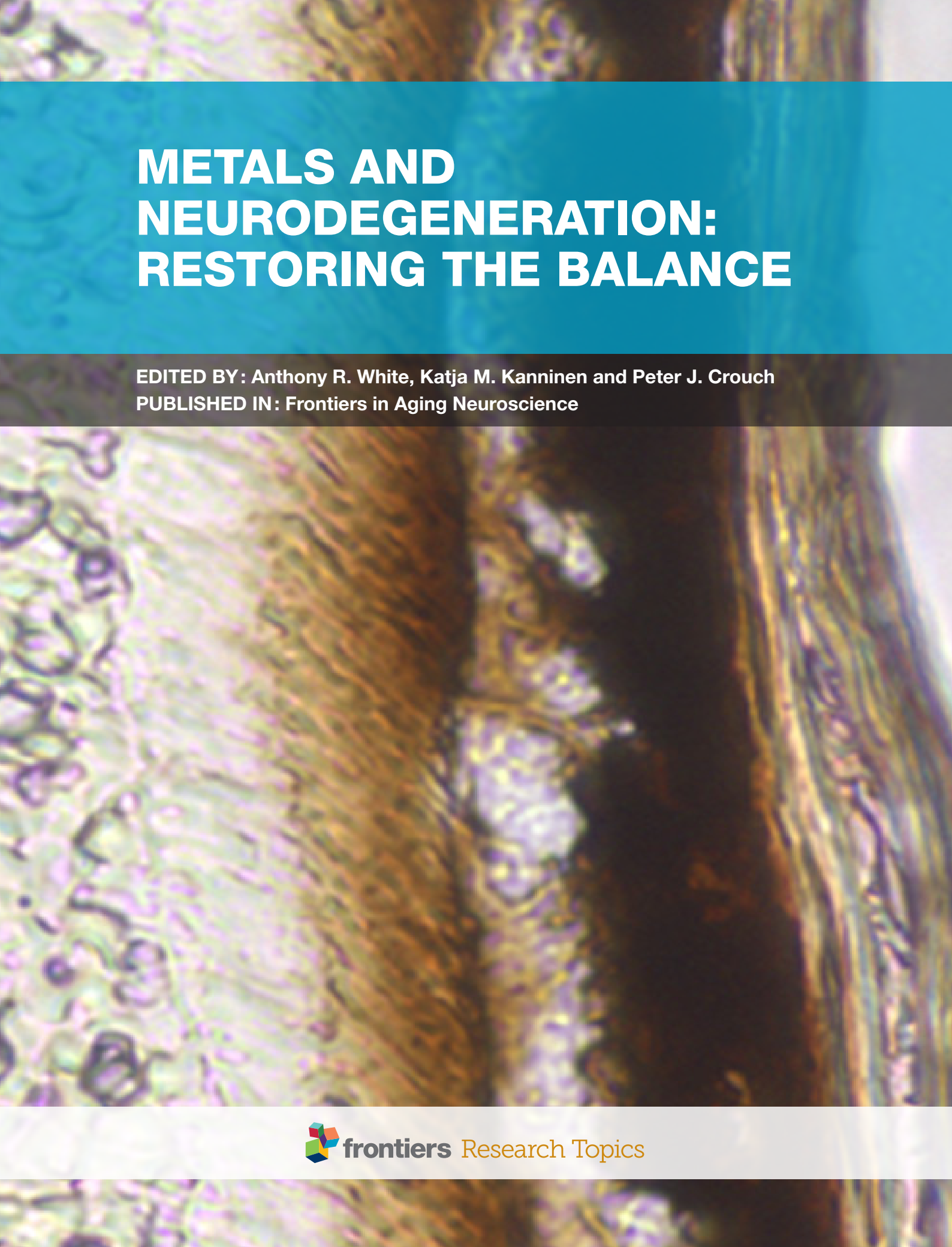

METALS AND NEURODEGENERATION: RESTORING THE BALANCE

EDITED BY: Anthony R. White, Katja M. Kanninen and Peter J. Crouch

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METALS AND NEURODEGENERATION: RESTORING THE BALANCE

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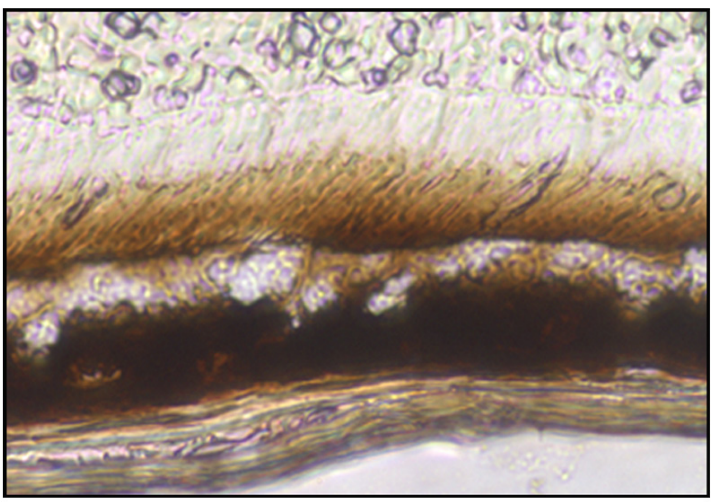

Light photomicrograph of 6-month-old Cp-/-Heph-/Y retina after DAB enhancement of Perls' stain for iron (brown). The image shows the critical role of ceruloplasmin (Cp) and hephaestin (Heph) in retinal iron regulation.
Taken from: Song D and Dunaief JL (2013) Retinal iron homeostasis in health and disease. Front. Aging Neurosci. 5:24. doi:10.3389/fnagi.2013.00024

Biometals such as copper, zinc and iron have key biological functions, however, aberrant metabolism can lead to detrimental effects on cell function and survival. These biometals have important roles in the brain, driving cellular respiration, antioxidant activity, intracellular signaling and many additional structural and enzymatic functions. There is now considerable evidence that abnormal biometal homeostasis is a key feature of many neurodegenerative diseases and may have an important role in the onset and progression of disorders such as Alzheimer's, Parkinson's, prion and motor neuron diseases. Recent studies also support biometal roles in a number of less common neurodegenerative disorders. The role of biometals in a growing list of brain disorders is supported by evidence from a wide range of sources including molecular genetics, biochemical studies and biometal imaging. These studies have spurred a growing interest in understanding the role of biometals in brain function and disease as well as the development

of therapeutic approaches that may be able to restore the altered biometal chemistry of the brain. These approaches range from genetic manipulation of biometal transport to chelation of excess metals or delivery of metals where levels are deficient. A number of these approaches are offering promising results in cellular and animal models of neurodegeneration with successful translation to pre-clinical and clinical trials. At a time of aging populations and slow progress in development of neurotherapeutics to treat age-related neurodegenerative diseases, there is now a critical need to further our understanding of biometals in neurodegeneration. This issue covers a broad range of topics related to biometals and their role in neurodegeneration. It is hoped that this will inspire greater discussion and exchange of ideas in this crucial area of research and lead to positive outcomes for sufferers of these neurodegenerative diseases.

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EDITORIAL
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Editorial: Metals and neurodegeneration: restoring the balance

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Keywords: metals, neurodegenerative diseases, Alzheimer's disease, Parkinson's disease (PD), copper, zinc, iron, iron chelating agents

There is considerable evidence that abnormal biometal homeostasis is a key feature of many neurodegenerative diseases and may have an important role in the onset and progression of disorders such as Alzheimer's (AD), Parkinson's (PD), prion, and motor neuron diseases. The role of biometals in a growing list of brain disorders is supported by evidence from a wide range of sources including molecular genetics, biochemical studies and biometal imaging. These studies have spurred a growing interest in understanding the role of biometals in brain function and disease as well as the development of therapeutic approaches that may be able to restore the altered biometal chemistry of the brain. In this Research Topic, Metals and Neurodegeneration: Restoring the Balance, we probe the biochemical basis of metal-mediated neurodegeneration, examine genetic links between metal dyshomeostasis and brain disorders, investigate metal trafficking and metal-synaptic interactions, and their role in neurodegeneration, and examine some of the key new approaches to understanding how metals drive neurodegenerative changes. We hope that these exciting insights will provide a strong platform to develop advances in therapeutics that will allow us to "restore the balance" in metal homeostasis in the brain.

One of the most prominent features across many neurodegenerative disorders is loss of metal homeostasis, and in many cases, the metal revealing the most substantial change is iron (Fe). Opening this Research Topic, Hare et al. (2013) provides an excellent overview of Fe transport and Fe-regulatory processes in the brain, and demonstrates clearly the complexity in these processes. The review describes how abnormalities in this complex process can lead to loss of Fe, which is associated with changes in neurotransmission, energy production and myelination, and is associated with diseases such as AD. Conversely, abnormal Fe handling can also lead to Fe accumulation, which is associated with AD and PD and is a major target of therapeutic developments based on Fe chelation. Expanding on Fe in neurodegeneration is the review by Muhoberac and Vidal (2013), who explore the genetic basis of Fe dyshomeostasis in hereditary ferritinopathy. The article provides a timely insight into the effects of abnormal Fe metabolism through loss of ferritin function, a key Fe-regulatory protein and how these changes can lead to ferritin accumulation, reactive oxygen species formation and oxidative stress. Mariani et al. (2013), further explores hereditary links between Fe and neurodegeneration. They describe links between Fe-specific gene variations (e.g., transferrin, hemochromatosis) and Fe regulatory proteins (ceruloplasmin and apolipoprotein E) in AD, PD and mild cognitive impairment. Despite a small cohort and rare alleles the studies provide important insights on altered Fe in neurodegeneration and illustrate that the role of metals in neurodegeneration must be examined in association with genetics, and disease sub-populations to gain a clear insight into the contributory role of metals in these diseases. When we think of metals and neurodegeneration, we often focus on the brain, but metal changes in the eye are also prominent in these disorders. Song and Dunaief (2013) describe

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the role of Fe in retinal degeneration in hereditary Fe overload disorders and the potential impact of Fe accumulation in acute macular degeneration.

Other metal changes also feature strongly in neurodegenerative diseases. Telianidis et al. (2013) provides an excellent overview of the key copper (Cu) transport proteins, ATP7a and ATP7b in cellular Cu homeostasis. They provide important insights into the cell fate when abnormal Cu trafficking occurs, as is evidenced by the genetic diseases, Menkes and Wilson disease, and more broadly in AD and prion diseases. Dringen et al. (2013) extends this to describe the essential role of Cu uptake, transport, metabolism, and export by astrocytes in the brain and how this has a major impact in neuronal survival and function. Cu is also an essential metal in synaptic function as illustrated by the exciting new research by Castro et al. (2014). They describe how Cu modulates zinc (Zn) homeostasis (another key metal involved in neurotransmission) in hippocampal neurons, and report that Cu has significant effects on expression of key synaptic proteins, synapsin, and dynamin. Zn and its contribution to neurodegeneration is also the topic covered by Szewczyk (2013), who describes how uncontrolled influx of Zn during traumatic brain injury and stroke, can exacerbate neuronal cell death. In contrast, Zn deficiency may also have a key role in neurodegeneration. This is covered by Szewczyk, with an insight into how changes to Zn transporters and metallothionein may contribute to altered Zn homeostasis in neurodegeneration.

As with Fe, Cu and Zn, less common biometals such as manganese (Mn) appear to have a growing role in neurodegenerative processes. Rather than intoxication from high doses, metals such as Mn can have extraordinary outcomes on complex cortical structures and associated cognitive function even at very low doses. This is covered in depth by Guilarte (2013). These effects may have an important role in neurodegenerative changes in AD and Parkinsonism.

Altered biometal homeostasis is not only a factor in the leading forms or neurodegeneration. They have a key role in many rarer forms of neurodegeneration including childhood neurodegenerative disorders. Parker et al. (2013), report on the role of metals in neuronal ceroid lipofuscinosis, neurodegeneration with brain iron accumulation (NBIA), and additional diseases, further extending the links between genetic mutations and metal abnormalities in these disorders.

Obviously, while this is a rapidly expanding field of research, as illustrated here, we still have much further to go to achieve a major understanding of where these biometal changes fit into the neurodegenerative disease process. Are they prime drivers of disease, significant contributors, a downstream outcome, or (likely) a mix of these? These questions will only be answered through the application of highly sensitive analytical and genetic approaches. Lothian et al. (2013), describe how this can be achieved with the relatively new and rapidly advancing field of metalloproteomics to dissect metal-protein interactions. Chen et al. (2013) then concludes with an excellent description of the powerful genetic model of *C. elegans* and how this is used to pin-point assessment of altered metal homeostasis and its associated genes in a simple but elegant model system.

It has been extraordinarily difficult to get the mainstream fields of neuroscience and medicine to understand the key role biometals have in neurodegeneration. We hope that this Research Topic will help to inform and expand the knowledge on how biometals contribute to neurodegeneration, and inspire others to enter this rapidly growing and exciting field of research. New insights are needed as a basis for innovative therapeutic approaches that hopefully will help to "restore the balance" in these widespread diseases.

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REVIEW ARTICLE
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A delicate balance: iron metabolism and diseases of the brain

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Iron is the most abundant transition metal within the brain, and is vital for a number of cellular processes including neurotransmitter synthesis, myelination of neurons, and mitochondrial function. Redox cycling between ferrous and ferric iron is utilized in biology for various electron transfer reactions essential to life, yet this same chemistry mediates deleterious reactions with oxygen that induce oxidative stress. Consequently, there is a precise and tightly controlled mechanism to regulate iron in the brain. When iron is dysregulated, both conditions of iron overload and iron deficiencies are harmful to the brain. This review focuses on how iron metabolism is maintained in the brain, and how an alteration to iron and iron metabolism adversely affects neurological function.

Keywords: iron regulation, Alzheimer's disease, Parkinson's disease, iron deficiency, iron chelation

INTRODUCTION

Iron is a fundamental requirement for most known life forms, and is likely to have played an integral role in the earliest development of life on this planet (Russell et al., 1993). Organisms have evolved to harness the unique chemistry of this highly abundant metal, which make it integral to a vast array of chemical reactions supporting cell division, oxygen transport and mitochondrial function. The iron redox couple mediates the transfer of single electrons through the reversible oxidation/reduction reactions of Fe^{2+} and Fe^{3+}. Iron is a *d*-block transition metal, and the unoccupied *d*-orbitals allow ionic iron (II), iron (III), and iron (IV) species to form ligands with both small and large biomolecules via oxygen, nitrogen, and sulfur atoms. The biological redox potential and electronic spin state, and thereby reactivity of iron, is determined by the nature of the ligand to which the species is bound. This configuration, along with the oxidation state of the iron itself, dictates whether an iron-based biomolecule is responsible for reactions involving oxygen transport and storage, electron transfer, or oxidation/reduction of other molecules (Beard, 2001). Reactions involving iron in the body are predominately redox-based, hydrolytic or involve polynuclear complex formation (Aisen, 2001).

Reliance upon iron for normal physiological function has thus necessitated a tightly regulated mechanism for ensuring the net turnover of dietary iron is essentially neutral (Crichton and Ward, 1992). This is especially important for the brain, where some of the highest concentrations of iron in the body are maintained (Gerlach et al., 1994). This review will provide an overview of how brain iron metabolism is regulated, and the consequences of perturbed iron homeostasis.

Abbreviations: AD, Alzheimer's disease, ADHD, attention deficit hyperactivity disorder; apo-Tf, apo-transferrin; ATP, adenosine-5′-triphosphate; BBB, blood–brain barrier; BCEC, brain capillary endothelial cells; BID, brain iron deficiency; cAMP, cyclic adenosine monophosphate; Cp, Ceruloplasmin; CREB, cyclic adenosine monophosphate response element-binding protein; CSF, cerebrospinal fluid; DMT1, divalent metal transporter 1; Fe, iron; Fe2Tf/holoTf, holo-transferrin; GWAS, genome-wide association study; HCP1, heme carrier protein 1; HFE, human hemochromatosis protein; HIF, hypoxia inducible factor; ICP-MS, inductively coupled plasma-mass spectrometry; IRE, iron responsive element; IRP1/2, iron regulatory proteins 1/2; LMW, low molecular weight; MMSE, mini-mental state examination; MPTP, methyl-4-phenyl-1,2,3,6-tetrahydropyridine; MRI, magnetic resonance imaging; mRNA, messenger RNA; NBIA, neurodegeneration with brain iron accumulation; NTBI, non-transferrin bound iron; 6-OHDA, 6-hydroxydopamine; PD, Parkinson's disease; RLS, restless legs syndrome; ROS, reactive oxygen species; SN, substantia nigra; STEAP 1-4, six-transmembrane epithelial antigen of prostate 1-4; TCS, transcranial sonography; Tf, transferrin; TFR1, transferrin receptor 1; TfR2, transferrin receptor 2; UTR, unstranslated region; MAPK, mitogen-activated protein kinase; Erk1/2, extracellular signal-regulated kinase 1/2; GSK-3, glycogen synthase kinase 3; Cdk-5, cyclin-dependent kinase 5; AMPA, α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid; Bcl-2, B-cell lymphoma 2; NMDA, *N*-methyl-D-aspartate.

IRON UPTAKE, TRANSPORT AND CELLULAR REGULATION

IRON CIRCULATION AND BRAIN UPTAKE

The major iron transporter protein in the body is the 80 kDa glycoprotein transferrin (Tf). Each bi-lobar molecule, consisting of two globular units at the N- and C-terminals has two iron-binding sites, which form a 4-atom tetradentate ligand via histidine, aspartate, and two tyrosine amino acid residues (Anderson et al., 1987). Almost all iron exchange and transport within the body is mediated by Tf (Finch and Huebers, 1982), with around 3–4 mg of iron typically circulating the healthy adult bound to Tf. Two Fe^{3+} ions oxidized by a ferroxidase and shunted into the interstitium by ferroportin are loaded onto a single Tf unit, and at any one time only around 30% of all circulating Tf units are occupied (only in cases of severe iron overload does Tf saturation occur; Aisen, 2001). Less than 1% of circulating iron is usually non-Tf bound. Non-Tf

bound iron (NTBI) is handled by a series of low molecular weight (LMW) ligands including citrate and ascorbate ions, as well as a possible small contribution from circulating albumin and ferritin proteins (Breuer et al., 2000) and ATP.

The hydrophobic barricade formed by the blood–brain barrier (BBB) prevents diffusion of hydrophilic Fe_2Tf into the nervous system, as well as prevent migration of NTBI. Moos et al. (2007) and Crichton et al. (2011) have recently published comprehensive pictures of iron trafficking within the brain, including uptake from the periphery. This step, where Fe_2Tf is transported across the BBB through brain capillary endothelial cells (BCECs). Tf-uptake into BCECs follows an endocytotic mechanism, where circulating Tf binds to Tf receptors which then internalize. It is a point of contention as to whether iron export from the endosome is mediated by the protein divalent metal transporter-1 (DMT1); conflicting reports have either identified (Burdo et al., 2001) or failed to identify (Moos and Morgan, 2004) DMT1 in rodent brain BCECs. An alternative hypothesis has suggested iron becomes segregated from Tf after liberation from the metal–protein complex in the endosome and is released independently of DMT1 (Moos et al., 2006). During development, when the BBB is not fully formed, there is a rapid influx of iron most likely stemming from NTBI; the developing rat brain shows a rapid intake of iron in line with increased expression of transferrin receptor 1 (TfR1) in BCECs, which in turn becomes the major iron regulatory mechanism once the BBB is sealed, after which iron intake slows (Taylor and Morgan, 1990). However, brain iron import is unlikely solely regulated by BCECs, like many other metabolic pathways redundancies are likely in place in the case one pathway breaks down. For instance, obstruction of BCEC TfR1 in mice and rats using intravenously administered monoclonal antibodies did not completely impede brain iron uptake (Ueda et al., 1993).

A possible alternative mechanism for the uptake of NTBI may be associated with the expression of ferroportin in the BBB (Wu et al., 2004) and circulating ferroxidases (enzymes that catalyze Fe^{2+} oxidation to Fe^{3+}) like ceruloplasmin (Cp; Osaki et al., 1966). It should be noted, however, that expression of ferroportin in BCECs has been disputed (Moos and Rosengren Nielsen, 2006). It is also unclear as to whether iron present in the BCEC endosome is in fact released into the cytosol. Moos et al. (2007) proposed that the possible lack of DMT1 is suggestive that the endosome traverses the BCEC cytosol intact (transcytosis) and releases Fe^{3+} directly into the brain for distribution to cells.

On the abluminal side of the BCEC astrocytes abut the cell membrane, forming part of either "neurovascular" or "gliovascular" units comprising of neurons, astrocytes, and BCECs (Abbott et al., 2006). Moos et al. (2007) suggested that astrocyte "end feet" surround the BCEC with a thin layer of interstitial fluid into which iron is released from endosomal Fe_2Tf–TfR1 complexes on the luminal membrane of the BCEC. Iron is then either re-complexed by Tf in the brain interstitium, or bound to LMW ligands released by the astrocyte. While the affinity of iron to small ligands is considerably smaller than that to Tf, it has been suggested that Tf saturation in the cerebrospinal fluid (CSF) is much higher than in the periphery, and that a larger proportion of NTBI circulates the nervous system (Leitner and Connor, 2012). Astrocytes also provide a source of Cp to ensure any circulating Fe^{2+} is quickly oxidized to Fe^{3+} to prevent unwanted reactive oxygen species (ROS) production through Fenton chemistry.

CELLULAR IRON TRAFFICKING IN THE BRAIN

Iron is released from Tf into cells via a particularly elegant mechanism. TfR1 is a ubiquitously expressed membrane protein with a dimeric structure and high affinity to Fe_2Tf, but at neutral pH, has a low affinity for apo-Tf (iron-free) so that the unligated Tf does not act as a competitive inhibitor of holo-Tf (iron-bound) uptake (Aisen, 2004). The Fe_2Tf forms a complex with the TfR1 receptor, which is then endocytosed. A proton pump mechanism is initiated to lower the pH within the endosome, which causes a conformational change to both the Fe_2Tf and TfR1 units, in turn resulting in release of the iron from its chaperoning protein (Hentze et al., 2004). The newly freed Fe^{3+} is quickly reduced by the six-transmembrane epithelial antigen of prostate 1-4 (STEAP 1-4), allowing export from the endosome into the cytosol by DMT1 (De Domenico et al., 2008). In the acidic endosome, apo-Tf has a strong affinity for the Tf receptor; this interaction prevents the degradation of free Tf when the endosome complexes with the lysosome before exocytosis. During exocytosis the pH returns to neutral, which causes dissociation of the apo-Tf from the TfR1, effectively recycling the Tf molecule for further use in iron circulation (Dautry-Varsat et al., 1983). In 1999, a homolog Tf receptor, TfR2, was identified (Kawabata et al., 1999), which initially showed expression only in hepatocytes, duodenal crypt cells, and erythrocytes. TfR2 has a 30-fold lower affinity to iron-bound Tf, yet mutations to the TfR2 gene results in hereditary hemochromatosis (Camaschella et al., 2000). TfR2 shares 45% amino acid identity with the ubiquitous TfR1 (Kawabata et al., 1999; Fleming et al., 2000). Interestingly, TfR2 has also been identified in dopaminergic neurons, and has been suggested to play a role in Fe_2Tf translocation to mitochondria (Mastroberardino et al., 2009).

Neurons express both TfR1 and DMT1 (Burdo et al., 2001), and therefore uptake iron via a receptor-mediated endocytotic mechanism (**Figure 1**), though it is likely that a small minority of iron uptake is sourced from NTBI *in vivo*. Astrocytes are devoid of TfR1, and NTBI is most likely their major iron source (Moos and Morgan, 2004). Oligodendrocytes, which require iron for myelin synthesis (see below; Connor and Menzies, 1998) also import iron through a mechanism independent of TfR1. Two noteworthy hypotheses have been proposed to explain how the comparatively high need for iron by oligodendrocytes is regulated without the major iron import mechanism present. Firstly, iron passes into the cytosol complexed with LMW ligands. Iron is then incorporated into Tf produced within the oligodendrocyte itself, where it is either used immediately or sequestered in ferritin for storage (Moos et al., 2007). Tf is not secreted by the oligodendrocyte itself (de Arriba Zerpa et al., 2000), presenting a fairly unique closed environment of iron regulation in what is predominately otherwise an intertwined regulatory system.

Once inside the cell, iron can follow multiple pathways dependent on need. Ferritins are responsible for iron storage and play an integral role in iron homeostasis, and are rarely saturated due to their large capacity for thousands of individual Fe^{3+} ions (Theil, 2004). Numerous other cytosolic proteins require iron for a variety

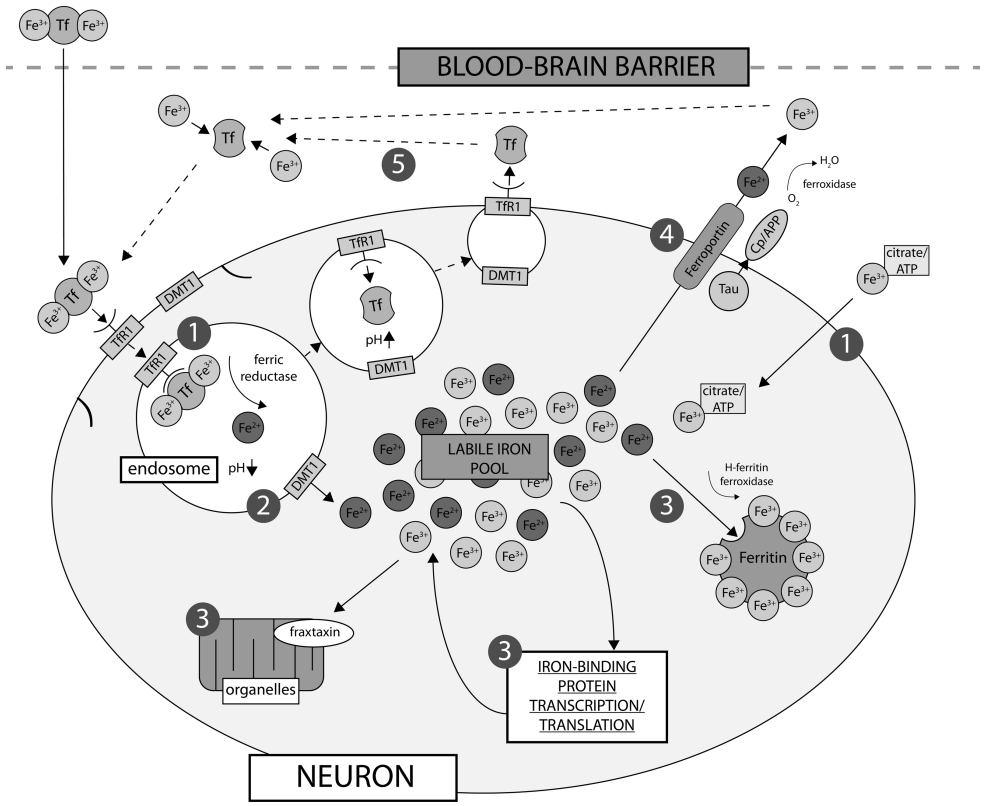

FIGURE 1 | Neuronal iron uptake and export. Iron is imported into the cell either as low molecular weight complexes with citrate/ATP, or via transferrin receptor (TfR1)-mediated endocytosis (1). Export of iron from the endosome via divalent metal transporter-1 (DMT-1) directly contributes to the labile iron pool (2), which constitutes the available iron content of the neuron and is regulated by cellular metal sensing and iron-binding protein expression, including ferritin, which is the major iron storage protein in neurons (3). Export of iron from neurons is regulated by intramembrane ferroportin, which is stabilized via a mechanism involving tau and ceruloplasmin (Cp)/APP (4). Iron is then recirculated by apo-Tf (5).

of normal functions. Iron is also important to mitochondrial functions, where it is incorporated into Fe–S clusters and heme proteins (Hentze et al., 2004). The mechanism for mitochondrial uptake has not been categorically confirmed, though the two proposed pathways involve either (i) diffusion of NTBI or (ii) direct translocation of extracellular Fe_2Tf via an endosomal pathway (Horowitz and Greenamyre, 2010). Within the mitochondria, the frataxin protein (implicated in Friedreich's ataxia) is suggested to act as a intramitochondrial iron chaperone (Richardson et al., 2010).

The only known export pathway in mammalian cells is mediated via ferroportin (Ganz, 2005). Ferroportin allows ferrous iron to be transported out of the cell (Donovan et al., 2005), and this process requires a ferroxidase to oxidize the ferrous iron to ferric, so that Tf can bind the exported iron. In the brain, ferroportin has been identified in both neurons (Abboud and Haile, 2000) and astrocytes (Dringen et al., 2007), as have the corresponding ferroxidases, the amyloid precursor protein (APP; Duce et al., 2010) and Cp (Texel et al., 2011).

REGULATION OF IRON-ASSOCIATED PROTEINS

Iron-associated proteins are regulated by iron status, therefore form a cycle to regulate iron metabolism (**Figure 2**). In cases of low cellular iron, two iron regulatory proteins (IRP1/2) are free to bind directly with iron responsive element (IRE) stem-loop structures within the mRNA of iron-binding proteins. The 3′ untranslated portion of, for example, TfR1 mRNA is sensitive to ribonuclease degradation, thus binding with IRP1/2 protects the mRNA and promotes TfR1 expression, increasing cellular iron uptake. Conversely, binding of IRP1/2 to the 5′ untranslated region (UTR) of, for example, ferritin mRNA prevents translation, reducing cytoplasmic ferritin expression, reducing the iron storage capacity of the cell, and increasing available iron (Aisen, 2001). When iron levels in the cell are high, labile iron binds with IRP1/2, preventing interactions between the regulatory proteins and the IREs in the mRNA of various iron regulating proteins (see **Figure 2**), eliciting the reverse cellular response to that observed in cases of iron deficit. The mechanism of iron-mediated inhibition of IRP/IRE binding depends on the protein involved: IRP1–Fe undergoes a conformational change that prevents IRE binding, whereas IRP2–Fe complexes undergo degradation via the ubiquitin proteasome pathway (Pantopoulos, 2004). Both IRP1 and IRP2 are present in the rat (Siddappa et al., 2003) and human brain (Connor et al., 1992c); IRP1 has been suggested as the primary regulatory protein in the human brain and is capable of forming a double IRP1/IRE complex (Hu and Connor, 1996).

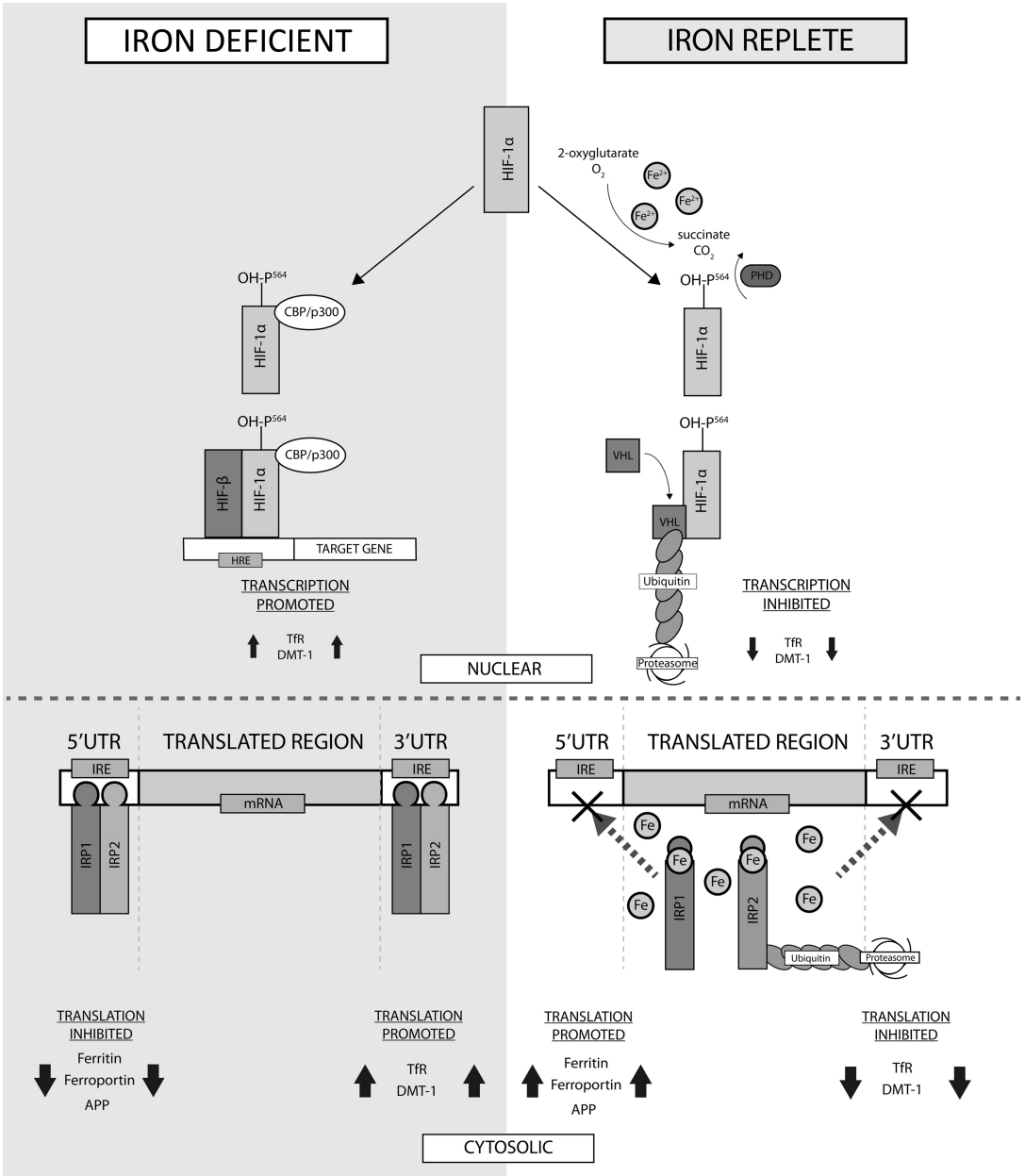

FIGURE 2 | Factors influencing cellular iron metabolism. Cellular oxygen homeostasis regulates iron metabolism in the nucleus via the hypoxia inducible factors (HIF-1α and -1β). In normoxia conditions, Fe^{2+} mediates the hydroxylation of proline residue 546 on HIF-1α by prolyl-4-hydroxylase (PHD), which enables ubiquitination via binding with the von Hippel–Lindau tumor suppressor gene product (VHL). Decreased HIF-1α inhibits transcription of proteins dictating iron uptake and export. In the iron-deficient cell, HIF-1α instead interacts with CREB binding protein/p300 (CBP/p300) and forms a heterodimer with HIF-1β, activating transcription of target genes possessing hypoxia response elements. In the cytosol, the iron replete cell prevents the binding of iron responsive proteins (IRP) -1 and -2 to the iron responsive elements (IREs) in the 5′ and 3′ untranslated region (UTR) of mRNA, inhibiting the transcription of uptake proteins and promoting expression of proteins involved in iron export. In cases of iron deficiency, IRPs directly interact with the 5′- and 3′-UTRs, eliciting the reverse effect. TfR, transferrin receptor; DMT-1, divalent metal transporter-1; APP, amyloid precursor protein.

Iron metabolism is also transcriptionally regulated by the action of hypoxia inducible factors (HIFs; see **Figure 2**), which consists of a cytosolic protein (HIF-1α) and a nuclear HIF-1β subunit that form a DNA binding heterodimer (Peyssonnaux et al., 2008). HIF-1α levels are dictated by cellular chemistry, and in normal conditions HIF-1α is hydroxylated by prolyl hydroxylase, marking the adduct for ubiquitination and degradation (Ratcliffe et al., 1999). This reaction requires oxygen, 2-oxoglutarate, ascorbate, and iron as cofactors. In hypoxic conditions HIF-1α ubiquitination is inhibited and translocation to the nucleus is increased, where dimerization with HIF-1β allows binding with cyclic adenosine monophosphate (cAMP) response element-binding protein

(CREB), which activates transcription of target genes with hypoxia response elements (HREs; Semenza, 2000). As iron is necessary for hydroxylation of HIF-1α, any decrease in cellular iron levels will increase dimerization of HIF-1α and β and downstream transcription of target genes, which, unsurprisingly, includes those responsible for Tf (Rolfs et al., 1997), TfR1 (Lok and Ponka, 1999), and DMT1 (Lis et al., 2005).

With such a sophisticated regulation mechanism for regulating iron-associated proteins transcription and translation, it is surprising diseases of iron overload and deficiencies exist. Some of these disorders arise from nutritional deficiency/excess or genetic mutation in one or more iron-associated proteins. But as will be described, the disorders of iron metabolism are invariably chronic disorders, not acute disorders. Possibly because there is complex regulatory mechanism that can resist, and then compensate for, short-term changes to iron levels.

IRON FLUX AND DEFICIENCY

Iron deficiency is a major nutrition deficiency most often observed in the developing world. Even in a developed nation, such as the USA, approximately 10% of toddlers and women of childbearing age are iron-deficient (Looker et al., 1997). As described above, the cellular iron regulatory mechanism is nuanced and robust, therefore allowing considerable divergence from the homeostatic norm of iron levels before disease precipitates. Disorders of iron homeostasis are thus invariably chronic, not acute diseases. This is especially true of the brain, which has more stable iron levels compared to the other organs (Youdim et al., 1989).

Peripheral iron deficiency can result in minor to severe symptoms. Anemia is an advanced iron deficiency syndrome caused by a number of factors ranging from dietary deficiency, blood loss, and metabolic lesions. The most common symptoms arising from anemia result from reduced oxygen transportation by hemoglobin. These symptoms include pallor, fatigue, faintness, shortness of breath, muscle weakness, angina pain, and elevated cardiac output (as compensation for reduced oxygen carrying capacity; Wood and Elwood, 1966).

While symptoms of anemia can arise over a period of weeks to months, iron in the brain is more resistant to dietary changes (Youdim et al., 1989) and indeed the brain may have critical periods that determine the level of iron in the brain throughout life (Dallman et al., 1975; Dallman and Spirito, 1977; Ben-Shachar et al., 1986). The brain accumulates iron during the weaning period, which establishes an iron "set point" for the brain. Perinatal pups on a restricted iron diet cannot recover their brain iron levels when supplemented later in life (Ben-Shachar et al., 1986). It has been proposed that the brain of a post-weaned mammal is impermeable to peripheral iron (Kaur et al., 2007), possibly explaining why brain iron levels are not easily altered with diet (Ben-Shachar et al., 1986). However, supplementation of isotopically enriched iron to mice reveals dietary iron incorporation into the brain at a similar rate to other organs (Chen et al., 2013). Peripheral iron is able to enter the brain by patterning with Tf, which can undergo receptor-mediated transcytosis to pass through the BBB (Fishman et al., 1987; Bradbury, 1997; Morgan and Moos, 2002). It is likely that brain iron is in constant flux with peripheral pools of iron. We speculate that brain iron levels do not tangibly deviate from the norm despite changes in the diet, because the brain iron levels are strictly governed by the iron homeostatic mechanism, and where the normal level of iron in the brain for an adult individual is likely determined in the critical weaning period of the mammal. This period can thus shape the iron biochemistry of the brain throughout the life of an individual, highlighting the urgency of addressing nutritional deficiency in infants raised in areas of poverty. Described below, chronic brain iron deficiency disrupts important process in the brain, altering neurochemistry that eventually leads to disease.

NEUROCHEMICAL EFFECT OF IRON DEFICIENCY

NEUROTRANSMITTER SIGNALING

Iron affects synthesis and signaling of the neurotransmitters dopamine, noradrenalin, adrenaline and 5-hydroxytryptamine, which are involved in emotion, attention, reward, movement, and various other functions. These neurotransmitters are synthesized by a number of iron-dependent enzymes including phenylalanine hydroxylase (Gottschall et al., 1982), tyrosine hydroxylase (Ramsey et al., 1996), and tryptophan hydroxylase (Kuhn et al., 1980). Brain iron deficiency (BID), however, rarely causes reduced expression or activity of these enzymes (Youdim et al., 1989). The conservation of iron in these enzymes under BID possibly reflects the importance of these enzymes to brain function.

In addition to neurotransmitter synthesis, iron impacts several other steps in neurotransmitter signaling, which are more vulnerable to changes in iron levels. Reduced neuronal uptake of the catecholaminergic neurotransmitters has been observed in several BID models (Burhans et al., 2005; Beard et al., 2006b; Bianco et al., 2008), and the extracellular concentration of neurotransmitters are elevated in BID rats (Beard et al., 1994). Dopaminergic signaling is further perturbed in iron deficiency by attenuating affinity and expression of D2 neurotransmitters (Youdim et al., 1989).

ENERGY PRODUCTION

The brain has a high energy demand, accounting for 20% of basal oxygen consumption (Halliwell, 2006) and thus requires high iron levels to generate ATP by the electron transport chain in the mitochondria. Various mitochondrial enzymes utilize iron as a cofactor including the mitochondrial ferredoxins (Redfearn and King, 1964), cytochromes (Slater, 1949), and aconitase (Dickman and Cloutier, 1950). Iron deficiency changes mitochondria morphology (Jarvis and Jacobs, 1974), impairs function (Masini et al., 1994), and damages mitochondrial DNA (Walter et al., 2002). Reduced mitochondrial efficiency possibly explains why iron deficiency results in elevation of oxidative stress markers (Knutson et al., 2000; Jeong et al., 2011; Wan et al., 2012a), despite loss of pro-oxidant iron.

MYELINATION

Myelin is the fatty "white matter" that insulates axons and preserves their signaling. Accordingly, under basal conditions, oligodendrocytes exhibit high levels of iron comparative to other brain cells (Benkovic and Connor, 1993; Connor et al., 1995). Iron treatment to cultured glial restricted precursor cells increases their differentiation into GalC1 oligodendrocytes, while treatment to cultured O2A oligodendrocytes progenitors increases their

proliferation without altering differentiation (Morath and Mayer-Proschel, 2001). Iron, therefore, has marked, but distinct effects on the temporal sequence of oligodendrocyte development. In a rat model, BID restricts both glia precursor cell proliferation and differentiation into oligodendrocytes (Morath et al., 2002) and decreases components of myelin: myelin basic protein, myelin proteolipid protein, galactolipids, phospholipids, and cholesterol (Yu et al., 1986; Ortiz et al., 2004). Lack of myelination causes slower neuronal conduction, evidenced by retardation of reflexes. In humans, iron deficiency is associated with abnormal reflexes in infants (Armony-Sivan et al., 2004) and in iron-deficient children, deficits in auditory brain stem potentials and visual evoked potentials have been observed (Roncagliolo et al., 1998; Algarin et al., 2003).

NEUROLOGICAL DISORDERS ASSOCIATED WITH IRON DEFICIENCY

FAILURE TO THRIVE

It is now widely recognized that BID in early life is associated with developmental delays in various brain faculties. Iron deficiency, characterized by anemia, has been associated with poorer fine and gross motor skills, visual-motor integration, language and global IQ, accompanying higher scores in anxiety and depression, social and attention problems (Palti et al., 1985; Lozoff et al., 1991; Hurtado et al., 1999) with some symptoms persisting 10 years after treatment for anemia (Lozoff et al., 2000). While the association between iron and various markers of developmental delay are unequivocal, the causal relationship is complicated by confounding socioeconomic variables that often accompany iron deficiency including generally poor nutrition, lack of stimulation in the home, lack of maternal warmth, poor maternal education, maternal depression, more absent fathers, parasitic infection, and low birth weight (Grantham-McGregor and Ani, 2001).

The importance of iron to neurodevelopment is thus unclear from observational human studies, which has necessitated study of iron-deficient experimental animal models. Agreeing with complementary human studies, BID in rats causes delayed behavioral milestones (Beard et al., 2006a), including impaired memory (Yehuda et al., 1986; Wachs et al., 2005) and motor function (Hunt et al., 1994). Symptoms resulting from dietary iron restriction in the first 21 days of the life of the rat are not recoverable even after 6 weeks of iron supplementation (Ben-Shachar et al., 1986). Combining the human and animal evidences strongly supports a critical role for iron in neurodevelopment, and since the symptoms are not readily correctable after the critical period, these also highlight the importance of monitoring and early dietary intervention.

ATTENTION DEFICIT HYPERACTIVITY DISORDER

Attention deficit hyperactivity disorder (ADHD) is a developmental disorder manifesting in symptoms of inattention, hyperactivity, and impulsiveness. ADHD is highly heritable, and several candidate disease-causing genes are involved in dopamine neurotransmission (*DAT1, DRD4, DRD5*; Elia and Devoto, 2007). Since iron interacts with multiple steps in dopamine neurotransmission, it is possible that BID might precipitate ADHD in idiopathic cases. While several studies showed reduced ferritin in children affected by ADHD (Konofal et al., 2004; Oner et al., 2008; Cortese et al., 2009; Juneja et al., 2010; Menegassi et al., 2010), the largest study (194 children), reported unaltered serum ferritin levels between ADHD patients and controls (Donfrancesco et al., 2012). As previously mentioned, iron status in children often co varies with multiple parameters of socioeconomic status, which might confound these studies. Further peripheral markers of iron do not often reflect the status of brain iron; therefore peripheral iron is not likely altered in ADHD. However, a recent study of 36 individuals reported reduced brain iron in the thalamic region as measured by magnetic resonance imaging (MRI) in ADHD patients (Cortese et al., 2012) suggesting a role for BID in the pathogenesis of this disease.

Could iron supplementation therefore be used as a treatment for ADHD? A case study reported a 3 year-old presenting with low serum ferritin (13 ng mL^{-1}) accompanying ADHD who was supplemented with ferrous sulfate (80 mg day^{-1}) and 8 months later was observed to exhibit various behavioral improvements (Konofal et al., 2005). This prompted a 12-week clinical trial of iron supplementation in ADHD, which recorded improvements in the ADHD rating scale for the treatment group (Konofal et al., 2008). These studies warrant further investigation into iron as a potential therapeutic, however, as discussed above, iron supplementation after a critical period is not effective in reversing cognitive symptoms of early BID in rats (Ben-Shachar et al., 1986), which might limit the use of this approach in ADHD.

RESTLESS LEGS SYNDROME

Restless legs syndrome (RLS) is a neurological disorder characterized by uncomfortable or odd sensations in the body (often legs) that prompt an incessant urge to move (Earley, 2003). The prevalence of RLS is estimated to be between 5 and 10% of the population (Lavigne and Montplaisir, 1994; Rothdach et al., 2000; Ulfberg et al., 2001). The disorder is associated with reduced dopamine uptake and reduced D2 receptor density (Staedt et al., 1995; Turjanski et al., 1999; Michaud et al., 2002), and is often treated with dopamine-based therapies (Hening et al., 1999; Allen et al., 2001). This neurochemical profile is consistent with BID (Youdim et al., 1989) of the nigrostriatal pathway. Indeed low ferritin and high Tf levels have been reported in CSF of RLS patients, while serum indices of iron metabolism were not altered (Earley et al., 2000; Clardy et al., 2006). Direct measurements of iron by post-mortem histological staining (Connor et al., 2003) and MRI (Allen et al., 2001; Earley et al., 2006) reveal decreased levels in the substantia nigra (SN) of affected patients.

NEURODEGENERATION

What are the lifetime consequences of BID? This remains an underexplored subject in brain iron research, which has historically focused on BID in neurodevelopment, and iron accumulation in neurodegeneration. Accordingly, to our knowledge, there has been no report of BID in a neurodegenerative disorder. Recently, a genetic mouse model of motor neuron iron deficiency ($IRP2^{-/-}$) exhibited reduced mitochondrial activity, hypomyelination, and neurodegeneration (Jeong et al., 2011), raising the possibility of BID-induced neurodegenerative disorders. Patients with the neurodegenerative disorder, dementia with Lewy bodies (DLB), have a threefold higher incidence of self-reported history of ADHD

symptoms. However, the status of iron in ADHD patients is only beginning to emerge, and the status of iron in DLB is also not known, so it is premature to mechanistically connect the two diseases via iron. Low iron levels impair mitochondrial function (Masini et al., 1994), and increase oxidative stress markers (Knutson et al., 2000), possibly by limiting the function of the iron-dependent antioxidant, catalase (Wan et al., 2012a). Longer-term studies of rodent models of BID will illuminate the neuroanatomical and neurobiochemical changes that result from low iron bioavailability.

IRON ACCUMULATION IN THE BRAIN

The sophisticated mechanisms that manage iron in the brain highlight the need for tightly controlled iron regulation, in order to exploit its utility in cellular operations, while preventing its deleterious capacity. Functional loss of IRPs by genetic mutations induces brain iron deposition, which is sufficient to cause neurodegeneration in diseases like aceruloplasminemia (Miyajima et al., 1987; Hochstrasser et al., 2004) and neuroferritinopathy (Feyt et al., 2001; Chinnery et al., 2007). This demonstrates the potential for iron elevation to participate in neuronal loss of more common neurodegenerative diseases [e.g., Alzheimer's (AD) and Parkinson's disease (PD)] where brain iron elevation features in both diseases.

