TGAAGAGGAAGAG AAACGGAGAAT	AGTTGATCTGTCT CCGCTTGGA
Ferritin light chain 1 *Ftl1*	AGGCCCTCTTGGAT CTGCAT	TCTTCTTGATGAGT TTCACCTCCTT
Glyceraldehyde-3-phosphate dehydrogenase *Gapdh*	CTGGAGAAACCTGC CAAGTA	CGTATTCATTGTC ATACCAGG
Insulin-like growth factor I receptor *Igf1r*	GCTTCTGTGAACCC CGAGTATTT	TGGTGATCTTCTCT CGAGCTACCT
Presenilin 1 *Psen1*	CTGATCGGCCTGTG CCTTA	AATCCGTGGCGAA GTAGAACA
Ribosomal protein L13A *Rpl13a*	GGTGGAAGTACCA GGCAGTGA	TTCCGTAACCTCA AGATCTGCTT

Table 2. Down-regulated pathways significantly enriched in *Tfr2*^mut brain transcriptome

Pathway	Fold Enrichment	*p* value	Benjamini *p* value
Long-term depression	6.8	5.4×10^{-7}	6.5×10^{-5}
Fc gamma R-mediated phagocytosis	3.8	0.004	0.229
Phosphatidylinositol signaling system	4.0	0.007	0.241
Glioma	3.9	0.008	0.207
Long-term potentiation	3.7	0.010	0.206
Aldosterone-regulated sodium reabsorption	5.6	0.011	0.192
ErbB signaling pathway	3.6	0.011	0.167
Pathways in cancer	2.0	0.013	0.174
Melanogenesis	3.4	0.014	0.174
Natural killer cell mediated cytotoxicity	3.3	0.017	0.187
Regulation of actin cytoskeleton	2.3	0.017	0.172
Calcium signaling pathway	2.4	0.029	0.254
GnRH signaling pathway	2.9	0.030	0.246
Insulin signaling pathway	2.6	0.032	0.244
Gap junction	2.8	0.035	0.247
Chemokine signaling pathway	2.3	0.040	0.265

Table 3. Down-regulated genes classified in the LTD, LTP or Glioma pathways

Gene name and symbol	Fold decrease	*p* value	Pathway(s)
Calcium channel, voltage-dependent, P/Q type, alpha 1A subunit *Cacna1a*	1.7	0.008	LTD
Calcium/calmodulin-dependent protein kinase II alpha *Camk2a*	1.5	0.043	LTP, Glioma
Guanine nucleotide binding protein, alpha O *Gnao1*	1.4	0.009	LTD
Glutamate receptor, ionotropic, AMPA2 (alpha 2) *Gria2*	1.3	0.036	LTD, LTP
Glutamate receptor, ionotropic, AMPA3 (alpha 3) *Gria3*	1.4	0.032	LTD
Guanylate cyclase 1, soluble, beta 3 *Gucy1b3*	1.4	0.001	LTD
Insulin-like growth factor I receptor *Igf1r*	1.5	0.044	LTD, Glioma
V-Ki-ras2 Kirsten rat sarcoma viral oncogene homolog *Kras*	1.4	0.044	LTD, LTP, Glioma
Phospholipase C, beta 1 *Plcb1*	1.4	0.042	LTD, LTP
Protein phosphatase 2 (formerly 2A), regulatory subunit A (PR 65), alpha isoform *Ppp2r1a*	1.4	<0.001	LTD
Protein kinase C, alpha *Prkca*	1.6	0.002	LTD, LTP, Glioma
Protein kinase C, gamma *Prkcc*	1.6	0.010	LTD, LTP, Glioma
V-raf-leukemia viral oncogene 1 *Raf1*	1.3	0.047	LTD, LTP, Glioma
Ribosomal protein S6 kinase, polypeptide 2 *Rps6ka2*	1.3	0.030	LTP

Table 4. Genes with significantly altered expression in all three models of iron loading