BRAIN IRON ACCUMULATION WITH AGING

Aging is an important risk factor for neurodegenerative diseases. Multiple failures of the iron regulatory system in disease could be contributed to by the aging process (Bartzokis et al., 1997; Martin et al., 1998; Pfefferbaum et al., 2009; Penke et al., 2012; Daugherty and Raz, 2013). Age-related iron retention can serve as predictors of behavioral deficits, such as cognitive decline (Penke et al., 2012) and motor impairment (Cass et al., 2007; Kastman et al., 2012), highlighting the possibility of its involvement in age-associated decline.

Brain iron elevation with age could be contributed to by changes in various proteins that comprise the iron regulation machinery. Ferritin is elevated during the aging process in both gray and white matter of occipital cortex (Connor et al., 1992b) and the SN (Zecca et al., 2004), but is unchanged in motor cortex and superior temporal gyrus (Connor et al., 1992b). Tf expression was found to be decreased in white matter of superior temporal gyrus, but elevated in white matter of occipital cortex (Connor et al., 1992b). Cp was found to be elevated in gray matter with aging, without changes in white matter (Connor et al., 1993), while another report observed that Cp was unchanged in SN (Zecca et al., 2004). In rat brains, iron and ferritin were found to increase with age, while Tf levels remain unchanged (Roskams and Connor, 1994). The mechanism of age-related iron accumulation is only beginning to be elucidated. The selective vulnerability of iron accumulation during aging could also explain why iron elevation is a feature of various neurodegenerative diseases.

ALZHEIMER'S DISEASE

Alzheimer's disease is the most prevalent neurodegenerative disease characterized clinically by progressive dementia, and pathologically by the presence of Aβ-containing plaques, and tau-containing neurofibrillary tangles in affected brain areas. Elevated iron is also a feature of AD-effected post-mortem brains (Zhu et al., 2009; Duce et al., 2010; Smith et al., 2010; Qin et al., 2011; Antharam et al., 2012; Loef and Walach, 2012). Iron accumulation occurs in AD cortex and hippocampus, but not cerebellum (Andrasi et al., 1995; Duce et al., 2010; Antharam et al., 2012), consistent with the pathological profile of neurodegeneration in AD. In addition, iron is accumulated in both plaques and tangles (Connor et al., 1992a; Smith et al., 1997; Meadowcroft et al., 2009), and is estimated to be three times that of the normal neuropil level in plaques (Lovell et al., 1998). The iron content in hippocampus of patients with AD was reported to correlate with the mini-mental state examination (MMSE) and the disease duration (Ding et al., 2009; Zhu et al., 2009), suggesting that iron can play a significant role in the disease progression.

Several genes of iron regulatory proteins are risk factors for sporadic AD, including Tf and human hemochromatosis protein (HFE). In a genome-wide association study (GWAS) study, Tf variant C2 positively correlates with AD risk with an OR of 1.21 (Bertram and Tanzi, 2008), which is supported by a number of independent studies (Van Landeghem et al., 1998; Schjeide et al., 2009; Kauwe et al., 2010) but was not confirmed in a recent large-scale GWAS study (Hollingworth et al., 2011). In addition, HFE mutations (H63D and C82Y) are risk factors for AD independently (Sampietro et al., 2001; Blazquez et al., 2007), and synergistically with *APOE* gene (Kauwe et al., 2010; Giambattistelli et al., 2011; Lehmann et al., 2012). Both of the genes are also shown to modulate iron content, and are implicated in the risk of cognitive impairment in normal aging (Bartzokis et al., 2011).

Iron accumulation can promote aggregation of both Aβ and tau, the key proteins involved in plaque and tangle formation, respectively. Three histidine residues of Aβ were suggested as the binding amino acids of iron, and this complex is redox-active (Nakamura et al., 2007; Bousejra-ElGarah et al., 2011). Recently it was found that iron delayed the amyloid fibril formation but enhanced the toxicity *in vitro*, suggesting the iron-bound Aβ oligomer could serve as a toxic species (Mantyh et al., 1993; Schubert and Chevion, 1995; Liu et al., 2011). These observations are relevant to disease since iron is concentrated in plaques (Meadowcroft et al., 2009; Gallagher et al., 2012), and increased iron content is prior to plaque formation in an animal model of AD (Leskovjan et al., 2011). Aβ–iron complex can induce ROS via Fenton chemistry (Rottkamp et al., 2001; Rival et al., 2009), and activate B-cell lymphoma 2 (Bcl-2) apoptosis pathway (Kuperstein and Yavin, 2003). Chelation of iron can prevent Aβ aggregation, and reverse the consequent memory loss in animal models of AD (Huang et al., 2004; Guo et al., 2013b).

Iron and tangles co-localized in AD (Smith et al., 1997) and tangles can bind iron in a redox-dependent manner, acting as a source for ROS within the neurons (Smith et al., 1997; Sayre et al., 2000). This process can also be removed by iron chelation (Shin et al., 2003). Fe(III), but not Fe(II), can induce tau aggregation *in vitro*, which again can be reversed by reducing Fe(III) to Fe(II) (Yamamoto et al., 2002) or iron chelators (Amit et al., 2008). Fe(II) can induce tau hyperphosphorylation (Lovell et al., 2004; Chan and Shea, 2006), via activation of extracellular signal-regulated kinase 1/2 (Erk1/2) pathway or the mitogen-activated

protein kinase (MAPK) pathway (Muñoz et al., 2006; Huang et al., 2007). Chelation therapies such as deferoxamine can inhibit iron-induced tau hyperphosphorylation *in vivo* (Guo et al., 2013a), and prevention of iron uptake can also inhibit this event by deactivating glycogen synthase kinase 3 (GSK-3) and cyclin-dependent kinase 5 (Cdk-5; Xie et al., 2012), two key tau kinases (Lei et al., 2011).

Understanding the cause of iron accumulation in AD might lead to new therapeutic opportunities. Multiple components of the iron regulatory system are altered in AD including IRP2 (Smith et al., 1998), ferritin (Connor et al., 1992a), and Tf (Loeffler et al., 1995). Of particular interest is APP, which is involved in Aβ production and iron homeostasis. APP expression is unchanged in AD cortex but its ferroxidase activity was reported to be decreased (Duce et al., 2010). This could prevent ferroportin-mediated iron export and Tf loading, which would lead to iron retention within neurons. APP-mediated iron export is also impacted by AD-associated tau protein (Lei et al., 2012). Soluble tau levels are reduced in AD brains compared to control brains (Ksiezak-Reding et al., 1988; Shin et al., 1992; Khatoon et al., 1994; Zhukareva et al., 2001, 2003; van Eersel et al., 2009); which might result from tau deposition into insoluble aggregates during the disease progress (Khatoon et al., 1994). Loss of functional tau could further perturb APP-mediated iron export by restricting the presentation of APP at the surface.

PARKINSON'S DISEASE

Parkinson's disease is the most prevalent movement disorder, caused by loss of dopaminergic neurons in the SN pars compacta. The brain of PD patients, and especially the SN, is decorated by Lewy body inclusions that are enriched with the α-synuclein protein. As early as 1924, iron deposition in the SN of PD patients was described (Lhermitte et al., 1924). Iron elevation within this nucleus has been consistently reported using multiple techniques such as inductively coupled plasma-mass spectrometry (ICPMS; Dexter et al., 1989; Lei et al., 2012), atomic absorption spectroscopy (Ayton et al., 2012b), X-ray fluorescence (Popescu et al., 2009), and MRI (Bartzokis et al., 1999).

Iron deposits have been found in Lewy Bodies in PD cases (Castellani et al., 2000), suggesting that α-synuclein may interact with iron at the biochemical level. Indeed, iron binds to α-synuclein (Bharathi and Rao, 2008; Peng et al., 2010), accelerates α-synuclein aggregation (Golts et al., 2002; Kostka et al., 2008), and causes toxic hydroxyl radical production *in vitro* (Turnbull et al., 2001). Treating iron to cells initiates α-synuclein aggregation (Ostrerova-Golts et al., 2000; Gault et al., 2010; Li et al., 2011), and the resultant oligomer promoted α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA)-receptor-mediated excitotoxicity (Huls et al., 2011). Iron administration to cells that overexpress a disease-related mutant form of α-synuclein, A53T, enhanced cytotoxicity of the protein via increasing the autophagic activity (Ostrerova-Golts et al., 2000; Chew et al., 2011), which could explain how iron causes toxicity in PD.

Iron content in SN is a risk factor and may serve as a biomarker of PD. Mutations in a number of iron-related proteins have been shown to associate with the risk of PD, including Tf (Borie et al., 2002), IRP2 (Deplazes et al., 2004), ferritin (Foglieni et al., 2007), and DMT1 (He et al., 2011). It has been debated whether iron accumulation in SN is a secondary effect of cell death in PD. However, recent developments in MRI and transcranial sonography (TCS) makes it possible to examine brain iron content in living patients. It has been shown using MRI that iron accumulates at the early stage of PD before the symptom onset (Bartzokis et al., 1999; Martin et al., 2008), and healthy individuals with increased SN iron content determined by TCS had 17 times higher risk of developing PD (Berg et al., 2011). The SN iron elevation in PD patients, shown by MRI, correlates with the disease susceptibility (Baudrexel et al., 2010), severity (Atasoy et al., 2004; Wallis et al., 2008) and duration of the disease (Kosta et al., 2006; Zhang et al., 2010). The early rise in iron, measured by TCS and MRI supports a role for iron in the pathogenicity of PD.

Iron accumulation is alone sufficient to cause parkinsonian neurodegeneration. Direct iron injection to rat brains can cause SN neuron loss (Ben-Shachar and Youdim, 1991), and feeding neonatal mice with iron can trigger later life parkinsonism and nigral degeneration (Kaur et al., 2007). Diseases primarily characterized by brain iron accumulation, including aceruloplasminemia (Miyajima et al., 1987; Hochstrasser et al., 2004; McNeill et al., 2008), neuroferritinopathy (Crompton et al., 2002; Chinnery et al., 2007), and iron accumulation (NBIA) (Schneider et al., 2012), often cause symptoms of PD. The observations from these diseases which are caused by rare loss-of-function mutations of IRPs indicate that a similar iron accumulation observed in idiopathic PD likely participates in the degenerative processes. Aceruloplasminemia can be recapitulated in mice that lack the Cp gene, and this can be rescued with iron chelation (Patel et al., 2002; Ayton et al., 2012b).

Modulation of iron shows beneficial effects on PD animal models. PD toxin model, 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) or 6-hydroxydopamine (6-OHDA) cause SN iron accumulation in mice, coincident with neuronal loss (Hare et al., 2013). These PD models can be rescued by iron chelation (Kaur et al., 2003; Mandel et al., 2004; Youdim et al., 2004a,b). Iron-mediated toxicity in these models can also be ameliorated by genetic or pharmacologically restoring ferritin (Kaur et al., 2003) and Cp (Ayton et al., 2012b).

Why does iron accumulate in PD? This could be contributed by a number of iron-related proteins that are changed in PD. Ferritin levels have been found to be decreased in post-mortem PD brains (Dexter et al., 1990; Werner et al., 2008); loss of iron storage capacity potentially makes free iron species more available for toxic interactions. Iron accumulation in PD might be caused by increased neuronal iron import. DMT1 is elevated in SN of PD patients (Salazar et al., 2008), which could promote iron import, but the levels of TfR1, which is required for DMT1-mediated iron import are unchanged when corrected for neuronal loss (Mash et al., 1991; Morris et al., 1994; Faucheux et al., 1997). Alternatively, iron accumulation in PD could also be attributed to reduced iron export. Cp levels in PD brains were unaltered, however, the activity is selectively reduced in SN, which could bottleneck iron export (Ayton et al., 2012b). Tau protein is also implicated in PD (Lei et al., 2010), and selective reduction of tau found in SN of PD patients may also contribute to iron accumulation by preventing APP-mediated iron export (Lei et al., 2012).

OTHER NEUROLOGICAL DISORDERS

Iron accumulation has been observed in affected brain regions of various diseases including progressive supranuclear palsy (Coffey et al., 1989; Dexter et al., 1991; Boelmans et al., 2012), Pick's disease (Ehmann et al., 1984), Huntington's disease (Dexter et al., 1991; Bartzokis et al., 2007; Jurgens et al., 2010; Rosas et al., 2012), prion disorders (Singh et al., 2009a, 2012), amyotrophic lateral sclerosis (Oba et al., 1993; Santillo et al., 2009; Langkammer et al., 2010), and multiple system atrophy with striatonigral degeneration (Dexter et al., 1991; Vymazal et al., 1999; von Lewinski et al., 2007; Wang et al., 2012). The iron accumulation in diseases such as prion disorders and Huntington's disease may result from ferritin accumulation (Simmons et al., 2007; Singh et al., 2012), but the cause or implications of iron elevation for these diseases is unclear at this stage.

Friedreich's ataxia is a disorder of iron metabolism more extensively studied. This autosomal recessive degenerative disease results from mutations in the mitochondrial protein frataxin (Campuzano et al., 1996; Carvajal et al., 1996). Friedreich's ataxia is characterized by degeneration of large sensory neurons and cardiomyopathy (Gordon, 2000), but brain atrophy and iron accumulation are also features of the disease (Synofzik et al., 2011). Recent studies suggested that the function of frataxin is related to the maintenance of iron homeostasis, acting as iron-storage protein in mitochondrial similar to ferritin, and also an intramitochondrial iron chaperone. It is also suggested to be involved in heme and iron sulfur cluster biogenesis. The frataxin mutant is unstable and severe reduction of the protein results in intramitochondrial iron accumulation and cytosolic iron deficiency in mice and humans, and is suggested to contribute to the pathogenesis of the disease (Gordon, 2000; Puccio et al., 2001; Richardson et al., 2010). Interestingly, a high iron diet limits some of the phenotypes in mouse models such as cardiac hypertrophy (Whitnall et al., 2012).

Like iron, copper also participates in neurodegenerative pathways. Copper is able to cause the aggregation of alpha synuclein (Bharathi and Rao, 2008), and copper is a co-factor of dopamine beta-hydroxylase, which is involved in dopamine synthesis (Ash et al., 1984). Copper is decreased in PD SN (Dexter et al., 1991; Ayton et al., 2012b) which might be a reason why the copper-dependent Cp protein is dysfunctional in the disease. Peripheral Cp is also depleted in Wilson's disease, which is primarily a disorder of copper homeostasis, caused by a genetic mutation to ATP7b (Bull et al., 1993). Copper accumulates in liver and brain, along with iron (Shiono et al., 2001; Litwin et al., 2013). Why does iron also accumulate as a result of the disease? Possibly reduced Cp levels in plasma reduce iron export in liver and brain, resulting in iron accumulation. Whatever the mechanism, Wilson's disease often presents as early-onset PD, possibly mediated by the elevation of copper and iron (Machado et al., 2006).

TOXICITY MECHANISMS OF IRON OVERLOAD IN DISEASES

Iron can induce neurotoxicity by its ability to promote the formation of ROS, a source of oxidative stress. Elevated iron is potentially neurotoxic, indeed the direct injection of iron into the rat brain causes neurodegeneration (Ben-Shachar and Youdim, 1991), possibly via an oxidative stress pathway which initiates several apoptotic signaling pathways (Ke and Ming Qian, 2003).

Recently, a type of RAS-related cell death pathway was shown to be linked with intracellular iron levels, termed ferroptosis (Dixon et al., 2012), which could be potentially responsible for cell death seen in iron overload diseases. This type of cell death pathway shared no markers of apoptosis (e.g., caspase activation, mitochondrial cytochrome c release), but could be prevented by iron chelation or iron uptake inhibition (Yagoda et al., 2007; Yang and Stockwell, 2008). This pathway is not induced by Fenton chemistry; rather it is related with iron-dependent enzymatic activities (Dixon et al., 2012). Indeed, inappropriate intracellular iron accumulation potentially damages a number of proteins such as Ca^{2+}-ATPase (Kaplan et al., 1997; Moreau et al., 1998), glutamate transporter (Gnana-Prakasam et al., 2009; Yu et al., 2009; Mitchell et al., 2011), Na^{+}/K^{+}-ATPase (Kaplan et al., 1997; Strugatsky et al., 2003), and *N*-methyl-D-aspartate (NMDA) receptor (Nakamichi et al., 2002; Munoz et al., 2011), as well as oxidizes lipid such as cholesterol (Kraml et al., 2005; Graham et al., 2010; Shinkyo and Guengerich, 2011), ceramides (Yurkova et al., 2005), and sphingomyelin (Jenkins and Kramer, 1988; Isaac et al., 2006); all of which were proposed to ultimately cause synaptic dysfunction and neuronal cell death (Mattson, 2004).

It is therefore not surprising that iron elevation observed in a number of neurodegenerative diseases, such as AD and PD, is proposed to be a key mediator in cell loss of these diseases (Ayton et al., 2012a; Lei et al., 2012). In neurodegenerative diseases, iron is also found to partner with disease-related proteins, such as β-amyloid, tau, prion, and α-synuclein, which form soluble and insoluble aggregates and activate cell death pathways (Chiti and Dobson, 2006). The presence of iron accelerates the aggregation process *in vitro* (Schubert and Chevion, 1995; Ostrerova-Golts et al., 2000; Rottkamp et al., 2001; Yamamoto et al., 2002; Khan et al., 2006), and aggravates the oxidative stress induced by the protein *in vivo* (Huls et al., 2011; Li et al., 2011; Wan et al., 2011).

Recently it has emerged that these disease-related proteins also participate in iron metabolism. The mRNA of APP has an IRE in its 5′-UTR (Rogers et al., 2002, 2008), and was found to facilitate iron export *in vitro* and *in vivo* (Duce et al., 2010). Suppression of APP expression in mice resulted in age-dependent iron accumulation (Duce et al., 2010), and overexpression of wild type APP resulted in iron reduction in SH-SY5Y neuroblastoma cells (Wan et al., 2012b). Interestingly, overexpression of a disease-related mutant form of APP, the Swedish mutant, in SH-SY5Y cells and *Caenorhabditis elegans* causes significant iron retention accompanied with elevated ROS (Wan et al., 2011). It was proposed by the authors that the observed iron change is due to the increased amount of Aβ (Wan et al., 2011), however, it can be alternatively explained by loss-of-APP function. Aβ oligomers were shown to decrease NTBI uptake, however, the disease relevance was unclear (SanMartin et al., 2012). Recently, tau protein was found to mediate APP trafficking, and reduction of tau blocked iron export, leading to intracellular iron accumulation (Lei et al., 2012). Tau knockout mice exhibited age-dependent neurodegeneration, which could be pharmacologically prevented by iron chelation therapy (Lei et al., 2012), supporting a function of tau in iron metabolism.

Other disease related proteins have been investigated less for their association with iron metabolism, but the emerging data could point to a role for these proteins in iron homeostasis. α-synuclein exhibits an IRE in its 5′-UTR mRNA (Friedlich et al., 2007), and is reported to be ferrireductase of unknown biological function (Davies et al., 2011). Recently, prion protein was also suggested to act as a functional ferrireductase, to modulate cellular iron uptake (Singh et al., 2009c, 2013). Loss of prion protein caused iron deficiency in mice, which can be reversed by expression of wild type prion protein (Singh et al., 2009b). In addition, huntingtin protein, involved in Huntington's disease, was also reported as an iron-responsive protein (Hilditch-Maguire et al., 2000). In huntingtin-deficient zebrafish, iron starvation was identified during development, and these zebrafish had decreased hemoglobin production (Lumsden et al., 2007).

THERAPEUTICS BASED ON IRON MODULATION

Since iron involves the pathogenesis of neurodegenerative disorders, chelation of iron therefore could be a therapeutic strategy. Currently, iron chelation is utilized in practice for transfusional iron overload and hemochromatosis (Nick, 2007). Treatment for this type of diseases requires selective iron chelators with high affinity, to facilitate bulk excretion of iron from the body (Positano et al., 2009; Meloni et al., 2010; Murphy and Oudit, 2010; Pietrangelo, 2010). Deferoxamine (Propper et al., 1976), deferiprone (Kontoghiorghes et al., 1987a,b) and deferasirox (Piga et al., 2006; Shashaty et al., 2006) have been tested for these diseases. However, neurodegenerative diseases that feature regional iron accumulation require therapeutic agents to cross blood–brain barrier, and target specific brain regions in preference to the rest of the body.

Several brain permeable iron chelators have been explored in pre-clinical models of AD and PD (Kontoghiorghes et al., 1987a; Ben-Shachar et al., 1992; Kaur et al., 2003; Youdim et al., 2004a; Liang et al., 2008; Gogoi et al., 2011) although none of these compounds have entered clinical trials so far. One pilot trial of deferiprone was reported to be beneficial for NBIA (Abbruzzese et al., 2011). The mechanisms for neuroprotection effects of iron chelators have been linked with suppression of apoptotic pathway (Youdim et al., 2005; Avramovich-Tirosh et al., 2007; Zhu et al., 2007; Amit et al., 2008; Gal et al., 2010), promoting survival pathways (Avramovich-Tirosh et al., 2010; Reznichenko et al., 2010), restoration of protein degradation (Zhu et al., 2007), and stabilization of mitochondrial function (Youdim et al., 2005).

Clioquinol is a moderate affinity iron chelator that has undergone extensive pre-clinical testing for neurodegenerative disorders, and a clinical trial (Cherny et al., 2001; Kaur et al., 2003; Ritchie et al., 2003; Lei et al., 2012). The therapeutic effects of clioquinol have often been attributed to its ionophore activity, which redistributes copper and zinc into the cell (Cherny et al., 2001; Nitzan et al., 2003; Adlard et al., 2008; Li et al., 2010; Crouch et al., 2011; Park et al., 2011). However, its ability to chelate iron is also likely involved in its neuroprotective properties. Iron binds to clioquinol (Tamura et al., 1973; Kidani et al., 1974; Ohtsuka et al., 1982), and several beneficial effects of clioquinol have reported to be iron-dependent (Felkai et al., 1999; Atamna and Frey, 2004; Choi et al., 2006; Rival et al., 2009). Treatment with clioquinol prevents the elevation of SN iron levels in MPTP-treated mice, which confers neuroprotection (Kaur et al., 2003). Similar treatment also prevented age-related nigra degeneration in tau knockout mice (Lei et al., 2012), highlighting a potential use of clioquinol as an iron-binding agent. These results suggest that clioquinol participates in iron redistribution, but more data is needed to confirm.

CONCLUSION

The tightly regulated nature of iron in the human brain protects against diseases associated with excess or deficiency. Disease manifests when these systems deteriorate or are overwhelmed. Iron deficiency is prevalent, particularly in underdeveloped societies, and causes long-term consequences to brain health. There is therefore urgent need to address nutritional deficiency in pregnancy and in infancy to prevent these long-term consequences. Iron elevation in the brain is a feature of several major neurodegenerative disorders. While the cause of this is unknown, it is noteworthy that a variety of neurodegenerative disease-associated proteins involved in iron metabolism through various mechanisms, supporting the hypothesis that iron and disease-related proteins participate in a toxic cycle. The involvement of iron in neurodegenerative diseases needs further elucidation, but iron overload in these disorders represents an attractive pharmacological target for disease modifying therapies.

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Abnormal iron homeostasis and neurodegeneration

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Abnormal iron metabolism is observed in many neurodegenerative diseases, however, only two have shown dysregulation of brain iron homeostasis as the primary cause of neurodegeneration. Herein, we review one of these - hereditary ferritinopathy (HF) or neuroferritinopathy, which is an autosomal dominant, adult onset degenerative disease caused by mutations in the ferritin light chain (FTL) gene. HF has a clinical phenotype characterized by a progressive movement disorder, behavioral disturbances, and cognitive impairment. The main pathologic findings are cystic cavitation of the basal ganglia, the presence of ferritin inclusion bodies (IBs), and substantial iron deposition. Mutant FTL subunits have altered sequence and length but assemble into soluble 24-mers that are ultrastructurally indistinguishable from those of the wild type. Crystallography shows substantial localized disruption of the normally tiny 4-fold pores between the ferritin subunits because of unraveling of the C-termini into multiple polypeptide conformations. This structural alteration causes attenuated net iron incorporation leading to cellular iron mishandling, ferritin aggregation, and oxidative damage at physiological concentrations of iron and ascorbate. A transgenic murine model parallels several features of HF, including a progressive neurological phenotype, ferritin IB formation, and misregulation of iron metabolism. These studies provide a working hypothesis for the pathogenesis of HF by implicating (1) a loss of normal ferritin function that triggers iron accumulation and overproduction of ferritin polypeptides, and (2) a gain of toxic function through radical production, ferritin aggregation, and oxidative stress. Importantly, the finding that ferritin aggregation can be reversed by iron chelators and oxidative damage can be inhibited by radical trapping may be used for clinical investigation. This work provides new insights into the role of abnormal iron metabolism in neurodegeneration.

Keywords: neurodegeneration, neuroferritinopathy, ferritin, inclusion bodies, iron, oxidative stress

INTRODUCTION

Hereditary ferritinopathy (HF) or neuroferritinopathy is an autosomal dominant, adult onset neurodegenerative disease caused by mutations in the ferritin light chain (FTL) gene (Curtis et al., 2001; Vidal et al., 2004a; Mancuso et al., 2005; Ohta et al., 2008; Devos et al., 2009; Kubota et al., 2009). The disease was first reported in members of two families from England and France and was named neuroferritinopathy (Curtis et al., 2001). Sequence analysis of the *FTL* gene in members of the English family disclosed an adenine duplication, which predicts alteration of the C-terminal FTL polypeptide sequence and length (Curtis et al., 2001). Thus, far, six different mutations in exon four of the FTL gene have been reported, all affecting the FTL C-terminus. The clinical phenotype of HF is characterized by a movement disorder, behavioral abnormalities, and cognitive impairment. The brain shows cerebral and cerebellar atrophy and cavitation of the putamen. The main neuropathologic findings are the presence of intranuclear and intracytoplasmic ferritin inclusion bodies (IBs) in glial cells and in some subsets of neurons, and abnormal iron deposition. Molecular level investigations of ferritin containing the mutant subunit reveal functional defects of iron mishandling, ferritin aggregation, and oxidative damage. These processes are linked to a structural defect in the FTL C-terminus leading to cellular dysfunction that can be broadly classified as a loss of normal function and gain of toxic function as discussed below.

FERRITIN STRUCTURE, IRON CHEMISTRY, AND PROTEIN AGGREGATION

Ferritin is uniquely suited for its crucial iron sequestration and storage function. Ferritin consists of 24 subunits that can self-assemble into a 480 kDa hollow sphere of ∼110 Å outer and ∼80 Å inner diameter (**Figures 1A, B**), which can store up to 4500 atoms of iron as a ferrihydrite biomineral (Vidal et al., 2004b; Crichton and Declercq, 2010). The exterior and interior of the ferritin shell are connected via channels (pores) along 3-fold and 4-fold symmetry axes at subunit junctions. There are eight 3-fold pores that are larger diameter and shorter relative to the six 4-fold pores. The 3-fold pores (**Figure 1C**), which are hydrophylic, have been implicated as the iron entry pathway in a number of studies, whereas the 4-fold pores (**Figure 1D**), which are hydrophobic, are smaller and considered essentially closed, especially to ions. Human ferritin is usually heteropolymeric with

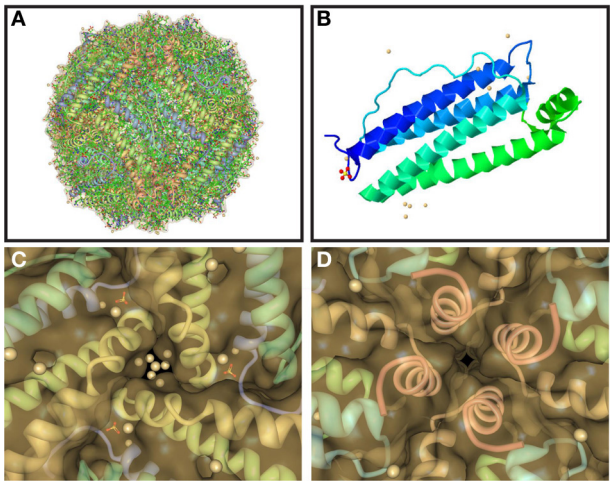

FIGURE 1 | Structure of ferritin, one of its 24 subunits, and its pores. Ferritin assembles spontaneously from 24 conformationally equivalent subunits into a hollow spherical shell **(A)** with 2-, 3-, and 4-fold symmetry axes. The subunits **(B)** are each composed of five α-helices with four of them parallel and tightly associated and the fifth (the E-helix) at an angle and pointed inwards. The junctions of the subunits form hydrophilic pores at the 3-fold axes **(C)**, which are implicated as the entry path for iron ions, and hydrophobic pores at the 4-fold axes **(D)**, which are smaller and considered closed, especially to ions. Pores are viewed from the ferritin shell interior with the inwardly pointing E-helices from the 4 subunits clearly visible in **(D)**. Structures were taken from RCSB (code 2FG8).

the 24-mer formed from two conformationally equivalent subunits with slightly different masses and 54% sequence identity. The two subunits are folded from the ferritin heavy chain (FTH1) and light chain (FTL) polypeptides with the former longer by 8 amino acids. Both subunits consist of four parallel α-helices (A–D) of ∼45 Å length and a shorter, carboxy terminal α-helix (E) at a 60° angle to the parallel helix bundle pointing into the interior of the shell (**Figure 1B**) (Crichton and Declercq, 2010). The 4-fold pores are formed from four hydrophobically-associated E-helices donated from four different subunits (**Figure 1D**), while 3-fold pores are formed from C- and D-helices from three subunits (**Figure 1C**). The FTH1 subunit contains the ferroxidase site in the interior of the parallel helix bundle, which converts Fe^{2+} to Fe^{3+} in the presence of O_2 on the pathway to biomineralization. Iron then migrates to the ferrihydrite nucleation site on the interior surface of the FTL subunit to complete biomineral formation. Interestingly, each organ fine tunes the ratio of FTH1 to FTL subunits, which can vary substantially, for optimal physiological function. No homopolymers of the FTH1 subunit have been reported, but homopolymeric ferritin formed from 24 FTL subunits is capable of a slow rate of iron sequestration and storage. The FTL subunit is more stable than the FTH1 subunit toward increasing temperature and denaturants, and when incorporated into the heteropolymer stabilizes it and hinders iron-induced aggregation (Santambrogio et al., 1992, 1993).

Properly functioning ferritin of the appropriate cellular concentration is essential for iron homeostasis, and a number of neurodegenerative diseases have links with misregulation of iron (Zecca et al., 2004; Berg and Youdim, 2006). The normal cellular response to abundant iron is to decrease the synthesis of the transferrin receptor, which transports iron into the cells, and to increase ferritin synthesis for appropriate iron sequestration and storage. During iron deficiency the synthesis of ferritin is inhibited in part by an increase in Iron Responsive Protein (IRP) binding to the Iron Responsive element (IRE) on target mRNAs. Clearly, iron must be made available to form the essential catalytic centers of neuroenzymes, e.g., tryptophan hydroxylase, which is required for serotonin synthesis, and tyrosine hydroxylase leading to dopamine. However, local iron concentration and its ligation (coordination) and oxidation state must all be carefully regulated to prevent cellular damage. Indeed, the chemistry that occurs at the iron can vary substantially depending on iron ligation, which could be provided by protein donors (e.g., certain amino acid side chains) or small molecules normally available in cells. Much cellular chemistry is driven by the process of redox change, and cells have available the redox drivers of reductants such as NADPH, glutathione, and ascorbate, as well as oxygen. Hydrogen peroxide and superoxide are generated in cells as part of several routine metabolic redox processes as well as by non-enzymatic reduced iron, and although they can deactivate a few enzymes, they are not particularly damaging at appropriate levels (Zecca et al., 2004). In fact, hydrogen peroxide has recently been characterized as a neuromodulator in striatal dopamine release (Rice, 2011). However, improperly coordinated iron has the potential to convert hydrogen peroxide and superoxide into the highly toxic hydroxyl radical, which is extraordinarily reactive. This radical indiscriminately attacks proteins, lipids, and DNA causing protein oxidation, fragmentation, and covalent crosslinking leading to their loss of function. Hydroxyl radical production occurs through redox change in metal-centered Fenton- and Haber-Weiss- type reactions, and when iron is coordinated by less than 6 strongly-bound ligands this conversion is facilitated (Graf et al., 1984). Hydrogen peroxide, superoxide, and the hydroxyl radical are generally termed reactive oxygen species (ROS), and cells produce enzymes (e.g., glutathione peroxidase, catalase, and superoxide dismutase) and small molecules (glutathione) to help control their levels (Murphy et al., 2011). The ability of ferritin to convert Fe^{2+} to Fe^{3+} and store it internally as a non-reactive biomineral can be considered a "detoxification" function by removing it from potentially inappropriate ligation and reactions. Although usually considered as antioxidants, glutathione and ascorbate have concentrations in the brain that are sufficiently high enough to provide reducing equivalents that can generate ROS and thus lead to cellular damage in the presence of improperly coordinated iron. Along these lines, during iron excess the likelihood of inappropriate iron coordination by normally available small molecule cellular constituents, which are not part of proteins and usually not problematic, is increased potentially causing the generation of ROS beyond basal levels and protein oxidation.

Proper disposal or reconstitution of damaged or misfolded proteins to avoid aggregation is an ongoing necessary cellular function, and protein aggregation is strongly linked with neurodegenerative diseases (Lansbury and Lashuel, 2006). Cells devote significant resources energetically through specialized

protein synthesis and transport toward the disposal or reconstitution of oxidized or misfolded proteins, which are generally prone to aggregate formation. The aggregation process is dependent upon a variety of factors besides the identity and extent of abnormality of the primary protein involved such as its concentration, cellular crowding by other proteins, and the presence of small molecules and metal ions. However, the aggregation process is generally thought to follow one of two paths. The misfolded protein first forms dimers, trimers, and oligomers with a structural rearrangement, and then forms highly ordered and symmetric structures, e.g., fibrils in Alzheimer disease. Alternately the aggregation process may involve less ordered clumping or packing together of proteins that leads to various tangles or IBs. Often the most toxic forms are the smaller oligomers and poorly ordered aggregates, and there is substantial evidence for this with the larger structures considered neuroprotective. However, the larger, microscopically visible IBs may not be inert cellular end products, but influence metabolism, transport and structure, perhaps even through causing detrimental mechanical crowding.

Transition metal ions such as iron, copper and zinc are often found at elevated levels in neurodegenerative diseases and are intimately connected with protein aggregation, misfolding and cellular dysfunction (Ayton et al., 2012). For example, the addition of iron to solutions of α-synuclein or amyloid β enhances the rate of aggregation and/or fibril formation even to the extent of influencing the structure of the aggregates formed, and elevated iron is associated with the pathology of these disorders in both animal models and humans. Metal ion binding can be causal to aggregation and misfolding, or be advantageous after they occur. However, metal ion-induced aggregated and misfolded proteins, while problematic for cellular processes and elimination, could foster an additional cellular dysfunction through providing binding sites that improperly ligate (coordinate) iron and thus generate ROS. The resulting oxidative damage itself can enhance protein aggregation. In Huntington disease IBs form iron-dependent centers of oxidative damage causing alterations in the cellular morphology of their surroundings (Firdaus et al., 2006). Cells devote resources to respond to damaged or aggregated proteins by synthesis of enzymes to repair or degrade them. As will be discussed below, mutant ferritin is intimately associated with iron mishandling, aggregation, and radical damage of proteins, which can be traced directly to a protein structural abnormality resulting from its disordered C-terminal helices and unstructured 4-fold pores (Baraibar et al., 2010).

CLINICAL PRESENTATION, GENETICS, AND PATHOLOGY OF HF

The neurodegenerative disease HF has been reported in members of Caucasian and Japanese families, being inherited in an autosomal dominant pattern. Linkage analysis, performed on members of an English family, linked the disease to a locus on chromosome 19q13.3, which contains the *FTL* gene. Affected individuals from this family were found to have an adenine insertion at position 460–461 of the *FTL* gene (*c.460dupA*) that was predicted to alter the carboxyl-terminal region of the protein (Curtis et al., 2001). Five additional mutations have been reported, all in exon 4 of the *FTL* gene, which consists of 4 exons and 3 introns. In all cases, the mutations (**Table 1**) affect the E-helix of the FTL polypeptide by altering both the C-terminal sequence and extending its length. In addition to these, two more cases of HF have been described. The first case was diagnosed pathologically and no genetic data is available (Schröder, 2005), while the second case was described as an individual with a missense mutation (A96T) in FTL (Maciel et al., 2005). In the later, it remains to be seen whether this case reflects a bigger spectrum of the disease or a different condition since the mother of the proband (also a carrier of the A96T mutation who displayed similar MRI findings) was asymptomatic (lack of autosomal dominant transmission), and the patient did not have significant involvement of the putamen, thalamus, and substantia nigra.

Clinically, HF presents as a movement disorder syndrome similar to Huntington disease or Parkinson disease. The disease may present with tremor, cerebellar signs, Parkinsonism, psychiatric problems, abnormal involuntary movements (dystonia, chorea), pyramidal syndrome, pseudo-bulbar symptoms, and cognitive deficit (Caparros-Lefebvre et al., 1997; Curtis et al., 2001; Vidal et al., 2004a; Mancuso et al., 2005; Chinnery et al., 2007; McNeill et al., 2008; Ohta et al., 2008; Devos et al., 2009; Kubota et al., 2009; Ory-Magne et al., 2009). The clinical presentation of HF has

Table 1 | Sequence alignment of WT- and MT-FTL polypeptides starting at residue 142.

Wild-type	L I K K M G D H L T N L H R L G G P E A G L G E Y L F E R L T L K H D	
p.Arg154LysfsX27	L I K K M G D H L T N L H K A G W P G G W A G R V S L R K A H S Q A R L R A F	1
p.His153GlufsX28	L I K K M G D H L T N L Q Q A G W P G G W A G R V S L R K A H S Q A R L R A F	2
p.His148ArgfsX34	L I K K M G D R P P D Q P P Q A G W P G G W A G R V S L R K A H S Q A R L R A F	3
p.His148ProfsX33	L I K K M G D P P D Q P P Q A G W P G G W A G R V S L R K A H S Q A R L R A F	4
p.Leu162ArgfsX24	L I K K M G D H L T N L H R L G G P E A G R P G G W A G R V S L R K A H S Q A R L R A F	5
p.Phe167SerfsX26	L I K K M G D H L T N L H R L G G P E A G L G E Y L S S K G S L S S T T K S L L S P A T S E G P L A K	6

Part of the helical domain D and the complete helical domain E of the WT-FTL subunit are shown above their respective sequences (Protein Data Bank code 2fg4). MT-FTL polypeptides have a C-terminus that is altered in sequence and length. References are: (1) Curtis et al., 2001; (2) Devos et al., 2009; (3) Kubota et al., 2009; (4) Mancuso et al., 2005; (5) Ohta et al., 2008; (6) Vidal et al., 2004a.

been reported to differ both within and between families (Ory-Magne et al., 2009), usually becoming evident in the third to fifth decade of life (depending on the specific mutation) and progressing unalleviated thereafter. Neuroimaging studies show abnormal signals in the globus pallidus and putamen, and cavitation of the putamen, while serum ferritin levels were reported to be decreased in some patients (Curtis et al., 2001; Ory-Magne et al., 2009). Neuropathological data is available for individuals with the *c.442dupC*, *c.460dupA*, and *c.497_498dupTC* mutations (Curtis et al., 2001; Vidal et al., 2004a; Mancuso et al., 2005). Examination of the brain shows mild cerebral and cerebellar atrophy as well as cavitations of the putamen. The main neuropathologic findings (**Figure 2**) are the presence of intranuclear and intracytoplasmic ferritin IBs in glial cells and in some subsets of neurons, substantial iron deposition, and mild to moderate nerve cell loss and gliosis. Glial cells containing IBs are mostly found in the caudate nucleus, putamen, and globus pallidus. These areas show severe nerve cell loss, extracellular ferritin deposits, and loss of neuropil. In the cerebral cortex, IBs are seen in perineuronal cells and in perivascular glia. The presence of IBs in neurons is clearly observed in the putamen, globus pallidus and thalamus, and in cerebellar granule cells and in Purkinje cells (Vidal et al., 2004a). Intranuclear inclusions are large enough to almost completely occupy the nucleus mechanically forcing chromatin against the nuclear membrane. IBs can be seen as homogenous, eosinophilic bodies, which can be labeled by antibodies against FTL and FTH1 polypeptides of ferritin and by antibodies specific for the mutant FTL polypeptide. Inclusions also contain Fe^{2+} and Fe^{3+}, as determined by Turnbull blue and by Perls' or Prussian blue, respectively. By transmission electron microscopy (TEM), nuclear IBs are seen as composed of small (∼100 Å) granular electron-dense particles that resemble ferritin and occupy a large portion of the nucleoplasm. IBs have been reported in the skin, kidney, liver, and muscle in affected individuals from French and American families (Vidal et al., 2004a; Mancuso et al., 2005). The presence of ferritin IBs in skin or muscle biopsies may help in the diagnosis of the disease.

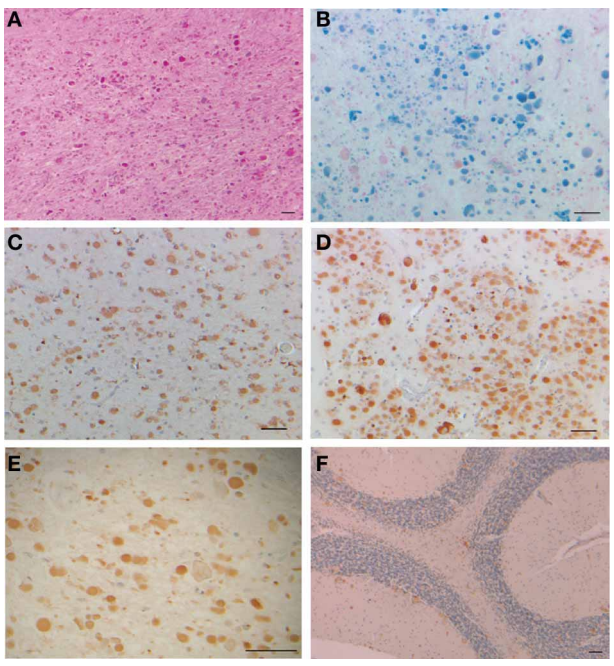

FIGURE 2 | Inclusion bodies, iron deposits, and immunohistochemistry from a patient with hereditary ferritinopathy. Sections of putamen show numerous ferritin IBs of various sizes **(A–E)**, which are ubiquitinated **(E)**. Ferritin IBs were also present in neurons and glial cells of the cerebellum **(F)**. Hematoxylin and eosin **(A)**; Perls' Prusian blue method for iron **(B)**; and immunohistochemistry using antibodies against mutant FTL **(C)**, wild-type FTL **(D)**, ubiquitin **(E)**, and FTH1 **(F)**. Scale bars: **(A–F)**, 50 μm.

FUNCTIONAL ABNORMALITIES IN MUTANT-CONTAINING FERRITIN

The isolation and biochemical analysis of IBs from individuals with HF identified wild-type FTL, FTH1 and mutant FTL polypeptides as the main components of IBs (Vidal et al., 2004a). Although ferritin is generally isolated as a heteropolymer of FTH1 and FTL subunits, initial structural and functional studies focused on the biological significance of the mutant as a 24-mer in its homopolymeric form. Thus, recombinant wild type (WT)- and mutant (MT)-FTL (p.Phe167SerfsX26) polypeptides were separately expressed in *E. coli*, reconstituted with all iron removed, and analyzed. Both polypeptides were soluble and assembled as 24-mer homopolymers by size exclusion chromatography (SEC). TEM analysis showed that the ferritin particle had a spherical shape and size (outer diameter ∼110 Å) similar to that of human ferritin (Baraibar et al., 2008). When WT-FTL apoferritin homopolymers (1 μM) were iron-loaded following a routine procedure by aerobic incubation with up to 4500 iron atoms per 24-mer of ferrous ammonium sulfate for 2 h, no precipitation was found. However, precipitation of the Mt-FTL homopolymer was observed to begin when the number of iron atoms was higher than ∼1500 iron atoms per ferritin 24-mer. By monitoring direct iron incorporation by native PAGE followed by Prussian blue stain, it was observed that at moderate iron loading of up to ∼1000 iron atoms per ferritin 24-mer and 2 h incubation, WT- and MT-apoferritin homopolymers incorporated very similar amounts of iron, which implies a degree of functionality for both. However, at higher iron to ferritin ratios, WT-FTL homopolymers continued incorporating iron, whereas incorporation by MT-FTL homopolymers dropped precipitously. The change in absorbance at 310 nm measured during the first 500 s after mixing iron and ferritin in a 1000 to 1 ratio was substantially larger for WT-FTL vs. MT-FTL homopolymers, uncovering a clear difference in iron handling between mutant and wild type ferritin at early times after mixing (Baraibar et al., 2010). When in separate solutions, both WT- and MT-FTL homopolymers showed significant ability to incorporate iron, but when in direct competition (in the same solution), there was complete absence of iron incorporation by MT-FTL. No precipitation was noted, which highlights the importance of direct iron mishandling by mutant ferritin without the effects of the iron-induced precipitation. The direct role of iron in the precipitation of MT-FTL homopolymers was further emphasized by using the iron chelator deferoxamine (DFX). Importantly, it was observed that greater than 50% of mutant homopolymers that were precipitated by the

addition of iron (3500 iron atoms per 24-mer) were re-solubilized by incubation with DFX (Baraibar et al., 2008), highlighting chelation as a major *in vitro* modulator of MT-FTL aggregation, which is an important marker for HF *in vivo*.

These homopolymeric studies were followed by examination of ferritin heteropolymers reconstituted with an equal number of mutant and wild type subunits in each 24-mer (Muhoberac et al., 2011). The intermediate mobility on SDS-PAGE and overlap in SEC profiles were indicative of appropriate co-assembly. It was found that both forms of heterepolymers containing the MT-FTL subunit (MT-FTL/FTH1, MT-FTL/WT-FTL) and again MT-FTL homopolymers themselves were significantly more susceptible to iron-loading induced precipitation than either the WT-FTL/FTH1 heterepolymer or the WT-FTL homopolymer, when analyzed by the same above-mentioned routine procedure. Thus, reconstitution of mutant with either wild type does not rescue ferritin from the iron-induced aggregative behavior (Muhoberac et al., 2011). Furthermore, a direct measure of iron incorporation using native PAGE and Prussian blue stain showed that WT-FTL/FTH1 was twice as capable of incorporating iron than MT-FTL/FTH1 heteropolymers. Importantly, some iron was still incorporated with mutant-containing ferritin suggesting some level of functionality.

Recently, oxidation of MT-FTL was observed to occur both *in vitro* and *in vivo* in individuals with HF (Baraibar et al., 2012). Incubation of MT-FTL homopolymers with physiological concentrations of iron and ascorbate resulted in shell structural disruption and polypeptide cleavage not found under the same conditions with WT-FTL. Along with the ∼21 kDa FTL polypeptide were found fragments of ∼ 6 and ∼14 K Da, as well as one of ∼27 kDa suggesting covalent crosslinking. Mutant ferritin also underwent a 2.5-fold increase in carbonyl group formation over wild type. Polypeptide cleavage and shell disruption was completely inhibited by addition of the radical trap 5,5-dimethyl-1-pyrroline N-oxide, indicating an enhanced propensity of MT-FTL 24-mers toward free radical-induced, oxidative damage *in vitro*. Importantly, IBs from a patient with HF exhibited extensive carbonylation together with an isolatable C-terminal MT-FTL fragment of ∼14 kDa, which are both indicative of *in vivo* oxidative ferritin damage. These data point toward a connection between oxidative damage, mutant ferritin, and HF, and suggest that radical scavengers (i.e., more generally antioxidants) and iron chelators have the potential to be therapeutic agents for treatment of HF.

E-HELIX DISRUPTION AND ENHANCED AGGREGATION IN MUTANT-CONTAINING FERRITIN

The structure of the spherical protein shell is maintained in mutant ferritin as was seen in the crystallographic structures of the MT-FTL (p.Phe167SerfsX26) homopolymers (Baraibar et al., 2010). However, a close up examination of the 4-fold pores showed remarkable disruption of the MT-FTL C-terminal helices making the pores unstable and leaky (**Figure 3** vs. **Figure 1D**). Because as many as the last 26 amino acids of MT-FTL remained unaccounted for crystallographically, mutant C-termini may extended and reach as far as the diameter of the ferritin shell itself. The C-terminal sequence of the mutant contains a number of groups known to bind iron, e.g., the C-terminal carboxylate, glutamate, tyrosinate and the hydroxyl groups of several serines and threonines. Considering the sensitivity of ferritin-containing MT-FTL to *in vitro* and *in vivo* (see below) iron-induced aggregation, a model was proposed in which iron binds to the unraveled and extended mutant C-termini on two different ferritin shells bridging them and initiating a gradual aggregation of ferritin and iron into a precipitate (**Figure 4**). Bridging is not necessarily restricted to C-termini and may become more general, e.g., between a C-terminal group and a surface amino acid which both have affinity for iron (Baraibar et al., 2008, 2010). This model has potential additional complexities because of (1) the very strong dependence of the strength of iron binding to certain groups on its redox state and (2) the existence of small iron hydroxide nucleation centers that may form in solution without the presence of protein.

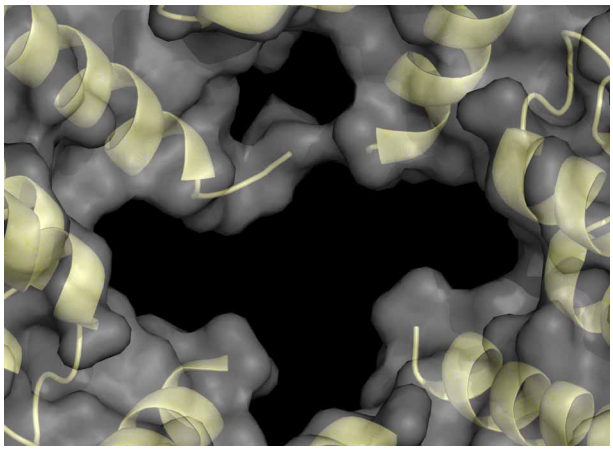

FIGURE 3 | Structural disruption of the 4-fold pores of ferritin caused by the C-terminal mutation. Each wild type 4-fold pore is formed from four tightly associated E-helices, one donated from each subunit, as seen in **Figure 1D**. With the mutant, the E-helices are unraveled causing significant disruption of the 4-fold pores and providing extended and disordered C-termini, which are not visible by X-ray crystallography. This MT-FTL 4-fold pore is an example of several, which vary somewhat in disruption details, and is viewed from the ferritin shell interior. The structure was taken from RCSB (code 3HX5).

The role of the mutant C-terminus and its protrusion above the protein shell in the iron-induced aggregation process was characterized further by comparing the iron loading of apoferritin homopolymers composed of WT-FTL, MT-FTL and a C-terminally truncated FTL polypeptide (p.S167X). The assembly status of the truncated FTL polypeptide as a homopolymer (24-mer) was verified by SEC and gel electrophoresis. In contrast to MT-FTL homopolymers, which began to precipitate at ∼1500 iron atoms per 24-mer, both the WT-FTL and truncated FTL homopolymers remained in solution up to a ratio of 4000 to 1. Thus, removal of the mutant portion of the C-terminus prevented iron-induced precipitation reinforcing the importance of the interaction between iron and the disordered C-terminus in the aggregation process (Baraibar et al., 2010).

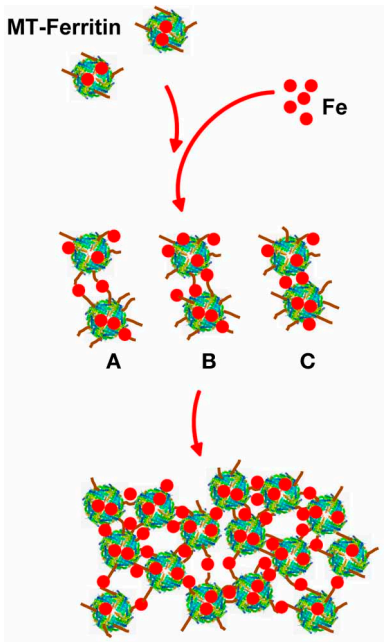

FIGURE 4 | Model of iron-induced aggregation of ferritin containing the C-terminal mutation. Unraveled MT-FTL C-terminal E-helices (brown lines) can extend a substantial distance from the ferritin shell surface into the solvent providing a number of groups that can coordinate iron. Addition of iron to a solution of MT-FTL-containing ferritin initiates bridging of C-termini by iron (or iron nucleation complexes) reducing their translational motion. Cross-linking may occur between two separate ferritin 24-mers through iron bridges between C-termini **(A)**, between a C-terminus and surface iron-binding amino acid side chain **(B)**, and/or eventually through surface amino acids that bind iron on both 24-mers **(C)** forming ferritin aggregates [adapted from Baraibar et al. (2008)].

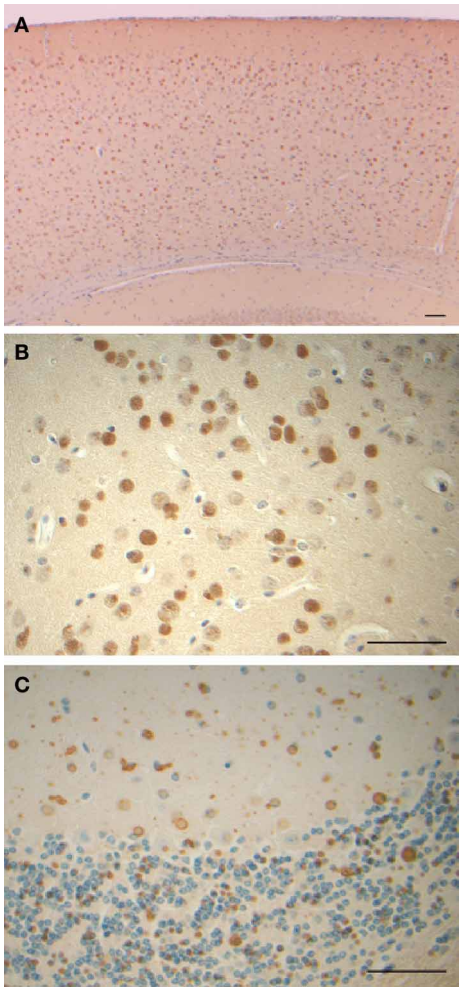

FIGURE 5 | Inclusion bodies and immunohistochemistry from a transgenic mouse model of hereditary ferritinopathy. Sections of cerebral cortex **(A)**, globus pallidus **(B)**, and cerebellum **(C)** of FTL-Tg mice show the presence of numerous ferritin IBs. Sections were from a 9 month old homozygous male **(A,B)** and an 11 month old heterozygous male **(C)**. Immunohistochemistry was performed using antibodies against the N-terminus of wild-type and mutant FTL **(A–C)**. Scale bars: **(A–C)**, 50 μm.

IRON CHELATION AND RADICAL TRAPPING IN ANIMAL AND CELLULAR MODELS OF HF

A transgenic animal model of HF (FTL-Tg) was generated in order to increase our understanding of the effects of MT-FTL on brain iron metabolism and ferritin expression and disposition. A human *FTL* cDNA carrying a thymidine and cytidine insertion at position 498 (*c.497_498dupTC*) was expressed in mouse, leading to iron mishandling, ferritin accumulation, and oxidative stress (Vidal et al., 2008). Expression of the transgene caused the formation of nuclear and cytoplasmic ferritin IBs in glia and neurons throughout the CNS (**Figure 5**), as well as in cells of other organ systems. The size and number of nuclear inclusions increased with age becoming large enough to cause mechanical crowding and displacement of chromatin, as was found in HF patients (Vidal et al., 2004a). FTL-Tg mice had a progressive neurological phenotype, a significant decrease in motor performance, and a shorter lifespan. These mutant mice showed an increase in brain iron and altered levels of associate proteins. Cytoplasmic FTL and FTH1 polypeptides increased and the transferrin receptor level decreased, as would be the expected in response to excess iron. Ubiquinated proteins and portions of the proteosome (20S, 11S, and 19S) accumulated in the IBs, implying cellular recognition of the presence of abnormal protein aggregates. FTL-Tg mice showed accumulation of oxidative DNA damage in brain mitochondria, but no significant damage to nuclear DNA (Deng et al., 2010). Furthermore, markers for oxidative stress such as protein carbonyl formation, nitrone-protein adducts, and lipid peroxidation, were found in the brain indicative of cellular damage by ROS (Barbeito et al., 2009).

Ferritin levels in primary cultures of astrocytes from the cerebral cortex of FTL-Tg mice respond dramatically to exposure to iron and chelators. Cell treatment with 50 uM ferric ammonium citrate (FAC) caused a switch of MT-FTL ferritin from the detergent-soluble to the detergent-insoluble fraction, strongly supporting a role for iron in the formation of IBs. After removal of FAC solution, addition of 50 uM of the lipophilic iron chelator 1,10-phenanthroline to the FAC-treated cells led to a large reduction in detergent-insoluble ferritin and

the reappearance of ferritin in the detergent-soluble fraction. Phenanthroline is a freely cell-permeable chelator and was chosen for the study over the weakly cell-permeable chelator deferroxamine (Baraibar et al., 2008). These studies show that IB formation is strongly dependent on iron levels and can be reversed by using iron chelators *in vivo*, which supports chelation therapy as a potential treatment to inhibit aggregation and reduce IB formation in HF.

Primary cultures of human skin fibroblasts from patients with HF were also used to characterize the effects of MT-FTL on cellular iron metabolism (Barbeito et al., 2010). These cells exhibited iron mishandling, ferritin accumulation, and evidence of oxidative stress, paralleling the dysfunction seen in both patients with HF and the mouse model. Mutant fibroblasts showed a significant increase in the level of total iron content under basal metabolic conditions when compared to wild type fibroblasts, but interestingly, without a significant difference in the level of the liable iron pool, which is the iron more readily available for metabolism. Cellular levels of MT-FTL, WT-FTL, and FTH1 polypeptides were all substantially increased in HF vs. wild type fibroblasts. IRE-IRP binding was reduced in HF fibroblasts consistent with the observed enhanced ferritin and decreased transferrin receptor-1 synthesis, and broadly consistent with higher total iron levels in HF fibroblasts. Significant higher levels of ROS were found in HF vs. non-HF fibroblasts (Barbeito et al., 2010), which supports antioxidant therapy as a potential treatment for HF.

ABNORMAL FERRITIN FUNCTION CAUSED BY THE MUTANT POLYPEPTIDE

Hereditary ferritinopathy provides a direct link between abnormal iron homeostasis and neurodegeneration. The pronounced cellular dysfunction in the pathogenesis of HF centers on three observable and well-characterized cellular abnormalities - iron accumulation, IB formation, and protein oxidation - as described in this review. All of these abnormalities involve a specific molecular-level defect of the MT-FTL C-terminal sequence causing (1) 4-fold pore disruption with reduced ability to store and sequester iron, and (2) unraveling and extension of the C-terminus causing iron-induced aggregation of ferritin. Both (1) and (2) contribute to increasing iron levels leading to the misdirected cellular response of synthesis of more ferritin to sequester iron, which in turn, creates a destructive positive feedback loop that accumulates ferritin and taxes cellular resources. Handling increasing levels of improperly sequestered and stored iron is problematic. Improperly ligated iron, whether this occurs on smaller ferritin aggregates, in IBs, or even through coordination by small molecule cellular constituents, produces ROS leading to protein oxidation, as is evident in the *in vitro* and *in vivo* studies reviewed here. Increasing levels of iron also enhance the aggregation of MT-FTL-containing ferritin, further exacerbating the situation. IBs contain both Fe^{2+} and Fe^{3+}, which suggests that ROS could be generated within them. Although HF is an autosomal dominant disease, the age at onset of the disease is not in early childhood, suggesting that the cells are apparently able to handle the many insults initially, but later succumb to cumulative damage. Taken together, a working hypothesis (**Figure 6**) for the role of MT-FTL in the pathogenesis of HF consistent with the results presented here implies both (1) a loss of normal ferritin function (decreased iron incorporation) that triggers intracellular iron accumulation and overproduction of ferritin polypeptides, and (2) gain of toxic function through radical production, ferritin aggregation, and oxidative stress. The concept of loss of normal function and gain of toxic function may have applicability to the understanding of other disease processes, especially when considering interactions with transition metal ions and available cellular reductants. More generally, a protein mutation or conformational change that leads to loss of normal function may also allow the ligation of transition metals to abnormal protein binding sites with the two major consequences of ROS generation and protein aggregation, which are both a gain of toxic function. It should be noted that zinc, although not redox active, can induce substantial protein aggregation, and may thus play an important role (Ayton et al., 2012). The loss of normal function could lead to a positive feedback loop in compensating cellular protein synthesis producing more abnormal protein metal binding sites, and could be further complicated if the protein in question normally handles transition metal ion transport or removal. It is somewhat ironic that the major iron storage protein ferritin acquires through mutation both the inability to properly handle iron and an enhanced aggregative sensitivity toward the metal that it is designed to keep in homeostasis.

OUTLOOK FOR TREATMENT

Currently there is no effective treatment for HF. Therapies aimed at decreasing iron levels or inhibiting ferritin synthesis would appear to be indicated in view of the pivotal roles played by ferritin and iron in cellular metabolism. Decreasing iron levels toward normal or eliminating mutant FTL polypeptide synthesis in HF patients by the more direct approach of gene therapy using viral transfer and expression that could influence iron or ferritin levels would be time consuming, costly to develop, and with some uncertainty of efficacy. Decreasing iron levels toward normal with appropriately designed chelators would reduce ROS production, pathological iron-induced aggregation, and IB formation. However, use of the iron chelators desferrioxamine and deferiprone (as well as venesection) was reported to cause profound and refractory iron depletion without clinical benefits (Chinnery et al., 2007). Although this initially may sound discouraging, the relatively limited number and variety of chelators examined until now as treatment for HF patients should be carefully considered. Chelators are characterized by a large number of molecular properties that need to be optimized to match both the complexities of the cellular system and the disease being treated. The choice of a chelator (and in the longer term its discovery and design) is difficult because optimization of one particularly molecular property to the cellular system and disease may adversely affect the optimization of another. For example, adding a particular group to the skeleton of a chelator to optimize lipid- vs. aqueous-solubility may adversely affect its redox properties or binding strength. Indeed, there are a number of considerations to chelator efficacy beyond facile blood-brain barrier penetration and increasing the binding strength as follows:

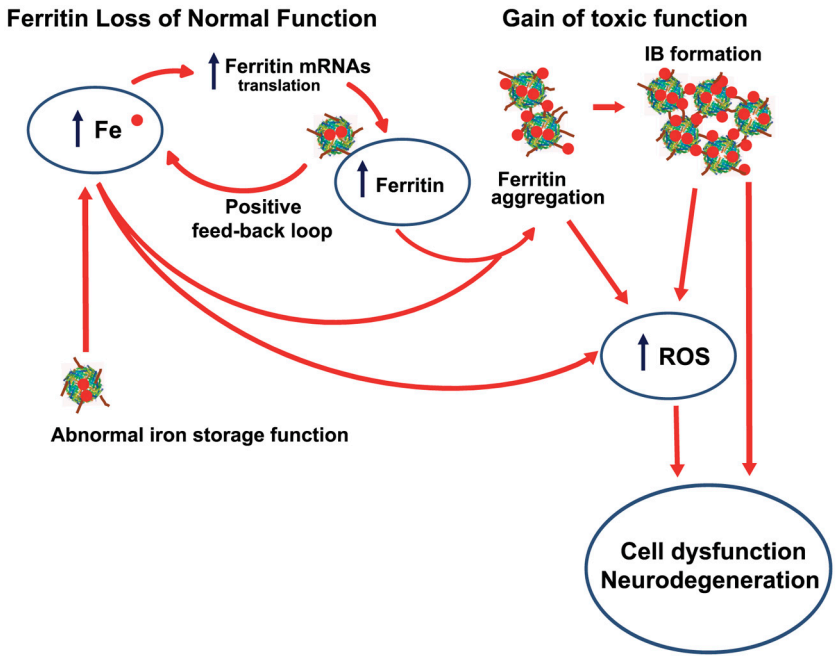

FIGURE 6 | Interrelationship between iron accumulation, ROS generation, inclusion body formation, and neurodegeneration in hereditary ferritinopathy. The pathogenesis of HF is consistent with (1) a loss of normal ferritin function through a decrease in iron incorporation into ferritin that triggers intracellular iron accumulation and overproduction of ferritin polypeptides (a positive feedback loop) and (2) a gain of toxic function through radical production, ferritin aggregation, and oxidative stress. ROS can form at improperly ligate iron bound to small ferritin aggregates and IBs, as well as to other cellular constituents (e.g., small molecules) because of elevated iron as represented by the lowest curved arrow in figure. The most toxic ROS is the hydroxyl radical that indiscriminately attack proteins, lipids and DNA causing extensive cellular damage.

(1) The chelator must selectively bind and release iron and not interfere significantly with the concentration, transport, and distribution of other metals. (2) Iron binding and removal must occur in a manner as not to sequester iron from functioning enzymes that require it for activity. (3) The chelator and iron-chelator complex must be hydrophobic enough to permeate multiple membrane barriers to redistribute iron for eventual excretion. (4) The iron-chelator complex should have redox and coordination properties such that it does not itself serve as a source of ROS using available oxygen and reducing equivalents in the brain. This list of considerations and the fine tuning of chelator molecular properties that it implies stand in contrast to the two chelators already explored to treat HF both in number and molecular composition. More generally this list is in contrast to the few chelators currently approved by the FDA for iron-overload therapy implying that chelation therapy for HF remains mainly unexplored.

Although additional *in vitro* characterization of mutant ferritin in combination with computational approaches may eventually prove useful in defining compounds with optimal molecular properties for drug candidates for HF therapy, it is important to consider application of currently available *in vivo* approaches employing existing animal and cellular models. One approach would be to develop a screening procedure using fibroblasts or astrocytes from HF patients against a small molecule library akin to the NIH Molecular Libraries Program or other similar programs. Whole animal studies using the mouse model would follow. In the best case scenario, the drug candidates would be the same or similar to an approved FDA drug. A more focused approach would perhaps be to screen approved drugs known to have iron deficiency as a side effect.

Although the preceding discussion targets the ability of chelators to remove and redistribute iron and to resolubilize or prevent ferritin aggregation, as was demonstrated to be operational both *in vitro* and *in vivo* by examples in this review, inhibition of oxidative damage by a free radical scavenger was also described. Damage by ROS could be reduced by removal of excess iron, but could also be reduced by the presence of radical scavengers. This points to the investigation of combined drug therapy for HF patients in the form of administering an optimal iron chelator simultaneously with a powerful antioxidant known to easily cross the blood brain barrier. The combined therapy as outlined here, especially if initiated early, may be more successful, not only for HF, but also for other neurodegenerative diseases characterized by brain iron deposition.

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Effects of hemochromatosis and transferrin gene mutations on peripheral iron dyshomeostasis in mild cognitive impairment and Alzheimer's and Parkinson's diseases

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Deregulation of iron metabolism has been observed in patients with neurodegenerative diseases. We have carried out a molecular analysis investigating the interaction between iron specific gene variants [transferrin (*TF*, P589S), hemochromatosis *(HFE)* C282Y and (H63D)], iron biochemical variables [iron, Tf, ceruloplasmin (Cp), Cp:Tf ratio and % of Tf saturation (% Tf-sat)] and apolipoprotein E (*APOE*) gene variants in 139 Alzheimer's disease (AD), 27 Mild Cognitive Impairment (MCI), 78 Parkinson's disease (PD) patients and 139 healthy controls to investigate mechanisms of iron regulation or toxicity. No difference in genetic variant distributions between patients and controls was found in our Italian sample, but the stratification for the *APOE*ε4 allele revealed that among the *APOE*ε4 carriers was higher the frequency of those carriers of at least a mutated *TF* P589S allele. Decreased Tf in both AD and MCI and increased Cp:Tf ratio in AD vs. controls were detected. A multinomial logistic regression model revealed that increased iron and Cp:Tf ratio and being man instead of woman increased the risk of having PD, that increased values of Cp:Tf ratio corresponded to a 4-fold increase of the relative risk of having MCI, while higher Cp levels were protective for PD and MCI. Our study has some limitations: the small size of the samples, one ethnic group considered, the rarity of some alleles which prevent the statistical power of some genetic analysis. Even though they need confirmation in larger cohorts, our data suggest the hypothesis that deregulation of iron metabolism, in addition to other factors, has some effect on the PD disease risk.

Keywords: iron, Alzheimer's disease, Parkinson's disease, *HFE*, transferrin

INTRODUCTION

It is now fairly well accepted that transition metals, such as iron and copper, are involved in the pathogenesis of some neurodegenerative disorders, like Alzheimer's disease (AD) and Parkinson's disease (PD). Specifically, an excessive iron accumulation has been reported in neuritic plaques, neurofibrillary tangles and in specific brain areas of patients with AD (Castellani et al., 2007). Moreover, abnormal exchange of cortical zinc has been described to link amyloid pathology with neuronal iron accumulation in AD (Duce et al., 2010) and, more recently, some data show that in AD, hippocampus damage occurs in conjunction with ferritin iron accumulation (Raven et al., 2013). This evidence is in disagreement with a recent meta-analysis, reported that iron is not increased in the AD brain (Schrag et al., 2011). However, neuroimaging *in vivo* as well as post-mortem examinations at autopsy have demonstrated that increased nigral iron content in patients with PD is a prominent pathophysiological feature (Dexter et al., 1987; Graham et al., 2000; Kaur and Andersen, 2004; Zecca et al., 2004; Berg et al., 2006; Rhodes and Ritz, 2008; Péran et al., 2010) and different findings suggest that decreased levels of serum ceruloplasmin may specifically exacerbate nigral iron deposition in PD patients (Bharucha et al., 2008; Jin et al., 2011, 2012; Martinez-Hernandez et al., 2011). We recently performed a meta-analysis of the published studies on serum to elucidate the possible role of systemic metabolism of iron in the risk for developing PD. Our results showed no change of iron levels in PD patients, but evidenced a higher heterogeneity that, although reducing the weight of result, stresses the attention on complex homeostasis of iron that play a crucial role in the pathogenesis of PD (Mariani et al., 2013).

The brain iron content increases with advancing age (Zecca et al., 2001) and could lead to enhanced oxidative stress through protein and lipid peroxidation, and DNA oxidation, causing, eventually, cellular and neuronal damage or death (Halliwell, 1992; Salvador et al., 2010). Brain iron homeostasis is regulated by different factors (Levenson and Tassabehji, 2004; Johnstone and Milward, 2010), among which the transferrin (Tf) and the hemochromatosis (*HFE*) proteins seem to play a key role. These proteins, encoded by the *TF* and *HFE* genes (Barry et al., 2005), compete for binding the Tf receptor (Tfr) (Feder et al., 1998).

Specifically, *HFE* is a membrane protein which controls iron absorption by regulating the affinity of Tfr on the cell membrane. *HFE* mutations cause hereditary hemochromatosis, a recessive autosomal disorder, with an increased absorption of dietary iron and its consequent overdeposition in tissues and organs (Phatak et al., 2002; Ajioka and Kushner, 2003; Barry et al., 2005). Concerning *TF* gene, there are different genetic variants due to amino acids substitution in the polypeptide chain. The *TF* C is the most common in Caucasian population and it has at least 16 variants (C1–C16). The C1 variant is the most prevalent (95%) and its encoding polymorphic gene has two allelic variants that produce transferrin C2 (proline in position 589 replaced by a serine, P589S). Both *TF* C2 (rs1049296) and the 2 *HFE*, C282Y (rs1800562) and H63D (rs1799945) gene variants, have been investigated as potential risk factors for neurodegenerative disorders. Studies analyzing the association between *HFE* mutations and AD came to different conclusions regarding the hypothesis that H63D mutation may anticipate the disease onset in sporadic AD (Sampietro et al., 2001; Candore et al., 2003). Still other authors evaluated the possible interaction between *TF* C2 and *HFE* C282Y, supporting the hypothesis that iron transport and regulation play a role in AD (Kauwe et al., 2010; Lehmann et al., 2012). Some authors suggested that the combination of *TF* C2 and *HFE* C282Y can lead to an excess of redox-active iron even in mild cognitive impairment (MCI) (Robson et al., 2004). Data available for PD, reported that the common variants in *HFE* might be a risk factor also for this disease (Guerreiro et al., 2006), even though the same authors did not confirm the risk for AD. Taken together these studies, although not always providing concordant results, seem to support the hypothesis that iron metabolism plays a role in some neurological disorders. To improve knowledge about the mechanism of iron regulation and toxicity in living AD, MCI, and PD subjects, a molecular analysis investigating the interaction between iron specific gene variants, iron biochemical assessments and apolipoprotein E (*APOE*) gene risk factor is reported herein.

MATERIALS AND METHODS

SUBJECTS

The subject sample consisted of 383 participants: 139 healthy controls (female (F)% 68, age mean ± standard deviation 63 ± 12 years), 139 AD (F% 71, age 73 ± 8), 27 MCI (F% 52, age 71 ± 8) and 78 PD patients (F% 33, age 67 ± 10; **Table 1**). Both patients and control subjects were recruited by two specialized dementia care Centers in Rome, Italy: the Department of Neuroscience of San Giovanni Calibita—Fatebenefratelli Hospital, and the Department of Neurology of Campus Bio-Medico University. The diagnosis of "probable AD" was posed according to NINCDS–ADRDA criteria (McKhann et al., 1984; Dubois et al., 2007). AD patients had a MMSE score ≤25 (Folstein et al., 1975). PD patients were diagnosed according to the UK PDS Brain Bank Criteria for the diagnosis of PD (Gibb, 1988) and the disease severity was classified on the basis of Unified Parkinson's Disease Rating Scale (UPDRS).

The inclusion and exclusion criteria for MCI were based on previous seminal studies (Albert et al., 1991; Petersen et al., 1999, 2001; Portet et al., 2006), defining elderly persons who do not meet the criteria for a diagnosis of dementia, with objective cognitive deficits, especially in the memory domain. Criteria were as follows: (I) objective memory impairment on neuropsychological evaluation, as defined by performances P1.5 standard deviation below the mean value of age and education- matched controls for a test battery including Buschke–Fuld and Memory Rey tests; (II) normal activities of daily living as documented by the history and evidence of independent living; and (III) clinical dementia rating score of 0.5. The exclusion criteria for MCI included: (I) mild AD; (II) evidence of concomitant dementia such as frontotemporal form, vascular dementia, reversible dementias (including pseudo-depressive dementia), fluctuations in cognitive performance, and/or features of mixed dementias; (III) evidence of concomitant extra-pyramidal symptoms; (IV) clinical and indirect evidence of depression as revealed by Geriatric Depression Scale scores higher than 13; (V) other psychiatric disorders, epilepsy, drug addiction, alcohol dependence, and use of psychoactive drugs including acetylcholinesterase inhibitors or other drugs enhancing brain cognitive functions; and (VI) current or previous uncontrolled or complicated systemic diseases (including diabetes mellitus) or traumatic brain injuries (Rossini et al., 2008).

The control sample consisted of healthy volunteers with no clinical evidence of neurological or psychiatric disease. Exclusion criteria for both patients and controls were conditions known to affect copper metabolism and biological variables of oxidative stress (e.g., diabetes mellitus, inflammatory diseases, recent history of heart or respiratory failure, chronic liver or renal failure, malignant tumors, and a recent history of alcohol abuse). All individuals included in this study are Caucasian. The study was

Table 1 | Demographic characteristics and mean values ± *SD* (standard deviation), or median value (minimum-maximum) of the biochemical parameters in study in controls, AD, MCI and PD patients.

	Controls	AD	MCI	PD
Age	63 (±12)	73 (±8)	71 (±8)	67 (±10)
sex (%F)	68%	71%	52%	33%
***APOE* (%ε4)**	11.4%	31.60%	–	–
Cp (mg/dL)	27.5 (10–60.2)	26.8 (±5.3) $p = 0.9$	24.4 (±5.1) $p = 0.1$	27.7 (±5.1) $p = 0.8$
Iron (μg/dL)	76.3 (11–190)	73.0 (11–441) $p = 0.9$	83.0 (±27.6) $p = 0.6$	74.84 (12–178) $p = 0.5$
Tf (g/L)	2.6 (1.6–4.8)	2.4 (±0.4) $p < 0.001$	2.4 (±0.6) $p = 0.04$	2.7 (±0.5) $p = 0.9$
Cp:Tf ratio * $10^{(-2)}$	10.3 (5–21.0)	11.4 (±2.7) $p = 0.002$	10.7 (±2.7) $p = 0.6$	10.8 (±2.3) $p = 0.5$
Tf saturation (%)	24.1 (1.8–76)	24.4 (2.25–126.0) $p = 0.469$	27.3 (10.9–51.8) $p = 0.652$	21.7 (2.5–58.8) $p = 0.203$

approved by the local IRB and all the subjects involved or legal guardians signed their informed consent.

GENOTYPING

Genomic DNA from fresh whole blood was prepared using the conventional method for DNA isolation (QLAamp DNA Blood Midi kit). Genotyping of SNPs rs1800562, rs1799945, rs1049296 were achieved by the TaqMan allelic discrimination assays from Applied Biosystems Inc. (Foster City CA). The predesigned SNPs genotyping assays ID are C_1085595_10, C_1085600_10, C_7505275_10 (Applied Biosystems). The total reaction volume per well was 20 μL, including 5 ng genomic DNA, 1 μL TaqMan SNP genotyping assay (containing two PCR primers and two dye (VIC or FAM) labeled TaqMan MGB probes) and 10 μL TaqMan Universal PCR Master Mix (Applied Biosystems), according to the manufacturer's manual. PCR was performed at 95°C for 10 min and 40 cycles at 95°C for 15 s and 60°C for 1 min. The samples were amplified, read, and analyzed using the ABI Prism 7900HT Sequence Detection Systemand ABI Prism SDS 2.4 software. Two blank controls in each 96-well-plate were used for the assay quality control. *APOE* genotyping was performed according to establish methods (Hixson and Vernier, 1990).

BIOCHEMICAL INVESTIGATIONS

Patients' fasting blood samples were collected in the morning and serum was rapidly stored at −80°C. Ceruloplasmin (Cp) was analyzed by immunoturbidimetry assay (Horiba ABX, Montpellier, France) (Wolf, 1982). Iron was analyzed using Ferene, a ligand capable of forming chelates with iron (II), (Higgins, 1981) and transferrin by mixing human serum with the antibody solution and measuring the resulting immune complexes by immunoturbidimetry assay (Skikne et al., 1990). The percentage of transferrin saturation (% transferrin-sat) was calculated by dividing serum iron by the total iron-binding capacity (TBC = Transferrin in mg/dL *1.25) and multiplying by 100. The ferritin was analyzed by immunoturbidimetry assay enhanced with latex (Simo et al., 1994). All reagents were ABX Pentra from Horiba ABX (Montpellier, France). Finally, we measured the Cp:Tf ratio [Cp/Tf *10 (−2)]. All biochemical measures were automated on a Cobas Mira Plus (Horiba ABX, Montpellier, France) and performed in duplicate.

STATISTICAL ANALYSIS

Demographic and clinical characteristics in our patient and control samples were described either in terms of mean ± SD if quantitative, or in terms of proportions. Student's *t*-test and the chi-square (χ^2) test, or a non parametric test when appropriate, were used to compare separately each diagnosis (AD, MCI and PD) group vs. healthy controls. The effects of both age and sex were considered in all statistical analyses. Diagnosis groups were compared using ANCOVA models adjusting for genetic variable as well as age and sex. A Multinomial Multiple Logistic Regression model was applied, considering the control group as the reference category, to evaluate the effects of the biological and genetic variables on the probability of being AD or PD or MCI. All tests were interpreted at the 0.05 level of significance. SPSS 16.0 software was used for all statistical analyses.

RESULTS

We screened participants for C282Y (rs1800562), H63D (rs1799945) *HFE* gene and P589S (rs1049296) *TF* gene mutations in order to test their association with AD, MCI and PD. Gene frequencies were in Hardy-Weinberg equilibrium in each group.

The analysis revealed that there was no significant difference in genotype or allele frequencies of C282Y and H63D (**Table 2**) *HFE* gene variants among AD, MCI and PD subjects vs. healthy controls, though a C282Y mutation higher frequency approached significance in the MCI group ($p = 0.06$; **Table 2**). The analysis of the *TF* C2 polymorphism revealed that the wild type genotype was carried by 64% of healthy controls vs. 54% of AD patients ($p = 0.08$; **Table 2**). Finally, the stratification for the *APOE*ε4 allele revealed a significant difference in the frequency of the mutated genotypes of the *TF* gene between *APOE*ε4 carriers and non-carriers ($p = 0.001$; **Table 3**).

Table 2 | Genotype and allelic frequencies for *HFE* (C282Y, H63D) and *TF* (P589S) mutations in controls, AD, MCI, and PD patients.

		Controls	AD patients	MCI patients	PD patients
***HFE* C282Y**					
Wt	GG	137 (98.6%)	138 (100%)	25 (92.6%)	70 (95.9%)
mutation	GA\AA	2 (1.4%)	–	2 (7.4%)	3 (4.1%)
			$p = 0.1$	$p = 0.06$	$p = 0.2$
allele	G	276 (99.3%)	276 (100%)	52 (96.3%)	143 (98%)
allele	A	2 (0.7%)	–	2 (3.7)	3 (2%)
			$p = 0.08$	$p = 0.06$	$p = 0.2$
***HFE* H63D**					
wt	CC	99 (71.2%)	99 (71.7%)	21 (77.8%)	53 (68.8%)
mutation	CG\GG	40 (28.8%)	39 (28.3%)	6 (22.2%)	24 (31.2%)
			$p = 0.4$	$p = 0.5$	$p = 0.5$
allele	C	236 (84.9%)	237 (85.9%)	48 (88.9)	130 (84.4%)
allele	G	42 (15.1%)	39 (14.1%)	6 (11.1%)	24 (15.6%)
			$p = 0.7$	$p = 0.7$	$p = 1$
***TF* C2**					
wt	CC	77 (65.3%)	59 (54.1%)	19 (70.4%)	47 (60.3%)
mutation	CT\TT	41 (34.7%)	50 (45.9%)	8 (29.6%)	31 (39.7%)
			$p = 0.08$	$p = 0.4$	$p = 0.2$
allele	C	194 (82.2%)	163 (74.8%)	45 (83.3%)	121 (77.6%)
allele	T	42 (17.8%)	55 (25.2%)	9 (16.7%)	35 (22.4%)
			$p < 0.0001$	$p = 0.8$	$p = 0.3$

Table 3 | Genotype associated with wild type *TF* C2 in AD patients with (*APOE*ε4+) and without (*APOE*ε4−) Apoε4 allele.

	TF C2		*p*-value
	CC	CT\TT	
*APOE*ε4+	12	22	
*APOE*ε4−	47	29	0.01

We measured iron, transferrin, ceruloplasmin, and calculated the Cp:Tf ratio and %Tf saturation in the whole study population. Some of these variables were not normally distributed and so we applied a Mann-Whitney non parametric test and these data are presented as median (min-max) in **Table 1**. We found a significant reduction in the concentration of Tf both in AD and in MCI and a significant increase of the Cp:Tf ratio in AD patients than in controls (**Table 1**). Conversely, adjusting for age and sex in an ANCOVA model we observed that there was a significative simple effect of the diagnosis factor on Tf values [$p = 0.008$, $F_{(3,\ 274)} = 4.055$]; in particular only the estimated parameter for AD diagnosis resulted significative (parameter estimate $= -0.197$, $t = -2.124$, $p = 0.035$), a significant simple effect of age was evident too [$p = 0.004$, $F_{(1,\ 274)} = 8.210$]. The simple effect of diagnosis was significant on Cp:Tf ratio ($p = 0.014$, $F = 3.603$, $df = 3{,}255$) but it was due to difference between MCI and controls (parameter estimate $= -1.666$, $t = -2.217$, $p = 0.028$). Also the simple effect of age [$p = 0.011$, $F_{(1,\ 255)} = 6.626$) and of sex [$p = 0.002$, $F_{(1,\ 255)} = 10.060$] were significant on Cp:Tf ratio.

Then, we again performed the ANCOVA analysis taking into account factors like diagnosis, age, sex and genetic variants and their possible interaction on concentration of biochemical variables under study. The analysis revealed a significant influence of the interaction between sex and *HFE* on iron concentrations [$p = 0.038$; $F_{(1,\ 243)} = 4.3524$] and, always on iron concentrations, a significant simple effect of sex [$p = 0.023$; $F_{(1,\ 243)} = 5.212$], age [$p = 0.028$ $F_{(1,\ 243)} = 4.876$]. The analysis reconfirmed the previous results on Cp:Tf ratio while the effect of *HFE* was not significant ($p > 0.20$). The effect of age was significant [$p = 0.016$, $F_{(1,\ 264)} = 5.886$] on the Tf values and also the effect of diagnosis factor [$p = 0.008$, $F_{(3,\ 264)} = 4.038$]. Finally, we observed the only significant effect of sex factor on Cp concentrations [$p < 0.001$, $F_{(1,\ 301)} = 35.886$]. The probability of having AD, PD or MCI was estimated with a multinomial logistic regression model considering the diagnosis as the dependent variable and the biochemical and genetic markers, as well as sex and age, as the independent variables and the control group was set as the reference category. These statistical analyses confirmed age as the main risk factor: the increase of age (1 year) increased of 14% the risk of being AD ($p < 0.001$. OR = 1.14; 95% C.I. 1.07–1.21). Concerning PD, increased of 1 μg/dL of iron ($p = 0.031$; OR = 1.06; 95% C.I. 1.01–1.11), of Cp:Tf ratio ($p = 0.016$; OR = 2.9; 95% C.I. 1.22–6.75) as well as being man instead of woman ($p < 0.001$; OR = 5.20; 95% C.I. 2.14–12.66) resulted in a higher risk of having PD, while the increase of Cp concentration (1 mg/dL) was a protection factor ($p = 0.042$; OR = 0.72; 95% C.I. 0.53–0.99). In MCI, we observed that increasing values of Cp:Tf corresponded to a 4-fold increase of the relative risk of having MCI ($p = 0.013$; OR = 4.27; 95% C.I. 1.374–13.32), and conversely that higher Cp levels resulted as a protection factor ($p = 0.009$; OR = 0.53; 95% C.I. 0.32–0.85).

DISCUSSION

Several studies investigated the role of the principal variants of *HFE* and *TF* genes on the risk of having neurodegenerative disorders, in particular AD, PD and MCI even though separately. The results reported are often controversial and this inconsistency suggests that, beside the complex genetic etiology, additional interactions can contribute to the disease, as for example, environmental factors, age, sex, and metal ion involvement or oxidative stress. Considering the current literature, we examined the contribution of *HFE* and *TF* mutations in PD, AD and MCI testing if single gene variants have an impact on iron metabolism. Even though our results don't support the hypothesis that these mutations are a genetic risk factor for Italian cohort they have been reported to have a strong effect on the disease risk in several populations (Buchanan et al., 2002; Combarros et al., 2003; Robson et al., 2004). Moreover, a direct significant synergy between the *TF* C2 allele and the GA and AA genotypes of C282Y of the *HFE* gene on the disease risk has been reported by Robson (Robson et al., 2004) and recently confirmed by two additional studies carried out on very large subject samples (Kauwe et al., 2010; Lehmann et al., 2012). Some of ours negative results can be explained on the basis of the fact that the C282Y *HFE* mutation is very rare in the Italian population (Sampietro et al., 2001), and specifically, in our AD sample there was no patient carrier of the homozygous genotype. Interestingly, while this mutation was rare also in the PD sample, the direct comparison between AD and PD revealed that PD patients had a significantly higher frequency. Even concerning these data, literature is controversial, in fact, Guerreiro and colleagues (Guerreiro et al., 2006) described a significant increase of the prevalence of homozygous C282Y carriers in PD patients compared with healthy controls but not in AD, concluding that it could be a risk factor for PD but not for AD in the Portuguese population. This result is confirmed by some (Dekker et al., 2003) but not all studies (Borie et al., 2002; Buchanan et al., 2002).

We found an interaction between *TF* C2 and *APOE*ε4 alleles (**Table 3**), evidence already described by some authors (Namekata et al., 1997) but not confirmed by others (Marklova et al., 2012) We observed that in AD patients with at least one copy of the *APOE*ε4 allele, the frequency of the CT or TT genotype of *TF* C2 was almost twice as that in the remaining AD non-*APOE*ε4 allele carriers. The underlying mechanisms of this interaction remain unknown but the effect of the combination between *TF* C2 and *APOE*ε4 in AD patients *is a* meaningful question that deserves further studies.

Concerning the biochemical analysis, we observed a significant decrease of Tf concentration in AD and MCI patients compared with healthy control subjects and an increase of the Cp:Tf ratios in AD in line with previous studies (Squitti, 2012). This finding is in line with the derangements of iron metabolism widely described in AD brain and also in MCI (Lavados et al., 2008; Smith et al., 2010). Altered patterns of *TF* gene expression and of Cp levels and activity in the AD brain have been reported (Loeffler et al., 1994, 1996; Castellani et al., 2007). Cp and Tf interact to limit concentrations of labile ("free") iron and copper species, and thus play an important role in antioxidant defense in serum. More precisely, the Cp:Tf system is involved in counteracting oxidative stress generated in different body districts by regulating the serum capacity to sequester exchangeable transition metals, which are particularly prone to partake in oxidative stress chain reactions (Kozlov et al., 1984; Hubel et al., 1996; Altamura et al., 2009).

The multinomial logistic regression model presented revealed the significant influence of iron and of Cp:Tf values on the probability of having PD, sustaining that an alteration on iron system is involved in the development of this disease. These results demonstrate that iron dysfunction has an effect on the PD risk, even though this diagnosis has not effect on the mean value of iron measured in general circulation, as demonstrated in a recent meta-analysis (Mariani et al., 2013) and confirmed by the ANCOVA results reported in our current study.

The effect of sex on iron levels in relation with PD is complex. In fact, even though in our recent meta-analysis (Mariani et al., 2013) we didn't find a significant effect of being women on iron circulating levels, the replication study reported in the same study (Mariani et al., 2013) pointed out that women had higher Tf serum levels, which can possibly modify iron internalization and tissue storage. In current study, we have again found a sex effect on the disease risk.

The activation of the Cp:Tf antioxidant system appears a mechanism of the early stages of some neurodegenerative diseases. Current result describes a 4-fold increased risk of having MCI in those subjects with higher values of the Cp:Tf ratio. This fact was document in a previous study demonstrating an increment in the ratio of serum copper to non-heme iron levels predicting which subjects with MCI would progress to dementia vs. those that would remain cognitively stable (Mueller et al., 2012).

Higher systemic Cp concentrations seem a protective factor for both MCI and PD subjects and our results about PD are in accord with recent findings reporting a loss of Cp ferroxidase activity in the substantia nigra of PD cases suggesting that intravenous Cp may have therapeutic potential in PD (Ayton et al., 2012). This evidence is consistent with the role of *Cp* that, oxidizing Fe (II) to Fe (III) allows iron binding to Tf and its mobilization from iron tissue storage. More specifically, the result that increased Cp concentrations are a protecting factor for PD is in line with the evidence that CP mutations have been associated with increased deposits of iron in SN (Hochstrasser et al., 2004, 2005).

A result worthy of interest is the effect of interaction between sex and *HFE*. As detailed in the ANCOVA model, the presence on an *HFE* gene mutation (H63D or C282Y) results in an increased absorption of iron in men. A "disease effect" seems essential to explain this relationship, since increased iron levels cannot be ascribed to the *HFE* change alone, but rather it is caused by the synergy between being man and *HFE* change on iron levels, sustaining previous results (Fargion et al., 1992; Niederau et al., 1996; Adams et al., 1997).

Our study has some limitations that need to be taken into account: the small size of the sample, and specifically the small number of MCI subjects included; one ethnic group considered; the rarity of some alleles which prevent the statistical power of some genetic analysis. Even though they need confirmation in larger cohorts, our data suggest that deregulation of iron metabolism, in addition to other factors, has some effect on the PD disease risk.

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Retinal iron homeostasis in health and disease

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Iron is essential for life, but excess iron can be toxic. As a potent free radical creator, iron generates hydroxyl radicals leading to significant oxidative stress. Since iron is not excreted from the body, it accumulates with age in tissues, including the retina, predisposing to age-related oxidative insult. Both hereditary and acquired retinal diseases are associated with increased iron levels. For example, retinal degenerations have been found in hereditary iron overload disorders, like aceruloplasminemia, Friedreich's ataxia, and pantothenate kinase-associated neurodegeneration. Similarly, mice with targeted mutation of the iron exporter ceruloplasmin and its homolog hephaestin showed age-related retinal iron accumulation and retinal degeneration with features resembling human age-related macular degeneration (AMD). *Post mortem* AMD eyes have increased levels of iron in retina compared to age-matched healthy donors. Iron accumulation in AMD is likely to result, in part, from inflammation, hypoxia, and oxidative stress, all of which can cause iron dysregulation. Fortunately, it has been demonstrated by *in vitro* and *in vivo* studies that iron in the retinal pigment epithelium (RPE) and retina is chelatable. Iron chelation protects photoreceptors and retinal pigment epithelial cells (RPE) in a variety of mouse models. This has therapeutic potential for diminishing iron-induced oxidative damage to prevent or treat AMD.

Keywords: iron, retina, age-related macular degeneration (AMD), chelator, oxidative stress, ferroportin, ceruloplasmin, hephaestin

INTRODUCTION

As an important part of many metabolic processes, iron is essential for life. However, excess iron can be toxic to tissues. Iron is a crucial component of enzymes of the citric acid cycle and electron transport chain, making iron essential for adenosine triphosphate (ATP) production (Wigglesworth and Baum, 1988; Poss and Tonegawa, 1997). Iron is also required by ribonucleoside reductase which is the rate-limiting enzyme of the first metabolic reaction in DNA synthesis (Wigglesworth and Baum, 1988). In the central nervous system (CNS), oligodendrocytes require iron for myelin synthesis and maintenance (LeVine and Macklin, 1990; Morris et al., 1992). Iron is an essential cofactor for synthesis of neurotransmitters, including dopamine, norepinephrine, and serotonin (Youdim, 1990). Thus, disruption of iron homeostasis may be involved in diseases of the CNS. Iron deficiency in children can cause auditory defects due to myelin disruption (Roncagliolo et al., 1998). Demyelinating diseases like multiple sclerosis are associated with cellular iron homeostasis dysfunction (Drayer et al., 1987).

In retina, iron is particularly critical for the visual phototransduction cascade. RPE65, an iron containing protein, is dependent on iron for isomerohydrolase activity. In the retinal pigment epithelium (RPE), this activity is important in catalyzing the conversion of all-trans-retinyl ester to 11-cis-retinol in the visual cycle (Moiseyev et al., 2005). 11-cis-retinol leads to 11-cis-retinaldehyde, the photosensitive component of rhodopsin. Photoreceptor cells continuously shed and synthesize their disc membranes, depending on iron-containing enzymes like fatty acid desaturase for biogenesis of lipids which is used in disc membrane replacement (Shichi, 1969). Although iron is essential for retinal function, excessive iron can be toxic. Ferrous iron can catalyze the conversion of hydrogen peroxide to hydroxyl radical, which is the most damaging of the reactive oxygen species. Oxidative damage caused by hydroxyl radical includes lipid peroxidation, DNA strand breakage and biomolecule degradation (Halliwell and Gutteridge, 1984). Hydroxyl radicals have been implicated in the pathogenesis of Alzheimer's and other CNS diseases (Smith et al., 1997). Since the main function of the RPE cells is phagocytize lipid-rich, easily oxidized photoreceptor outer segments in a high oxygen tension environment, the retina and RPE are particularly prone to oxidative damage. Therefore, iron must be carefully regulated in the eye to provide necessary iron without giving rise to oxidative damage. Retinal iron deficiency has not been found to be a clinical problem, perhaps because the retina is very efficient at taking up whatever iron is available in the circulation, and/or because retinal iron stores significantly exceed iron needs.

GENERAL IRON HOMEOSTASIS

In the circulation, iron exists in two forms: heme and non-heme iron. The majority of non-heme iron is bound to transferrin (Baker and Morgan, 1994), which is capable of binding two molecules of ferric iron. Adults have approximately 3 mg of circulating non-heme iron, only saturating 30% of transferrin binding sites. However, transferrin cannot cross the tight junctions of the blood brain barrier (BBB); therefore, cells that comprise the BBB must import iron and transfer it into neural tissue. Iron-laden transferrin binds to transferrin receptor (TfR) on the cell surface,

and this complex is internalized into endosomes. In low pH, iron is released (Sipe and Murphy, 1991) and exported by endosomes for use, storage in ferritin, a multisubunit protein consisting of H- and L-chains (Aisen et al., 2001) or export. Upon iron delivery, transferrin is recycled to the surface (Hunt and Davis, 1992).

Ferroportin exports ferrous iron that is not utilized or stored in the cell (Abboud and Haile, 2000; Donovan et al., 2000; McKie et al., 2000), but exported iron must be oxidized to be accepted by transferrin in the circulation. Oxidation of iron is accomplished by the ferroxidases such as, ceruloplasmin (Osaki et al., 1966), hephaestin (Heph) (Vulpe et al., 1999), amyloid precursor protein (APP) (Duce et al., 2010) or zyklopen (Chen et al., 2010). These enzymes are crucial for iron export and when disrupted result in cellular iron accumulation and degeneration. Ceruloplasmin, a copper binding protein, contains over 95% of copper found in plasma. Mutation of a copper transporter in Wilson's disease causes decreased serum ceruloplasmin levels resulting from failure of copper incorporation into ceruloplasmin. Hephaestin is 50% identical to ceruloplasmin at the amino acid level. In the sex-linked anemia mouse, *heph* has been identified as the disrupted gene product. In these mice, failure of hephaestin to export iron from the enterocyte into the circulation results in low serum iron and enterocyte iron increase (Vulpe et al., 1999). Unlike ceruloplasmin, which is a secreted plasma protein or glycosylphosphatidylinositol (GPI) membrane-anchored protein (Patel and David, 1997), Heph is present only as a membrane-bound protein. APP, a type I transmembrane protein precursor, was found to be a functional ferroxidase. Both full-length and soluble APP species can interact with ferroportin to facilitate iron export from neurons and other cells (Duce et al., 2010). In Alzheimer's disease, iron export by APP is inhibited by elevated extracullular Zn^{2+} dissociating from β-Amyloid. The APP domain responsible for ferroxidase activity has been debated (Ebrahimi et al., 2012). In 2010, zyklopen was identified as another multicopper ferroxidase and plays a role in placental iron transport. Immunostaining of zyklopen identified its expression in the brain, kidney, testes and retina but not in the liver or intestine (Chen et al., 2010). Our unpublished data also suggests that zyklopen is expressed in brain and neural retina, but not RPE cells.