Gene symbol	Gene name	Fold change (vs. control , $p<0.05$)		
		$Tfr2^{mut}$	$Hfe^{-/-}$	Iron Diet
2310003H01Rik	RIKEN cDNA 2310003H01 gene	1.3	1.6	1.2
2310022B05Rik	RIKEN cDNA 2310022B05 gene	1.3	1.5	1.2
4833439L19Rik	RIKEN cDNA 4833439L19 gene	-1.4	-1.8	-1.3
5133401N09Rik	RIKEN cDNA 5133401N09 gene	1.3	1.5	1.2
Apoa1bp	Apolipoprotein A-I binding protein	1.3	1.9	1.2
Arfip2	ADP-ribosylation factor interacting protein 2	-1.3	-3.3	-1.2
Atp2a1	ATPase, Ca++ transporting, cardiac muscle, fast twitch 1	2.0	2.4	1.5
Chst10	Carbohydrate sulfotransferase 10	1.5	1.9	1.2
Coro2b	Coronin, actin binding protein, 2B	-2.0	-3.1	-1.7
Doc2b	Double C2, beta	1.6	2.0	1.4
Efemp2	Epidermal growth factor-containing fibulin-like extracellular matrix protein 2	1.5	1.7	1.3
Fam108c	Family with sequence similarity 108, member C	1.6	1.9	1.4
Fam109a	Family with sequence similarity 109, member A	1.4	1.3	1.3
Frs3	Fibroblast growth factor receptor substrate 3	1.4	1.8	1.2
Ftl1	Ferritin light chain 1	1.3	1.3	1.2
H1f0	H1 histone family, member 0	2.2	3.0	1.6
Hsdl1	Hydroxysteroid dehydrogenase like 1	1.3	1.8	1.1
Klhdc1	Kelch domain containing 1	-1.4	-1.9	-1.3
Litaf	LPS-induced TN factor	1.3	1.4	1.2
Mpv17	MpV17 mitochondrial inner membrane protein	-1.3	-1.4	-1.3
Mrpl13	Mitochondrial ribosomal protein L13	1.5	2.5	1.3
Mt3	Metallothionein 3	-1.5	-2.1	-1.4
Nkain4	Na+/K+ transporting ATPase interacting 4	1.6	2.3	1.5
Pak4	P21 protein (Cdc42/Rac)-activated kinase 4	1.4	2.2	1.2
Plcb1	Phospholipase C, beta 1	-1.4	-1.4	-1.3
Polr1c	Polymerase (RNA) I polypeptide C	1.4	2.1	1.2
Psip1	PC4 and SFRS1 interacting protein 1	-1.6	-4.7	-1.5
Rbm4b	RNA binding motif protein 4B	1.3	1.9	1.2
Slc16a2	Solute carrier family 16 (monocarboxylic acid transporters), member 2	1.8	2.1	1.3
Smoc1	SPARC related modular calcium binding 1	1.4	1.6	1.3
Spast	Spastin	1.7	2.6	1.2
Spsb4	SplA/ryanodine receptor domain and SOCS box containing 4	1.5	2.2	1.2
Ube2ql1	Ubiquitin-conjugating enzyme E2Q family-like 1	-1.3	-2.1	-1.2
Zdhhc4	Zinc finger, DHHC domain containing 4	1.5	1.8	1.3

Decreased expression relative to control is presented as a negative fold change, increased expression relative to control is presented as a positive fold change.

Table 5. Select significant microarray findings common to *Tfr2*[mut] and *Hfe*^{-/-} mice

Gene name and symbol	Gene functions	Fold change (vs. wildtype, $p<0.05$)	
		Tfr2[mut]	*Hfe*^{-/-}
Calcium channel, voltage-dependent, P/Q type, alpha 1A subunit *Cacna1a*	LTD, linked to familial hemiplegic migraine	↓ 1.7	↓ 8.9
Calcium/calmodulin-dependent protein kinase IIα *Camk2a*	LTP, synaptic plasticity, learning and memory	↓ 1.5	↓ 1.9
FBJ osteosarcoma oncogene *Fos*	Transcription factor, marker of neuronal activation	↑ 2.4	↑ 3.7
Insulin-like growth factor 1 receptor *Igf1r*	LTD, brain development, anti-apoptotic properties	↓ 1.5	↓ 11.5

↑ = increased transcripts relative to wildtype control, ↓ = decreased transcripts relative to wildtype control.