Iron levels are managed by an iron-responsive mechanism involving iron regulatory proteins (IRPs, IRP1 and IRP2) which serve to register intracellular iron status. This regulation allows individual cells to regulate iron uptake, sequestration and export depending on their iron status. Under conditions of iron starvation, IRP1 and IRP2 are activated for high affinity binding to multiple "iron-responsive elements" (IREs) in the 3′-untranslated region of TfR mRNA and to a single IRE in the 5′-untranslated region of the mRNAs encoding both H- and L-ferritin chains. This stabilizes TfR mRNA (Binder et al., 1994) and inhibits ferritin mRNA translation (Muckenthaler et al., 1998). Conversely, failure of IRPs to bind to cognate IREs in iron-replete cells leads to degradation of TfR mRNA and synthesis of ferritin.

IRON IMPORT INTO THE RETINA

The blood–retinal barrier isolates the retina from the bloodstream. The intercellular tight junctions of the neuroretinal vasculature and RPE resulting in independent barriers prevent intercellular diffusion, thereby protecting both sides of the retina from systemic circulation.

TRANSFERRIN MEDIATED TRANSPORT

When bound to transferrin in the choroidal circulation, iron is taken up by high affinity TfR at the basolateral surface of the RPE. Then, this complex is internalized into a low pH endosome where iron is dissociated from transferrin. After export from the endosome, iron enters a cytoplasmic pool of labile iron where it can bind to ferritin or be transported for further use. Whether RPE cells transport iron apically toward the retina is not clear. A potential alternative route for iron transport into the retina is through the retinal vascular endothelial cells. Evidence suggests that ferroportin is expressed by these cells, which may transport iron into the retina in a process regulated by hepcidin (Hadziahmetovic et al., 2011a). Once iron enters the retina, it is likely to bind transferrin. The vitreous humor and aqueous fluid contain large amounts of transferrin (Hawkins, 1986; Yu and Okamura, 1988; Tripathi et al., 1990). Experiments with intravitreal injection of labeled fucose or tyrosine suggest that the vitreous transferrin is partially synthesized locally in the eye (Laicine and Haddad, 1994), in part by the ciliary body (Rodrigues et al., 1998). The iron-transferrin complex can then be taken up by the TfRs on the photoreceptor inner segments (Yefimova et al., 2000). The photoreceptor inner segments of adult rat retinas are immunolabled for TfR, which probably bind and internalize the iron-transferrin complex in the photoreceptor matrix.

DIVALENT METAL TRANSPORTER-1

Divalent metal transporter-1 (DMT1), a proton symporter, moves one atom of ferrous iron and a proton in the same direction (Gunshin et al., 1997). DMT1 is located on the apical surface of intestinal epithelial cells and transports dietary free iron, upon reduction from the ferric to ferrous form, from the luminal surface into the enterocytes (Rouault and Cooperman, 2006). DMT1 is located on the endosome and transports iron from the endosome into the cytoplasm in most other cells in the body (Rouault and Cooperman, 2006). Some studies have localized DMT1 in rat brain blood vessels (Burdo et al., 2001), while others have detected it in neurons but not vascular cells (Gunshin et al., 1997). While this issue remains controversial, its potential localization in blood vessel suggests that DMT1 may mediate iron transport to and/or from the brain. Belgrade rats with a mutation in DMT1 have a hypochromic, microcytic anemia and less iron in their brains, consistent with a role for DMT1 in brain iron transport (Burdo et al., 1999). In the retina, we detected strong DMT1 immunolabel in rod bipolar cell bodies, rod bipolar cell axons, horizontal cell bodies, and photoreceptor inner segments. However, the importance of DMT1 for iron import into these cells is currently unclear.

DEXRAS

Dexras1, a 30 kDa G-protein with close homology to the Ras subfamily and localized predominantly to the brain, can be induced by the activation of glutamate-NMDA receptors to signal the uptake of iron in neurons (Cheah et al., 2006). The activation of glutamate-NMDA receptors stimulates nitric oxide

synthase which binds to the nitric oxide synthase 1 adapter protein, CAPON, and delivers nitric oxide to Dexras1 causing S-nitrosylation of Dexras1. In the down-stream pathway, Dexras1 interacts with peripheral benzodiazepine receptor-associated protein (PAP7), which binds to DMT1 to induce iron uptake. It has been hypothesized that NMDA neurotoxicity is caused by increased iron uptake following induction of this signaling cascade, since iron chelation in neuronal cultures protects against NMDA neurotoxicity. Consistent with this mechanism, Dexras1 knockout mice are protected against retinal ganglion cell death induced by intravitreal NMDA injection (Chen et al., 2013).

IRON STORAGE

FERRITIN

Iron is primarily stored as cytosolic ferritin which is capable of holding as many as 4500 iron molecules in the ferric state in its central core. H-ferritin has ferroxidase activity allowing it to rapidly accumulate Fe^{3+} in its central core (Aisen et al., 2001) while L-ferritin does not (Levi et al., 1994). The distribution of iron and ferritin has been characterized in the adult rat retina. Proton-induced X-ray emission identified the largest amounts of heme and non-heme iron in the inner segments of photoreceptors, the RPE, the choroid, the inner nuclear layer, and the ganglion cell layer (Yefimova et al., 2000). Iron was present in somewhat lesser, but still significant amounts in the photoreceptor outer segments (Yefimova et al., 2000; Ugarte et al., 2012). Interestingly, immunohistochemistry studies have demonstrated a similar distribution pattern of retinal ferritin. The exception is that iron, but not much ferritin, is contained in the photoreceptor outer segment. Also, ferritin levels are regulated by IRPs (Hentze and Kühn, 1996). IRPs inhibit ferritin translation by binding to ferritin mRNA in iron-depleted cells to regulate ferritin levels.

MITOCHONDRIAL FERRITIN

Mitochondrial ferritin (TfR) is structurally similar to H-ferritin but localized to the mitochondria (Levi et al., 2001). MtF was detected by immunohistochemistry in the photoreceptor inner segments and diffusely throughout the inner retina (Hahn et al., 2004b). In order to confirm mitochondrial localization, anti-MtF was co-labeled with an antibody specific for the ATPase in Complex V of the electron transport chain of mitochondria. Label co-localized to the inner segment ellipsoids, the location of the mitochondria, but not the inner segment myoid.

IRON EXPORT

CERULOPLASMIN

Ceruloplasmin, a multicopper oxidase with ferroxidase activity, functions as an antioxidant by oxidizing iron from its Fe^{2+} to Fe^{3+} state, thus preventing oxidative damage induced by Fe^{2+} mediated free radical generation by the Fenton Reaction (Osaki, 1966). Additionally, by oxidizing iron from its Fe^{2+} to Fe^{3+}, ceruloplasmin facilitates iron export out of the cell since only ferric iron can be taken up by transferrin in the circulation (Osaki, 1966); extracellular ferroxidases including ceruloplasmin are important in the ferroxidation which is thus necessary to initiate efficient iron export, probably through the generation of an ion gradient (Sarkar et al., 2003).

Two forms of ceruloplasmin have been identified: the secreted form and the membrane-anchored GPI-linked form. The secreted form is the predominant form made by the liver while the GPI-linked form is the predominant form found in the brain (Patel et al., 2000). Both forms of ceruloplasmin are present in mouse and human retina (Chen et al., 2003). The secreted form was also identified in the mouse and human RPE (Hahn et al., 2004b). Immunohistochemistry demonstrated that ceruloplasmin protein is located diffusely throughout the retina. The strongest signal in mouse retina was found in the Müller cells. As confirmed by Western analysis, ceruloplasmin is also present in the aqueous, vitreous and retina of a normal human eye (Chen et al., 2003).

Ceruloplasmin has been found to be upregulated in the retina in multiple pathological conditions including light damage, optic nerve crush, glaucoma, and diabetes (Levin and Geszvain, 1998; Chen et al., 2003; Miyahara et al., 2003; Farkas et al., 2004; Gerhardinger et al., 2005). In 10-week-old male BALB/c mice exposed to bright fluorescent light or room light for 7 h, retinal ceruloplasmin levels were increased in light exposed retinas compared to controls. Again, the strongest label was observed in the Müller cells (Chen et al., 2003). It was postulated that upregulation of ceruloplasmin following light damage serves to protect the eye from light-induced oxidative attack. Both the direct antioxidant effect of ceruloplasmin as well as its iron export function may be important for protection against light damage.

In rats subjected to intraorbital optic nerve crush, ceruloplasmin mRNA levels were found to be upregulated relative to controls. Ceruloplasmin mRNA localized to the inner nuclear and ganglion cell layers demonstrated by *in situ* hybridization. Immunohistochemistry localized ceruloplasmin protein in sporadic cells within the nerve fiber layer of untreated retinas (Levin and Geszvain, 1998). In monkeys with experimental glaucoma, microarray assay has demonstrated ceruloplasmin upregulation and immunohistochemical studies have localized the ceruloplasmin to Müller cells (Miyahara et al., 2003). In human retinas with glaucoma, there are increased ceruloplasmin levels in the inner plexiform layer, ganglion cell layer, and nerve fiber layer compared with controls by immunohistochemistry (Farkas et al., 2004).

In addition, ceruloplasmin has also been shown to be upregulated in the diabetic rat retina (Gerhardinger et al., 2005). Similarly, ceruloplasmin is upregulated in the aqueous and vitreous of rabbit eyes with endotoxin-induced ocular inflammation (McGahan and Fleisher, 1986). Ceruloplasmin is additionally upregulated secondary to oxidative stress to the lens, as confirmed by exposure of immortal murine lens epithelial cells to the oxidant *tert*-butyl hydroperoxide (Li et al., 2004).

HEPHAESTIN

Hephaestin (Heph), another multicopper ferroxidase, is a homolog of ceruloplasmin with 50% amino acid identity (Vulpe et al., 1999). Similar to ceruloplasmin, Heph facilitates iron export by oxidizing ferrous iron to its ferric state thereby facilitating its transport across the plasma membrane and uptake by transferrin in the extracellular space (Vulpe et al., 1999). Vulpe et al identified Heph through characterization of sex-linked anemia (sla) mouse which has a Heph mutation that severely decreases its ferroxidase activity. In this mouse, decreased Heph ferroxidase activity

resulted in reduced iron export from intestinal epithelial cells, yielding an anemic mouse.

The presence of *heph* mRNA has been confirmed by RT-PCR in mouse and human RPE cells, and the localization of Heph protein also has been demonstrated by immunohistochemistry in Müller cells in the retina with the greatest amounts in the Müller endfeet adjacent to the internal limiting membrane (Hahn et al., 2004b). The importance of ceruloplasmin and Heph in iron export from the retina is demonstrated in mice with combined deficiency of these two enzymes that results in age-dependent iron accumulation in both RPE and retina (Hahn et al., 2004b). In these mice, Perls' staining revealed iron accumulation in the photoreceptor outer segments and RPE, and electron micrographs showed electron-dense vesicles in the RPE (**Figure 1**). These vesicles were likely lysosomes or endosomes, sometimes fused with melanosomes. X-ray spectroscopy demonstrated that these vesicles had four times more iron content compared to other intracellular structures. Immunohistochemistry revealed H- and L-ferritin were present in rod bipolar cell termini in the inner plexiform layer. Additionally, L-ferritin was found in the inner sections of the outer plexiform layer and the RPE. H-ferritin label showed strongest signal in the photoreceptor inner segments and axons in the outer part of the outer plexiform layer (Hahn et al., 2004a). In contrast to cytosolic H- and L-ferritin, MtF has not been shown to be IRP-regulated. But MtF was increased in the photoreceptor inner segments of $Cp^{-/-}$ and $Cp^{-/-}Heph^{-}$/Y mice (Hahn et al., 2004a). In $Cp^{-/-}$ mice, MtF colocalized with a mitochondria-specific antibody to the inner segment ellipsoid rather than the inner segment myoid. The elevated iron level in these mice causes oxidative stress (detected by increased isoprostane levels) resulting in retinal degeneration characterized by RPE hypertrophy and autofluorescence, sub-RPE deposits, photoreceptor death, and subretinal neovascularization. This degeneration is prevented by the oral iron chelator deferiprone (Hadziahmetovic et al., 2011b).

FERROPORTIN AND HEPCIDIN

Evidence suggests that Ferroportin (Fpn), a transmembrane protein, exports iron out of the cell in cooperation with ferroxidases Cp or Heph which convert it to ferric form (Harris et al., 1999; Vulpe et al., 1999). Ceruloplasmin increased iron export from *Xenopus* oocytes in the presence of Fpn (Donovan et al., 2000; McKie et al., 2000). This effect was observed when ceruloplasmin was added to the medium (McKie et al., 2000). Ceruloplasmin also exports iron from macrophages *in vitro*, but only when tissue culture oxygen levels mimick *in vivo* tissue oxygen levels rather than atmospheric oxygen levels (Sarkar et al., 2003). Furthermore, stable expression of Fpn in J774 cells, a macrophage cell line, increases iron efflux after erythrophagocytosis (Knutson et al., 2005). Conditional knockout of Fpn in mouse villus enterocytes has shown that the protein functions as the major, if not only, iron exporter from the duodenum to the circulation (Donovan et al., 2005).

In the mouse retina, ferroportin immunolocalizes to the RPE, photoreceptor inner segments, inner and outer plexiform layers, and ganglion cell layer. In mice deficient in Cp and Heph, ferroportin immunoreactivity increases, presumably due to an iron-mediated increase in levels of ferroportin transcription (Hahn et al., 2004a).

Hepcidin (Hepc) is a 25 amino acid peptide found in human plasma and urine and produced by the liver (Krause et al., 2000; Park et al., 2001). It was shown that Hepc has anti-microbial activity, and its expression is upregulated by infection, inflammation and iron (Pigeon et al., 2001; Nicolas et al., 2002). Hepc binds to ferroportin, triggering its internalization and lysosomal-dependent degradation (Nemeth et al., 2004; Knutson et al., 2005). Liver secretes Hepc into the circulation when iron is in excess. The Hepc then triggers degradation of ferroportin in enterocytes and macrophages, maintaining homeostasis by preventing iron release into the circulation. In the retina, Hepc can also be synthesized by Muller cells, photoreceptors and RPE and is upregulated by inflammation (Gnana-Prakasam et al., 2008) and elevated iron levels (Hadziahmetovic et al., 2011b). Ferroportin degradation in vascular endothelial cells and RPE is triggered by Hepc, preventing further iron transport into the neural retina, which is consistent with Hepc knockout mice exhibiting increased retinal iron and subsequent retinal degeneration (Hadziahmetovic et al., 2011b). Bmp6 is crucial for regulation of systemic iron homeostasis through Hepc. Bmp6 was detected in RPE and its receptors are expressed in the neurosensory retina. In cultured RPE cells, Bmp6 was down-regulated by oxidative stress and up-regulated by iron. Bmp6 secreted from the RPE cells likely binds Bmp6 receptors in the neurosensory retina up-regulating Hepc, because both intravitreal and subretinal injection of Bmp6 up-regulated Hepc within the neurosensory retina but not within the RPE. $Bmp6^{-/-}$ mice had age-dependent retinal iron accumulation and degeneration. *Postmortem* RPE from patients with early AMD exhibited decreased Bmp6 levels. The diminished Bmp6 levels observed in RPE cells in early AMD may contribute to iron build-up in AMD. This may in turn propagate a vicious cycle of oxidative stress and iron accumulation, exacerbating AMD and other diseases with hereditary or acquired iron excess (Hadziahmetovic et al., 2011c).

DISRUPTION OF IRON HOMEOSTASIS AND OXIDATIVE DAMAGE

Increased intraocular iron has been shown to cause oxidative injury to the retina as shown when intravitreous iron sulfate is administered to adult C57BL/6 mice, resulting in increased superoxide radicals in photoreceptor inner segments, lipid peroxidation of the photoreceptors and retinal degeneration (Rogers et al., 2007). With a higher concentration of iron sulfate injection, these mice exhibited an irregular outer border of the outer nuclear layer suggesting photoreceptor damage. Ceruloplasmin-deficient mice have hereditary iron overload and also show features of retinal degeneration. Eighteen-month-old $Cp^{-/-}$ mouse retinas showed mild degeneration of the inner nuclear layer including condensed chromatin and dark cytoplasm in the cells (Patel et al., 2002). Patients with ceruloplasmin deficiency from the hereditary disease aceruloplasminemia also have retinal iron accumulation with retinal degeneration (Dunaief et al., 2005). The degeneration primarily involves RPE cells, with hypo and hyperpigmentation, autofluorescence, sub-RPE drusen and subretinal drusenoid deposits (Wolkow et al., 2011).

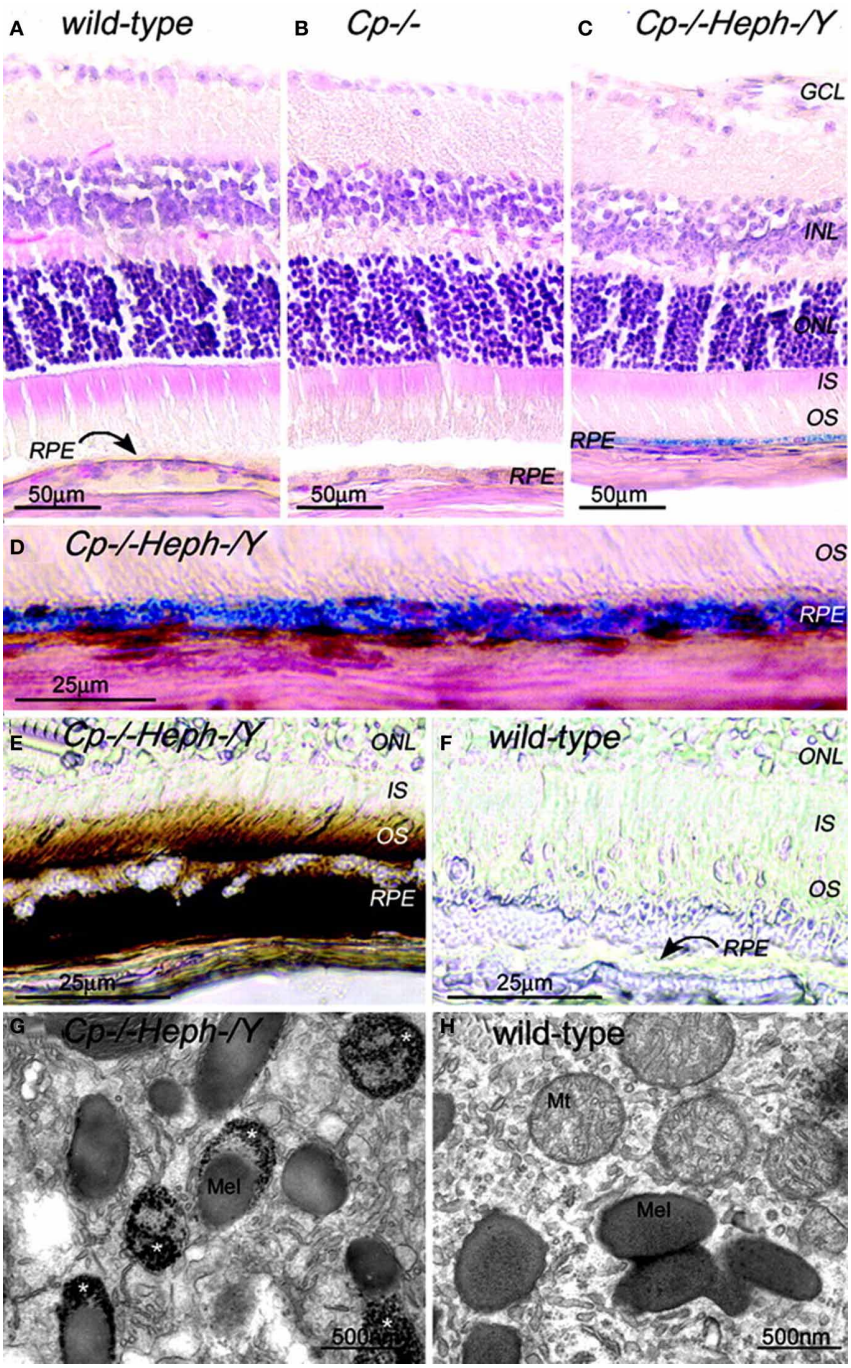

FIGURE 1 | Adult (6-month-old) $Cp^{-/-}Heph^{-}/Y$ RPE and photoreceptors accumulate iron. (A–C) 6-month-old WT **(A)**, $Cp^{-/-}$ **(B)**, and $Cp^{-/-}Heph^{-}/Y$ **(C)** retinas Perls' stained for iron (blue) and counterstained with hematoxylin/eosin. **(D)** High magnification of Prussian blue Perls' label in 6-month-old $Cp^{-/-}Heph^{-}/Y$ RPE. (**E** and **F**) Light photomicrographs of 6-month-old $Cp^{-/-}Heph^{-}/Y$ **(E)** and WT **(F)** retinas after DAB enhancement (brown) of Perls' stain. (**G** and **H**) Electron micrographs of RPE from 6-month-old $Cp^{-/-}Heph^{-}/Y$ **(G)** and WT **(H)** eyes. Only the $Cp^{-/-}Heph^{-}/Y$ RPE **(G)** contains electrondense vesicles (*) sometimes fused with melanosomes. Reprinted from Hahn et al. (2004b).

Our team has found that mice with age-dependent iron accumulation from combined deficiency of Cp and Heph also exhibit retinal degeneration (Hahn et al., 2004b). In these mice, RPE cells in up to 75% of a histologic section across the entire retina were severely hypertrophic, hyperplastic, and necrotic with local photoreceptor loss and subretinal neovascularization revealed by light microscopy. Electron microscopy of the hypertrophic RPE revealed excessive accumulation of phagosomes and lysosomes which appear to contain undigested outer segments (**Figure 2**).

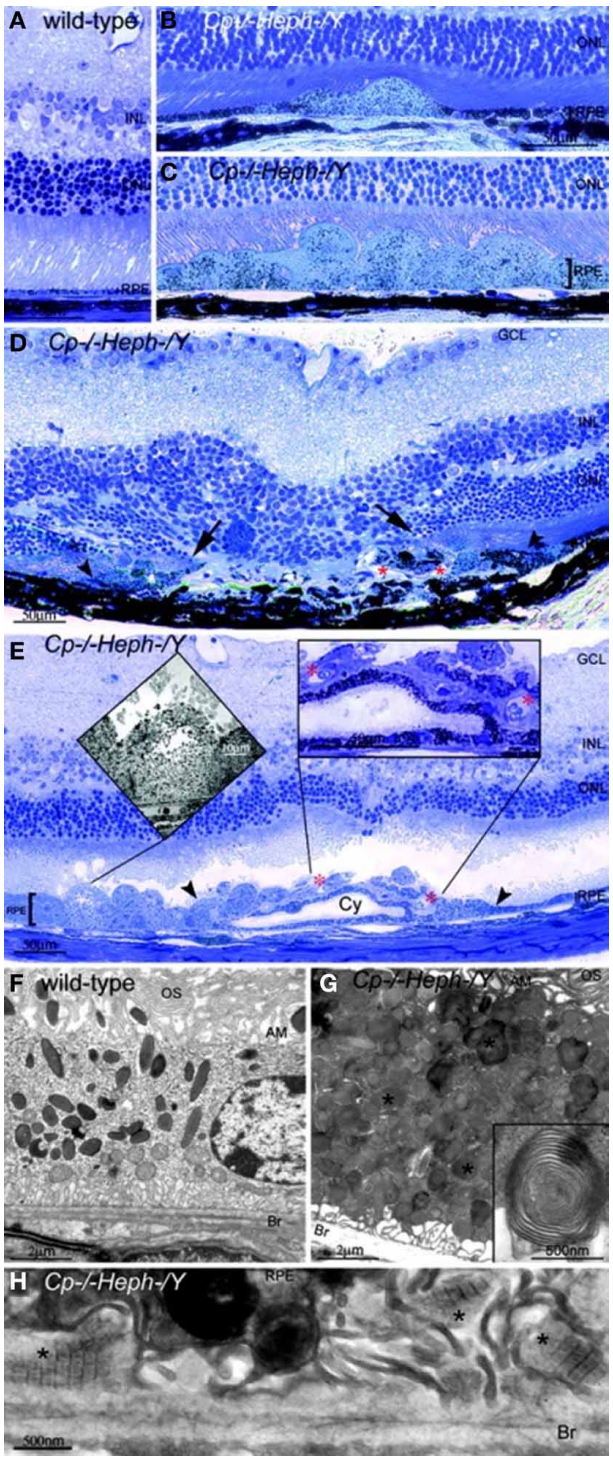

FIGURE 2 | $Cp^{-/-}Heph^{-}/Y$ (9-mon-old) mice have retinal degeneration. **(A)** Light photomicrograph of WT retina. (**B** and **C**) $Cp^{-/-}Heph^{-}/Y$ retina has focal patches of hypertrophic RPE cells in some regions **(B)** and confluent hypertrophic RPE cells in other areas **(C)**. **(D)** In an area of RPE hyperplasia (demarcated by arrowheads), $Cp^{-/-}Heph^{-}/Y$ retinas have local photoreceptor degeneration [demarcated by arrows in the outer nuclear layer (ONL)] and subretinal neovascularization (red*). **(E)** In an area of hypertrophic, hyperplastic (area demarcated by arrowheads) RPE cells, *(Continued)*

FIGURE 2 | Continued
a necrotic RPE cell also observed by electron microscopy (Left Inset) is present. Within the area of RPE hyperplasia, there is local photoreceptor thinning and subretinal neovascularization (red*) visible as small vessels containing erythrocytes (Right Inset). The hyperplastic RPE have formed a localized cyst (Cy). **(F)** Electron micrograph of WT RPE. Br, Bruch's membrane; AM, apical microvilli; OS, photoreceptor outer segments. **(G)** Electron micrograph of $Cp^{-/-}Heph^{-}/Y$ RPE overloaded with phagosomes and lysosomes containing photoreceptor outer segments at various stages of digestion. Some of these lysosomes (*) contained multilamellar structures characteristic of outer segment membranes (Inset). **(H)** Electron micrograph of $Cp^{-/-}Heph^{-}/Y$ deposits between RPE and Bruch's membrane containing wide-spaced collagen (*). (Scale bars: **A–E**, 50 μm; **F** and **G**, 2 μm; **H**, 500 nm.) Reprinted from Hahn et al. (2004b).

Disrupted RPE-photoreceptor interactions can cause iron overload that probably exacerbates retinal degeneration (Yefimova et al., 2002). In Royal College of Surgeons (RCS) rats with a mutation in the receptor tyrosine kinase Mertk, the RPE is unable to phagocytise shed outer segments, resulting in a layer of undigested outer segment tips in the subretinal space (Yefimova et al., 2002). This disrupts the normal function of RPE-photoreceptor interactions, including transferrin diffusion, and leads to increased non-heme iron levels in the interphotoreceptor debris layer, and diminished transferrin levels in the photoreceptor layer, both of which promote photoreceptor loss at *post-natal* day 20.

RETINAL DISORDERS RESULTING FROM ABNORMAL RETINAL IRON METABOLISM

Disrupted iron homeostasis in hereditary and acquired diseases can result in iron overload and retinal disease. Putative retinal iron transport mechanisms and several diseases affecting them are summarized in **Figure 3**.

AGE-RELATED MACULAR DEGENERATION

AMD is the leading cause of irreversible blindness in people over age 65 in developed nations (Leibowitz et al., 1980; Klein et al., 1995). In early stage, manifestations of the disease include drusen deposits associated with the RPE. As the disease progresses, patients may develop geographic atrophy in dry AMD or non-exudative AMD; choroidal neovascularization in wet or exudative AMD. Oxidative stress and radical mediated damage are implicated in the pathogenesis of AMD (Beatty et al., 2000; Zarbin, 2004). In a large clinical trial, patients with intermediate stage AMD given dietary supplements of antioxidants including beta-carotene, vitamin C, vitamin E, and zinc, experienced reduced progression to advanced AMD. This suggests oxidative stress is an important factor involved in AMD pathogenesis (AREDS, 2001).

Iron may be a major source of oxidative stress in AMD. Perls' stain detected increased iron in AMD-affected maculas compared to healthy age-matched maculas (Hahn et al., 2003), specifically in the RPE and Bruch's membrane of early AMD, geographic atrophy, and exudative AMD patients. Analysis of the *postmortem* macula of a 72-year-old white male with advanced geographic atrophy demonstrated iron overload not only in the RPE but also in the photoreceptor layer. Perls' stain detected iron in the photoreceptors and internal limiting membrane along with elevated

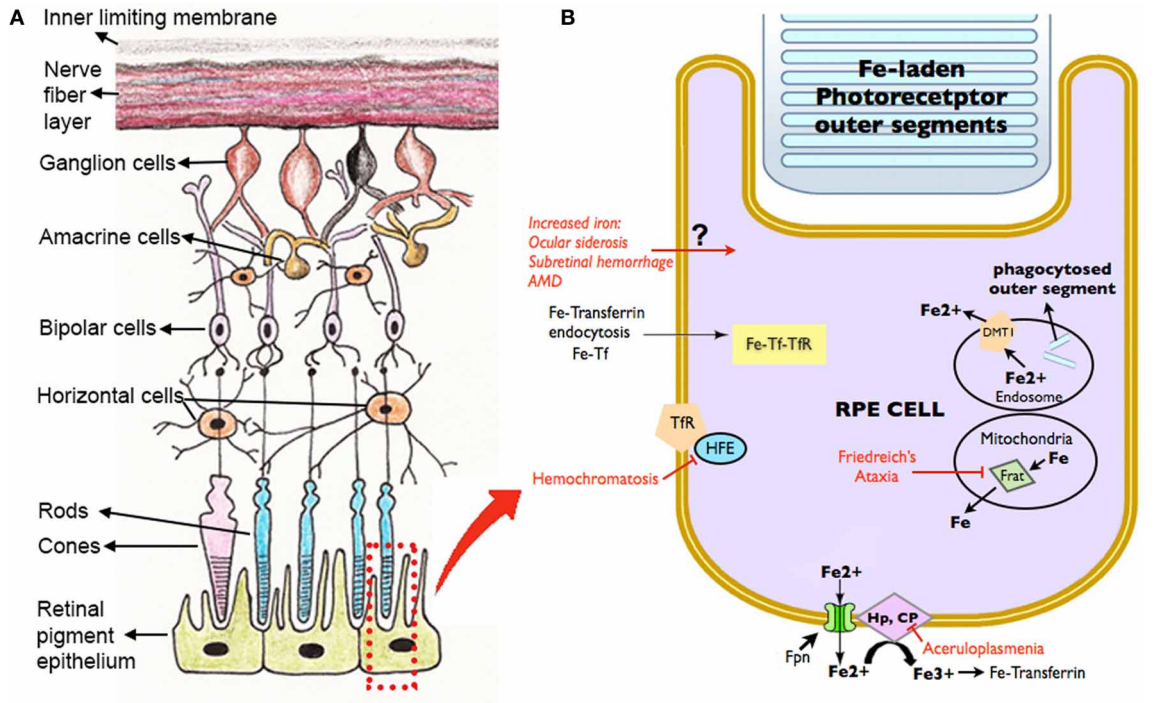

FIGURE 3 | Schematic diagram showing the retinal layers (A), with enlarged view of the RPE and potential routes of iron traffic (B). Several diseases that disrupt retinal iron transport are indicated in red. Proteins involved in iron transport are illustrated: Cp, ceruloplasmin; DMT1, Divalent metal tranporter-1; Fe, iron; Fe^{2+}, ferrous iron; Fe^{3+}, ferric iron; Fpn, ferroportin; Frat, Frataxin; HFE, histocompatability leukocyte antigen class I-like protein involved in iron homeostasis; Hp, Hephaestin; Tf, transferrin; TfR, Transferrin receptor.

levels of ferritin and ferroportin in the photoreceptors and internal limiting membrane whereas the normal maculas were only weakly labeled with anti-ferritin and anti-ferroportin antibody (Dentchev et al., 2005). Mean aqueous humor iron levels were elevated by more than two-fold in dry AMD patients undergoing cataract surgery relative to controls, supporting the assertion that iron levels are elevated in AMD eyes (Jünemann et al., 2013).

Although iron accumulation has been detected in AMD retinas, whether it is a cause or consequence of AMD remains elusive. However, there are several lines of evidence indicating that iron directly contributes to AMD pathogenesis. First, iron accumulates with age increase (Hahn et al., 2006). Eyes from younger donors (less than 35 years old) and older donors (older than 65 years old) were dissected and separated into the retina and RPE/choroid. Atomic absorption spectrophotometry of retinas showed a significant increase in iron levels in the retinas of older compared to younger eyes. Much of this iron in the neural retina is likely to be in the photoreceptor layer, which is the region containing the most iron in rat retinas (Ugarte et al., 2012).

Second, iron is a potent generator of radicals and results in photoreceptor/RPE toxicity when locally elevated in conditions such as ocular siderosis. Although siderosis causes pan-retinal degeneration but not drusen, geographic atrophy, or CNV, this variation may be attributed to the differences in route of iron delivery and spatial and temporal patterns of iron accumulation. Third, mice with double deficiency of Cp and Heph and subsequent age dependent retinal iron accumulation develop a retinal degeneration with several features of AMD (Hahn et al., 2004b). These features include sub-RPE wide-spaced collagen that is normally found in association with drusen in humans, RPE lysosomal inclusions, and RPE death. Histologically, these mice show focal photoreceptor loss and subretinal neovascularization similar to the advanced stage of AMD (**Figure 2**). Fourth, a patient with iron overload resulting from aceruloplasminemia had early-onset subretinal deposits similar to drusen found in AMD patients (Dunaief et al., 2005). Finally, transferrin is upregulated in AMD patients suggesting altered iron regulation or levels (Chowers et al., 2006). Microarray analysis and RT-PCR on *postmortem* retinas of AMD patients and age-matched controls found a 3.5-fold increase in transferrin mRNA levels in dry AMD relative to retinas without AMD and 2.1-fold increase in wet AMD vs. retinas without AMD. Western blot analysis showed a 2.1-fold increase in transferrin levels in AMD retinas compared to retinas without AMD. In these AMD patients, immunohistochemistry detected more transferrin signal in AMD retinas, especially in the photoreceptors, Müller cells, and drusen.

While the mechanism of iron accumulation in AMD is unknown, plausible culprits include inflammation, hypoxia, and oxidative stress, all of which contribute to AMD pathogenesis. Inflammation can cause cellular iron sequestration through IL-6 mediated upregulation of hepcidin (Ganz and Nemeth, 2009). Hypoxia can lead to increased iron uptake through HIF-mediated upregulation of DMT1, a cellular iron importer (Wang et al.,

2010). Oxidative stress can upregulate TfR, another cellular iron importer (Fonseca et al., 2003). Further, hemorrhage in wet AMD causes iron transfer from degraded red blood cells to the retina (Bhisitkul et al., 2008).

ACERULOPLASMINEMIA

Aceruloplasminemia is a rare adult-onset autosomal recessive disease caused by mutations in the ceruloplasmin gene on chromosome 3q (Harris et al., 1995). Iron export from certain tissues is disrupted in these patients because ceruloplasmin normally facilitates iron export by converting it from Fe^{2+} to Fe^{3+}, the form of iron that can be loaded onto transferrin. The retina, brain, and pancreas become iron overloaded in patients with aceruloplasminemia. Clinically, aceruloplasminemia includes the triad of retinal degeneration, dementia, and diabetes (Yamaguchi et al., 1998). The retinal findings in aceruloplasminemia have only been reported in a few patients. All of them have showed retinal pigmentary abnormalities, and one had subretinal macular deposits similar to drusen (Dunaief et al., 2005). Fluorescein angiography (FA) showed RPE atrophy resulting in window defects in the macula. The drusen-like opacities seen by ophthalmoscopy caused blocked fluorescence on the FA. The drusen-like deposits were first observed at age 47 and became smaller and spread out in a centrifugal fashion over the subsequent 9 years. Interestingly, the patient had yellow pingueculae in both eyes and Perls' Prussian blue staining revealed iron overloaded epithelial cells in the pinguecula. Histopathology of this case revealed RPE iron accumulation, hypertrophy, hyper and hypo-pigmentation, autofluorescence, sub-RPE and sub-retinal drusen.

Similarly, histopathology in mice with combined deficiencies of Cp and Heph has been studied (Hahn et al., 2004b). By 6–9 months, these mice demonstrated RPE iron accumulation and hypertrophy. Focal RPE hyperplasia and necrosis, local photoreceptor loss, and subretinal neovascularization were also observed in some mice. Electron microscopy of hypertrophic RPE showed increased phagosomes, lysosomes, and sub-RPE deposits of wide-spaced collagen, resembling some hallmarks of AMD (**Figure 2**).

HEMOCHROMATOSIS

There are two types of hemochromatosis: primary and secondary. As a hereditary disease, primary hemochromatosis is characterized by excessive iron accumulation in the heart, pancreas, liver, and other tissues (Pietrangelo, 2006). Clinically, this results in cardiomyopathy, diabetes, cirrhosis, arthritis, testicular failure, and darkening of the skin. Several mutations can lead to hereditary hemochromatosis. The most common mutation resides in the histocompatibility leukocyte antigen class I-like protein involved in iron homeostasis (HFE) gene product (Feder et al., 1998). This protein normally binds the TfR and forms a stable complex, thus lowering the affinity of the receptor for transferrin. The mutation affects complex formation, resulting in more transferrin binding to TfR and subsequently more iron import into the cell. HFE also regulates the expression of Hepc. Another genetic form of hereditary hemochromatosis results from mutation in ferroportin, reducing iron export from cells (Pietrangelo, 2004). Hereditary hemochromatosis can also result from mutations in Hepc, hemojuvelin (responsible for juvenile hemochromatosis), or TfR 2 (Pietrangelo, 2005; Nemeth and Ganz, 2006). In three cases with hereditary hemochromatosis, retinal abnormalities include drusen and iron in the peripapillary RPE, ciliary epithelium, and sclera (Roth and Foos, 1972).

Evidence from mice supports the possibility that primary hemochromatosis may result in retinal iron accumulation and degeneration. The mouse retina expresses HFE and HJV (Martin et al., 2006; Gnana-Prakasam et al., 2009b), and in knockouts of each of these genes, the retina accumulates iron and exhibits degeneration (Gnana-Prakasam et al., 2009a, 2012). In addition, the RPE exhibits a proliferative phenotype in mice lacking these genes.

Secondary hemochromatosis, or acquired hemochromatosis, results from iron intake during the process of multiple transfusions that are administered to patients with sickle cell anemia and thalassemia. Defects in Bruch's membrane underlying the RPE, called angioid streaks, can also be observed in these patients. However, it is difficult to differentiate the effects of iron overload, intraocular hemorrhage, and chelation therapy on the retina. It is possible that these patients have retinal iron overload. Alternatively, if local control of iron homeostasis in the retina prevents excess iron accumulation despite systemic iron overload, retinal iron level may not be affected. Retinal iron quantification and localization in *postmortem* eyes will help to address these issues.

FRIEDREICH'S ATAXIA

Friedreich's ataxia (FRDA) is an autosomal recessive neurodegenerative disease characterized by progressive ataxia. In most patients with FRDA, a gene encoding the mitochondrial protein frataxin contains a mutation that leads to progressive iron accumulation in mitochondria. Most patients with Friedreich's ataxia can develop a late-onset progressive optic neuropathy, indicating ganglion cell involvement (Porter et al., 2007). A published case in a 59-year-old woman with FRDA documented severe optic neuropathy with rapid-onset catastrophic vision impairment (Porter et al., 2007). Color fundus photos showed a pale optic disc and scattered fleck-like yellow deposits throughout the macula. These yellowish deposits were auto-fluorescent, suggesting the presence of lipofuscin. Additionally, a pigmentary retinopathy has also been associated with FRDA.

PANTOTHENATE KINASE-ASSOCIATED NEURODEGENERATION (PKAN)

Pantothenate kinase-associated neurodegeneration (PKAN), an autosomal recessive neurodegenerative disorder which used to known as the Halervorden-Spatz syndrome, is characterized by iron accumulation in the brain resulting in dystonia, choreoathetosis, rigidity, spasticity, tremor, dementia, or psychomotor retardation (Koeppen and Dickson, 2001). The disease is due to mutations in the pantothenate kinase 2 gene which produces phosphopantothenate (Zhou et al., 2001). Phosphopantothenate condenses with cysteine in the production of coenzyme A. The mutation results in deficient phosphopantothenate and increased cysteine which binds iron. As a result, iron accumulates in areas of the brain normally containing the most iron, including

the medial globus pallidus and subtantia nigra. In the retina where coenzyme A is in the highest demand, disrupted membrane biosynthesis is likely to occur. Rod photoreceptors are constantly shedding outer segments and synthesizing new membrane discs and deficit of CoA may explain the retinopathy resulting from PKAN. Clinical features of PKAN include childhood onset, dystonia or psychomotor retardation (Koeppen and Dickson, 2001). Retinal features in these patients include a pigmentary retinopathy with attenuated arterioles, foveal hyperpigmentation, posterior pole depigmentation, bone spicules, bull's eye maculopathy, and flecked retina (Newell et al., 1979; Luckenbach et al., 1983). The flecks and macular annulus correlated to melanolipofuscin-contaning macrophages. Nearby RPE cells were hypertrophic with aggregated melanolipofuscin inside. The bone-spicule pattern seen by ophthalmoscopy corresponded to melanin pigment in RPE cells found around the retinal vasculature. Histology demonstrated total loss of photoreceptor outer and inner segments. Mice with a knockout of pantothenate kinase 2 also show photoreceptor degeneration (Kuo et al., 2005).

SIDEROSIS

Ocular siderosis is a sight-threatening condition resulting from deposition of iron in intraocular tissue that leads to oxidative damage from radicals generated by ferrous iron. The most common cause is an intraocular foreign body, but it can also due to an intraocular hemorrhage. Clinical manifestations of this disease include corneal iron deposition, iris heterochromia, pupillary mydriasis, failure of accommodation, anterior subcapsular cataract, or lens discoloration. If there is involvement of the trabecular meshwork and Schlemm's canal, secondary glaucoma can develop (Cibis et al., 1959; Talamo et al., 1985; Sneed, 1988). In the retina, RPE clumping and atrophy can occur as well as retinal arteriolar narrowing and retinal detachment. Initially, electroretinography (ERG) a- and b- wave amplitude may increase, but as the siderosis progresses and photoreceptors degenerate, there is gradual decrease in amplitude (Knave, 1969).

Animal models of siderosis have shown both histopathologic and functional abnormalities in the retina. After 10 days of solid iron foreign body insertion into rabbit vitreous, degeneration of the outer nuclear layer and RPE was observed (Declercq et al., 1977). Consistent with morphological change, ERG measurements also showed a reduction in both the a- and b-wave amplitudes under both scotopic and photopic conditions. Cibis et al. studied pathological specimens of patients who had ocular siderosis and hemosiderosis. Sequelae of intraocular iron overload include contraction bands in the vitreous body and inner surface of the retina, proliferation and obliteration of blood vessels, retinal detachment, and retinal degeneration (Cibis et al., 1959).

In ocular siderosis, iron accumulates in the eye because it has been introduced acutely. With aging and disease where iron transport is dysregulated, iron accumulation is slower and more chronic. Yet, whether the iron accumulation is acute or chronic, the photoreceptors and RPE are the cell types most susceptible to iron-induced damage or death.

SUBRETINAL HEMORRHAGE

Subretinal hemorrhage in the macula may disrupt vision function in a number of diseases including AMD, myopic degeneration, angioid streaks, and ocular histoplasmosis. In a study of patients with intraretinal and subretinal hemorrhage, it was shown that the visual acuity loss depends on hemorrhage size and ability of the tissue to clear the blood (Gillies and Lahav, 1983).

The possible mechanisms for vision dysfunction caused by subretinal hemorrhage include direct iron toxicity to photoreceptors, iron toxicity to the RPE, isolation of the photoreceptors from the RPE, cell migration and proliferation in the subretinal space, or proliferation of a fibrovascular membrane (Gillies and Lahav, 1983). Autologous blood injection into the subretinal space of albino rats and rabbits resulted in progressive degeneration of the photoreceptor with edematous change, and iron accumulation in the RPE and photoreceptor outer segments (Glatt and Machemer, 1982). Deferoxamine, an iron chelator, has been shown to protect retina from toxicity caused by subretinal blood in these rats (Youssef et al., 2002). In a similar experimental model using rabbits, iron was identified with Perls' staining in RPE and photoreceptors, and triamcinolone reduces photoreceptor apoptosis (Bhisitkul et al., 2008). Oxyhemoglobin is believed to be a mediator responsible for the pathology of blood in the retina. *In vitro*, experiments with oxyhemoglobin demonstrated that, when elevated, led to lipid peroxidation in retinal tissues (Ito et al., 1995). The hemoglobin-binding protein hemopexin may protect retina from heme-mediated toxicity. RPE cells bind and internalize the heme–hemopexin complex from the retina and thus facilitate clearance of sub- or intra-retinal blood (Hunt et al., 1996).

POTENTIAL THERAPEUTICS

Since iron-mediated oxidative damage may be involved in the pathogenesis of AMD, it is reasonable to assume antioxidants and iron chelators may reduce the incidence and progression of AMD. It has been shown by Age-Related Eye Disease Study (AREDS) that supplemental zinc, vitamin C, vitamin E, and β-carotene can reduce the risk of AMD progression, and it is likely that additional antioxidants may further protect or slow the AMD progression. Given that iron is one of the most potent sources of oxidative damage via hydroxyl radical production in the Fenton reaction, and since the antioxidants used in the AREDS study may not be able to eliminate all of the hydroxyl radical generated by iron, iron chelators may prove to be a useful complement to AREDS vitamins. Several reports indicate that iron chelation may be beneficial in neurological diseases such as Alzheimer's disease and Parkinson's disease, Huntington's disease and FRDA (Richardson, 2004; Zheng et al., 2005). It is plausible that iron chelation may also be effective in treating retinal disease associated with iron overload. Iron-binding proteins could also be therapeutically useful, as transferrin has been shown to protect against hereditary retinal degeneration in mice (Picard et al., 2010).

However, there are challenges with choosing clinically available iron chelators. Ideally, the iron chelator for retinal degeneration therapy should be absorbed easily in sufficient volume through the GI tract, and transit the blood–retinal barrier efficiently. Such chelators are likely to be uncharged, lipid soluble, and of small molecular size to facilitate passage through the blood–retinal

barriers (Maxton et al., 1986; Kalinowski and Richardson, 2005). Additionally, the ideal chelator might exclusively bind iron but no other biologically important divalent metals such as Zn^{2+} (Liu and Hider, 2002).

Currently, the clinically available iron chelators include deferoxamine, deferiprone, and deferasirox. Another potentially therapeutic iron chelator is salicylaldehyde isonicotinoyl hydrazone (SIH). The advantages and disadvantages of each of these chelators are summarized by Mehta (Mehta and Dunaief, 2012). Previously, *in vitro* experiments demonstrated iron in the RPE and Bruch's membrane can be chelated with deferoxamine. A recent study (Obolensky et al., 2011) demonstrated that treatment with zinc-deferoxamine reduced retinal oxidative stress and enhanced photoreceptor survival, leading to both functional and structural rescue in the rd10 model of retinitis pigmentosa. However, deferoxamine is a cumbersome iron chelator requiring subcutaneous or intravenous administration due to its poor absorption by the GI system. In addition, deferoxamine has serious systemic side effects including pulmonary toxicity, bony changes, growth failure, and promotion of *Yersinia enterocolitica* infections (De Virgiliis et al., 1988; Brill et al., 1991; Tenenbein et al., 1992). Deferoxamine can also cause retinotoxicity. Haimovici et al. describe macular and peripheral pigmentary changes, as well as reduction in retinal function as indicated by reduction in ERG amplitude and electrooculogram light-peak to dark-trough ratios (Haimovici et al., 2002).

In contrast, deferiprone can be administered orally with fewer systemic side effects which can be prevented by careful monitoring. Oral deferiprone was found to be effective in decreasing retinal iron levels and oxidative stress in mice with age-dependent iron accumulation from combined Cp and Heph deficiency (Hadziahmetovic et al., 2011b). Unlike deferoxamine, deferiprone was not found to be toxic to the mouse retina. The iron chelator salicylaldehyde isonicotinoyl hydrazone was also found to reduce levels of reactive oxygen species and prevent RPE cell death in human RPE cell lines with exposure to oxidative stress (Kurz et al., 2009; Lukinova et al., 2009). In the experiments conducted by Lukinova et al., the RPE cells treated with SIH were also resistant to oxidative stress induced by staurosporine, anti-Fas, and exposure to A2E plus blue light (Lukinova et al., 2009). We also found that deferiprone can rescue light-damage-induced photoreceptor death in which iron dysregulation is not the primary cause of the degeneration (Song et al., 2012). Iron chelators, such as deferiprone, which have a lower affinity for iron than deferoxamine, may have less potential to cause side effects by removing iron from proteins that require an iron cofactor. Taken together, these results indicate that iron chelation could protect the retina against a broad range of insults. This has promising implications for the treatment of retinal diseases.

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Role of the P-Type ATPases, ATP7A and ATP7B in brain copper homeostasis

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Over the past two decades there have been significant advances in our understanding of copper homeostasis and the pathological consequences of copper dysregulation. Cumulative evidence is revealing a complex regulatory network of proteins and pathways that maintain copper homeostasis. The recognition of copper dysregulation as a key pathological feature in prominent neurodegenerative disorders such as Alzheimer's, Parkinson's, and prion diseases has led to increased research focus on the mechanisms controlling copper homeostasis in the brain. The copper-transporting P-type ATPases (copper-ATPases), ATP7A and ATP7B, are critical components of the copper regulatory network. Our understanding of the biochemistry and cell biology of these complex proteins has grown significantly since their discovery in 1993. They are large polytopic transmembrane proteins with six copper-binding motifs within the cytoplasmic N-terminal domain, eight transmembrane domains, and highly conserved catalytic domains. These proteins catalyze ATP-dependent copper transport across cell membranes for the metallation of many essential cuproenzymes, as well as for the removal of excess cellular copper to prevent copper toxicity. A key functional aspect of these copper transporters is their copper-responsive trafficking between the *trans*-Golgi network and the cell periphery. ATP7A- and ATP7B-deficiency, due to genetic mutation, underlie the inherited copper transport disorders, Menkes and Wilson diseases, respectively. Their importance in maintaining brain copper homeostasis is underscored by the severe neuropathological deficits in these disorders. Herein we will review and update our current knowledge of these copper transporters in the brain and the central nervous system, their distribution and regulation, their role in normal brain copper homeostasis, and how their absence or dysfunction contributes to disturbances in copper homeostasis and neurodegeneration.

Keywords: copper, copper homeostasis, ATP7A, ATP7B, Menkes disease, Wilson disease, occipital horn syndrome, ATP7A-related motor neuropathy

INTRODUCTION

Copper is indispensable for normal development and function of the central nervous system (CNS). The copper concentration of the human adult brain is significant, estimated at 7–10% of total body copper, similar to that of the liver, the major organ that regulates the copper status of the body (Cartwright and Wintrobe, 1964; Linder, 1991). Regional variance in brain copper concentrations reflects differing metabolic requirements for copper, which change during development (reviewed in Lutsenko et al., 2010). Copper is required as a cofactor for numerous critical enzymes that are involved in vital CNS processes such as respiration, neurotransmitter synthesis, activation of neuropeptides and hormones, protection from oxidative damage, myelination, pigmentation, and iron metabolism among others. The redox cycling of copper between Cu^{2+} and Cu^{+} oxidation states is utilized by enzymes involved in these processes for catalytic reactions. However, this redox activity also can lead to elevated reactive oxygen species (ROS) and corruption of critical proteins by adventitious binding of copper ions (Halliwell and Gutteridge, 1984). High oxygen consumption in the brain (20% of total body oxygen), coupled with low levels of antioxidants and antioxidant enzymes, and high levels of metal ions, mean the brain is particularly susceptible to ROS-induced oxidative stress. Hence, precise regulation of brain copper is essential to ensure appropriate levels and distribution for the maintenance of brain function, without risking inadvertent interactions with other cellular components.

Much of what is currently known about brain copper regulation comes from studies of diseases where copper dysregulation is associated with neurodegeneration. Menkes disease (MD; OMIM 309400) is an X-linked inherited disorder with serious neurological symptoms and neurodegeneration resulting from severe copper deficiency. Occipital horn syndrome (OHS; OMIM 304150) is an allelic variant of MD with milder neurological symptoms and predominantly connective tissue defects. In Wilson disease (WD; OMIM 277900), an inherited, autosomal recessive copper toxicosis disorder, patients present with hepatic and neurological symptoms (reviewed in Danks, 1995). There is mounting evidence that copper dysregulation plays a key role in

more common neurodegenerative diseases such as Alzheimer's, Parkinson's, Huntington's, motor neuron, and prion diseases (reviewed in Scheiber et al., 2013).

The mechanisms of brain copper import, distribution, and export are now beginning to be elucidated. The exchange of copper between the periphery and the brain is highly regulated by the brain barriers. The copper concentration of cerebrospinal fluid (CSF; ∼0.25 μM; Kjellin, 1963; Lentner, 1981) is up to 100-fold lower than that in the plasma (11–25 μM; Tietz, 1987). In a rat brain perfusion study that compared copper uptake into brain capillaries, parenchyma, choroid plexus, and CSF, non-protein bound free copper ion was the predominant copper species that entered the brain via both the blood–brain barrier (BBB) and the blood–CSF barrier (BCB; Choi and Zheng, 2009). The higher rate of copper transport into the brain parenchyma compared to the CSF suggests that the BBB is the main site through which copper enters the brain. Copper influx into the brain parenchyma and CSF is regulated by copper transporters CTR1, ATP7A, and ATP7B, which are highly expressed in the brain capillaries and choroid plexus (Iwase et al., 1996; Qian et al., 1998; Kuo et al., 2006; Niciu et al., 2006; Choi and Zheng, 2009; Donsante et al., 2010; Davies et al., 2013).

Significant insight into the mechanisms controlling brain copper homeostasis began two decades ago with the identification of the genes encoding the essential copper-transporting ATPases, ATP7A (Chelly et al., 1993; Mercer et al., 1993; Vulpe et al., 1993) and ATP7B (Bull et al., 1993; Petrukhin et al., 1993; Yamaguchi et al., 1993). Mutations in *ATP7A* and *ATP7B* underlie MD and WD, respectively. *ATP7A* is located on chromosome Xq13.2-13.3 and comprises 23 exons that span approximately 150 kb[1]. ATP7B is located on chromosome 13q14.3 and comprises 21 exons that span approximately 80 kb[2]. Transcripts of approximately 7.5–8.5 kb are produced from both genes and contain coding regions of 4.5 kb, which are translated to produce proteins of 180 and 165 kDa, respectively. ATP7A and ATP7B are members of the P_{1B}-subfamily of the P-type ATPases. They undergo ATP-dependent cycles of phosphorylation and dephosphorylation to catalyze the translocation of copper across cellular membranes. Their structure and biochemistry was thoroughly reviewed by Lutsenko et al. (2007). They are highly related in structure and function with approximately 60% amino acid identity. They have eight transmembrane domains that form a path through cell membranes for copper translocation; and a large N-terminus with six metal-binding domains (MBDs), each comprising approximately 70 amino acids and the highly conserved metal-binding motif GMxCxxC (where x is any amino acid). Other highly conserved domains include the intramembrane CPC motif that is required for copper translocation through the membrane, the N-domain containing the ATP-binding site, the P-domain containing the conserved aspartic acid residue and the A-domain comprising the phosphatase domain. Copper-binding together with other N- and C-terminal signals regulate their activity, intracellular location, and copper-induced intracellular trafficking (see below and reviewed in La Fontaine and Mercer, 2007; Lutsenko et al., 2007; **Figure 1**).

ATP7A and ATP7B possess dual functions, delivering copper for incorporation into copper-dependent enzymes, and removal of excess copper from cells. These functions are largely regulated by their sub-cellular localization (see below). *ATP7A* is ubiquitously expressed in extrahepatic cells and tissues, which explains the systemic defects caused by its absence or inactivation in MD and points to a house-keeping role for ATP7A. *ATP7B* has a more limited expression pattern, with the highest expression level in the liver, and lower levels in the kidney, placenta, brain, heart, and lungs (Bull et al., 1993; Tanzi et al., 1993; Vulpe et al., 1993). This restricted expression suggests more specialized functions for ATP7B in regulating copper physiology, such as biliary copper excretion (Terada et al., 1999). ATP7B also has a biosynthetic role, supplying copper to cuproenzymes such as ceruloplasmin (Terada et al., 1998). In cells where ATP7B is co-expressed with ATP7A, it often has a specific and distinct role (Veldhuis et al., 2009a; La Fontaine et al., 2010), for example in copper secretion into milk during lactation (Michalczyk et al., 2008), and in fine-tuning the intracellular copper balance in the kidney (Linz et al., 2008; Barnes et al., 2009). The expression of both copper-transporting P-type ATPases (copper-ATPases) in the brain and the severe neurological symptoms that arise from a deficiency of either transporter, suggest that they play key roles in regulating brain copper homeostasis. This review will summarize our current knowledge of the expression, localization, and contribution of ATP7A and ATP7B to maintaining and managing copper levels within the brain.

EXPRESSION AND LOCALIZATION OF ATP7A AND ATP7B IN THE BRAIN

ATP7A

The *ATP7A* gene is transcribed to produce an 8.5 kb transcript that is expressed in all tissues examined except for the liver (Chelly et al., 1993; Vulpe et al., 1993). In the mouse brain, the *Atp7a* transcript is expressed in the cerebrovascular endothelial (CVE) cells that form the BBB (Qian et al., 1998), and is strongly expressed in the choroid plexus (Kuo et al., 1997; Murata et al., 1997; Nishihara et al., 1998; Choi and Zheng, 2009), a structure that forms the BCB and regulates the concentration of substances in the CSF (**Figure 2A**). Choi and Zheng (2009) further showed that *Atp7a* is more highly expressed in the brain barriers (BBB and BCB), the brain capillaries and choroid plexus, than in brain parenchyma. Comparing the two barriers, *Atp7a* mRNA expression is 3.4-fold higher in the choroid plexus than in the cerebral capillaries. This observation is consistent with the finding that the Atp7a protein levels in the developing and adult mouse brain are highest in the choroid plexus/ependymal cells of the lateral and third ventricles (Niciu et al., 2006).

Based on abnormal copper accumulation in cultured astrocytes from the macular mouse, a model of MD, Kodama et al. (1991) proposed that astrocytes play an important role in copper transport from the blood and CSF toward neurons, and that this pathway is disturbed in MD and animal models (**Figure 2B**). Kaler and Schwartz (1998) later confirmed the expression of mouse and rat *Atp7a* in astrocytes from various brain regions (cerebral cortex,

[1]http://asia.ensembl.org/Homo_sapiens/Gene/Summary?g=ENSG00000165240;r=X:77166194-77305892

[2]http://asia.ensembl.org/Homo_sapiens/Gene/Summary?g=ENSG00000123191;r=13:52506809-52585630

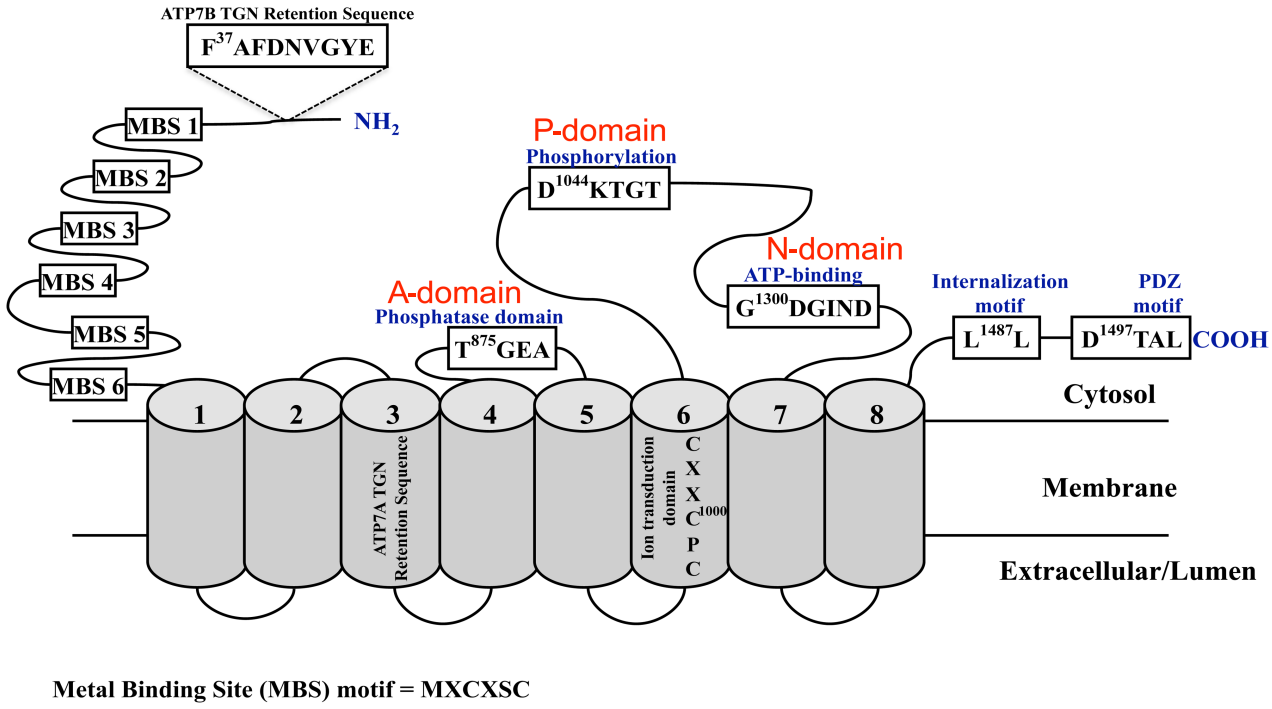

FIGURE 1 | Schematic diagram of the copper-ATPases, ATP7A and ATP7B. Shown are the highly conserved domains: the N-terminal copper-binding domain, the phosphatase (A-domain), phosphorylation (P-domain), and ATP-binding (N-domain) domains; and the motifs and sequences required for their localization and trafficking. The cylindrical regions labeled 1-8 represent the transmembrane domains.

corpus striatum, and cerebellum). Therefore, ATP7A is postulated to play a key role in the copper distribution from astrocytes to neurons. Importantly, the successful treatment of MD mouse models by intravenous administration of copper earlier than postnatal day 7 (P7; Mann et al., 1979) was proposed to be a consequence of the immaturity of the BBB, which includes astrocytes (Kodama et al., 1991). This immaturity allows the penetration of copper that is prevented in these ATP7A-deficient models once development of the BBB is complete.

Early *in situ* hybridization studies in mouse brain showed significant levels of the *Atp7a* transcript in the hippocampal CA1 region, the dentate gyrus, the cerebellar granular layer and the olfactory bulb, and lower levels in the hippocampal CA3 region and Purkinje neurons (Iwase et al., 1996). *Atp7a* mRNA was detected also by RT-PCR in mouse and rat cerebral cortex and cerebellum, and in isolated rat cerebellar granule neurons (Kaler and Schwartz, 1998). Consistent with these observations, a detailed immunohistochemical study by Niciu et al. (2006) demonstrated the presence of the Atp7a protein in most CNS cell types at P11 in the developing mouse brain and in the adult brain. In transgenic mice that overexpressed human ATP7A, the protein is primarily produced in the CA2 region of the hippocampus, the Purkinje neurons of the cerebellum, and in the choroid plexus (Ke et al., 2006). The overexpression of ATP7A resulted in an overall reduction of brain copper concentrations (Ke et al., 2006), which is consistent with ATP7A in the choroid plexus functioning to efflux copper back into the circulation (Choi and Zheng, 2009). In more recent studies of human brain tissue, ATP7A protein levels are most prominent in the cerebellum and substantia nigra (Davies et al., 2013). The significance of ATP7A expression in these brain regions is poorly understood.

ATP7A expression is developmentally regulated (Kuo et al., 1997; Barnes et al., 2005; El Meskini et al., 2005; Niciu et al., 2006). The widespread expression of *Atp7a* mRNA in neurons and ependymal cells during embryonic and postnatal development in the mouse (Kuo et al., 1997; Murata et al., 1997) suggests a house-keeping role for ATP7A in the brain and CNS. Interestingly, *Atp7a* is not detectable by RT-PCR in embryonic day 20 rat astrocytes, but it is detectable in P3, P8, and adult astrocytes (Kaler and Schwartz, 1998). The study by Niciu et al. (2006) showed that Atp7a protein levels are most abundant in the early postnatal period, peaking in the neocortex and cerebellum at P4. From birth (P0) to adulthood, there is a decline in Atp7a levels in most brain regions, and this decline is more pronounced in the hippocampus and cerebellum than in the hypothalamus (Niciu et al., 2006). During this postnatal period, despite a general decline in Atp7a levels, there is increased Atp7a expression in CA2 hippocampal pyramidal cells and cerebellar Purkinje neurons. The authors proposed that since the CA2 region is resistant to epileptogenesis, the increase in CA2 Atp7a levels may contribute to seizure resistance (Niciu et al., 2006). The observed increase in Atp7a levels in Purkinje neurons is consistent with significant levels of ATP7A in human cerebellar Purkinje neurons (Davies et al., 2013), but it is inconsistent with other reports of a postnatal decline in mouse Atp7a mRNA and protein levels in these cells (Iwase et al., 1996; Barnes et al., 2005). Although Barnes et al.

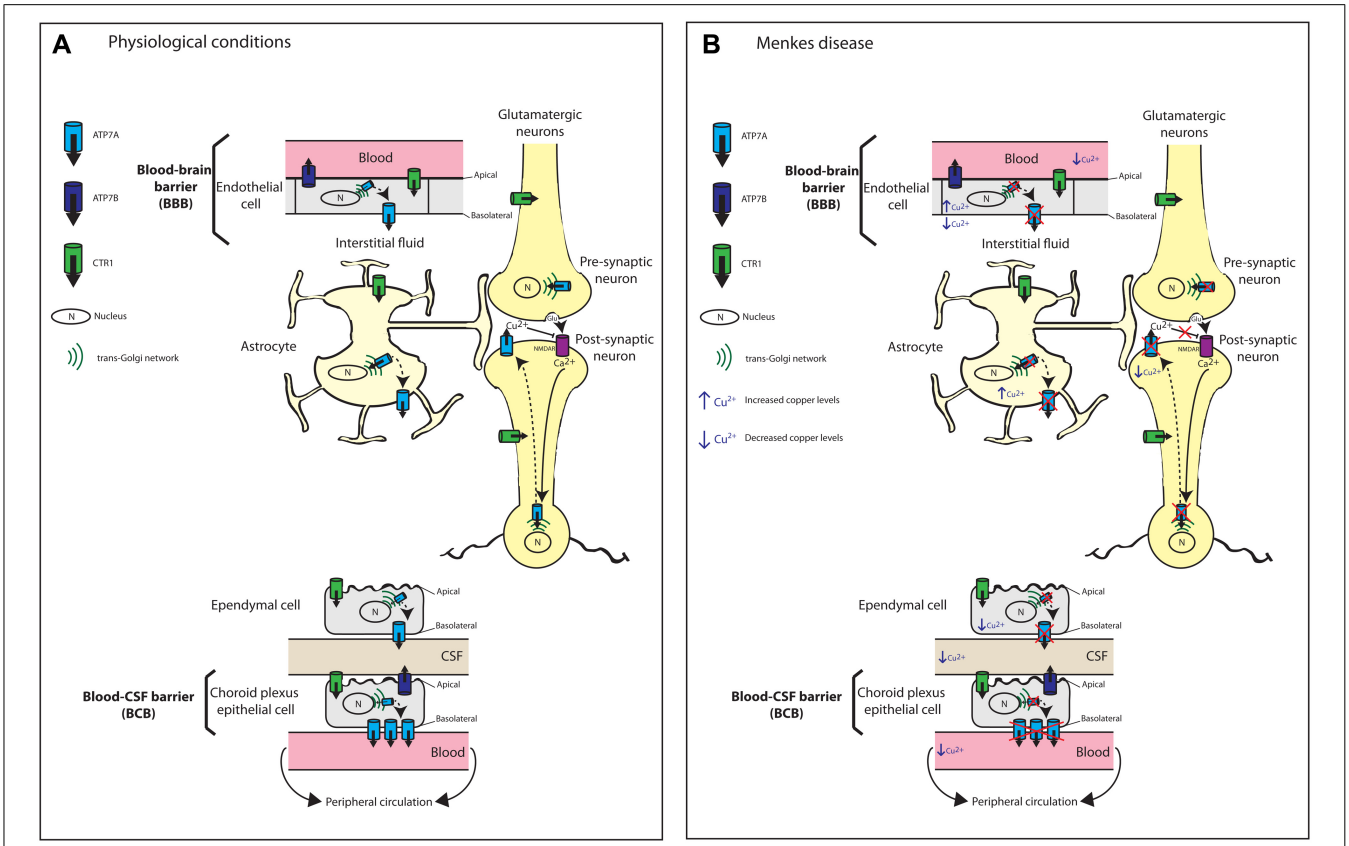

FIGURE 2 | Schematic diagram illustrating the proposed mechanisms of copper transport across the blood brain barrier (BBB) and blood cerebrospinal fluid barrier (BCB). (A) Copper transport under physiological conditions. Copper import into cells is via the major copper import protein CTR1. The proposed locations and orientation of ATP7A and ATP7B are shown. ATP7A is expressed in cerebrovascular endothelial cells that form the BBB but its expression is 3.4-fold higher in the choroid plexus than in the cerebral capillaries. In both choroid plexus epithelial cells and capillary endothelial cells of the brain, ATP7A predominantly localizes to the basolateral membrane. ATP7A facilitates copper transport from the blood across the BBB to the brain parenchyma. At the choroid plexus, ATP7A facilitates the removal of excess copper from the brain into the blood. Therefore, the BCB serves as the main pathway for eliminating excess brain copper. In contrast, ATP7B is concentrated at the apical membrane. At the CSF-facing apical membrane of choroid plexus epithelial cells, ATP7B may contribute to copper transport into the CSF and copper sequestration in the choroid plexus. Astrocytes are the first brain parenchyma cells to encounter metal ions that cross the BBB and play an important role in copper transport from the blood and CSF toward neurons. Copper transport in glutamatergic neurons is depicted. Copper-independent and reversible trafficking of ATP7A is stimulated by *N*-methyl-D-aspartate receptor (NMDAR) activation that leads to Ca^{2+} influx and is associated with the rapid release of copper from neurons. The released copper down-regulates NMDAR activity thus protecting neurons from excitotoxicity. **(B)** Perturbed copper transport in Menkes disease due to the absence or inactivation of ATP7A. The predicted consequences of ATP7A inactivation in relation to copper levels in brain barrier cells, astrocytes and neurons are shown. Any copper that reaches the BBB accumulates within endothelial cells leading to reduced copper transport to the brain parenchyma, astrocytes and neurons. Any copper that reaches astrocytes also will accumulate. There is reduced copper transport across the BCB. Excitotoxicity due to impaired synaptic copper release from glutamatergic neurons, which down-regulates NMDAR activity, can contribute to seizures and neuronal degeneration in Menkes disease.

(2005) reported a postnatal switch in the expression of Atp7a from Purkinje neurons to Bergmann glia in the rodent brain, in human brain tissue, ATP7A could not be detected in the Bergmann glia (Davies et al., 2013). This discrepancy may allude to the possibility that different mechanisms regulate human and rodent cerebellar copper homeostasis. The increased ATP7A expression in the cerebellum may explain the increased sensitivity of this region, and in particular Purkinje neurons, to copper deficiency as observed in MD (Menkes et al., 1962; Kumode et al., 1993, 1994; Iwase et al., 1996; Geller et al., 1997; Liu et al., 2005a; Niciu et al., 2006).

In the olfactory system, neuronal Atp7a localization correlates with neuronal maturation. Atp7a is initially concentrated in neuronal cell bodies at early embryonic stages, then shifts to the extending axons during the postnatal period (El Meskini et al., 2005). Atp7a levels peak prior to synaptogenesis, which occurs postnatally. Similarly, increased expression levels precede synapse formation following injury-stimulated neurogenesis. Together with a follow-up study of the mottled brindled mouse model of MD ($Mo^{Br/y}$), these data suggest a role for ATP7A in axon extension and synaptogenesis, the absence of which may contribute to the neurodegeneration evident in MD and its mouse models (El Meskini et al., 2007).

The increase in ATP7A during the early postnatal period indicates a crucial role for copper during early development, and particularly in synaptogenesis. This may underlie the critical window during which some human MD patients and mouse models

(the mottled mouse mutants) respond favorably to postnatal copper injections, especially when administered in the early postnatal period (Mann et al., 1979; Fujii et al., 1990; Niciu et al., 2006, 2007; Tang et al., 2008). Kaler et al. (2008) reported that this successful copper treatment depends on the presence of a mutant ATP7A protein with some residual copper transport function. Hence, the therapeutic efficacy of copper injections may be a consequence of increased levels of mutant ATP7A, albeit with significantly reduced catalytic activity (La Fontaine et al., 1999; Kaler, 2011).

A zebrafish mutant, *calamity*, with an embryonic-recessive lethal mutation in the *ATP7A* ortholog, shows impaired copper homeostasis with absent melanin pigmentation, defective notochord formation, and neurodegeneration in the hindbrain region of the developing brain (Mendelsohn et al., 2006; Madsen et al., 2008). These authors proposed a developmental hierarchy of copper metabolism, where notochord formation is preferentially preserved during copper limitation, potentially explaining some of the vascular and neurologic abnormalities observed in MD. *Drosophila* has a sole ortholog of *ATP7A*, *DmATP7*, which appears to play an important role in the developing *Drosophila* brain (Norgate et al., 2007). It is strongly expressed in the larval brain at different developmental stages (Burke et al., 2008). Strong *DmATP7A* expression is observed in the ventral ganglion but it is absent from most of the optic lobes (Burke et al., 2008).

ATP7B

Although ATP7B is expressed in the brain (Bull et al., 1993; Tanzi et al., 1993), its expression patterns and contribution to brain copper homeostasis are less well characterized than that of ATP7A. In the brain of the developing mouse embryo, *Atp7b* mRNA cannot be detected, suggesting that there may be no prenatal expression or that expression is below the detection limits (Kuo et al., 1997). Alternatively, consistent with the possibility that significant expression begins postnatally, brain copper levels in an *Atp7b* null mouse continues to increase slightly throughout adult life (Buiakova et al., 1999). In a study of Atp7b protein and mRNA distribution in the rat brain, Atp7b is detected in the hippocampus, the granular cells of the dentate gyrus and pyramidal cells of the CA1 to CA4 layers, the glomerular cell layer of the olfactory bulbs, Purkinje neurons of the cerebellum, pyramidal neurons of the cerebral cortex, and cores of several nuclei (e.g., pontine nuclei and lateral reticular nuclei) in the brainstem (Saito et al., 1999). In these brain regions, both *Atp7b* mRNA and protein correlate with copper distribution as determined by staining with the copper chelator bathocuproine disulfonic acid (BCS). Based on similar distribution patterns of cuproenzymes such as dopamine-β-hydroxylase (DBH) and Cu–Zn superoxide dismutase (Cu–Zn SOD), as well as abnormal catecholamine synthesis in the Long-Evans Cinnamon (LEC) rat model of WD (Saito et al., 1996; Okabe et al., 1998), these authors speculated that ATP7B-mediated control of copper homeostasis in these brain regions is important in regulating DBH activity.

In contrast to mouse Atp7a, there is continuous Atp7b expression in the adult mouse cerebellar Purkinje neurons, and an age-dependent down-regulation (Barnes et al., 2005). Based on kinetic studies and experiments in mice lacking Atp7b, these authors proposed a homeostatic role for ATP7A in maintaining intracellular copper at a certain level, and a biosynthetic role for ATP7B mediating the synthesis of copper-dependent enzymes such as ceruloplasmin (Barnes et al., 2005).

In the human brain, immunohistochemical staining reveals expression of the ATP7B protein in the visual cortex, anterior cingulate cortex, body of caudate, putamen, substantia nigra, and cerebellum, with the most significant levels of ATP7B detected in the cerebellum, anterior cingulated cortex, and caudate putamen (Davies et al., 2013). Strong staining of ATP7B in Purkinje neurons and not the Bergmann glia is consistent with the findings in the adult rat and mouse brains. However, there is no correlation between ATP7A and ATP7B protein levels and copper levels in the brain regions investigated (Davies et al., 2013).

FUNCTION AND REGULATION OF THE COPPER-ATPases IN CNS CELL TYPES

REGULATION OF ATP7A AND ATP7B

Much of what is currently known about the copper-ATPases derives from studies in peripheral cells and tissues. Emerging studies of copper transport and the copper-ATPases in the brain and certain CNS cell types both support our current understanding of their mechanism of action and regulation, but also provide new insights into the complexity of copper's role in the CNS.

ATP7A and ATP7B have a dual role in cells; a biosynthetic role, delivering copper to the secretory pathway for metallation of cuproenzymes, and a homeostatic role, exporting excess copper from the cell. Under normal, physiological conditions, ATP7A and ATP7B are localized at the *trans*-Golgi network (TGN) where they provide copper to copper-dependent enzymes synthesized in the secretory pathway. ATP7A has a role in the metallation of enzymes such as peptidylglycine α-amidating monooxygenase (PAM; El Meskini et al., 2003; Steveson et al., 2003), tyrosinase (Petris et al., 2000; Setty et al., 2008), extracellular SOD3 (Qin et al., 2005), DBH (Saito et al., 1996), and lysyl oxidase, all of which are expressed in the CNS (Lutsenko et al., 2010). ATP7A-mediated copper delivery to lysyl oxidase is proposed based on similar temporal expression of these two proteins in the developing rat brain and the sensitivity of lysyl oxidase-dependent processes to ATP7A inactivation as evident in MD and OHS (Royce et al., 1980; Kuivaniemi et al., 1985; Kaler et al., 1994; Kaler, 1998; Tchaparian et al., 2000; Lutsenko et al., 2007). Copper delivery to apo-ceruloplasmin in hepatocytes (Terada et al., 1998) and mouse cerebellum (Barnes et al., 2005) is mediated by Atp7b, and by Atp7a in macrophages in response to hypoxia-mediated increased copper uptake (White et al., 2009).

The key mechanism by which the copper-ATPases regulate cellular copper levels involves alteration of their cellular localization in response to changes in cytoplasmic copper concentration. When intracellular copper levels are elevated, ATP7A and ATP7B traffic from the TGN to the cell periphery to export excess copper from cells. The copper-induced trafficking of ATP7A was first described in Chinese hamster ovary (CHO-K1) cells (Petris et al., 1996), and since then, it has been reported in a wide range of non-polarized (e.g., human fibroblasts, HeLa, and mammary carcinoma cells; Yamaguchi et al., 1996; Ackland et al., 1997; Dierick et al., 1997; Francis et al., 1998; La Fontaine et al., 1998a,b; Qi and Byers, 1998) and polarized cell types (Madin–Darby canine kidney (MDCK),

mouse enterocytes, Caco-2; Greenough et al., 2004; Monty et al., 2005; Nyasae et al., 2007). Trafficking of ATP7B also has been observed in non-polarized hepatoma, human bladder carcinoma, and CHO-K1 cells (Hung et al., 1997; Yang et al., 1997; Forbes and Cox, 2000; La Fontaine et al., 2001; Huster et al., 2003), and in polarized hepatic cells (HepG2 and WIF-B; Roelofsen et al., 2000; Guo et al., 2005; Cater et al., 2006).

In polarized cells, ATP7A traffics from the TGN to a rapidly recycling vesicular pool located near the basolateral membrane (Monty et al., 2005; Nyasae et al., 2007), whereas ATP7B traffics to sub-apical vesicles (Roelofsen et al., 2000; Guo et al., 2005; Cater et al., 2006). At these locations they sequester and mediate the export of excess copper. When copper levels return to normal, ATP7A and ATP7B recycle back to the TGN (Petris and Mercer, 1999; Cater et al., 2006; Nyasae et al., 2007). The trafficking and recycling of the copper-ATPases requires specific signal sequences (**Figure 1**). For both ATP7A and ATP7B, only MBD 5 or 6 is necessary and sufficient to mediate copper-stimulated trafficking from the TGN to the cell periphery (Strausak et al., 1999; Cater et al., 2004). C-terminal di- and tri-leucine motifs in ATP7A and ATP7B, respectively, are required for retrograde trafficking of the proteins to the TGN when copper levels return to normal (Petris et al., 1998; Cater et al., 2006; Braiterman et al., 2011), and for basolateral targeting of ATP7A in polarized MDCK cells (Greenough et al., 2004). TGN retention of ATP7A is mediated by a 38 amino acid sequence contained within transmembrane domain three (Francis et al., 1998) whereas ATP7A retention at the basolateral surface may require the PDZ-binding motif (D^{1497}TAL) within the C-terminus (Greenough et al., 2004). In ATP7B, a nine amino acid sequence (F^{37}AFDNVGYE) in the N-terminus is essential for TGN retention under low copper conditions and for apical targeting in polarized hepatocytes when copper levels are elevated (Braiterman et al., 2009). Although details of the trafficking machinery involved in these processes remain to be fully elucidated, interacting partners that may play a role include AIPP1, a PDZ domain-containing protein that may bind to the PDZ motif in ATP7A (Stephenson et al., 2005), and dynactin subunit p62 that may be involved in ATP7B trafficking (Lim et al., 2006b).

In addition to the N- and C-terminal signals that control the copper-ATPase response to elevated copper, other processes also contribute to regulating copper-ATPase activity. They include, posttranslational modifications such as transient autophosphorylation at the invariant aspartate residue (D^{1044}KTGT; **Figure 1**) to form an acyl-phosphate intermediate, a process that is characteristic of the P-type ATPase catalytic cycle (Voskoboinik et al., 1998, 2001a,b; Petris et al., 2002; Tsivkovskii et al., 2002; Cater et al., 2007), copper-stimulated phosphorylation by kinases (Vanderwerf et al., 2001; Voskoboinik et al., 2003; Veldhuis et al., 2009b), and protein interactions. ATOX1 was the first protein shown to interact in a copper-dependent manner with the N-terminal domain of both proteins for delivery of copper to the secretory pathway (Hamza et al., 1999; Larin et al., 1999). MBD 1–4 is most important for this interaction, in particular MBD 2 and 4 (Strausak et al., 2003; Walker et al., 2004; Achila et al., 2006). This is supported by studies in the *Atox1* null mice, which demonstrated a critical requirement for Atox1 in Atp7a function and copper homeostasis (Hamza et al., 2001, 2003). A 45 kDa isoform of the promyelocytic leukemia zinc finger (PLZF) protein, a transcriptional repressor of Hox genes, interacts with the C-terminus of ATP7B (Ko et al., 2006). Both proteins co-localized within the TGN, and based on experiments in HepG2 cells and *Drosophila*, this interaction is proposed to have a role in regulating ERK signal transduction. However, the functional consequences of these associations remain to be elucidated. The glutaredoxin1 (GRX1)/glutathione (GSH) system may play a key role in regulating the trafficking and activity of the copper-ATPases by regulating their redox state through glutathionylation and deglutathionylation by GRX1 (Lim et al., 2006a; Singleton et al., 2010). GRX1 protects proteins from oxidative damage (Mieyal et al., 2008), and it is expressed throughout the brain with highest activity in the hippocampus, cortex and midbrain (Balijepalli et al., 1999; Ehrhart et al., 2002; Aon-Bertolino et al., 2011). Hence, GRX1/GSH may preserve the integrity and function of ATP7A and ATP7B during oxidative stress, which accompanies neuropathological processes. Clusterin (ApoJ) and COMMD1 regulate the degradation of misfolded and mutant copper-ATPases via the lysosomal and proteasomal pathways, respectively (de Bie et al., 2007; Materia et al., 2011, 2012).

ROLE OF THE COPPER-ATPases IN CNS CELL TYPES

Information on the activity of the copper-ATPases in specific CNS cell types is limited and somewhat fragmentary, but emerging evidence is revealing specific roles for ATP7A and ATP7B in brain and CNS copper homeostasis. Peripherally, ATP7A and ATP7B mostly have cell type-specific expression patterns and where co-expressed in certain cells and tissues (e.g., kidney, placenta, and mammary gland), they have distinct roles (reviewed in La Fontaine and Mercer, 2007 and Veldhuis et al., 2009a). Similarly in the CNS, ATP7A and ATP7B may be uniquely expressed in certain cell types and co-expressed in others.

Choroidal epithelial cells and brain capillary endothelial cells

The BBB and BCB regulate the movement of copper between the brain and peripheral circulation. In both choroid plexus epithelial cells and capillary endothelial cells of the brain, ATP7A predominantly localizes to the basolateral membrane, which is consistent with its location in other polarized epithelial cell types such as intestinal enterocytes (Monty et al., 2005; Nyasae et al., 2007; Davies et al., 2013). In contrast, ATP7B is concentrated at the apical membrane, again consistent with its localization in polarized hepatocytes (Roelofsen et al., 2000; Guo et al., 2005; Cater et al., 2006; Davies et al., 2013). This distinct membrane localization of ATP7A and ATP7B combined with their discrete enzyme kinetics may be a mechanism to ensure strict control over copper transport across the BCB and BBB (Tsivkovskii et al., 2002; Barnes et al., 2005; Hung et al., 2007). The kinetically slower ATP7B at the CSF-facing apical membrane of choroid plexus epithelial cells may explain the slower rate of copper transport into the CSF relative to the choroid plexus copper uptake rate, and may contribute to copper sequestration in the choroid plexus (Barnes et al., 2005, 2009; Choi and Zheng, 2009). In contrast, the basolateral location of the kinetically faster ATP7A facilitates the removal of excess copper from the brain into the blood (Davies et al., 2013). This arrangement of the copper-ATPases is similar to that in other tissues such as

the mammary epithelium or the kidney where ATP7A serves to protect from copper excess while ATP7B serves a biosynthetic role (La Fontaine and Mercer, 2007; Veldhuis et al., 2009a). Therefore, the BCB serves as the main pathway for eliminating excess brain copper (**Figure 2**). In a further study that utilized cultured choroidal epithelial Z310 cells, siRNA-mediated knockdown of ATOX1 and ATP7A resulted in increased cellular copper retention, confirming the involvement of ATP7A in copper transport across the choroid plexus and hence in regulating copper homeostasis of the CSF (Monnot et al., 2012). The BBB, which regulates copper influx into the brain, has lower levels of ATP7A at the basolateral surface of brain capillary endothelial cells to enable rapid but limited transport of copper into the brain to accommodate sudden changes in brain copper concentration (Choi and Zheng, 2009).

Astrocytes

Astrocytes play a pivotal role in brain copper homeostasis. They are strategically sandwiched between endothelial cells of the BBB and neurons, hence, they are the first brain parenchyma cells to encounter metal ions that cross the BBB. Astrocytes are able to efficiently take up and store copper, and with greater resistance to copper-induced toxicity, they protect neurons from copper toxicity (reviewed in Tiffany-Castiglioni et al., 2011; Scheiber and Dringen, 2012). Atp7a has been detected in rodent astrocytes in culture and in brain tissue (Qian et al., 1997; Barnes et al., 2005; Niciu et al., 2007; Scheiber et al., 2012). These studies implicate Atp7a in copper export from astrocytes, which is consistent with the early observations by Kodama et al. (1991) of copper accumulation in astrocytes of the macular mouse (**Figure 2B**). In astrocytes, similar to peripheral cell types, Atp7a localizes to a perinuclear region and undergoes copper-responsive trafficking between the TGN and the cell periphery. Therefore, it was proposed that Atp7a mediates copper export from astrocytes, which is time-, concentration- and temperature-dependent (Scheiber et al., 2012). However, in contrast to previous studies, copper export from cultured astrocytes is non-saturable and does not follow the established Michaelis–Menten kinetics for ATP7A-dependent copper transport (Scheiber et al., 2012). Therefore, additional ATP7A-independent mechanisms may be involved in copper export from astrocytes. The rate of copper export is proportional to the increase in copper content after copper exposure, which led Scheiber et al. (2012) to propose that the copper export rate is determined by the trafficking or fusion of Atp7a-containing vesicles with the plasma membrane, rather than by ATP7A-mediated transport of copper into vesicles.

Neurons

Cerebellar Purkinje neurons are highly sensitive to copper deficiency during development (Lyons and Prohaska, 2009). In the human brain, ATP7A and ATP7B are co-expressed in Purkinje neurons (Davies et al., 2013), and in the mouse brain, for a postnatal period of up to 2 weeks (Barnes et al., 2005). Co-expression of both copper-ATPases may be a mechanism to achieve a delicate balance of copper that is critical to cerebellar development and function. Based on the faster kinetics of copper transport by Atp7a relative to Atp7b, a homeostatic role is proposed for Atp7a in maintaining the intracellular copper level, and a biosynthetic role for Atp7b to mediate the synthesis of copper-dependent enzymes such as ceruloplasmin (Barnes et al., 2005).

Hippocampal ATP7A plays a pivotal role in learning and memory. In mature rat primary hippocampal neurons, Atp7a undergoes both copper-dependent and -independent redistribution from the TGN to dendrites and axons (Schlief et al., 2005). The study by Schlief et al. (2005) provides the first evidence of copper-independent and reversible trafficking of Atp7a that (i) is stimulated by *N*-methyl-D-aspartate (NMDA) receptor activation, (ii) requires Ca^{2+} influx through the NMDA receptor, and (iii) is associated with the rapid release of copper from hippocampal neurons (**Figure 2A**). The inability to detect Atp7a at the cell surface prompted the suggestion that copper may be released by exocytosis from vesicles once Atp7a has recycled back to the TGN. This possibility is consistent with the rapid efflux of copper following NMDA receptor activation. The authors suggest that NMDA receptor activation-mediated trafficking of copper-loaded, ATP7A-associated vesicles provides a readily available and releasable pool of copper following Ca^{2+} influx (Schlief et al., 2005). In the hippocampus, following neuronal depolarization, about 15 μM of copper is released into the glutamatergic synaptic cleft from synaptic vesicles (Rajan et al., 1976; Hartter and Barnea, 1988; Kardos et al., 1989; Barnea et al., 1990; Hopt et al., 2003). While the physiological significance of synaptic copper release is not yet clear, it is proposed that copper down-regulates NMDA activity through redox processes that involve *S*-nitrosylation of specific cysteine residues within NMDA subunits NR1 and NR2A, thus protecting hippocampal neurons from excitotoxicity (Schlief et al., 2006). These findings link ATP7A and copper with modulating memory and learning processes that depend on precise regulation of NMDA receptor activity (Schlief et al., 2006). In MD, deficiencies in both brain copper and ATP7A activity will prevent copper release, thus impairing NMDA receptor modulation. The subsequent NMDA-mediated excitotoxicity may contribute to the pathological features, seizures and neuronal degeneration, that are characteristic of MD (Schlief et al., 2006). In Alzheimer's disease (AD), the combination of increased copper and Aβ [the amyloidogenic cleavage product of the amyloid precursor protein (APP)] in the synaptic cleft may promote the formation of neurotoxic Aβ-copper oligomers contributing to the neuropathology of AD.

Motor neurons

The recent identification of a new ATP7A-related distal hereditary motor neuropathy (dHMN), caused by mutations in *ATP7A* (see below), has drawn attention to the role of ATP7A in motor neurons (Kennerson et al., 2010; Yi et al., 2012). The cell bodies of motor neurons are located within the anterior horn of the spinal cord, and their axons extend for long distances to distal limb muscles. Hence, both the CNS and peripheral nervous system are affected in motor neuron disorders (Harding and Thomas, 1980). In undifferentiated NCS-34 cells (a motor neuron-enriched cell line), Atp7a is localized at the TGN and traffics to the cell periphery with elevated copper, as it does in most other cell types. In differentiated NSC-34 cells, Atp7a can be detected along the full length of Tau-1-positive neuritic projections, with localization to the axonal membrane when copper levels are elevated (Yi et al., 2012). Based on the data presented, ATP7A was postulated to

traffic along axons, and to mediate copper release from the axonal membrane of motor neurons.

Retinal pigment epithelium

Retinal degeneration is observed in both MD and WD (Ferreira et al., 1998). The essential requirement for copper and ATP7B for retinal structure integrity is underscored by reduced thickness of total macula as well as ganglion cell and inner plexiform layer in WD patients. Up to 50% of WD patients have abnormal visual evoked potentials (VEPs; Albrecht et al., 2012). Immunohistochemical studies of mouse and human retina identified the presence of ATP7A in the retinal pigment epithelium (RPE), outer plexiform layer (OPL), and ganglion cell layer (GCL) of the BALB/c mouse retina (Krajacic et al., 2006). In contrast, ATP7B expression is restricted to the RPE. Within the RPE, both copper-ATPases localize to a perinuclear region that overlap with TGN and Golgi markers, consistent with their biosynthetic role in delivering copper to tyrosinase for melanogenesis, and to the iron homeostatic proteins ceruloplasmin and hephaestin. Increased copper levels trigger a redistribution of ATP7B to a diffuse cytoplasmic compartment in an immortalized human RPE cell line.

Microglia

Activated microglial cells concentrate at sites of neuronal damage and inflammation. Atp7a expression is elevated in activated microglia surrounding Aβ plaques in the TgCRND8 transgenic mouse model of AD (Zheng et al., 2010). Data from cultured mouse BV-2 microglial cells suggest Atp7a expression and trafficking is responsive to stimulation by the pro-inflammatory cytokine interferon-gamma (IFN-γ). Concomitant copper accumulation due to upregulation of the copper import protein, Ctr1 and copper uptake, suggests a role for copper in the pro-inflammatory pathway. Furthermore, the IFN-γ-stimulated ATP7A redistribution differs from that induced by copper, and appears to mediate copper sequestration in cytoplasmic vesicles rather than copper export. This mechanism is postulated to represent a protective mechanism by activated microglia at sites of amyloidogenesis to reduce aberrant copper-Aβ interactions in the extracellular milieu (Zheng et al., 2010).

Pineal gland

Pineal night-specific ATPase (PINA) is a novel splice variant of ATP7B, lacking the N-terminal MBDs and the first four transmembrane domains. It has a predominant nocturnal expression in the pineal gland and retina, under the control of the retina-specific protein, cone rod homeobox (CRX; Li et al., 1998). Despite a large N-terminal truncation, PINA retains a weak copper transport function in a *Saccharomyces cerevisiae ccc2*Δ complementation assay (Borjigin et al., 1999). This finding implicates a yet-to-be-defined role for PINA-mediated copper metabolism in pineal and retinal circadian function (Li et al., 1998; Borjigin et al., 1999).

Neuroblastoma cells

Neuroblastoma cells have a high demand for copper for proliferation. High concentrations of copper in neuroblastoma cells are likely achieved via down-regulation of ATP7A expression and thus, reduced copper efflux. Retinoid treatment of neuroblastoma cells causes transcriptional upregulation of *ATP7A* and an increase in ATP7A protein levels (Liu et al., 2005b; Bohlken et al., 2009). Ectopic expression of the retinoic acid receptor β_2 subtype (RARβ_2) also induces ATP7A expression, which is associated a growth inhibitory effect. Conversely, knockdown of ATP7A is associated with reduced copper efflux and increased viability of retinoid-treated cells. These data support a model where malignant neuroblastoma cells have a high copper-dependency for viability and proliferation, and copper depletion by retinoid/RARβ_2-induced ATP7A upregulation offers therapeutic benefit (Bohlken et al., 2009).

FUNCTIONAL CONTRIBUTION OF ATP7A AND ATP7B TO BRAIN COPPER HOMEOSTASIS

An appreciation of the role of ATP7A and ATP7B in brain copper management derives largely from the functional consequences of their absence or inactivation in the genetically inherited diseases, MD, OHS, WD, and the recently identified ATP7A-related motor neuropathy. The importance of their correct function is also emerging in the context of other neurological diseases where there is perturbed copper metabolism, such as the prion diseases and AD. Our current understanding of the copper-ATPase contribution to brain copper homeostasis and the consequences of their absence or impaired function is summarized below.

MENKES DISEASE AND OCCIPITAL HORN SYNDROME

The widespread expression of *ATP7A* accounts for the systemic defects that arise from mutation of this gene in MD. This X-linked disease presents in males within the first few months of life, and in severe cases, it is fatal within 2–3 years. The severity of the clinical presentation can vary, but commonly includes abnormal neurodevelopment, seizures associated with cerebral atrophy and dysmyelination, a range of connective tissue and vascular abnormalities, fragile bones, an unusual kinky hair structure (pili torti), hair and skin pigmentation defects and failure to thrive (reviewed in Danks, 1995; Kaler, 2011). These symptoms arise from impaired intestinal copper absorption leading to systemic copper deficiency and consequently, reduced activity of critical copper-dependent enzymes. Paradoxically, copper retention is evident in certain tissues exposed to the limited copper that reaches them and in MD patient skin fibroblast cells (Camakaris et al., 1980; Danks, 1995). Histochemical analysis of brain tissue from affected MD patients revealed neurodegeneration in the cerebral cortex, cerebellum, and hippocampus (Okeda et al., 1991) where ATP7A is enriched (see above).

Occipital horn syndrome is also caused by mutations in *ATP7A*, but it is a milder disease with primarily connective tissue defects and moderate neurological symptoms (Kaler et al., 1994; Kaler, 1998). Causative mutations are often splice site mutations that result in reduced levels of normal *ATP7A* mRNA (reviewed in Kaler, 2011). The milder phenotype suggests that sufficient residual functional ATP7A is produced, but the prominent connective tissue defects indicate that copper delivery to lysyl oxidase is severely disrupted (Kaler, 2011; Scheiber et al., 2013).

The neurological symptoms of MD and OHS have been attributed to impaired ATP7A-mediated copper transport across the BBB (**Figure 2B**), leading to deficiencies of enzymes such as cytochrome *c* oxidase, SOD1, BDH, PAM, lysyl oxidase, and

tyrosinase, some of which require ATP7A for metallation in the TGN. The bioactivity of many neuropeptides is dependent on their amidation by PAM, while DBH is important for the production of the neurotransmitter norepinephrine from dopamine (reviewed in Lutsenko et al., 2010; Kaler, 2011). However, impairment of other ATP7A-mediated functions also is likely to contribute to the neurodegeneration in MD. For example, excitotoxicity due to impaired synaptic copper release, which down-regulates NMDA receptor activity, can contribute to seizures and neuronal degeneration (Schlief et al., 2005, 2006; **Figure 2**). In $Mo^{Br/y}$ mice, the absence of a functional ATP7A protein results in degeneration of Purkinje neurons, cytoskeletal abnormalities, and impaired synaptogenesis and axonal outgrowth (Niciu et al., 2006; El Meskini et al., 2007; Niciu et al., 2007).

There are a number of MD mouse models. As noted in the preceding sections, these mouse models have been invaluable in revealing insights into the critical role of ATP7A in the brain. The mottled mice are a series of mouse mutants with mutations in the murine ortholog of *ATP7A*. They exhibit a range of phenotypes that recapitulate the variable clinical severity of the human disease (Levinson et al., 1994; Mercer et al., 1994; Mercer, 1998). The $Mo^{Br/y}$ mouse model resembles most closely classical MD with neurological deficits and early postnatal lethality. This mouse expresses close to normal levels of Atp7a that has severely reduced copper transport activity due to an in-frame deletion of six nucleotides, leading to the loss of highly conserved amino acids A^{799} and L^{800} (Grimes et al., 1997). Studies in this mouse elegantly demonstrated the importance of Atp7a in supplying copper to PAM for the amidation of neuropeptides (Steveson et al., 2003), in axon extension and synaptogenesis, and in compensatory mechanisms to facilitate copper transport across the BBB. The latter includes upregulation of the mutant Atp7a in endothelial cells, as well as increased association of $Mo^{Br/y}$ astrocytes and microglia with the BBB, and these mechanisms possibly contribute to the effectiveness of early copper treatment (El Meskini et al., 2005, 2007; Niciu et al., 2007). The severe copper deficiency in the brain of $Mo^{Br/y}$ mice can be rescued by transgenic expression of the human *ATP7A*. Expression of the *ATP7A* transgene was detected in the cerebellar Purkinje cell layer, the CA1 and CA2 regions of the hippocampus, the mitral layer of the olfactory bulb, the vascular endothelium, and the choroid plexus, but it could not be detected in astrocytes (Llanos et al., 2006). The gene correction significantly improved the survival of the ATP7A-expressing transgenic $Mo^{Br/y}$ mice compared with non-transgenic animals. Furthermore, a recent study demonstrated rescue of neonatal $Mo^{Br/y}$ mice following lateral ventricle injections of adeno-associated virus serotype 5 (AAV5) harboring a truncated *ATP7A* cDNA, in combination with copper treatment (Donsante et al., 2011). The authors proposed different effects of the two treatments: the copper injections increased the amount of brain copper available to levels that were 25–50% of the wild type, while the AAV5 gene delivery improved copper utilization. This was associated with enhanced activity of DBH and correction of brain pathology (Donsante et al., 2011). These findings further illustrate the critical role of copper and ATP7A for brain function and highlight the potential for gene therapy in the treatment of MD patients.

ATP7A-RELATED MOTOR NEUROPATHY

Recently, missense mutations in *ATP7A* were found to cause a form of dHMN (Kennerson et al., 2010; Yi et al., 2012), which represents the third clinical phenotype associated with mutations in *ATP7A*. dHMNs are a clinically and genetically heterogeneous group of diseases characterized by lower-motor neuron weakness and muscular atrophy (Rossor et al., 2012). Merner et al. (2010) proposed that a common factor in the disease mechanism may be altered copper homeostasis. The phenotype of ATP7A-related motor neuropathy is clinically distinct from that of MD, with variable age of onset that ranges from the first to the sixth decade of life, no overt abnormalities of copper metabolism, and typically distal muscle weakness and atrophy of the lower extremities leading to hand and foot deformities (Kaler, 2011). Nerve conduction studies suggest that the disease causes gradual degeneration of motor neuron axons in the limbs, beginning in the distal portion and progressing toward the cell body (Takata et al., 2004; Kennerson et al., 2009, 2010). The late-onset nature of the disease suggests that these mutations in *ATP7A* have subtle effects on ATP7A function that can take years to manifest clinically.

The missense mutations within *ATP7A* that result in T994I and P1386S substitutions were identified in two families with multiple affected males (Kennerson et al., 2010; Yi et al., 2012). These mutations are outside of the conserved functional domains and they cause abnormal ATP7A trafficking, affecting specifically motor neuron function. An abnormal interaction between ATP7A (T994I) and valosin-containing protein (p97/VCP) was demonstrated, the latter, an ATPase with functions that include vesicular trafficking and protein degradation by the ubiquitin (Ub)-proteasome system (UPS; Watts et al., 2004; Yi et al., 2012). Mutations in p97/VCP are also associated with other diseases that involve motor neuron degeneration (Watts et al., 2004). The significance of this interaction in mediating the disease phenotype awaits further investigation. Potential mechanisms that can lead to axonal degeneration as a result of these *ATP7A* mutations include copper deficiency, oxidative damage from mislocalized copper, and/or slowed synaptic copper release, resulting in inefficient down-regulation of the NMDA receptor and hence excitotoxicity leading to neuronal damage and axonal dieback.

WILSON DISEASE

In a significant proportion of WD patients, neurodegeneration and neurological presentation reveals an important role for ATP7B in maintaining neuronal copper homeostasis. WD manifests primarily in the liver and brain. Mutations that inactivate ATP7B lead to impaired biliary copper excretion (Terada et al., 1999) and consequently, hepatic copper overload, apoptotic cell death, liver damage, and spillage of copper into the plasma and CSF (Weisner et al., 1987; Kodama et al., 1988; Strand et al., 1998; Gitlin, 2003). Hence, copper also accumulates in extrahepatic tissues, notably the brain, kidneys, and cornea (Danks, 1995; Culotta and Gitlin, 2001).

Approximately 40–50% of WD cases present with neurological symptoms and these patients typically have a later onset than those with the liver disease, presenting in the second or third decade (Das and Ray, 2006; Gouider-Khouja, 2009). Although ATP7B is expressed in several brain regions, brain copper accumulation in

WD appears to be secondary to the liver disease, because it can be reversed by transplantation (Emre et al., 2001; Schumacher et al., 2001). The psychiatric symptoms also are reversible with chelation therapy (Madsen and Gitlin, 2007). The main areas of the brain affected in WD patients include the thalamus, subthalamic nuclei, brainstem, cerebellum, and frontal cortex (Das and Ray, 2006). The precise mechanisms mediating neuronal injury in WD are not clear, but potentially involve increased extracellular copper combined with impaired copper homeostasis in those regions of the brain suffering loss of ATP7B function. For example, impaired DBH synthesis may explain the predominant abnormalities of the basal ganglia that result in Parkinsonian symptoms such as rigidity and tremor (Das and Ray, 2006; Pfeiffer, 2007).

The toxic milk mouse (*tx*) and the LEC rat are mouse models of WD that harbor a point mutation (Theophilos et al., 1996) or deletion (Wu et al., 1994), respectively, in *Atp7b*. Studies of the neurological symptoms and neurodegenerative processes in these models have been lacking until recently. One study found that despite an increase in brain copper levels of the *tx* mouse, there are no neurological or behavioral symptoms (Allen et al., 2006). In contrast, a more recent study reported copper deposition in the striatum and hippocampus of *tx* mice associated with an inflammatory response in these tissues, as well as motor and cognitive disturbances and impaired spatial memory (Terwel et al., 2011). The reason for the discrepancy between these two studies is not clear but may relate to the genetic background of the *tx* mice used.

ALZHEIMER'S DISEASE

Alzheimer's disease is a progressive neurodegenerative disorder occurring late in life. Patients suffer memory loss and cognitive decline. Key pathological hallmarks include intra- and extracellular proteinaceous deposits (senile plaques comprising the Aβ peptides derived from the processing of APP and neurofibrillary tangles (NFTs) composed of hyperphosphorylated tau). The role of copper in AD is gaining prominence with the discoveries that: (i) it is increased and/or mis-localized in the AD brain; (ii) enriched in extracellular plaques; (iii) deficient in Aβ plaque neighboring brain regions; and (iv) disease-linked proteins, APP, Aβ, tau, and BACE1, are copper-binding proteins with key roles in brain metal regulation (reviewed in Kozlowski et al., 2009; Hung et al., 2010). These observations and findings of dysregulation of other metals such as iron and zinc have led to the "metal theory of AD" with the coining of the term, "metallostasis." This represents the fatigue of brain metal regulation and distribution, leading to Aβ aggregation and deposition, intraneuronal iron accumulation, and consequently, oxidative injury and neurodegeneration (Roberts et al., 2012; Bush, 2013).

Genetic analysis of AD patients and healthy controls uncovered *ATP7B* as a genetic risk factor for AD. A number of single nucleotide polymorphisms (SNPs) in *ATP7B* are associated with increased risk for AD (Bucossi et al., 2011, 2012; Squitti et al., 2013). These changes occur in either a MBD, the ATP-binding N-domain or transmembrane domains, which may negatively affect ATP7B function in relation to metal-binding, ATP hydrolysis or copper translocation across the membrane. SNPs in the transmembrane domains present the strongest association for AD risk (Squitti et al., 2013). These observations further support a crucial role for ATP7B in maintaining brain copper homeostasis and a potential role in AD pathogenesis. In contrast to these observations, transgenic APP mice (CRND8) that are homozygous for the tx^J mutation, and therefore lacking a functional ATP7B protein, exhibit elevated brain copper levels, but a markedly reduced number of amyloid plaques and decreased plasma Aβ levels (Phinney et al., 2003). The authors postulated that the mechanism of this beneficial effect of the tx^J mutation involves increased clearance of peripheral Aβ pools. Alternatively, elevated intracellular copper due to the tx^J mutation may correct the copper-deficient phenotype of the CRND8 mice. The copper-induced retention of APP at the cell surface, leading to reduced Aβ production and interaction with copper in lipid rafts may explain the decrease in Aβ and amyloid plaques (Hung et al., 2009; Acevedo et al., 2011).

APOE and *CLUSTERIN* (*APOJ*) polymorphisms represent the strongest and third strongest genetic risk factors for AD, respectively (Bertram et al., 2007). The encoded proteins have long associations with AD (Mahley et al., 2006; Nuutinen et al., 2009; Yu and Tan, 2012). ApoE and clusterin are well-known for their function as secreted extracellular chaperones with key roles in lipid transport. They have neuroprotective functions, cooperatively regulating the deposition and clearance of Aβ (DeMattos et al., 2004; Wilson et al., 2008). In humans, there are three common *APOE* alleles, ε*2,* ε*3,* and ε*4. APOE-ε4* confers increased risk for AD. The presence of *APOE-ε4* in WD patients with the common H1069Q mutation in *ATP7B* is associated with an earlier onset of symptoms (Litwin et al., 2012). In contrast, *APOE-ε3* is associated with a significant delay in the onset of WD symptoms compared with *APOE-ε4* carrying patients (Schiefermeier et al., 2000; Wang et al., 2003). This difference may be linked to the isoform-specific antioxidant activity of the ApoE isoforms (Miyata and Smith, 1996). ApoE4 is less effective than ApoE2/ApoE3 as an antioxidant, which may explain the greater susceptibility of ApoE4 patients to copper toxicity. While the exact mechanism remains unclear, these studies suggest a role for ApoE in copper regulation and in influencing WD phenotypes.

We recently demonstrated that clusterin interacts with ATP7A and ATP7B, and this interaction appears to facilitate the degradation of misfolded copper-ATPase molecules predominantly via the lysosome (Materia et al., 2011, 2012). Both MD and WD exhibit a high degree of clinical variability (de Bie et al., 2007; Tumer and Moller, 2009), with reports of identical mutations, even among siblings, conferring variable clinical expression (Duc et al., 1998; Borm et al., 2004). Hence, these observations implicate other factors in determining the clinical phenotype. Based on functional similarities between clusterin and ApoE, the association between ApoE isoforms and WD onset, and the role of clusterin in copper-ATPase degradation, these molecules potentially could play a role in modifying the expression of neurological disease such as AD, MD, and WD. Conceivably, genetic variations in the *clusterin* and *ApoE* alleles, together with environmental factors, could contribute to the variability in the clinical expression of MD and WD.

PRION DISEASE

Prion diseases are characterized by the continual conformational change of the normal prion protein (PrP^C) to an infectious,

protease-resistant, β-sheet-rich form of the protein (PrP^{Sc}; Prusiner, 1982). The resultant toxic PrP^{Sc} aggregates can disrupt axonal transport and synaptic transmission and/or trigger apoptosis, leading to the neurodegenerative pathologies that are collectively termed transmissible spongiform encephalopathies (TSE) (reviewed in Davies and Brown, 2013).

Prion protein is a membrane glycoprotein with four N-terminal octameric repeats and a nearby site that binds copper (Hornshaw et al., 1995a,b; Brown et al., 1997). This protein is ubiquitously expressed but enriched in neurons and concentrated at the synapse (Sales et al., 1998). Its normal physiological role still remains to be fully elucidated but insight into the cell biology and biochemical properties of PrP^{C} is revealing some clues regarding its function. Various studies have demonstrated that copper-binding induces PrP^{C} internalization (Davies and Brown, 2013) prompting the suggestion that PrP^{C} may be involved as a receptor for copper uptake or efflux (Brown and Harris, 2003). More recently, You et al. (2012) confirmed previous findings that copper modulates NMDA receptor activity. They further demonstrated that this occurs through a copper-dependent interaction between PrP^{C} and the GluN1 subunit, which reduces glycine affinity for the receptor, thus suppressing its activity. The role of ATP7A-mediated synaptic copper release may be important in this context but whether it plays a direct role in PrP^{C}-copper interactions is not clear.

In contrast to the beneficial role of copper in PrP^{C} function noted above, other studies reported that copper binding to PrP^{C} increases its conversion to PrP^{Sc}, and that copper chelation delays the onset of prion disease (Sigurdsson et al., 2003). In support of these studies, disruption of copper homeostasis due to a hypomorphic mutation in *ATP7A* delays the onset of prion disease in mice (Siggs et al., 2012). Copper levels in the brain are reduced by 60% and the amount of the disease-causing PrP^{Sc} is significantly lower than that of the controls. The controversies over the role of copper in PrP^{C} function and prion disease remain to be clarified.

CONCLUDING STATEMENT

Copper plays a central role in a complex network of signaling pathways that regulate a host of physiological and pathophysiological processes. The crucial role of copper in brain and CNS development and function was highlighted three decades ago when the connection was made between infants with MD and copper deficiency (Danks et al., 1972). However, despite its importance, detailed knowledge of the mechanisms controlling brain copper homeostasis remains limited. Significant advances have been made with the subsequent discovery of key components of the copper regulatory network. The copper-ATPases, ATP7A and ATP7B are among these and since their discovery two decades ago, significant progress has been made toward understanding their pivotal role in normal copper homeostasis. Emerging data now reveal a complex and varied role for copper in the brain, and that the copper-ATPases are integral to the regulation and maintenance of copper-mediated processes within the brain. The consequences of their malfunction are clearly illustrated by the severe neurological deficits and neurodegeneration that accompany the disorders of copper transport, MD and WD. More subtle functional defects in the copper-ATPases or in factors that regulate their activity and/or stability are likely to contribute to other neurodegenerative diseases where copper is dysregulated, such as Alzheimer's, motor neuron and prion diseases. Given the complexity of the CNS, much remains to be learned about the role of ATP7A and ATP7B in neurological development and neurodegenerative processes. Current ongoing research into the factors that affect the regulation of their expression, post-translational modification and activity will continue to provide new insights into their involvement and adaptive capacity during neuropathological processes associated with aging and disease.

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Copper metabolism of astrocytes

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This short review will summarize the current knowledge on the uptake, storage, and export of copper ions by astrocytes and will address the potential roles of astrocytes in copper homeostasis in the normal and diseased brain. Astrocytes in culture efficiently accumulate copper by processes that include both the copper transporter Ctr1 and Ctr1-independent mechanisms. Exposure of astrocytes to copper induces an increase in cellular glutathione (GSH) content as well as synthesis of metallothioneins, suggesting that excess of copper is stored as complex with GSH and in metallothioneins. Furthermore, exposure of astrocytes to copper accelerates the release of GSH and glycolytically generated lactate. Astrocytes are able to export copper and express the Menkes protein ATP7A. This protein undergoes reversible, copper-dependent trafficking between the *trans*-Golgi network and vesicular structures. The ability of astrocytes to efficiently take up, store and export copper suggests that astrocytes play a key role in the supply of neurons with copper and that astrocytes should be considered as target for therapeutic interventions that aim to correct disturbances in brain copper homeostasis.

Keywords: ATP7A, astroglia, copper export, Ctr1, metallothioneins, oxidative stress, toxicity, transport

INTRODUCTION

Astrocytes have many important functions in the brain, including the maintenance of extracellular ion homeostasis, the modulation of synaptic transmission and plasticity, the supply of metabolites, and the defense of the brain against oxidative stress and toxins (Parpura et al., 2012; Schmidt and Dringen, 2012). In addition, astrocytes are considered as key regulators of the homeostasis of the redox-active metals iron and copper in the brain (Dringen et al., 2007; Tiffany-Castiglioni et al., 2011; Scheiber and Dringen, 2013). Copper is essential for brain cells as a cofactor and structural component of various enzymes that are involved in important biochemical pathways such as the respiratory chain, the antioxidative defense and the iron metabolism (Scheiber and Dringen, 2013). However, excess of copper in cells is harmful, since copper in redox-active form can catalyze the production of hydroxyl radicals in a Fenton-like reaction, thereby inducing oxidative stress and cell damage (Halliwell and Gutteridge, 2007). Thus, a tight regulation of cellular copper metabolism is required in order to ensure sufficient availability of copper for essential enzymes without concomitant copper-induced oxidative damage.

The presence of many copper-containing enzymes in astrocytes (Scheiber and Dringen, 2013) demonstrates that brain astrocytes require copper as essential element. Histochemical staining of brain sections revealed that copper is present in astrocytes in normal brain (Szerdahelyi and Kasa, 1986). Elevated astrocytic copper levels have been found in the brains of North Ronaldsay sheep, an animal model for copper toxicosis (Haywood et al., 2008). Astrocytes have been reported to be remarkably resistant against copper-induced toxicity (Chen et al., 2008; Reddy et al., 2008; Scheiber and Dringen, 2013). Thus, these cells appear to be equipped with the machinery to deal well with even large amounts of copper. This short review will summarize the current knowledge on the uptake, storage and export of copper in cultured astrocytes and will describe alterations of basal metabolic pathways of astrocytes after exposure to copper. Finally, we will address the potential roles that astrocytes may play in the copper homeostasis of the normal brain and in the dysregulation of copper homeostasis that has been connected with a number of human diseases.

UPTAKE STORAGE AND EXPORT OF COPPER

COPPER UPTAKE

Several groups have reported that cultured astrocytes efficiently accumulate copper (Brown, 2004; Scheiber et al., 2010a,b, 2012; Qian et al., 2012). The apparent K_M value for copper uptake of about 10 μM as well as the expression of the copper transporter receptor 1 (Ctr1) in cultured astrocytes (Scheiber et al., 2010a) suggest that this transporter is responsible for the high affinity copper uptake into astrocytes (**Figure 1**). However, the inhibition of copper accumulation in astrocytes by zinc (Scheiber et al., 2010a,b) suggests that Ctr1-independent transport processes also contribute to the astrocytic copper accumulation. Transporters that could mediate Ctr1-independent copper uptake into astrocytes include the divalent metal transporter 1 (DMT1) and members of the Zrt/IRT-like protein (ZIP) family (Scheiber et al., 2010a; Scheiber and Dringen, 2013). Since Cu^+ has been reported to be the copper species transported by both Ctr1 and DMT1 (Lee et al., 2002; Arredondo et al., 2003), an ecto-cuprireductase on the plasma membrane of astrocytes and/or

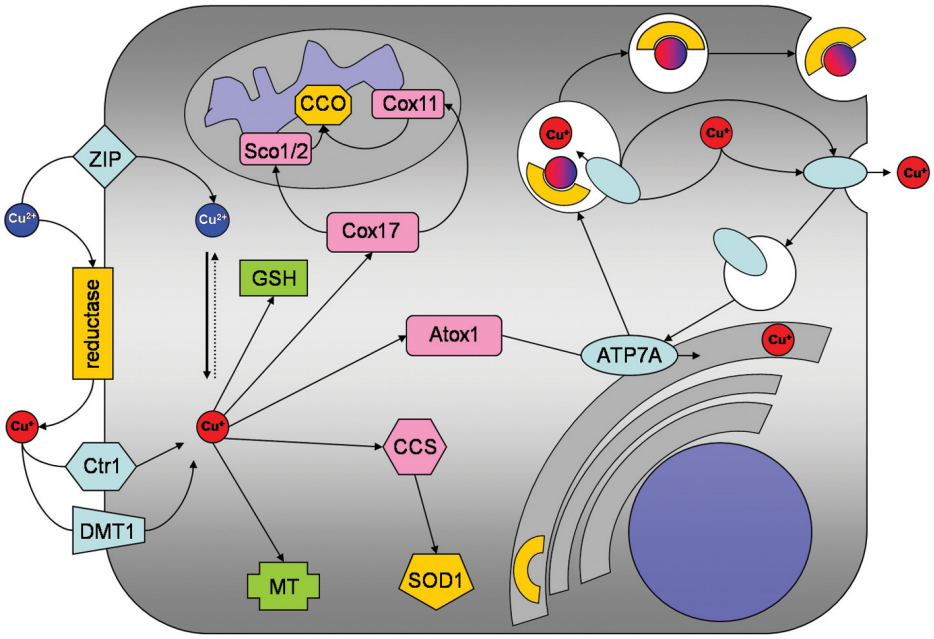

FIGURE 1 | Copper metabolism of astrocytes. Copper is taken up into astrocytes by the copper transporter receptor 1 (Ctr1) and also by Ctr1-independent mechanisms which may include the divalent metal transporter 1 (DMT1) or members of the ZIP family of metal transporters. An ecto-cuprireductase and/or extracellular ascorbate will provide the reduced copper species for astrocytic uptake by Ctr1 or DMT1. Accumulated copper is stored in astrocytes as complex with GSH or in metallothioneins (MT). In addition, copper is shuttled to its specific cellular target, by the copper chaperones CCS to superoxide dismutase 1 (SOD1), by Cox17 to Sco1/2 and Cox11 for subsequent incorporation into cytochrome *c* oxidase and by antioxidant protein 1 (Atox1) to ATP7A. ATP7A transports copper into the *trans*-Golgi network for subsequent incorporation into copper-dependent enzymes. When the cellular copper level rises above a certain threshold, ATP7A translocates reversibly via vesicles to the plasma membrane to export copper.

astrocyte-derived ascorbate (Scheiber and Dringen, 2013) may be involved in reducing extracellular Cu^{2+} to the Cu^{+} that is subsequently taken up into astrocytes (**Figure 1**). An additional protein that has been suggested to contribute to the astrocytic copper uptake or export is the prion protein (Brown, 2004).

STORAGE AND INTRACELLULAR TRAFFICKING OF COPPER

Despite of their efficient accumulation of exogenous copper, astrocytes have been reported to be remarkably resistant to copper-induced toxicity (Chen et al., 2008; Reddy et al., 2008; Scheiber and Dringen, 2013). The most likely reason for this resistance is that astrocytes have a high capacity to store excess copper as complex with glutathione (GSH) and in metallothioneins (MT; **Figures 1**, **2**) which keep the excess copper bound in a non-toxic form. When exposed to copper cultured astrocytes elevate their cellular levels of GSH (Scheiber and Dringen, 2011a) and MT (Scheiber and Dringen, 2013), thereby increasing their storage capacity for copper.

For incorporation into copper-dependent enzymes, copper is shuttled to its target proteins by specific copper chaperones (Robinson and Winge, 2010). The copper chaperone for Cu/Zn superoxide dismutase (CCS) that delivers copper to Cu/Zn superoxide dismutase is expressed in astrocytes (Rothstein et al., 1999). In contrast, expression of Atox1 and Cox17, which deliver copper to ATP7A and to cytochrome c oxidase, respectively, has not been reported so far for astrocytes. However, as astrocytes express functional ATP7A (Scheiber et al., 2012) and cytochrome c oxidase (Bolaños and Heales, 2010), Atox1 and Cox17 are likely to be expressed in these cells.

EXPORT OF COPPER

Cellular export of copper is mediated by the copper ATPases ATP7A and ATP7B which are mutated in the human disorders Menkes disease and Wilson's disease, respectively (Huster, 2010; Kaler, 2011). Cultured astrocytes have recently been shown to export copper in a time-, concentration- and temperature-dependent manner (Scheiber et al., 2012). This copper export is most likely mediated by ATP7A (**Figure 1**) as this protein is expressed in astrocytes in culture and in brain (Barnes et al., 2005; Niciu et al., 2007; Scheiber et al., 2012). The copper-dependent trafficking of ATP7A between the *trans*-Golgi network and sites close to the plasma membrane (Scheiber et al., 2012) strongly suggests a contribution of ATP7A in copper export from astrocytes. This view is also supported by the marked accumulation of copper in astrocytes derived from the macular mouse model of Menkes disease (Kodama et al., 1991). The potential of astrocytes to export copper suggests that these cells can supply other types of brain cells with copper and that impaired copper supply from astrocytes contributes to the neuronal copper deficiency in Menkes disease (**Figure 2**).

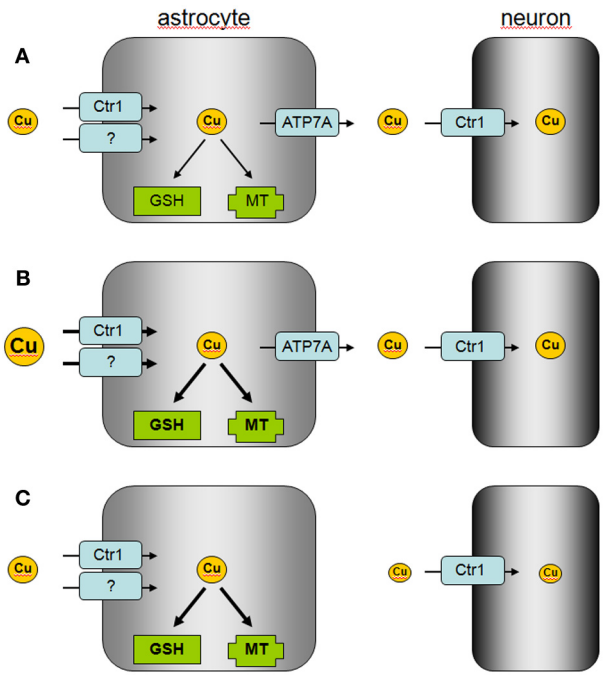

FIGURE 2 | Proposed model of astrocytic copper supply to neurons. **(A)** In the normal brain, copper is efficiently taken up by astrocytes via Ctr1 and other transporters which are so far not identified. Excess of copper is stored by astrocytes in MT or as GSH complex and copper is released via ATP7A to supply neurons with copper. **(B)** In copper overload conditions, excess of copper is efficiently taken up into astrocytes and stored in MT or as GSH complex to prevent copper-induced neurotoxicity. **(C)** In Menkes disease, astrocytes accumulate and store copper, but the mutated ATP7A is unable to mediate astrocytic copper export and copper supply to neurons.

MODULATION OF ASTROCYTIC METABOLISM BY COPPER

Elevation of the cellular copper content of cultured astrocytes caused a time- and concentration-dependent acceleration in glucose consumption and lactate release (Scheiber and Dringen, 2011b). This consequence of copper treatment appears not to be mediated by mitochondrial impairment, but was prevented by inhibition of protein synthesis (Scheiber and Dringen, 2011b). Thus, the copper-induced stimulation of glycolytic flux in astrocytes is likely to depend on transcription and/or translation. Copper-activated transcription factors such as the nuclear factor kappa B or the metal transcription factor 1 have been suggested to contribute to the copper-induced stimulation of glycolytic flux in astrocytes (Scheiber and Dringen, 2013).

Elevated copper content also affects the GSH metabolism of astrocytes. Exposure to copper chloride markedly increased the cellular GSH content of cultured astrocytes (Scheiber and Dringen, 2011a). Copper-induced increase in γ-glutamate cysteine ligase activity by a post-translational mechanism and increased uptake of the GSH precursors cysteine or cysteine into astrocytes have been suggested as potential reasons for the increased specific GSH content of copper-treated astrocytes (Scheiber and Dringen, 2011a, 2013). A compromised GSH export which has been reported to increase cellular GSH contents in astrocytes (Minich et al., 2006) can be excluded as a contributor to the increased cellular GSH content of copper-treated astrocytes, as the GSH export is accelerated in copper-loaded astrocytes (Scheiber and Dringen, 2011a). This accelerated, temperature-sensitive GSH export is mediated by the multidrug resistance protein 1 and is considered to be a direct consequence of the increased cellular GSH concentration in copper-treated astrocytes (Scheiber and Dringen, 2011a).

FUNCTIONS OF ASTROCYTES IN THE COPPER METABOLISM OF NORMAL AND DISEASED BRAIN

The ability of astrocytes to efficiently take up, store and export copper suggests that these cells serve as key regulators of the copper homeostasis in the brain (Tiffany-Castiglioni et al., 2011; Scheiber and Dringen, 2013). Efficient copper uptake and storage in astrocytes serves to protect other brain cells against copper toxicity. The known high antioxidative potential of astrocytes (Dringen et al., 2005) is likely to protect these cells from acute copper toxicity, while the upregulation of the cellular copper storage capacity by induction of MT synthesis (Scheiber and Dringen, 2013) and by elevated GSH synthesis (Scheiber and Dringen, 2011a) will contribute to the prolonged resistance of astrocytes against copper-induced toxicity. In addition, the ability of astrocytes to export copper suggests that astrocytes are able to provide copper to neurons and other neighboring cells in the brain (**Figure 2**). Further studies are required to investigate how signals derived from neurons and/or other types of brain cells will affect the basal copper metabolism of astrocytes and whether such signals could modulate the copper export from astrocytes.

Disturbances in copper homeostasis in brain occur in both Menkes disease and Wilson's disease (Kaler, 2011; Kodama et al., 2011), but also in other neurodegenerative disorders such as Alzheimer's disease and Parkinson's disease (Rivera-Mancia et al., 2010; Double, 2012; Greenough et al., 2013; Scheiber and Dringen, 2013). The role of astrocytes in the copper homeostasis of the brain in these disorders should be considered. The impaired supply of copper from astrocytes to neurons due to mutations of the Menkes protein ATP7A may foster the neuronal copper deficiency in Menkes disease, while the capacity of astrocytes to uptake and store excess of copper may be insufficient to protect neurons against the excess of copper present in the brain in Wilson's disease (**Figure 2**). In addition, the disturbances in the distribution of brain copper in Alzheimer's disease (Greenough et al., 2013) may reflect impaired supply of copper from astrocytes to neurons (Scheiber and Dringen, 2013). The ability of astrocytes to efficiently accumulate, store and export copper makes this brain cell type to an interesting target for therapeutic strategies that aim to correct the observed disturbances in copper metabolism in human disorders (Greenough et al., 2013; Liddell et al., 2013).

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Copper-uptake is critical for the down regulation of synapsin and dynamin induced by neocuproine: modulation of synaptic activity in hippocampal neurons

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Extracellular and intracellular copper and zinc regulate synaptic activity and plasticity, which may impact brain functionality and human behavior. We have found that a metal coordinating molecule, Neocuproine, transiently increases free intracellular copper and zinc levels (i.e., min) in hippocampal neurons as monitored by Phen Green and FluoZin-3 fluorescence, respectively. The changes in free intracellular zinc induced by Neocuproine were abolished by the presence of a non-permeant copper chelator, Bathocuproine (BC), indicating that copper influx is needed for the action of Neocuproine on intracellular Zn levels. Moreover, Neocuproine decreased the mRNA levels of Synapsin and Dynamin, and did not affect the expression of Bassoon, tubulin or superoxide dismutase (SOD). Western blot analysis showed that protein levels of synapsin and dynamin were also down regulated in the presence of Neocuproine and that these changes were accompanied by a decrease in calcium transients and neuronal activity. Furthermore, Neocuproine decreased the number of active neurons, effect that was blocked by the presence of BC, indicating that copper influx is needed for the action of Neocuproine. We finally show that Neocuproine blocks the epileptiform-like activity induced by bicuculline in hippocampal neurons. Collectively, our data indicates that presynaptic protein configuration and function of primary hippocampal neurons is sensitive to transient changes in transition metal homeostasis. Therefore, small molecules able to coordinate transition metals and penetrate the blood-brain barrier might modify neurotransmission at the Central Nervous System (CNS). This might be useful to establish therapeutic approaches to control the neuronal hyperexcitabiltity observed in brain conditions that are associated to copper dyshomeotasis such as Alzheimer's and Menkes diseases. Our work also opens a new avenue to find novel and effective antiepilepsy drugs based in metal coordinating molecules.

Keywords: copper, zinc, neocuproine, synaptic activity, dynamin, synapsin, hyperexcitability, epileptiform-like activity

INTRODUCTION

Zinc (Zn) and copper (Cu) ions have a key physiological importance in mammalian tissue (Mathie et al., 2006). Zn and Cu are abundant trace elements in the human brain (Takeda et al., 2001; Tarohda et al., 2004) and they participate in the regulation of brain physiology, being key structural components of several proteins and co-factors for enzymes that are critical for brain function, including enzymes involved in antioxidant defense and cellular respiration (Mathie et al., 2006). Increasing evidence suggests that Zn and Cu ions function as signaling molecules in the nervous system (Mathie et al., 2006). Moreover, these metals are released from the synaptic terminals of certain neurons, affecting postsynaptic receptors and regulating

Abbreviations: BAPTA, 1,2-bis(o-aminophenoxy)ethane tetraacetate; BC, bathocuproine; CNS, central nervous system; DMSO, dimethyl sulfoxide; EDTA, Ethylenediaminetetraacetic acid; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; ROS, Reactive Oxygen Species; SOD, Superoxide Dismutase; sPSCs, spontaneous postsynaptic currents; TPEN, N,N,N,N′-tetrakis-(2-Pyridylmethyl)ethylenediamine; TTX, tetrodotoxin.

neuronal excitability (Hartter and Barnea, 1988; Kardos et al., 1989; Trombley and Shepherd, 1996; Vlachova et al., 1996; Weiser and Wienrich, 1996; Hopt et al., 2003; Peters et al., 2011; Opazo et al., 2014). Recently, some reports have described the effects of Cu at the synaptic level, where it modulates complex parameters such as LTP (Goldschmith et al., 2005; Gaier et al., 2013, 2014a,b) and receptor pharmacology (Peters et al., 2011; Marchetti et al., 2014). On the other hand, Zn has been considered to play a protective role, because zinc deficiency in the diet can cause malfunctions of several organs and physiological functions (Fukahori and Itoh, 1990). Zn-deficient animals display abnormalities in behavior, which is associated to the deregulation of Zn-binding ProSAP/Shank family members (Grabrucker et al., 2014). In addition, Zn can regulate different intracellular pathways that may explain the effect of Zn-deficiency in brain development (Mackenzie et al., 2011; Nuttall and Oteiza, 2012).

Metal chelators are usually used to evaluate the effect of metals on cell cultures (Calderaro et al., 1993; Göçmen et al., 2000). For example, Neocuproine, a membrane permeable Cu (I) chelator, has been frequently used as a protective agent against oxidative stress caused by Cu (Calderaro et al., 1993). Moreover, Neocuproine can inhibit the relaxation of electrically stimulated mouse corpus cavernosum (Göçmen et al., 2000) and the facilitation of bladder contraction induced by purinergic nerve stimulation (Göçmen et al., 2005).

Considering that hippocampal formation is enriched in transition metals (Tarohda et al., 2004), we evaluated the effect of Neocuproine on different synaptic parameters of primary hippocampal cultures. We observed that Neocuproine acutely increased intracellular Cu and Zn levels that lead to a concomitant decrease in the number of active neurons, an effect which was dependent of copper influx from the extracellular space. These changes correlated with a decrease in levels of synapsin and dynamin. Moreover, Neocuproine blocked the epileptiform-like activity induced by bicuculline in hippocampal neurons. Thus, the use of molecules that can modulate the free levels of Cu and Zn ions could have potential roles on the physiopathology of the central nervous system (CNS). For example, the information presented in this paper can be useful to establish therapeutic approaches to control hyperexcitability observed in brain conditions associated to copper dyshomeostasis such as Alzheimer and Menkes disease (Palop et al., 2003; Amatniek et al., 2006; Prasad et al., 2011; Schrag et al., 2011; James et al., 2012). This work also opens new venues to find novel and effective antiepilepsy drugs (Cully, 2014) based in metal coordinating molecules.

MATERIALS AND METHODS

PRIMARY CULTURES OF RAT HIPPOCAMPAL NEURONS

Hippocampal neurons were obtained from 18-day old pregnant Sprague-Dawley rats and maintained for 10–14 days *in vitro* (DIV) as previously described (Peters et al., 2011). Animals were obtained from the animal house of Catholic University of Chile (Santiago, Chile). All animals were handled in strict accordance with NIH recommendations and approved by the appropriate committee of University of Concepción (Concepción, Chile).

ELECTROPHYSIOLOGY

Experiments were performed in the "whole-cell" configuration using the internal and external solutions described below. Synaptic activity was recorded after a stable baseline was reached. Recording pipettes were pulled from borosilicate glass (WPI, Sarasota, FL) in a horizontal puller (Sutter Instruments, Novato, CA). Membrane currents were measured using an Axopatch-200B amplifier (Axon Instruments, Inc., Burlingame, CA) and an inverted microscope (Nikon, Eclipse, TE200-U, Japan). Data was collected, stored and analyzed using a data acquisition system card (Axon Instruments, Inc.) and the pClamp9 software (Axon Instruments, Inc.). For synaptic activity records, data was analyzed using the Minianalysis software, obtaining the frequency, amplitude and decay time of the records. All experiments were performed at room temperature (20–25°C) using a holding potential of −60 mV. Data are given as means ± S.E.M. and are obtained from at least 3 experiments.

SOLUTIONS AND DRUGS

The intracellular medium contained (in mM): 120 KCl, 2 $MgCl_2$, 2 ATP-Na_2, 10 BAPTA, 0.5 GTP, 10 HEPES (pH 7.4). The extracellular medium contained (in mM): 150 NaCl, 5.4 KCl, 2 $CaCl_2$, 1 $MgCl_2$, 10 glucose and 10 HEPES (pH 7.4).

IMMUNOFLUORESCENCE

Hippocampal cultures were grown in glass coverslips precoated with poly-lysine (1%). After the treatments, hippocampal neurons were washed with PBS (pH 7.4), and then fixed with paraformaldehyde (4%) at RT for 10 min. Then, the dish was washed again in PBS and neurons were permeabilized and blocked for 30 min with PBST (PBS + triton 0.1%) and BSA 10%. Then, cells were incubated with the following primary antibodies for 16 h: Anti-synapsin and anti-MAP 2 antibody (Chemicon) were used in a 1:100 dilution. Secondary antibodies conjugated with FITC or Cy3 were used for fluorescent staining (Jackson ImmunoResearch Laboratories, PA). All of them were used at 1:500 for 2 h. Finally, samples were mounted in fluorescent mounting medium (DAKO, CA, USA) and images were obtained under a Nikon Eclipse confocal microscope (Nikon, Japan) (60×, water immersion, NA 1.4).

RT-PCR

Total RNA was extracted from primary rat hippocampal cell cultures using RNAsolv (Omega Biotek). Two μg of RNA were used to prepare cDNA using Stratascript kit (Stratagene). PCR was performed with specific forward and reverse primers (Genbank) (**Table 1**), using 20–40 cycles in a Biorad Thermal Cycler.

WESTERN BLOT

Hippocampal cells were incubated with Neocuproine (0–30 μM), culture media was removed and cells were washed with PBS and then homogenized in sample buffer containing: SDS (4%), Glycerol (12%), Phenol Red (0.0025%) and 10% β-mercaptoethanol in Tris-HCl (450 mM, pH 8.45). 20 μl of each homogenate (≈ 10 μg of total protein) was submitted to SDS electrophoresis

Table 1 | The list and sequence of primers used for RT-PCR analysis.

Protein	Forward primer	Reverse primer
Bassoon	5′-CCCCCAACCACTGCTAACTA-3′	5′-CGAGCACAGAGGGGAAGTAG-3′
Snap29	5′-AGAGCTGTGGGCAGAGTGTT-3′	5′-ACTCCATGCACACAAACCAA-3′
Synapsin	5′-CACCGACTGGGCAAAATACT-3′	5′-TGTGCTGCTGAGCATCTCTT-3′
Dynamin I	5′-CAGGACAGGCCTCTTCACTC-3′	5′-CCGATGTTGTTGATGGTCAG-3′
Dynamin II	5′-ACCCCACACTTGCAGAAAAC-3′	5′-GGCTCTTTCAGCTTGACCAC-3′
Tubulin	5′-GCACTCTGATTGTGCCTTCA-3′	5′-ACTGGATGGTACGCTTGGTC-3′
SOD1	5′-GTCGTCTCCTTGCTTTTTGC-3′	5′-CACCTTTGCCCAAGTCATCT-3′

in 10–20% Tricine gels. Protein bands were transferred onto nitrocellulose membranes, blocked with 5% milk and incubated with the following primary antibodies: monoclonal anti-synapsin, anti-dynamin (1:500, Chemicon) and α-tubulin (1:1000, Sigma) as internal control. Immunoreactive bands were detected with secondary antibodies conjugated with HRP (Santa Cruz Biotechnology) and visualized with ECL Plus Western Blotting Detection System (PerkinElmer).

ZN^{2+} AND CU^{2+} INTRACELLULAR MEASUREMENTS

Hippocampal cells were loaded for 30 min at 37°C with FluoZin-3-AM (1 μM) or Phen Green (5 μM) (Invitrogen, USA) to measure Zn^{2+} or Cu^{2+}, respectively. Then, cells were washed twice with external solution as described above and incubated under control or experimental conditions for a maximal time of 30 min at 37°C. Confocal images were acquired on middle cellular plane, with 20× and 60× magnification. Intracellular fluorescent signals were recorded with a CCD camera (SensiCam camera, PCO, Germany), with 200 ms of exposition and 2 s acquisition (Ex:Em; 490:510 nm).

ANALYSIS OF INTRACELLULAR CALCIUM TRANSIENTS

Hippocampal cells were loaded with Fluo-3 AM (1 μM in pluronic acid/DMSO, Molecular Probes, Eugene, OR, USA) for 30 min at 37°C and then washed twice with external solution, as described above, and incubated under control or experimental conditions for a maximal time of 30 min at 37°C. Neurons were mounted in a perfusion chamber that was placed on the stage of an inverted fluorescent microscope (Eclipse TE, Nikon), equipped with a CCD camera (SensiCam camera, PCO, Germany) and xenon excitation lamp. Cells were subsequently excited for 200 ms each 2 s intervals and recorded during 5 min, using a Lambda 10-2 filter wheel (Sutter Instruments) and regions of interest (ROI) were simultaneously selected on several neuronal somata on each plate (Ex:Em; 480:510 nm) recording more than 10 cells in each experiment. Finally, calcium transients, as defined by their TTX sensitivity (Gu et al., 1994), were acquired and analyzed off line with Axon Instruments Workbench 2.2 software.

DATA ANALYSIS

Results are expressed as the mean ± SEM. Statistical significance was determined using One-way ANOVA. A level of * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$ was considered statistically significant followed by the Bonferroni post test.

RESULTS

NEOCUPROINE INDUCES A COPPER-MEDIATED INTRACELLULAR ZINC INCREASE

Zn and Cu may regulate the neurotransmission of CNS neurons by a presynaptic or postsynaptic mechanism (Goldschmith et al., 2005; Mathie et al., 2006; Leiva et al., 2009; Peters et al., 2011). Therefore, pharmacological intervention with compounds that change the balance of these metals at the neuronal level may result in changes in synaptic function. To test this idea we exposed primary rat hippocampal neurons (10–14 DIV) to Neocuproine (Neo), a high affinity copper chelator (Göçmen et al., 2000). First, we measured the changes of intracellular Cu using Phen Green, a fluorescent probe that decreases its fluorescent signal (quenching) when coordinates Cu (Chavez-Crooker et al., 2003). As it was expected, neurons loaded with Phen Green under control conditions displayed a stable basal fluorescent signal (**Figure 1A**). However, when they were exposed to $CuCl_2$ (3 μM), the fluorescent signal was decreased, and a subsequent addition of EDTA (10 μM) to the bath recovered the signal to the basal levels (**Figure 1A**). Interestingly, when Neocuproine (10 μM) was added alone to the bath, a small decrease in the intracellular fluorescence was also observed, indicating that Neocuproine was inducing an increase of free intracellular copper (**Figure 1A**). This effect was observed in all neuronal cells exposed to Neocuproine and the changes were detected both in soma and neuronal projections (**Figures 1Bb,d**). These results suggest that changes in free intracellular Cu could be explained by a Cu influx from the extracellular space, occurring at different cell membrane domains, or by a Cu release from organelles or intracellular proteins, which are widely distributed at the intracellular space. In order to evaluate the effect of Neocuproine on the intracellular levels of other transition metal, we performed similar experiments using a specific fluorescent probe for Zn, FluoZin-3, which increases its fluorescence signal in the presence of Zn (Sensi et al., 2011). In fact, when Zn is co-applied with pyrithione (a Zn chelator) (Pyr/*Zn* = 30/10 μM) a rapid increase was observed (**Figure 1C**), which was abolished by a subsequent application of TPEN, a cell-permeant Zn chelator (Sensi et al., 2011; **Figure 1C**). Interestingly, when hippocampal cultures pre-loaded with Fluo-Zinc were exposed to Neocuproine (10 μM), the fluorescent signal rapidly increased (**Figures 1D,E**) in the soma and neuronal projections (Zoom on **Figure 1D**). We confirmed that this signal was associated with changes in free intracellular zinc, since the increase in the fluorescent signal induced by Neocuproine was abolished by a subsequent application of TPEN (**Figure 1F**). Therefore,

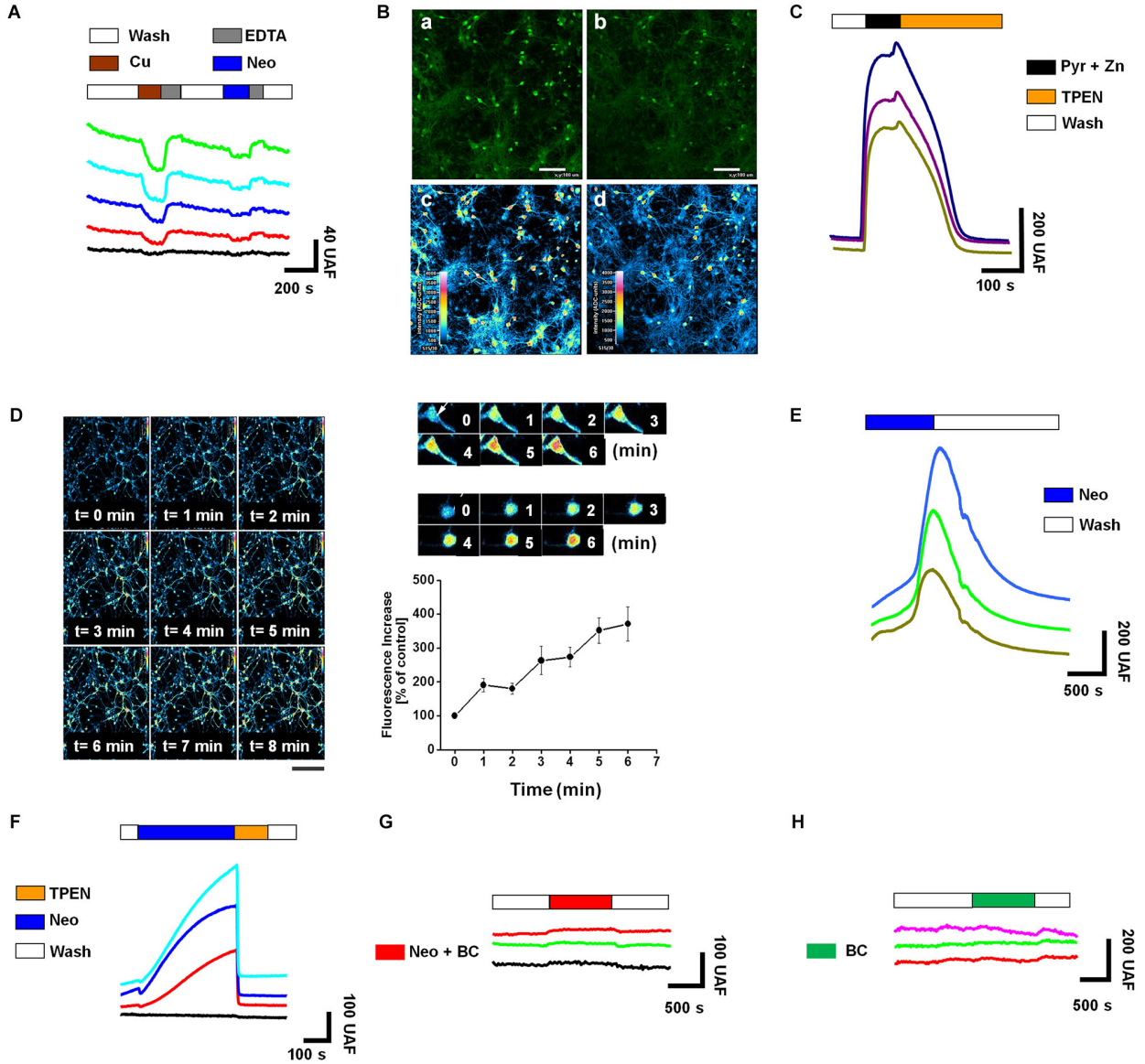

FIGURE 1 | Neocuproine increases intracellular Zn^{2+} $[Zn^{2+}]_i$ by promoting copper influx in hippocampal neurons. **(A)** The traces show the intracellular changes of Phen Green fluorescence after extracellular acute application of $CuCl_2$ (3 μM) (quenching), EDTA (10 μM) (recovery) or Neocuproine (Neo, 10 μM) (quenching). Traces in color correspond to the signal recorded in a single cell. The black trace corresponds to the typical background signal recorded outside of cells. **(B)** Confocal microphotographs of hippocampal cultures loaded with Phen Green, untreated (a and c) or treated with Neo (10 μM) for 10 min (b and d). Pseudocolor images shown in "**c**" and "**d**", correspond to fluorescence photographs shown in "**a**" and "**b**", respectively. Bar represents 100 μm. **(C)** The traces show the intracellular changes of FluoZin-3 fluorescence under control conditions or after the acute application of the agents indicated in the figure (Pyr/Zn = 30/10 μM; TPEN = 10 μM). Each trace corresponds to the signal recorded in a single cell. **(D)** Confocal microphotographs of hippocampal cultures loaded with FluoZin-3, before (time = 0) and after of a short application (up to 8 min) of Neo (10 μM). Scale bar, 250 μm. At the right of this panel is shown a confocal zoom of two neurons from the experiment described in "**(D)**". The graph summarizes the intracellular Zn^{2+} changes recorded in the soma of single neurons treated as shown in "**(D)**". Values are mean ± SEM (N = 3). **(E)** The traces show the rapid intracellular changes of FluoZin-3 fluorescence after the acute application of Neocuproine (Neo, 10 μM). **(F–H)** Each colored trace corresponds to the FluoZin-3 fluorescent signal recorded on the soma of single hippocampal neuron under the treatments indicated in the figures (Neocuproine, Neo = 10 μM; Bathocuproine, BC = 10 μM; TPEN = 10 μM). The black traces correspond to the typical background signal recorded outside of cells.

Neocuproine increases simultaneously free intracellular Cu and Zn. Considering that Neocuproine has a higher affinity for Cu than Zn and also that the application of Neocuproine was at the bath solution, we hypothesized that Neocuproine facilitated the entry of copper from the extracellular space, which in turns displaced Zn from intracellular reservoirs. To test this hypothesis, we evaluated the effect of Bathocuproine (BC), a selective non cell-permeant Cu chelator (Mohindru et al., 1983), on the

intracellular Zn changes induced by Neocuproine. In agreement with this hypothesis, we found that the presence of BC completely abolished the changes in Zn levels induced by Neocuproine (**Figure 1G**), indicating that Neocuproine facilitates the influx of copper, which then allows the rise of free intracellular Zn. We confirmed that BC itself was not changing the basal levels of intracellular Zn (**Figure 1H**). Altogether, these results indicate that it is possible to modify free intracellular Zn levels by using specific molecules that facilitate copper influxes from the extracellular space.

ACUTE APPLICATION OF NEOCUPROINE DOES NOT AFFECT SYNAPTIC ACTIVITY

To evaluate if rapid intracellular changes in Cu and Zn induced by Neocuproine affects neurotransmission, we studied the synaptic activity of neurons under the same experimental conditions. Spontaneous calcium transients were recorded in the presence of the Fluo3-AM probe, allowing us to assess the changes on intracellular calcium as a reflection of synaptic and neuronal network activity (Peters et al., 2011). We observed no significant differences between control and acutely Neocuproine-treated neurons (**Figure 2A**). Moreover, when we evaluated the electrophysiological synaptic activity of these neurons by whole cell patch clamp recordings, the result correlated with calcium signals, since no differences were observed between control and Neocuproine-treated neurons (**Figure 2B**), in the frequency (control, 2.4 ± 0.6 Hz; Neocuproine, 2.5 ± 0.3 Hz) (**Figure 2C**) or amplitude (control, 280 ± 38 pA; Neocuproine, 190 ± 47 pA) (**Figure 2D**) of the currents. The patch clamp recordings also indicated that the control and Neocuproine-treated neurons presented an active neurotransmission.

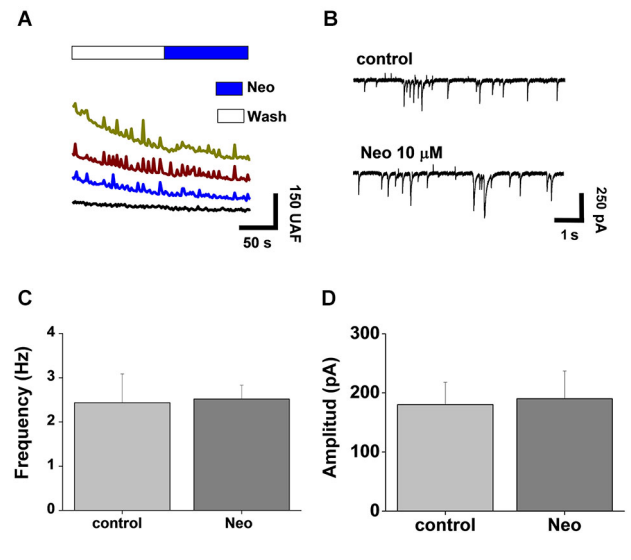

FIGURE 2 | Acute application of Neocuproine does not alter synaptic transmission. **(A)** Calcium transients of hippocampal neurons in the absence or presence of Neocuproine (Neo, 10 μM). **(B)** Typical miniature synaptic currents obtained in the absence or presence of Neocuproine (Neo, 10 μM). The graphs summarize the frequency **(C)** and the amplitude **(D)** values of miniature synaptic currents recorded in three independent experiments performed under the conditions described in "**(B)**." In both cases values are mean ± SEM ($N = 3$).

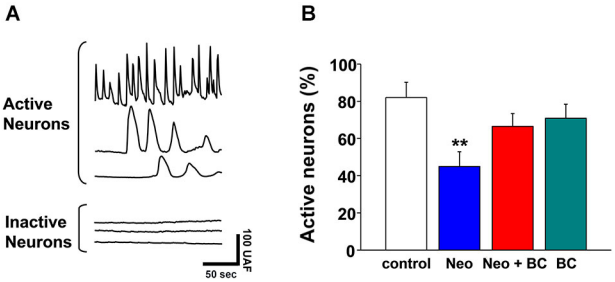

FIGURE 3 | Chronic application of Neocuproine decreases the number of active neurons. **(A)** The top traces show typical calcium transients observed in active hippocampal neurons in culture. The bottom traces show the lack of calcium transients in inactive hippocampal neurons in culture. Each trace corresponds to the signal recorded in a single cell. **(B)** The graph summarizes the percentage of active hippocampal neurons under different treatments: untreated (control) or treated with Neocuproine (Neo, 10 μM) in the absence or presence of Bathocuproine (BC, 10 μM) for 12 h. Values are mean ± SEM ($N = 3$). **, $p < 0.01$.

SUB-CHRONIC APPLICATION OF NEOCUPROINE DECREASES THE NUMBER OF ACTIVE NEURONS

To analyze if Neocuproine could induce changes in synaptic activity after sub-chronic incubations, hippocampal cultures were exposed to Neocuproine (10 μM) for 12 h and then the synaptic activity was evaluated measuring calcium transients. We found that the number of active neurons was decreased in the presence of Neocuproine, and that the effect was inhibited by the presence of BC in the cell media (**Figures 3A,B**), indicating that Neocuproine decreased the synaptic activity by a mechanism involving copper entry from the extracellular space.

SUB-CHRONIC APPLICATION OF NEOCUPROINE DOWN-REGULATES SYNAPSIN AND DYNAMIN

We reasoned that the reduction on the number of active neurons after the treatment with Neocuproine should be accompanied with changes in the machinery responsible for neurotransmission, including scaffold and synaptic proteins. First, we analyzed the mRNA levels of dynamin I, dynamin II, synapsin, Bassoon, SNAP29, Superoxide Dismutase (SOD) and tubulin in rat hippocampal cultures treated in the absence or presence of 10 μM Neocuproine for 12 h. We found that the mRNA of dynamin (I and II) and synapsin, but not the mRNA of Bassoon and SNAP29 were decreased. We also did not observe changes in SOD or tubulin mRNA levels (**Figure 4A**). Then, hippocampal cultures were incubated with increasing concentrations of Neocuproine (up to 30 μM) for 12 h. Under these conditions protein levels for dynamin I and synapsin (**Figures 4B–D**) were decreased in the presence of Neocuproine in a concentration dependent manner. A similar pattern was also observed by immunofluorescence analysis (**Figure 5A**), indicating that Neocuproine decreased the signal of specific synaptic proteins such as synapsin. To evaluate if the change in synapsin levels induced by Neocuproine was also mediated by the uptake of

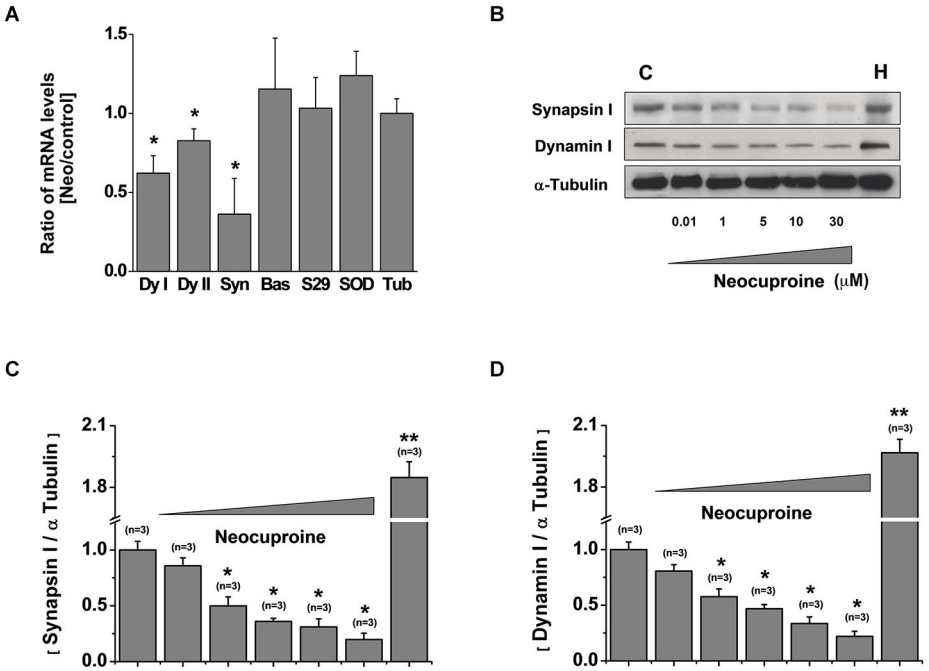

FIGURE 4 | Chronic application of Neocuproine down-regulates synapsin and dynamin. (A) mRNA levels of dynamin I (dyI), dynamin II (dyII), synapsin, Bassoon (Bas), SNAP29, Superoxide Dismutase (SOD) and Tubulin (Tub) expressed by rat hippocampal cultures (11 DIV) treated in the absence or presence of Neocuproine (Neo, 10 μM) for 12 h. Values are mean ± SEM ($N = 3$). *, $p < 0.05$. **(B)** Protein levels of synapsin and dynamin I expressed by rat hippocampal cultures (11 DIV) treated in the absence or presence of Neo (up to 30 μM) for 12 h. Whole brain homogenate (H) of 15 days post-natal rat was used as positive control. **(C,D)** The graphs summarize the data obtained in experiments described in "**(B)**." Values are mean ± SEM ($N = 3$). *, $p < 0.05$; **, $p < 0.01$.

copper from the extracellular media, we performed experiments in the presence of BC (**Figures 5B,C**). In agreement with this hypothesis, BC blocked the effect Neocuproine on synapsin levels, indicating that copper uptake was critical for the decrease in synapsin levels.

NEOCUPROINE BLOCKS THE EPILEPTIFORM-LIKE ACTIVITY INDUCED BY BICUCULLINE IN HIPPOCAMPAL NEURONS

Considering that Neocuproine modulates neurotransmission we evaluated whether Neocuproine down-regulates the epileptiform-like activity in hippocampal neurons. To evaluate the chronic effect of Neocuproine on the neuronal activity we used whole cell patch clamp technique (−60 mV) and measured miniature postsynaptic currents in primary hippocampal neurons treated with bicuculline for 24 h, which induce epileptiform-like activity in neurons (Carrasco et al., 2007; Sepúlveda et al., 2009). As shown in **Figure 6**, we found that the frequency of miniature postsynaptic currents was significantly lower after chronic administration (24 h) of Neocuproine (10 μM) compared to control (Control, 0.352 ± 0.045 Hz, n =12 vs. Neocuproine, 0.125 ± 0.016 Hz, $n = 11$; $p < 0.01$). However, no significant differences were observed in the amplitude of the miniature postsynaptic currents (control, 35.81 ± 5.22 pA, $n = 12$ vs. Neocuproine, 22.08 ± 3.72 pA, $n = 11$). These results correlate with the decrease in the number of active neurons (**Figure 3**) and the decrease on the levels of presynaptic proteins dynamin (I and II) and synapsin observed after sub-chronic treatments with Neocuproine (**Figure 4**). Interestingly, chronic treatments with Neocuproine blocked the epileptiform-like activity induced by bicuculline (5 μM; 24 h) (**Figure 6**; Control, 0.352 ± 0.045 Hz, $n = 12$; bicuculline, 0.744 ± 0.104; Neocuproine + bicuculline 0.197 ± 0.047; Neocuproine, 0.125 ± 0.016 Hz, $n = 11$; $p < 0.01$), suggesting that Neocuproine can abolish epileptogenic-like activity in CNS neurons.

DISCUSSION

Zn and Cu are abundant trace elements involved in the regulation of human brain physiology (Takeda et al., 2001; Tarohda et al., 2004), being key structural components of several proteins and co-factors for enzymes that are critical for brain function (Mathie et al., 2006). In fact, increasing evidence suggests that Zn and Cu ions function as signaling molecules in the nervous system (Mathie et al., 2006). Moreover, these metals are released from the synaptic terminals of certain CNS neurons, affecting post-synaptic receptors and regulating neuronal excitability (Hartter and Barnea, 1988; Kardos et al., 1989; Trombley and Shepherd, 1996; Vlachova et al., 1996; Weiser and Wienrich, 1996; Hopt et al., 2003; Peters et al., 2011; Sensi et al., 2011). Therefore, pharmacological strategies that interfere the homeostasis of these transition metals may modulate synaptic function. In this regard, this is the first time that Neocuproine, a high affinity copper chelator (Göçmen et al., 2000), is shown to decrease synaptic

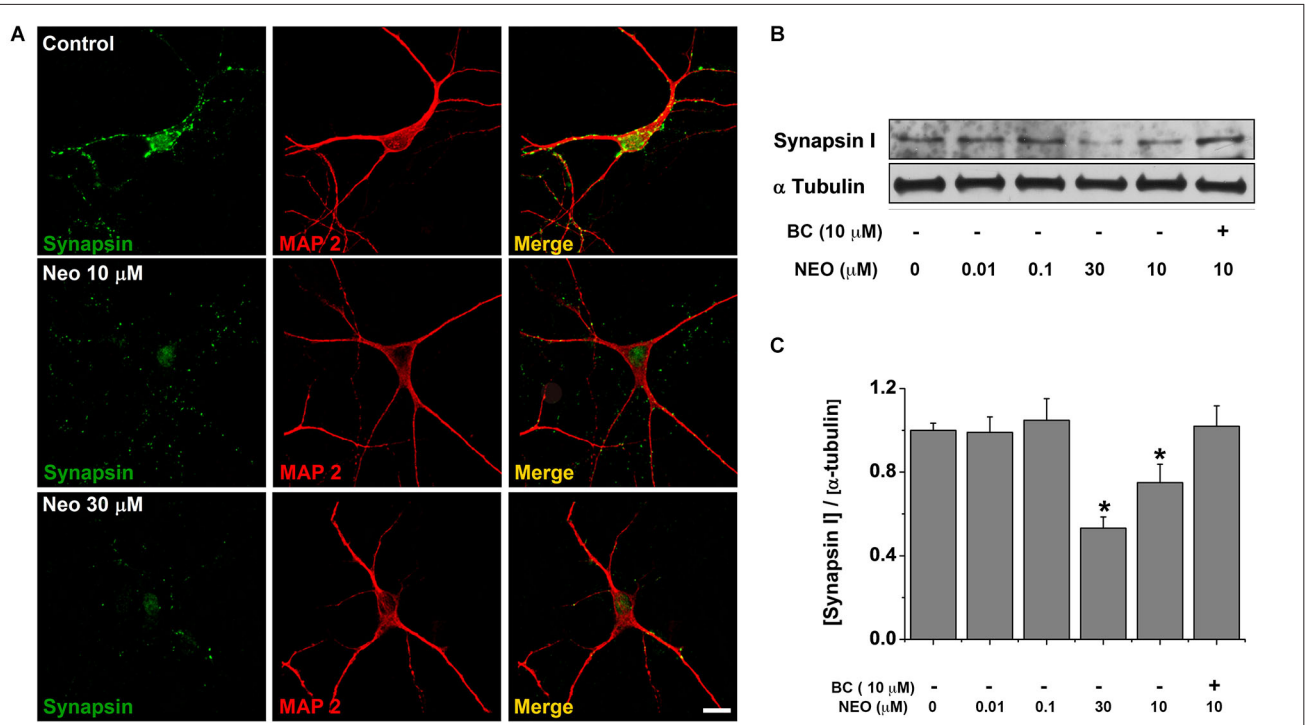

FIGURE 5 | Bathocuproine blocks the decrease of synapsin induced by Neocuproine treatments. **(A)** Microphotographs of rat hippocampal neurons treated with Neocuproine (Neo, 10 μM and 30 μM) for 12 h. Synapsin immunoreactivity is shown in green, and MAP2 in red. Scale bar, 25 μm. **(B)** Protein levels of synapsin expressed by rat hippocampal cultures (11 DIV) treated in the absence or presence of Neo (up to 30 μM), and Neo (10 μM) + Bathocuproine (BC, 10 μM) for 12 h. **(C)** The graph summarize the data obtained in the experiment described in "**(B)**". Values are mean ± SEM ($N = 3$). * $p < 0.05$.

activity in hippocampal neurons (**Figure 3**), by a mechanism that involves the influx of Cu from the extracellular space that in turn induces the subsequent mobilization of intracellular Zn from proteins and/or organelles that were not identified in this study. According to our results, the presence of BC, a selective non cell-permeant Cu chelator (Mohindru et al., 1983), completely abolished the changes in the intracellular Zn levels induced by Neocuproine (**Figure 1**), indicating Neocuproine facilitated the influx of copper, which subsequently drove the rise of free intracellular Zn. In our working hypothesis, after Neocuproine-Cu complexes get across the plasma membrane Neocuproine donates Cu to Zn-proteins that interchange Zn and Cu in the intracellular space, thus releasing the Zn to the cytosol. We hypothesized that the rise of Zn induced by Neocuproine is occurring through a competitive mechanism. However, we could not discard that Neocuproine-Cu complexes or free Cu donated by Neocuproine-Cu complexes can inhibit, at the extra/intracellular space, Zn pumps or active Zn transporters that explain the rise in cytosolic Zn (Sensi et al., 2011). Moreover, all these changes in Cu and Zn homeostasis could promote the formation of reactive oxygen species (ROS), which may also participate in the regulation of synaptic proteins. We believe that our experimental conditions do not favor the formation of toxic levels of ROS, because the neuronal viability was not affected in the presence of Neocuproine. We acknowledge that different results can be expected in neuronal viability if exogenous Cu is added to the media, because it has been show that Neocuproine-Cu complexes are toxic to cultured astrocytes (Chen et al., 2008). However, we can not ruled out that non-toxic levels of ROS were produced in the presence of Neocuproine. Therefore, future studies are needed to better understand the mechanisms behind the effect of Neocuproine on living neurons, which may lead to establish protocols to change the neuronal activity. This information may be useful to prevent or to treat copper-related neurodegenerative diseases and neurological conditions, which remain untreatable to date (Bush, 2003). Interestingly, the effect of Neocuproine on free intracellular Cu and Zn was observed in cells maintained in cell media that was not supplemented with extra Cu or Zn and contained in total 1 μM of total Cu. Altogether, these results indicate that it is possible to modify the free intracellular Zn levels by using specific molecules that facilitate copper influxes from the extracellular space under basal conditions. These results also suggest that the alteration of the homeostasis of one transition metal can affect the homeostasis of another for a simple mechanism of competition.

We further found that the number of active neurons was decreased in primary cultures treated with Neocuproine, which was blocked by the presence of BC in the cell media (**Figure 3**), indicating that Neocuproine decreased the synaptic activity by a mechanism that involves the entry of copper to the cell from the extracellular space. Interestingly, the reduction on the number of active neurons after the treatment with Neocuproine was

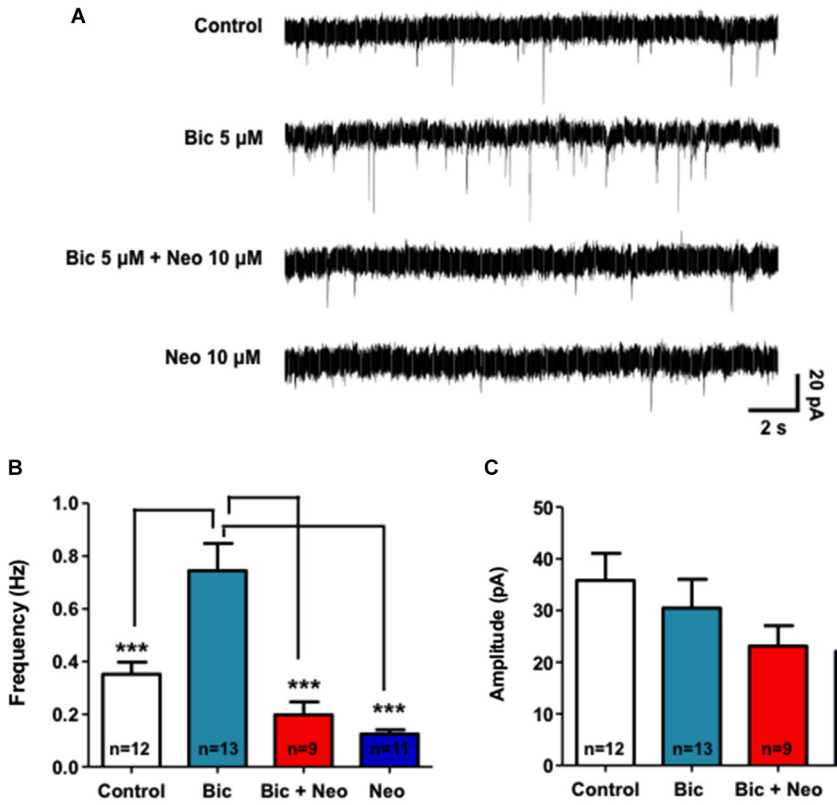

FIGURE 6 | Neocuproine blocks the epileptiform-like activity induced by bicuculline in hippocampal neurons. (A) Representative traces of miniature postsynaptic currents illustrate the effect of control and chronic (24 h) bicuculline (5 μM) treatment in the absence or presence of Neocuproine (Neo, 10 μM) in hippocampal neurons. **(B,C)** The graphs summarize the effect of the different treatments on the frequency (Hz) and amplitude (pA) of the records obtained in three independent experiments performed under the conditions described in "**(A)**". Values are mean ± SEM obtained from the indicated number of neurons. ***, $p < 0.001$.

accompanied with changes in synapsin and dynamin I, both important proteins in the presynaptic machinery responsible for neurotransmission, through a direct or indirect mechanism that may lead to a reduction in neurotransmitter release. For example, Zn, Cu or Neocuproine-Cu complexes could directly interact with these proteins, decreasing their protein stabilities or/and their synthesis or/and increasing their degradation by the proteasome (Colledge et al., 2003). Because, we have recently found that Neocuproine does not change the ubiquitination pattern of neuronal cells (data not shown), a mechanism involving the ubiquitin-proteasome system can be partially ruled out, suggesting that the down-regulation effect of Neocuproine on dynamin and synapsin might be explained by changes in protein expression. All these possible mechanisms deserve further examination. In this regard, a protein involved in synaptic vesicle dynamic, SV2A, has been recently found to be an effective target for epilepsy (Gillard et al., 2011) supporting the presynapse as a target to control hyperexcitability.

In summary, in this work we have shown that Neocuproine, in time, concentration and copper dependent manner promotes changes on neurotransmission, suggesting that changes in transition metal might be required by neuronal cells to maintain adequate synaptic function (Peters et al., 2011). In fact, an increase in the brain concentration of copper, as well as a decrease in the levels of this metal, can lead to serious illness (Bush, 2003). Interestingly, Neocuproine blocks the epileptiform-like activity induced by bicuculline in hippocampal neurons, suggesting that Neocuproine might be a prototype drug to control the hyperexcitability observed in brain disorders such as Alzheimer and Menkes disease (Palop et al., 2003; Amatniek et al., 2006; Prasad et al., 2011), which are also characterized by brain copper dyshomeostasis (Schrag et al., 2011; James et al., 2012). Our data suggest that Neocuproine blocks the epileptiform-like activity by reducing the levels of synapsin and dynamin. However, we can not discard the participation of other presynaptic and postsynaptic targets that may be involved in the effect of Neocuproine on synaptic activity. Further *ex vivo* and *in vivo* studies are needed to confirm that Neocuproine is really effective to control hyperexcitability in humans. The origin of the brain overexcitation in these diseases is not well understood, but it might be related to changes in brain bioavailable copper. Interestingly, Tg2576 mice that overexpress APP and have lower levels of brain copper (Maynard et al., 2002) are more susceptible to chemical-induced seizures (Westmark et al., 2008), suggesting an association between brain copper homeostasis and brain overexcitation.

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Zinc homeostasis and neurodegenerative disorders

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Zinc is an essential trace element, whose importance to the function of the central nervous system (CNS) is increasingly being appreciated. Alterations in zinc dyshomeostasis has been suggested as a key factor in the development of several neuropsychiatric disorders. In the CNS, zinc occurs in two forms: the first being tightly bound to proteins and, secondly, the free, cytoplasmic, or extracellular form found in presynaptic vesicles. Under normal conditions, zinc released from the synaptic vesicles modulates both ionotropic and metabotropic post-synaptic receptors. While under clinical conditions such as traumatic brain injury, stroke or epilepsy, the excess influx of zinc into neurons has been found to result in neurotoxicity and damage to postsynaptic neurons. On the other hand, a growing body of evidence suggests that a deficiency, rather than an excess, of zinc leads to an increased risk for the development of neurological disorders. Indeed, zinc deficiency has been shown to affect neurogenesis and increase neuronal apoptosis, which can lead to learning and memory deficits. Altered zinc homeostasis is also suggested as a risk factor for depression, Alzheimer's disease (AD), aging, and other neurodegenerative disorders. Under normal CNS physiology, homeostatic controls are put in place to avoid the accumulation of excess zinc or its deficiency. This cellular zinc homeostasis results from the actions of a coordinated regulation effected by different proteins involved in the uptake, excretion and intracellular storage/trafficking of zinc. These proteins include membranous transporters (ZnT and Zip) and metallothioneins (MT) which control intracellular zinc levels. Interestingly, alterations in ZnT and MT have been recently reported in both aging and AD. This paper provides an overview of both clinical and experimental evidence that implicates a dysfunction in zinc homeostasis in the pathophysiology of depression, AD, and aging.

Keywords: zinc, zinc transporters, metallothioneins, depression, aging, Alzheimer's disease, neurodegeneration

INTRODUCTION

Knowledge about zinc has rapidly evolved over the years with the last two decades having brought, interesting new insights about the role of zinc in molecular and cellular processes as well as health and disease. Zinc is one of the most prevalent trace elements in the human body. It is a key structural component of a great number of proteins, and a co-factor of more than 300 enzymes that regulate a variety of cellular processes and cellular signaling pathways essential for both brain and systemic physiology (Takeda, 2000). In the brain, zinc is also present in its free ionic form (Zn^{2+}) within synaptic vesicles, mostly at the glutamatergic terminals (Frederickson et al., 2000; Paoletti et al., 2009; Sensi et al., 2011). Synaptically released zinc, during neuronal activity, affects the activity of N-methyl-D-aspartate (NMDA) and α-amino-3-hydroxyl-5-methyl-4-isoxazole-propionate (AMPA) glutamate receptors, $GABA_A$ and glycine inotropic receptors (Smart et al., 2004). It has also been found to activate a specific metabotropic Zn2+-sensing receptor GPR39 (Besser et al., 2009). In physiological concentrations zinc exhibits neuroprotective activity, although high concentrations of zinc are neurotoxic (Choi et al., 1988; Perry et al., 1997; Cote et al., 2005; Plum et al., 2010). Therefore, an imbalance of zinc homeostasis will have complex implications in a number of brain processes then leading to the onset of chronic pathologies such as depression, schizophrenia, Alzheimer's disease (AD), Parkinson's disease, aging, or amyotrophic lateral sclerosis (ALS). Given the complex nature of zinc homeostasis in the brain, it is not surprising that several different groups of proteins are involved in managing its cellular levels. The first group consists of are membranous transporters (ZnTs) mediating the zinc efflux from cells or influx into cellular compartments or organelles (Huang and Tepaamorndech, 2013). The second group is members of the Zip family (zinc-regulated and iron-regulated transporter proteins) that promote zinc transport from the extracellular space or from intracellular vesicles to the cytoplasm (Cousins et al., 2006). So far, 10 members of the ZnT and 14 members of the ZIP protein families have been identified (Lichten and Cousins, 2009). The third group of these zinc homeostasis-regulating proteins is metallothioneins (MTs)—a group of low-molecular-weight metal-binding proteins that have a high affinity for zinc (Krezel et al., 2007). Four MT isoforms have been described so far; MT-I and MT-II are expressed in many tissues; MT-IV is exclusively expressed in some stratified squamous epithelia (Quaife et al., 1994) and MT-III. MT-III is a brain-specific member of the MTs protein family, found exclusively in neurons, and localized predominantly in neurons that

sequester zinc in synaptic vesicles (Masters et al., 1994). MT-III mRNA has been found in the cortex, hippocampus, amygdala, and cerebellum (Masters et al., 1994). The role of MTs is to buffer cytoplasmic zinc following its influx into the cytoplasm, and so far it seems that temporary cellular zinc storage is the exclusive function of MTs (Krezel et al., 2007). MTs play a crucial protective role (due to their redox properties) in the presence of radiations, heavy toxic metals, lipid peroxidation, or reactive oxygen species (ROS) (Sato and Kondoh, 2002).

The understanding of the physiological functions of zinc transporters and MTs has grown dramatically during recent decade and their involvement in the pathogenesis of neurodegenerative diseases more apparent than previously. This review focuses on depression, AD, and age related pathologies, in which a specific role for zinc dyshomeostasis has been reported. Also, disease associated alterations in proteins responsible for zinc transport and zinc storage will be discussed.

THE ROLE OF ZINC IN MODULATING SYNAPTIC FUNCTION

There are several important aspects associated with zinc depletion; supplementation and delivery of zinc to the brain. Experimentally, zinc deficiency is reached by partaking in a diet that contains 0.5 mgZn/kg–6 mgZn/kg for at least 2–4 weeks (Tamano et al., 2009; Mlyniec et al., 2012). Zinc overdoses can be obtained at 100 mgZn/kg–180 mgZn/kg (Yang et al., 2013). The most common way for assessing the zinc level is by measuring the serum or plasma zinc. Unfortunately, elevated or lowered serum zinc does not correspond with the elevated or lowered brain zinc total. This suggests that the brain zinc total is strictly controlled and may not be easily influenced by peripheral zinc level. The other problem is the lack of sensitive methods to measure alterations in the extra or intracellular zinc levels. Available data indicates that the hippocampus seems to be the most responsive both to the deficiency as well as an overdose of zinc (Takeda et al., 2005; Suh et al., 2009; Yang et al., 2013). Because the hippocampus is the region of the brain which plays a critical role in memory, learning and neurogenesis, the impact of zinc deficiency or zinc supplementation on these processes will be critical. Indeed, it was found that a zinc deficient diet, decreases the number of progenitor cells and immature neurons in the dentate gyrus (DG) in rodents and that reversal to a normal diet containing zinc restored a number of these cells (Gao et al., 2009; Suh et al., 2009). Reduced progenitor cells were also found after zinc chelator treatment and in ZnT3 KO mice (a lack of zinc in the synaptic vesicles) (Suh et al., 2009). A growing body of evidence indicates that dietary zinc deficiency influences hippocampal learning and memory in an age-dependent manner. It was found that a decrease in dietary zinc during early development produces an irreversible deficit of learning and memory, while zinc deficient induced impairments in young adult rats can be reversed by feeding them with an adequate diet (Takeda, 2000; Keller et al., 2001). Recent data published by Gao et al. (2009, 2011) showed that the zinc-deficiency induced hippocampal learning and memory impairments is in part due to the disruption of the calmodulin (CaM), CaM-dependent protein kinase II (CaMKII), and cAMP-responsive element binding protein (CREB) signaling pathway. As was mentioned above zinc was found to modulate neural transmission through the GPR39 Zn2+-sensing receptor. Recent studies showed a significant reduction in the GPR39 protein level in the frontal cortex in mice receiving the zinc deficient diet (Mlyniec et al., 2013). This study provides evidence that the GPR39 Zn2+-sensing receptor may be involved in the pathomechanism of depression. This hypothesis was further supported by data indicating the up-regulation of the GPR39 receptor after chronic antidepressant treatment (Mlyniec and Nowak, 2013).

The other mechanism by which zinc can modulate synaptic functions is the transactivation of the tropomyosin-related kinase B (TrkB) receptor and activation of brain-derived neurotrophic factor BDNF signaling in a neurotrophic—independent manner (Huang et al., 2008). Zinc can affect BDNF signaling also by promoting the maturation of pro-BDNF to BDNF throughout the activation of metalloproteinases (MMPs) (Hwang et al., 2005).

Zinc also appears to have an effect of oxidative stress. It was found that both high and extremely low concentrations of zinc are associated with increased oxidative and nitrosative stress [by increasing the expression of neuronal nitric oxide synthase (nNOS) and NADPH oxidase] (Noh and Koh, 2000; Aimo et al., 2010), however, intermediate concentrations was found to be neuroprotective (Aimo et al., 2010). This demonstrates once again the importance of zinc homeostasis in normal brain function. Although, the effect of zinc deficiency on the brain zinc homeostasis and learning and memory has been well studied, the effect of a zinc overdose on these processes is poorly described and the data are rather conflicting. First, the effect of zinc supplementation on learning and memory impairments is dose dependent. Generally, zinc supplementation in a low dosage seems to improve the performance of animals in spatial memory tasks (Piechal et al., 2012) or the contextual discrimination task (Yang et al., 2013). However, memory deficits in rats after low dose of zinc supplementation were also observed (Flinn et al., 2005; Railey et al., 2010). Interestingly Yang et al. (2013) reported that zinc supplementation in high doses induce a dramatic decrease in hippocampal zinc levels, especially in the CA3 and DG, and impaired learning and memory due to a decreased availability of synaptic zinc and BDNF deficits.

ZINC AND DEPRESSION

Depression is a common mental disorder associated with functional impairment, significant disability, morbidity and mortality. Despite the extensive research that has so far been carried out on depression, its pathophysiology is still poorly understood. One of the many hypotheses proposed for depressive disorder indicates that depression is characterized by an enhanced neurodegeneration and decreased neurogenesis (Maes et al., 2009). On the other hand, there is increasing evidence linking depression or depression-related changes in brain function or cognitive performance to zinc ion availability.

ZINC LEVELS IN DEPRESSION (TABLE 1)

Clinical studies demonstrate significantly lower serum zinc levels in patients suffering from major depression or unipolar depression than that in non-depressed patients (McLoughlin and Hodge, 1990; Maes et al., 1994, 1997; Nowak et al., 1999). In some

patients, a negative correlation between the serum zinc level and severity of depression was found (Maes et al., 1994; Nowak et al., 1999). A lower serum zinc level was also found to accompany antepartum and postpartum depression. In this study the level of zinc was also negatively correlated with the severity of depressive symptoms (Wojcik et al., 2006). Low serum zinc levels have also been noted in depressed patients with end-stage renal disease undergoing hemodialysis (Roozbeh et al., 2011). Moreover, treatment-resistant depressed patients have been shown to exhibit much lower serum zinc concentrations than their non-treatment resistant depressed counterparts (Siwek et al., 2010). Thus far, only two studies have reported no differences in the zinc level between depressed and non-depressed patients (Narang et al., 1991; Irmisch et al., 2010). The paper published by Irmisch et al. (2010), however indicated that zinc concentrations might differ dependent on comorbid disorders and severity of depression. Similarly, Narang et al. (1991) reported no significant difference between control and depressed patients, however, they found that the values were significantly higher in recovered patients compared to patients with depression. Although these results do not confirm the general hypothesis of a lack of zinc in depressive disorders, however favor the existence of correlation between severity of depression or status of patients and zinc concentration.

ZINC DEFICIENCY AND DEPRESSION

There is a paucity of clinical studies that have examined the relationship between dietary zinc intake and depressive symptoms (**Table 1**). One study, carried out by Amani et al. (2010) showed that both daily zinc intake and the serum zinc levels in young depressed women were about two thirds of that observed in healthy volunteers. Moreover, an inverse correlation was found between serum zinc concentrations and depression scale scores. In another study, conducted among a group of pregnant women, the relationship between dietary zinc intake, psychosocial stress and sociodemographic factors and depression was examined. Analysis of the results showed that lower zinc intake, higher stress and social disadvantage were associated with the occurrence of depressive symptoms, which were in turn attenuated by higher zinc intake (Roy et al., 2010). Data coming from animal studies further support the hypothesis that a deficiency in zinc can lead to the induction of depressive behavioral symptoms (**Table 1**). Studies have shown that zinc-deficient mice exhibit an increased immobility time in the forced swim test (FST) and tail suspension test (TST) (Whittle et al., 2009; Mlyniec and Nowak, 2012; Mlyniec et al., 2012). Pro-depressive-like behavior (increased immobility in the FST or anhedonia) was also found in rats subjected to zinc-deprivation (Tassabehji et al., 2008; Tamano et al., 2009; Watanabe et al., 2010)

ZINC TREATMENT/SUPPLEMENTATION IN DEPRESSION

Some clinical studies have shown the beneficial effect of zinc supplementation in the treatment of depression (**Table 1**). One such study by Nowak et al. (2003a), was conducted in depressed patients, treated with tricyclic antidepressants and selective serotonin reuptake inhibitors supplemented with zinc or a placebo. Analysis of the Hamilton Depression Rating Scale (HDRS) and Beck Depression Inventory (BDI) scores revealed that patients who received the zinc supplementation of antidepressant treatment displayed much lower scores than patients treated with placebos and antidepressants. A beneficial effect of zinc as an adjunct agent was also found in treatment-resistant patients (Siwek et al., 2009). In this placebo-controlled, double blind study patients were randomized into two groups: the first were treated with imipramine and received one daily placebo and the second were treated with imipramine supplemented with zinc. It was found that zinc supplementation significantly reduced the depression scores [measured by Clinical Global Impression (CGI); Montgomery-Asberg Depression Rating Scale (MADRS); BDI and HDRS] and facilitated the effect of the treatment in antidepressant treatment resistant patients. No significant differences in the CGI, MADRS, BDI, and HDRS scores were demonstrated between zinc and placebo- supplemented antidepressant treatment non-resistant patients. The benefit of zinc supplementation in patients with major depression has been recently reported by Ranjbar et al. (2013). This randomized, double-blind, placebo-controlled trial is the next clinical study indicated that zinc supplementation in conjunction with antidepressants might be beneficial for reducing depressive symptoms.

The other study published by Sawada and Yokoi (2010) showed that young women taking multivitamins and zinc supplements exhibited a significant reduction in depression and anxiety symptoms than women taking only multivitamins. In 2012, Sandstead published the results from six randomized controlled comparative treatment experiments in Chinese and Mexican-American low-income children, aged 6–9 years; middle-income US premenopausal women; middle income US adolescents and middle-income US men, illustrating that subclinical zinc deficiency changes the brain function and that zinc and micronutrient treatment improves altered brain functions (Sandstead, 2012). Two studies have so far shown no effect of zinc supplementation on the improvement of depressive symptoms (Nguyen et al., 2009; DiGirolamo et al., 2010). However, these studies differ significantly from that previously described with respect to both the patients and the length and quality of applications. The first study by DiGirolamo et al. (2010) examined the effect of six months of zinc supplementation on the mental health of school-age children. The second study, investigated the impact of combinations of micronutrient supplements on symptoms of depression rather than effect of zinc supplementation as a stand-alone. Because of these methodological limitations in existing studies, further well-designed, adequately powered research is required.

The beneficial effects of zinc treatment have been also reported in preclinical studies (**Table 1**). Zinc administration induced an antidepressant-like effect (reduction in immobility time) in both the FST and TST (Kroczka et al., 2000, 2001; Nowak et al., 2003b; Rosa et al., 2003; Cunha et al., 2008; Franco et al., 2008). Zinc was also active in different models of depression. In the olfactory bulbectomy (OB) a reduction in the number of trials in the passive-avoidance test and a decreased OB-induced hyperactivity in rats after zinc treatment was observed (Nowak et al., 2003b). While in the chronic mild stress (CMS) model of depression; zinc reversed the CMS-induced reduction in the consumption of sucrose in rats (Sowa-Kucma et al., 2008). In chronic unpredictable stress (CUS) in turn, zinc treatment prevented deficits

Table 1 | Summary of the main clinical and preclinical findings supporting the involvement of zinc in depression.

Serum/plasma zinc status—human data	References
↓ Major depressed patients; negative correlation between the serum zinc and severity of depression	Maes et al., 1994; Nowak et al., 1999
↓ Depressed patients vs. control	Siwek et al., 2010
↓ Patients with affective disorders	McLoughlin and Hodge, 1990
↓ Women with antepartum and postpartum depressive symptoms	Wojcik et al., 2006
↓ Depressed patients with end-stage renal disease undergoing hemodialysis	Roozbeh et al., 2011
↔ Depressed patients; zinc concentrations differ dependent on comorbid disorders and severity of depression	Irmisch et al., 2010
↔ Depressed patients; significantly higher zinc level in recovered patients compared to patients with depression	Narang et al., 1991
Effect of zinc deficiency—human study	
Correlation between dietary zinc intake and the serum zinc concentrations; the inverse correlation between serum zinc levels and depression scales	Amani et al., 2010
Zinc intake moderates the association between stress and depressive symptoms	Roy et al., 2010
Effect of zinc deficiency—animal study	
↑ Immobility time in FST in rats	Tassabehji et al., 2008; Tamano et al., 2009; Watanabe et al., 2010
↑ Immobility time in FST in mice	Whittle et al., 2009; Mlyniec et al., 2012
↑ Immobility time in TST in mice	Whittle et al., 2009; Mlyniec and Nowak, 2012
↓ Saccharin preference in rat	Tassabehji et al., 2008
Effect of zinc treatment/supplementation in depression—clinical trials	
Zinc supplementation (25 mg/12 weeks) significantly reduced scores in HDRS and BDI when compared with placebo treatment	Nowak et al., 2003a
Zinc supplementation (25 mg/12 weeks) augments the efficacy and speed of onset of therapeutic response to imipramine treatment, particularly in patients previously non-responsive to antidepressant pharmacotherapies	Siwek et al., 2009
Zinc supplementation (25 mg/12 weeks) significantly reduced HDRS compared to placebo	Ranjbar et al., 2013
Women who took multivitamins and zinc (7 mg/10 weeks) showed a significant reduction in anger-hostility score and depression-dejection score in the Profile of Moods State (POMS)	Sawada and Yokoi, 2010
Zinc deficiency changes the brain function but zinc and macronutrient treatment improves altered brain functions	Sandstead, 2012
Zinc supplementation (10 mg/6 months) did not induce differences in mental health outcomes between zinc and placebo groups, however, increases in serum zinc concentrations were associated with decreases in internalizing symptoms (depression and anxiety)	DiGirolamo et al., 2010
No effect of zinc supplementation on the improvement of depressive symptoms	Nguyen et al., 2009
Effect of zinc treatment—animal study	
↓ In immobility time in both FST and TST	Kroczka et al., 2000, 2001; Nowak et al., 2003b; Rosa et al., 2003; Cunha et al., 2008; Franco et al., 2008
↓ Reduction in the number of trials in the passive-avoidance test in OB model; ↓ OB- induced hyperactivity in open field test in OB model	Nowak et al., 2003b
Zinc reversed the CMS-induced reduction in the consumption of sucrose	Sowa-Kucma et al., 2008
Zinc prevented deficits in the fighting behavior in CUS model	Cieslik et al., 2007
Zinc intensifies the effects of standard antidepressants in FST, TST, and CUS	Szewczyk et al., 2002, 2009; Rosa et al., 2003; Cieslik et al., 2007; Cunha et al., 2008

in the fighting behavior of chronically stressed rats (Cieslik et al., 2007). Moreover, zinc has been found to intensify the effects of standard antidepressants (IMI, fluoxetine, paroxetine, bupropion, or citalopram) in the FST, the TST, and CUS (Szewczyk et al., 2002, 2009; Rosa et al., 2003; Cieslik et al., 2007; Cunha et al., 2008).

Presented above data strongly indicated the importance of zinc deficiency in human depression and indicated the benefit

of zinc supplementation in both the efficacy and the speed of the therapeutic response to antidepressants treatment. Thus, the understanding of the mechanisms involved in the antidepressant activity of zinc might contribute to the development of a new therapeutic strategy for the treatment of depression or depression-related diseases. Published so far data points out that the modulation of glutamatergic neurotransmission (via the NMDA or AMPA glutamate receptors), serotonergic transmission (especially via the 5-HT1A receptor) and regulation of BDNF level seems to be the most important interactions involved in the antidepressant-like activity of zinc (Nowak et al., 2004; Sowa-Kucma et al., 2008; Cichy et al., 2009; Szewczyk et al., 2009, 2010).

ZINC AND ALZHEIMER'S DISEASE

AD is a chronic neurodegenerative disorder and the most common cause of dementia. It is estimated that AD represents 60–80% of all dementia cases (Daviglus et al., 2010). The clinical features of AD vary from stable performance and cognitive health with only a gradual decline in the short-term memory to a serious state of cognitive impairment and into different forms of dementia (deterioration of memory, learning, orientation) (Daviglus et al., 2010). On the other hand the pathological features of AD is the accumulation of β-amyloid (Aβ) and the aggregation of Aβ is suggested as the cause of neurodegeneration observed in AD (Small and Cappai, 2006).

Although the key role of Aβ in the pathogenesis of AD is strongly established now, the mechanism by which Aβ induces toxicity or the causes and factors associated with the risk or progression of AD is still poorly understood. One of the several hypotheses proposed for the pathophysiology of AD is the trace elements hypothesis, with zinc taking the center stage. Zinc was first described as a possible factor leading to dementia by Burnet (1981) and, since then, the knowledge base regarding the role of zinc in the pathogenesis and therapy of AD has evolved rapidly.

ZINC LEVELS IN AD

Serum, cerebrospinal fluid (CSF) and brain zinc levels have been investigated in patients diagnosed with AD (**Table 2**). Several of these studies investigating serum zinc levels have shown either divergent data with no differences (Shore et al., 1984; Haines et al., 1991), a significant decrease (Jeandel et al., 1989; Baum et al., 2010; Brewer et al., 2010; Vural et al., 2010) or a significant increase (Gonzalez et al., 1999; Rulon et al., 2000) when compared to matched controls. The main problem associated with these clinical studies is that different methodologies and different selections of patients were used meaning that the end result could account for the various divergent data obtained in the studies. Studies looking at CSF zinc levels also showed some discrepancies. For instance, Hershey et al. (1983) and Sahu et al. (1988) found no differences in CSF levels of zinc in patients with dementia of the Alzheimer type relative to a matched group of healthy controls. In contrast, Molina et al. (1998) found a significant decrease in CSF zinc levels in AD patients than the control subjects.

Studies investigating zinc content in brain tissue suggests that an alteration in the zinc level seems to be fraction/region specific. Studies involving whole tissue samples have shown no differences in brain zinc levels between AD and the control subjects. Although, some alterations in the brain zinc levels were found when tissue was sub-fractionated (a decrease in nuclear but not in mitochondrial or microsomal fractions) or when different brain regions were analyzed separately (Wenstrup et al., 1990). Indeed, decreased zinc levels have been found in the neocortex, medial temporal gyrus, thalamus, and hippocampus (Corrigan et al., 1993; Panayi et al., 2002), whilst increased levels were found in the amygdala, hippocampus, cerebellum, olfactory areas and superior temporal gyrus (Thompson et al., 1988; Samudralwar et al., 1995; Danscher et al., 1997; Religa et al., 2006). The above mentioned data, even though inconsistent, strongly support the hypothesis that a deregulated zinc homeostasis is involved in the pathophysiology of AD.

ROLE OF ZINC IN AD—POSSIBLE MOLECULAR MECHANISMS

Amongst all the multiple roles of zinc in the pathogenesis of AD, the most widely studied is the involvement of zinc in the accumulation of Aβ. Post-mortem studies using different imaging techniques for zinc analysis have demonstrated significant increases in zinc levels in neuropil and plaques present in the brain of AD patients when compared to normal age-matched controls (Lovell et al., 1998; Suh et al., 2000; Dong et al., 2003; Miller et al., 2006). On the other hand, lack of synaptic zinc prevents Aβ deposition (Lee et al., 2002).

Aβ is the product of proteolytic cleavage from the amyloid precursor protein (APP) by the enzyme known as β-secretase or β-site APP cleaving enzyme-1 (BACE-1) (Masters et al., 1985). Several pathways for the involvement of zinc in APP processing or Aβ aggregation has been suggested. It was found that APP synthesis is regulated by zinc-containing transcription factors NF-$_{\kappa}$B and sp1 (Grilli et al., 1996). Zinc is also involved in processing of APP protein (Lee et al., 2009). The processing of APP relies on a number of activities by enzymes secretases (α-,β-, and γ-). The predominant route by which APP is processed in the brain is cleavage by the α-secretase, within the Aβ region, producing sAPP (soluble amyloid precursor peptide) (Ling et al., 2003). Further processing by the β-secretase and γ-secretase leads to the formation of Aβ peptide (Wilquet and De, 2004). It was found that APP contains a specific zinc binding site localized in the cysteine-rich region of the APP ectodomain (spanning the α-secretase position) (Bush et al., 1994b) and it is suggested that changes in the intracellular concentration of zinc may influence the relative activities of APP secretases (Bush et al., 1994a,b). However, is worth mentioning that zinc is clearly not the only factor influencing APP processing and its role has not been fully determined.

Recent evidence suggests that oxidative stress is an additional factor contributing to the progression of AD (Butterfield et al., 2001; Jomova et al., 2010) and that ROS or exogenous oxidants are able to promote a harmful zinc release from MTs (Aizenman et al., 2000; Bossy-Wetzel et al., 2004). Zinc accumulation can in turn, induce mitochondrial dysfunction and further ROS generation (Sensi et al., 2003). Results presented by Sensi et al. (2008) indicate that such ROS-dependent intraneuronal zinc rises are particularly high in AD neurons expressing mutant APP, presenilin-1 (PS-1) and tau (Sensi et al., 2008).

Table 2 | Summary of the main clinical and preclinical findings supporting the involvement of zinc in AD.

Serum/CSF/brain zinc status—human data	References
↔ Serum zinc level in patients with and without cognitive impairment in the community	Haines et al., 1991
↔ Serum and hair zinc concentration in patients with AD	Shore et al., 1984
↑ Serum zinc level in AD epsilon 4 apoE allele carriers	Gonzalez et al., 1999
↑ Zinc serum in AD subjects compared with age-matched control subjects-postmortem study	Rulon et al., 2000
↓ Serum zinc level in AD patients	Baum et al., 2010
↓ Blood zinc in patients with AD than in controls	Brewer et al., 2010
↓ Serum zinc level in patients with senile dementia of the Alzheimer type (SDAT) when compared to control subjects	Jeandel et al., 1989
↓ Plasma zinc level in patients with AD compared with controls	Vural et al., 2010
↔ In CSF zinc level in patients with dementia of the Alzheimer type	Hershey et al., 1983; Sahu et al., 1988
↓ CSF zinc levels in AD patients as compared with controls	Molina et al., 1998
↓ Hippocampal zinc concentration in patients with AD—postmortem study	Corrigan et al., 1993
↓ Zinc level in both hemispheres of the superior frontal gyrus, the superior parietal gyrus, the medial temporal gyrus, the hippocampus and the thalamus in the AD patients—postmortem study	Panayi et al., 2002
↑ Zinc level in hippocampus and amygdala in AD patients—postmortem study	Danscher et al., 1997
↑ Tissue zinc in the AD-affected cortex compared with the control group	Religa et al., 2006
↑ Zinc in olfactory regions of AD patients as compared to control subjects	Samudralwar et al., 1995
↑ Zinc in amygdala and hippocampus in AD patients as compared to controls	Thompson et al., 1988
Zinc dyshomeostasis as a new therapeutic target in AD—animal study	
Administration of DP-109 (the lipophilic metal chelator) reduced the aggregation of Aβ protein and deposition of amyloid plaques in aged female hAbetaPP-transgenic Tg2576 mice, compared to animals receiving vehicle treatment	Lee et al., 2004
Clioqunol (metal chelator) reduced zinc accumulation in the neuritic plaques and inhibit amyloidogenic AβPP processing in the AβPP/PS1 mouse brain	Wang et al., 2012
Carnosine supplementation in 3 × Tg-AD mice promotes a strong reduction in the hippocampal intraneuronal accumulation of Aβ and completely rescues AD and aging-related mitochondrial dysfunctions	Corona et al., 2011
Selective intracellular release of zinc ions from bis(thiosemicarbazonato) complexes reduces levels of Alzheimer disease amyloid-beta peptide	Donnelly et al., 2008
Presenilins are important for cellular zinc turnover and has the potential to indirectly impact β-amyloid aggregation through metal ion clearance	Greenough et al., 2011
Zinc supplementation delays hippocampal-dependent memory deficits and reduces both Aβ and tau pathology in the hippocampus	Corona et al., 2010
Zinc dyshomeostasis as a new therapeutic target in AD—human study	
PBT2 (copper/zinc ionophore) lowered CSF levels of Aβ and significantly improved cognition in AD patients	Lannfelt et al., 2008; Faux et al., 2010

ZINC DYSHOMEOSTASIS AS A NEW THERAPEUTIC TARGET IN AD

Considering the fact that zinc contributes to the aggregation of the Aβ protein and deposition of amyloid plaques in AD, research has been focused on the use of metal complexation ability as therapeutic agents in AD (**Table 2**). Indeed, the metal chelator, clioquinol (CQ) and zinc modulator—DP-109 were found to significantly decrease the formation of amyloid plaques in the brains of APP/PS1 double transgenic mice and aged female hAbetaPP-transgenic Tg2576 mice, respectively (Lee et al., 2004; Wang et al., 2012). Faux et al. (2010) describes a successful phase 2 clinical trial of the quinoline derivative, PBT2 in AD (Faux et al., 2010). This randomized, placebo controlled trial found that this metal-protein attenuating compound (MPAC) that affects the Cu2(+)-mediated and Zn2(+)-mediated toxic oligomerization of Abeta seen in AD significantly lowered the CSF levels of Aβ and significantly improved cognition in AD patients (Lannfelt et al., 2008; Faux et al., 2010). The other possible therapeutic compound for AD, suggested recently is carnosine—a

peptide with cooper/zinc chelating properties (Trombley et al., 1998). Corona et al. (2011) found that dietary supplementation of carnosine reduces hippocampal intraneuronal accumulation of Aβ and rescues mitochondrial dysfunctions in triple-transgenic AD mice (3 × Tg-AD) but does not affect the development of the tau pathology and only slightly reduces cognitive deficits (Corona et al., 2011). Furthermore, the paper of Donnelly et al. (2008) demonstrated the beneficial effect of the selective intracellular delivery of zinc using bis(thiosemicarbazonato) complexes in the reduction of the extracellular levels of Aβ and suggested the role of these metal-loaded compounds as potential therapeutic agents for AD (Donnelly et al., 2008). In turn Greenough et al. (2011) reported that presenilin, which mediates the proteolytic cleavage of the β-amyloid precursor protein to release β-amyloid, is important for cellular cooper/zinc turnover and has the potential to indirectly impact on amyloid aggregation through zinc ion clearance (Greenough et al., 2011).

Interestingly, a delay in hippocampal-dependent memory deficits and reduction of both the Aβ and tau pathology in the hippocampus in 3 × Tg-AD mice was also observed after zinc supplementation (Corona et al., 2010). This study also indicated the involvement of the BDNF-tyrosine kinase type B (TrkB) receptor pathway in the mechanism of the beneficial effect of zinc supplementation in this AD model (Corona et al., 2010).

All of these data further emphasizes the integral role of zinc in the mechanism of AD and support the hypothesis that restoring zinc homeostasis might be beneficial in the treatment of AD, although it also indicated the complex interactions between AD and zinc.

ROLE OF METALLOTHIONEINS AND ZINC TRANSPORTERS IN AD

There are several proteins/pathways that interact with zinc and that are also relevant to AD (**Figure 1**). One of these is MT. As noted earlier, MTs are zinc- binding proteins involved in the regulation of the transport, storage and transfer of zinc to various enzymes and transcription factors (Liuzzi and Cousins, 2004; DiGirolamo et al., 2010). The involvement of MTs in the regulation of zinc homeostasis makes it important in the context of the Zinc hypothesis of AD. Indeed, there are a number of studies that have reported increases, decreases and no change in MT isoforms in the brain. The study published by Adlard et al. (1998) showed a significant increase in MT I/II in the gray matter of preclinical AD cases when compared to non-AD cases. The authors suggested that the increase in MT I/II might be associated with the initial stages of AD processes due to the oxidative stress or alterations in the metabolism of heavy metals (Adlard et al., 1998). MT-III, also known as the growth inhibitory factor (GIF) was found to be down-regulated in the AD cortex (Uchida et al., 1991; Tsuji et al., 1992; Cuajungco and Lees, 1997; Yu et al., 2001), although no changes in the MT-III level in AD was also observed (Erickson et al., 1994; Amoureux et al., 1997). These discrepancies across different studies may result from the stage of the disease or cellular zinc status. Another important group of proteins involved in the homeostasis

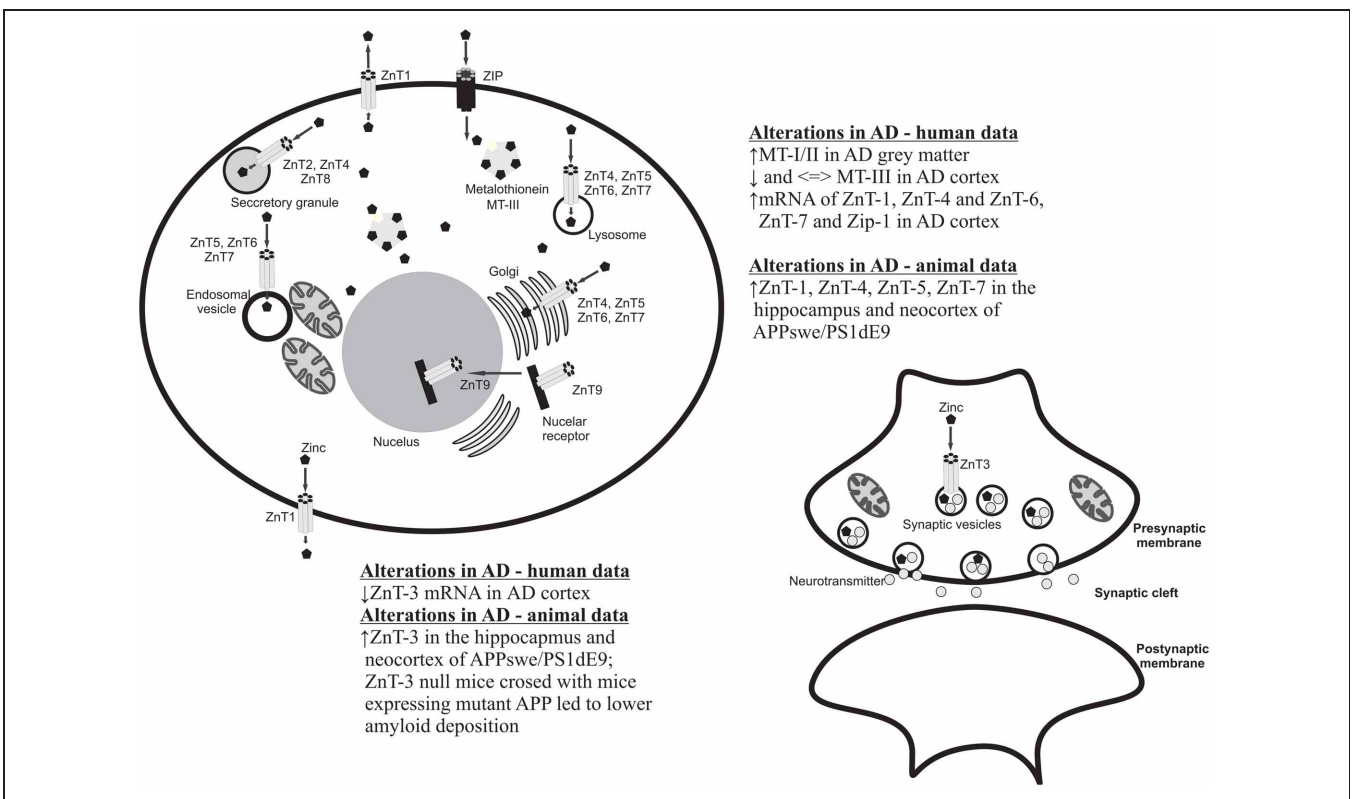

FIGURE 1 | Cellular localization of zinc transporters and metallothioneins and summary of the main clinical and preclinical findings supporting the involvement of these proteins in AD.

of zinc and the pathogenesis of AD are zinc transporter (ZnTs) proteins. Zhang et al. (2008) showed that six ZnTs such as: 1–7 are extensively present in the Aβ, being therefore positive plaques in the cortex of human AD brains. Recent studies showed alterations in levels of ZnTs proteins in the brain of subjects diagnosed with the preclinical stage of AD (PCAD), mild cognitive impairment (MCI), early (EAD), and the late (LAD) stage of AD when compared to the control subjects (Lovell et al., 2005, 2006; Smith et al., 2006; Lovell, 2009; Lyubartseva et al., 2010; Lyubartseva and Lovell, 2012). Human postmortem brain tissue from Braak-staged individuals with AD displayed a reduced expression of ZnT-3 mRNA (Beyer et al., 2009) and increased mRNA levels of the other more established zinc transporters, such as LIV1, ZIP1, ZnT1, ZnT6 in the AD cortex (Beyer et al., 2012). Also animal studies have linked dyshomeostasis in the brain zinc level to the pathogenesis and progression of AD. Lee et al. (2002) using a ZnT-3 null mice crossed with mice expressing mutant APP showed that the absence of synaptic zinc reduces the plaque load and increases the ratio of soluble/insoluble Aβ species. As such, this data suggested that synaptic zinc plays a key role in Aβ aggregation and plaque accumulation. Other studies also reported that with aging, female mice exhibit higher levels of synaptic, insoluble Aβ and plaques than males and that these sex differences disappeared in ZnT-3 knockout mice, correlating with the well described age-adjusted increase incidence for AD in females rather than males (Katzman et al., 1989). Recent studies published by Zhang et al. (2010) showed significant increases of ZnT-1, ZnT-3, ZnT-4, ZnT-6, and ZnT-7 in the hippocampus and neocortex of APPswe/PS1dE9 transgenic mice which corresponding to a form of early onset AD. Lang et al. (2012) in turn demonstrated that over-expression of Drosophila homolog of human Zip1 results in zinc accumulation in Aβ42–expressing fly brains and that inhibition of Zip1 expression induces a reduction of Aβ42 fibril deposits and improves cognition (Lang et al., 2012).

ZINC IN BRAIN AGING

Aging is an inevitable process associated with progressive pathological features such as: oxidative stress, altered cell metabolism, damaged of nucleic acid, or deposition of abnormal forms of proteins. In the brain aging is characterized by neuronal loss, cognitive impairment, and susceptibility to neurological disorders (Mocchegiani et al., 2005).

Recent progress in studies involving age related processes provide evidence that changes occurring in the brain during aging are related to zinc homeostasis and that zinc deficiency is a common cause of morbidity among the elderly (Mocchegiani et al., 2005). In aging, zinc deficiency is usually the result of an inadequate zinc dietary intake. It has been reported that only 40% of elderly people have a sufficient intake of zinc (Andriollo-Sanchez et al., 2005; Mocchegiani et al., 2008). Studies comparing old and young mice fed with low dietary zinc indicated that zinc is an important nutritional factor for a proper inflammatory/immune response (Kelly et al., 1996). Accordingly, zinc has anti-inflammatory properties and a low zinc status is associated with increased susceptibility to infection plus intracellular zinc has been found to play a key role in signaling in immune cells (Haase and Rink, 2009; Hasan et al., 2012). On the other hand aging is characterized by the progressive dysregulation of immune responses. Therefore, zinc has been suggested as a good factor in providing the remodeling of some age-associated changes and also as leading to healthy ageing through the reduction of inflammation (Kahmann et al., 2008). The study by Wong et al. (2013) suggests that age-related epigenetic dysregulation in ZnT expression may change cellular zinc levels and increase inflammation with age. They found that reduced Zip6 expression enhanced proinflammatory responses and that this age-induced Zip6 dysregulation correlated with an increased Zip6 promoter methylation. Interestingly, dietary supplementation reduced aged-associated inflammation (Wong et al., 2013). The other mechanism linking age, zinc and inflammation is associated with MTs. It was found that ageing is associated with a higher MT expression and consequently, low availability of intracellular zinc for normal immune responses. On the other hand, the supplementation of zinc in aging improves immune function and leads to decreased mortality from infections (Mocchegiani et al., 2010). In another study, Mocchegiani et al. (2011) showed evidence that zinc deficiency and an altered immune response is more evident in people with a polymorphism in IL-6 and metal-response element binding transcription factor-1 (MT1A) and that these individuals will benefit more from zinc supplementation.

CONCLUSIONS

From the foregoing results, it is obvious that zinc homeostasis may play a major role in the initiation and propagation of the pathological features of psychiatric and neurodegenerative disorders. However, more studies are needed to explain the exact mechanisms linking zinc and processes related to these diseases.

First, since zinc deficiency is prevalent in patients with psychiatric and neurodegenerative disorders, the appropriate preventive measures should be considered especially in the elderly. Conversely, even if the beneficial effects of zinc supplementation were reported either in treatment or in the prevention of depressive or aging symptoms, zinc supplement users should be overly cautious and avoid overdosing.

Some of the studies presented above suggest that zinc can be useful not only by itself but in combination with other drugs used in treatment. Other important aspects in the context of zinc and treatment of patients are metal chelating drugs, for which the positive effect was particularly emphasized in AD. The weakness in most of these drugs, however, are the side effects caused by the chelation of other important divalent metal ions in the brain. Chelation should thus be used only when the brain zinc level is expected to have neurotoxic effects.

Recently, the zinc-homeostasis regulating proteins such as transporters and MTs have been gaining more prominence in related literature indicating they may be very important players in the pathophysiology of neurodegenerative disorders. Therefore, more studies are needed to fully understand the influence of peripheral zinc deficiency or an overdose on these proteins.

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Manganese neurotoxicity: new perspectives from behavioral, neuroimaging, and neuropathological studies in humans and non-human primates

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Manganese (Mn) is an essential metal and has important physiological functions for human health. However, exposure to excess levels of Mn in occupational settings or from environmental sources has been associated with a neurological syndrome comprising cognitive deficits, neuropsychological abnormalities and parkinsonism. Historically, studies on the effects of Mn in humans and experimental animals have been concerned with effects on the basal ganglia and the dopaminergic system as it relates to movement abnormalities. However, emerging studies are beginning to provide significant evidence of Mn effects on cortical structures and cognitive function at lower levels than previously recognized. This review advances new knowledge of putative mechanisms by which exposure to excess levels of Mn alters neurobiological systems and produces neurological deficits not only in the basal ganglia but also in the cerebral cortex. The emerging evidence suggests that working memory is significantly affected by chronic Mn exposure and this may be mediated by alterations in brain structures associated with the working memory network including the caudate nucleus in the striatum, frontal cortex and parietal cortex. Dysregulation of the dopaminergic system may play an important role in both the movement abnormalities as well as the neuropsychiatric and cognitive function deficits that have been described in humans and non-human primates exposed to Mn.

Keywords: manganese, neurotoxicity, Parkinson's disease, dopamine, motor function, cognitive function, working memory

INTRODUCTION

Manganese (Mn) is an essential trace metal that is required for a number of enzymes important for normal cellular functions (Aschner and Aschner, 2005). However, excess accumulation of Mn in the brain results in a neurological syndrome with cognitive, psychiatric and motor abnormalities (Pal et al., 1999; Olanow, 2004; Perl and Olanow, 2007; Guilarte, 2010). Following excess exposure to Mn, the highest concentrations of Mn in the brain occur in the basal ganglia, specifically in the globus pallidus, caudate/putamen, and substantia nigra (Dorman et al., 2006; Guilarte et al., 2006a). These same studies have shown that Mn also accumulates in other brain structures within the cerebral cortex and in white matter (Dorman et al., 2006; Guilarte et al., 2006a). The accumulation of Mn in the basal ganglia is likely to be responsible for a form of parkinsonism with overlapping, but distinct clinical features with those seen in idiopathic Parkinson's disease (PD) (see below). Recently, there has been a great deal of debate in the scientific literature regarding the possibility that Mn may have an etiological role in idiopathic PD or accelerate the expression of PD (Racette et al., 2001, 2005). From a different perspective during the last decade there is mounting experimental evidence that exposure to Mn, at lower doses than those needed to produce motor function deficits, has a significant effect on executive function and cognition (Klos et al., 2006; Schneider et al., 2006, 2009; Roels et al., 2013). In this review, I examine the available evidence from human and non-human primate studies on the impact of elevated Mn exposures and its effects on motor function and cognitive domains.

MANGANESE-INDUCED PARKINSONISM

The first description of Mn-induced parkinsonism goes back to 1837 when Couper provided the sequelae of workers employed in the grinding of Mn oxide ore (Couper, 1837). In more modern times, there has been a number of reports describing clinical expression of parkinsonism in occupationally exposed workers (Mena et al., 1967; Cook et al., 1974; Huang, 2007; also see studies in Perl and Olanow, 2007 and in Guilarte, 2010) with clear evidence that excess exposures to Mn produces motor function deficits in humans and non-human primates that resemble some aspects to those expressed in idiopathic PD (Perl and Olanow, 2007; Guilarte, 2010) as well as more subtle effects on motor function, specifically fine motor control depending upon the level of exposure (Perl and Olanow, 2007; Guilarte, 2010). However, there are clear differences between Mn-induced parkinsonism and idiopathic PD from a clinical perspective and in the underlying neuropathology (Perl and Olanow, 2007; Guilarte, 2010) (see next section).

The most compelling human evidence of Mn-induced parkinsonism in the last decade comes from a very unfortunate human experiment in which young drug users inject very high levels

of Mn from use of home-made psychostimulant preparations (ephedron, also called methcathinone) (de Bie et al., 2007; Meral et al., 2007; Sanotsky et al., 2007; Sikk et al., 2007, 2010, 2013; Selikhova et al., 2008; Stepens et al., 2008, 2010; Varlibas et al., 2008; Colosimo and Guidi, 2009; Yildirim et al., 2009; Iqbal et al., 2012). These cases of young drug users with clinical parkinsonism as a result of drug abuse are reminiscent of young addicts injecting 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) and expressing clinical parkinsonism in the early 1980s (Langston et al., 1983). The ephedron home-made preparations is the result of using potassium permanganate to oxidize ephedrine or pseudoephedrine and it is injected with minimal purification; thus, users inject very high doses of Mn. These ephedron users exhibited clinical parkinsonism that is not responsive to L-dopa therapy (Sanotsky et al., 2007; Selikhova et al., 2008; Stepens et al., 2008; Colosimo and Guidi, 2009; Sikk et al., 2013). This clinical observation suggests that the underlying neurobiology associated with Mn-induced parkinsonism is different from the well-recognized loss of dopamine neurons in the substantia nigra pars compacta (SNpc) that is responsive to L-dopa therapy in idiopathic PD patients (Savitt et al., 2006) and in MPTP subjects (Forno et al., 1993; Forno, 1996) and MPTP exposed non-human primates (Nerastet et al., 1994).

The etiological role of Mn in producing the motor function deficits in these relatively young ephedron users can be confirmed by the extremely high levels of Mn measured in their blood (Selikhova et al., 2008; Stepens et al., 2008; Sikk et al., 2010, 2013) and the bilateral hyperintensive signal in the basal ganglia observed in T1-weighted magnetic resonance imaging (MRI) consistent with excess accumulation of Mn in the brain (Selikhova et al., 2008; Stepens et al., 2008; Sikk et al., 2010, 2013). Importantly, in Eastern European countries in which there is expression of Mn-induced parkinsonism in ephedron drug users, the synthesis of the ephedron uses potassium permanganate to oxidize ephedrin or pseudoephedrin. On the other hand, in the United States drug users make the same ephedrone preparation, however, they oxidize the ephedrine with chromate and there is no evidence of parkinsonism (Stepens et al., 2008). This provides compelling evidence that the culprit in the home-made ephedron preparations used in Eastern European countries is the high levels of Mn that are injected by these individuals.

MANGANESE-INDUCED PARKINSONISM: DEGENERATION OR DYSFUNCTION OF DOPAMINERGIC NEURONS?

HUMAN STUDIES

In the last decade there has been a great deal of debate in the scientific literature about the potential role of Mn on the etiology of idiopathic PD. Epidemiological studies indicate that long-term exposure (>20 years) to Mn is associated with idiopathic PD (Gorell et al., 1999). Studies in welders have suggested that Mn exposure precipitates an earlier expression of idiopathic PD (Racette et al., 2001, 2005). However, the studies in welders have been criticized from several perspectives (see Ravina et al., 2001; Guilarte, 2010) and a confounding problem in human studies is that workers occupationally exposed to Mn could have an underlying susceptibility to develop PD. Thus, it is difficult to know whether the Mn exposure is the etiological agent that induces idiopathic PD or whether there is a coincidental Mn exposure in individuals that are destined to express the disease. In an effort to examine the potential role of Mn in idiopathic PD, a recent study used [^{18}F]-Fluoro-L-Dopa Positron Emission Tomography ([^{18}F]-FDOPA PET) imaging on 20 asymptomatic welders (exposed to welding fumes containing Mn), 20 subjects with idiopathic PD and 20 normal controls (Criswell et al., 2011). [^{18}F]-FDOPA PET is a non-invasive neuroimaging method to assess presynaptic dopamine terminal activity *in vivo* and has been used in idiopathic PD patients as a marker of dopamine terminal integrity (Gallagher et al., 2011; Jaimini et al., 2013). Notably, [^{18}F]-FDOPA uptake is dramatically decreased with a distinct regional pattern in the caudate and putamen of idiopathic PD patients (Morrish et al., 1996; Nurmi et al., 2001; Hilker et al., 2005; Gallagher et al., 2011). An important aspect of the investigation by Criswell and colleagues is that welders were relatively young (mean age 45.2 years), apparently asymptomatic and in good health, thus reducing the possibility of expressing an underlying idiopathic PD etiology in order to minimize the likelihood of coincidental Mn exposure with idiopathic PD. The authors found that welders expressed significantly elevated levels of blood Mn and a higher pallidal index (the pallidal index is a measure of Mn accumulation on the globus pallidus as a ratio of the signal intensity in the globus pallidus over the intensity in the frontal white matter using T1-weighted MRI) than controls and subjects with idiopathic PD; thus confirming that the welders were actively exposed to Mn. Upon neurological examination, welders demonstrated a slightly elevated average United Parkinson's Disease Rating Scale-subscale 3 (UPDRS3) score relative to controls indicative of subtle effects of Mn on motor function while the idiopathic PD subjects had a much higher score consistent with their diagnosis. The results of the [^{18}F]-FDOPA-PET studies indicated that the welders had a small (10%) but significantly lower level of [^{18}F]-FDOPA uptake in the caudate nucleus relative to controls but no effect on the anterior or posterior putamen (Criswell et al., 2011). On the other hand, idiopathic PD patients expressed the expected pattern of [^{18}F]-FDOPA uptake deficits in the caudate and putamen relative to controls. That is, idiopathic PD subjects had marked reductions in [^{18}F]-FDOPA uptake in the putamen (∼52% in the posterior putamen and 35% in the anterior putamen) with a smaller reduction in the caudate nucleus (∼17%) (Criswell et al., 2011). This study showed that the pattern of the impairment in dopamine terminal function in welders actively exposed to Mn-containing welding fumes is not the same to that observed in idiopathic PD. It should also be noted that the interpretation of the decrease in [^{18}F]-FDOPA uptake in the caudate in the welders exposed to Mn should not necessarily be interpreted as representative of dopamine terminal degeneration as is the case in idiopathic PD. It is possible that Mn exposure could alter enzymes that are responsible for [^{18}F]-FDOPA metabolism and this possibility needs to be ruled out. Other types of PET studies should also be performed that would be more representative of dopamine terminal integrity and are less likely to be influenced by changes in dopamine metabolizing enzymes and/or changes in dopamine levels. For example, [^{11}C]-dihydrotetrabenazine (DTBZ) PET for vesicular monoamine trasnporter type-2 (VMAT-2) is more

likely to represent structural changes in dopamine terminals than [^{18}F]-FDOPA PET when it relates to studies with Mn.

The findings of Criswell et al. (2011) do suggests that the small but significant Mn-induced decrease in [^{18}F]-FDOPA uptake in the caudate nucleus may be associated with potential effects on cognitive domains since the caudate nucleus has extensive connections to cortical structures, especially to frontal cortical areas that are involved in executive function (see below). Consistent with this hypothesis, several studies in early idiopathic PD patients show that reductions in [^{18}F]-FDOPA uptake in the caudate nucleus are associated with deficits in working memory performance and executive function (Rinne et al., 2000; Jokinen et al., 2009, 2013), effects that were not associated with reduction in [^{18}F]-FDOPA uptake in the putamen.

This recent study using state-of-the-art PET instrumentation and analysis provides evidence of a relative lack of dopamine neuron terminal degeneration in welders expressing small increases on the UPDRS3 scale. Previous studies in smelter workers with clinical parkinsonism have reported normal [^{18}F]-FDOPA-PET in the striatum (Huang, 2007). Further, neuroimaging studies performed in the ephedrone users indicating normal levels of dopamine terminals, based on dopamine transporter (DAT) levels, in the striatum using SPECT imaging despite the fact that they express clinical parkinsonism (Selikhova et al., 2008; Colosimo and Guidi, 2009; Sikk et al., 2010, 2013; Iqbal et al., 2012). Thus, the most recent human studies with state-of-the-art neuroimaging methodologies indicate that there is a relative lack of dopamine neuron terminal degeneration in the caudate and putamen as a result of Mn exposure. These findings raise the important question, what is the underlying neurobiological deficit in dopaminergic neurons in Mn-induced parkinsonism?

NON-HUMAN PRIMATE STUDIES

During the last decade, our laboratory in collaboration with a multidisciplinary group of investigators has been studying the neurological consequences of chronic exposures to moderate levels of Mn (Guilarte et al., 2006a,b, 2008a,b; Burton and Guilarte, 2009; Burton et al., 2009; Verina et al., 2011, 2013; Schneider et al., 2006, 2009). These on-going studies use research naïve *Cynamolgos macaques* (5–6 years of age at the initiation of the study) in which there is extensive behavioral and neuroimaging assessment prior to (baseline) and at two different time points after initiation of Mn administration (Guilarte et al., 2006b, 2008a). After the animals have gone through the behavioral and neuroimaging protocols [the latter includes T1-weighted MRI (MRI), Magnetic Resonance Spectroscopy (MRS), PET and currently Diffusion Tensor Imaging (DTI)] *ex vivo* neurochemical and neuropathological confirmation of the PET findings as well as other neurochemical and neuropathological outcomes are performed. One of the neuroimaging studies performed is to assess DAT levels as a putative synaptic marker of dopamine terminal integrity in the caudate and putamen using [^{11}C]-methylphenidate PET. Another PET study uses a continuous infusion of [^{11}C]-raclopride (a D2-dopamine receptor ligand) with amphetamine challenge in order to measure both D2-dopamine receptor (D2R) levels and *in vivo* dopamine release (Laruelle, 2000; Zhou et al., 2006). Importantly, the imaging studies provide an internal control since each animal receives a "baseline" (prior to Mn exposure) imaging set (MRI/MRS/DTI/PET). In addition, to the Mn-exposed animals, an "imaged-control" group was used. This group of animals goes through the same imaging protocol, but they do not receive Mn. A second "naïve controls" group was also used for the neuropathological endpoints and this group of animals does not receive Mn exposure nor does it go through the imaging protocol.

The results of our PET studies demonstrate that chronic exposure to moderate levels of Mn does not produce the loss of dopamine terminals, i.e., there was a lack of dopamine terminal degeneration in the caudate and putamen based on [^{11}C]-methylphenidate PET for DAT under our experimental Mn dose and exposure conditions (Guilarte et al., 2006b, 2008a). On the other hand, we found a highly significant effect of Mn on dopamine terminal dysfunction since there was a marked (∼60% from baseline) and progressive decrease of *in vivo* dopamine release in the striatum of Mn-exposed animals measured by PET (Guilarte et al., 2006b, 2008a). This effect was not observed in the "imaged-control" group. Therefore, the impairment of *in vivo* dopamine release was the direct result of the Mn administration (Guilarte et al., 2008a).

One potential explanation for the impairment of *in vivo* dopamine release measured by PET in the Mn-exposed animals is that Mn produces a decrease in the synthesis of dopamine, thus resulting in lower levels of synaptic (vesicular) dopamine available for release. To answer this questions, a number of *ex vivo* neurochemical studies were performed in the caudate and putamen of the same animals in which PET studies were performed. The results show that when all control groups were combined (imaged-controls and naïve controls) and used as a referent group, there were no significant differences on the levels of dopamine and metabolites in the caudate and there was only an effect of Mn on dopamine levels in the putamen when compared to the naïve controls only (Guilarte et al., 2008a). A similar effect was observed for DAT and vesicular dopamine transporter-2 (VMAT-2) in the caudate and putamen. Lastly, there was no effect of Mn-exposure on DAT or tyrosine hydroxylase (TH) immunostaining in the caudate and putamen. In summary, the non-human primate studies performed under highly controlled experimental and Mn dosing conditions indicate that exposure to moderate levels of Mn does not result in dopamine neuron degeneration as in idiopathic PD but it produces significant dopamine neuron dysfunction. We have proposed that the subtle fine motor control deficits observed in these animals is the result of a dopamine release deficit (Guilarte et al., 2006b, 2008a; Guilarte, 2010). Our non-human primate findings are consistent with the most recent neuroimaging studies in humans indicating a lack of dopamine neuron terminal degeneration in subjects with clinical parkinsonism resulting from ephedrone use (Selikhova et al., 2008; Colosimo and Guidi, 2009; Sikk et al., 2010; Iqbal et al., 2012).

While the current review does not include rodent studies, there is recent evidence in the literature that rodents exposed to Mn also have impairment in dopamine release with no change in total tissue dopamine levels, dopamine neuron terminals in the striatum, or TH-positive dopaminergic cell bodies in the SNpc

(Vidal et al., 2005; Peneder et al., 2011). Combined these studies provide evidence that Mn-induced parkinsonism may be the result of the inability of dopamine neuron terminals to release dopamine rather than a decrease of dopamine synthesis in intact terminals and/or the loss of dopamine as a result of terminal degeneration. These findings provide a logical explanation to the evidence that Mn-induced parkinsonism is not responsive to L-dopa therapy (Lu et al., 1994; Sanotsky et al., 2007; Selikhova et al., 2008; Stepens et al., 2008; Colosimo and Guidi, 2009; Sikk et al., 2013) as is idiopathic PD since in Mn-induced parkinsonism there is no apparent loss of dopamine terminal or dopamine levels in the striatum. Our findings in non-human primates that Mn impairs dopamine release needs to be confirmed in humans exposed to Mn. Collectively, our PET findings implicate a novel mechanism by which dopamine neuron dysfunction, that is, the inability to release dopamine, rather than a degenerative process can result in clinical parkinsonism as a result of Mn exposure.

EFFECTS OF MANGANESE EXPOSURE ON NEUROPSYCHIATRIC SYMPTOMS AND COGNITIVE FUNCTION

The clinical expression of Mn-induced neurotoxicity in humans has been described as a continuum with different stages with distinct clinical manifestations (Mergler et al., 1999). Humans exposed to Mn express changes in sleep patterns and mood with uncontrollable laughter and crying, euphoria, aggressiveness, hallucinations and psychosis (Donaldson, 1987). An acute effect of Mn intoxication has been described as a clinical condition with symptoms reminiscent of schizophrenia and amphetamine-induced psychosis (Donaldson, 1987; Perl and Olanow, 2007). Although the current knowledge on the psychiatric aspects of chronic Mn exposure are limited, recent studies indicate that humans with increased exposure to Mn (Bowler et al., 2003, 2006, 2007a,b; Josephs et al., 2005; Park et al., 2009) and from medical conditions that results in increased Mn accumulation in the brain (Mirowitz et al., 1991; Klos et al., 2006) express impairments in attention and learning and memory function suggestive of frontal lobe and subcortical dysfunction. Studies have shown that workers occupationally exposed to Mn have a higher incidence of neuropsychiatric symptoms than referents (Bouchard et al., 2007) and elevated levels of Mn markedly increase neuropsychiatric symptoms associated with alcohol abuse (Sassine et al., 2002). An increasing number of reports also indicate effects on working memory (Bowler et al., 2003, 2006, 2007a,b; Klos et al., 2006) and poor cognitive performance (Mergler and Baldwin, 1997; Santos-Burgoa et al., 2001; Bowler et al., 2003, 2007a,b; Klos et al., 2006). Importantly, the effects of Mn on working memory points to deficits in frontal lobe function, a brain region known to be involved in neuropsychiatric illnesses such as schizophrenia (Goldman-Rakic, 1999; Abi-Dargham et al., 2002). A growing number of reports in children with elevated exposures to Mn indicate below average performance in verbal and visual memory tests (Woolf et al., 2002; Wright et al., 2006) and intellectual function (Wasserman et al., 2006; Claus Henn et al., 2010; Bouchard et al., 2011; Menezes-Filho et al., 2011; Khan et al., 2012). Children followed from birth through the early years have cord blood Mn concentrations that were negatively correlated with scores on attention, non-verbal memory and hand skills (Takser et al., 2003). Despite these studies, basic knowledge on mechanism(s) by which Mn produces psychiatric symptoms and cognitive impairment is lacking. Therefore, a great deal can be learned not only from Mn effects on basal ganglia function but also from effects on cognitive domains associated with the frontal cortex and other cortical and subcortical structures.

THE CEREBRAL CORTEX—A NOVEL TARGET OF MANGANESE NEUROTOXICITY

There is a paucity of knowledge on the neuropathological consequences of excess Mn accumulation in cortical regions and specifically in the frontal cortex. This is based in part on the fact that: (1) most studies on Mn-induced neurochemical and neuropathological changes have been focused on basal ganglia structures due to its association with movement abnormalities and parkinsonism, and (2) Mn accumulates to a high degree in the basal ganglia. Besides the suggestion from neuropsychological and cognitive tests of frontal cortex involvement in Mn-induced neurological dysfunction, a review of the literature brings to light a lack of neuropathological studies in which Mn effects on the cerebral cortex have been performed. It is only recently when neuroimaging studies have interrogated cortical regions to examine their susceptibility to Mn-induced neurotoxicity. In this context, our recent studies in non-human primates have reported proton MRS metabolite changes in Mn-exposed animals (Guilarte et al., 2006a). This includes a decrease in N-acetylaspartate (NAA) to creatine (Cr) ratio (NAA/Cr) in the parietal cortex with a nearly significant decrease ($p = 0.055$) in frontal white matter (Guilarte et al., 2006a). A decrease in the NAA/Cr ratio is representative of neuronal dysfunction and/or neuronal loss (Clark, 1998; Block et al., 2002). Since this original publication, two human studies have described effects of Mn on brain metabolites in the cerebral cortex. Chang et al. (2009) have shown that cognitive decline in welders was associated with a decrease in myoinositol/creatine (mI/tCr) ratio in the anterior cingulate cortex indicative of glial involvement. More recently, another MRS study in smelters showed a small but significant decrease in NAA/tCr ratio in the frontal cortex that was strongly correlated with cumulative Mn exposure (Dydak et al., 2011). Therefore, there is emerging evidence that exposure to Mn results in altered levels of brain metabolites in the cerebral cortex that reflect neuronal loss or dysfunction and glial cell activation. The only other evidence describing cortical involvement with brain Mn accumulation is a case report of an individual exhibiting progressive dementia, and extrapyramidal syndrome with an elevated Mn body burden (Banta and Markesbery, 1977). Brain biopsy and examination of cortical tissue revealed numerous neuritic plaques and neurofibrillary tangles in the right frontal lobe typical of Alzheimer's disease (AD) (Banta and Markesbery, 1977).

NEUROPATHOLOGICAL CHANGES IN THE FRONTAL CORTEX OF MN-EXPOSED NON-HUMAN PRIMATES

Previous reports from our on-going studies on the neurological effects of Mn in non-human primates have provided compelling evidence of Mn-induced pathology in the frontal cortex of young, research naïve animals (Guilarte et al., 2008b; Verina

et al., 2013). Using microarray technology in frontal cortex tissue from Mn-exposed and control animals, we found significant alterations in genes with biological functions associated with: (1) cholesterol metabolism and transport, (2) axonal/vesicular transport, (3) inflammation and the immune response, (4) cell cycle regulation and DNA repair, (5) and proteasome function and protein folding and turnover. The most highly upregulated gene was β-amyloid precursor-like protein 1 (APLP1), a member of the amyloid precursor protein (APP) family associated with AD (Guilarte et al., 2008b). The increase in APLP1 gene expression was confirmed at the protein level using immunohistochemistry. We also found diffused β-amyloid plaques (6E10 antibody immunohistochemistry) in the frontal cortex from Mn-exposed animals that were not observed in age-matched controls. These findings were unexpected as these were young adolescent animals and normally non-human primates do not express β-amyloid diffuse plaques at an early age, although there is evidence of diffused β-amyloid plaques in aged (>20 years of age) non-diseased monkeys (Kimura et al., 2003, 2005). Examination of frontal lobe tissue also provided evidence of cortical and subjacent white matter degeneration based on silver staining. In the gray matter, histological staining provided evidence of neurons with a significant degree of intracytoplasmic vacuolization. In some of the animals, we observed neurons with hypertropic nuclei, a condition that has been associated with the early stages of AD (Iacono et al., 2008, 2009). Histological assessment of the frontal cortex also showed cells with apoptotic stigmata and astrocytosis in both the gray and white matter. More recently, we have reported evidence of α-synuclein aggregation in the frontal cortex gray and white matter from the same Mn-exposed animals (Verina et al., 2013). As noted earlier, these Mn-exposed animals expressed a near significant ($p = 0.05$) decrease in NAA/Cr ratio in the frontal cortex white matter (Guilarte et al., 2006a) consistent with the observation of white matter degeneration in post-mortem brain tissue. Therefore, our studies provided the first evidence of significant pathology in the frontal and parietal cortex of non-human primates exposed to Mn.

Recent human studies also support neurodegenerative changes resulting from Mn exposure in frontal cortex white matter. Stepens et al. (2010) report that individuals injecting ephedron-containing Mn express white matter abnormalities based on DTI. The authors describe evidence of diffuse white matter changes reflected by reductions in fractional anisotropy (FA) in the ephedron users. They also find effects specific to white matter underlying the right ventral premotor cortex and the medial prefrontal cortex. The authors indicate that the clinical features of these ephedron users point to a disorder of higher-level motor programming and that the pattern of motor function deficits resemble executive function deficits similar to those displayed by patients with prefrontal cortex lesions (Stepens et al., 2010). Another human study examining white matter ultrastructural integrity in welders also reveal white matter changes measured by DTI (Kim et al., 2011). They show that FA was significantly reduced in the corpus callosum and frontal white matter of welders. The FA values in these white matter regions was significantly associated with blood Mn levels and pallidal index. Importantly, the degree of FA disruption was associated with impaired attention, lower working memory and deficits in executive function tests (Kim et al., 2011).

These findings provided strong evidence that the frontal cortex gray matter and subjacent white matter are vulnerable, but previously unrecognized targets for Mn-induced neurotoxicity despite the fact that Mn accumulates in cortical structures at significantly lower concentrations than in the basal ganglia. These observations suggest that the neurotoxicological effects of Mn are not solely based on the degree to which Mn accumulates in different brain regions but they are also based on the vulnerability of a specific brain region to Mn-induced neurotoxicity. The emerging evidence in humans and non-human primates suggest that future studies on subjects with environmental and occupational exposures to Mn or in patients with medical conditions in which excess brain Mn accumulation occurs should be tested for neuropsychiatric symptoms and cognitive function deficits.

EFFECTS OF MANGANESE EXPOSURE ON WORKING MEMORY

In the previous section evidence is provided that exposure to elevated levels of Mn results in detrimental effects on cortical structures, specifically the frontal and parietal cortex. Recent human and non-human primates studies suggest that a resulting effect of Mn-induced neuropathology in the frontal cortex is working memory deficits. Chang et al. (2010) report that welders with chronic Mn exposure express increased brain activity measured by functional MRI in working memory networks during the 2-back verbal working memory task. They interpret these findings as the welders requiring more neural resources in working memory networks to compensate for subtle deficits in working memory. In another study, Wasserman et al. (2011) found significant associations between Mn levels in drinking water and reductions in Perceptual Reasoning and Working Memory scores.

Our non-human primate studies were the first to provide initial evidence of Mn effects on working memory under highly controlled experimental conditions (Schneider et al., 2006, 2009). We showed that chronic Mn exposure resulted in deficits in non-spatial and spatial working memory. Non-spatial working memory assessed by delayed matching to sample performance appeared to be more affected than spatial working memory using a variable delayed response task (Schneider et al., 2009). In general, the human and non-human primate studies provide substantial evidence for impairments of cognitive domains that are mediated by the frontal cortex. Further, the non-human primate findings also implicate brain metabolite changes in the parietal cortex, a brain region that is important for working memory performance and plays an important role in integrating sensory information and visuo-spatial processing (Constantinidis and Wang, 2004; Seger, 2006; Linden, 2007).

CAN DOPAMINE NEURON DYSFUNCTION IN THE STRIATUM AND/OR FRONTAL CORTEX EXPLAIN THE WORKING MEMORY DEFICITS OBSERVED IN Mn EXPOSED NON-HUMAN PRIMATES?

Working memory is closely associated with frontal cortex function (Constantinidis and Wang, 2004; Linden, 2007) and dopamine neurotransmission in the striatum (Rinne et al., 2000;

Sawamoto et al., 2008; Jokinen et al., 2009) and the frontal cortex (Brozoski et al., 1979; Rotaru et al., 2007). The dopamine cell bodies located in the SNpc project to the caudate and putamen and this nigrostriatal system is involved in motor control. In addition, there are direct mesolimbic dopaminergic projections from the ventral tegmental area to the frontal cortex (Bjorklund and Dunnett, 2007). The caudate nucleus receives dopaminergic input from the SNpc and it can influence frontal cortex function via well-defined frontostriatal circuits (Alexander et al., 1986; Seger, 2006). Human and non-human primates studies show that the dorsolateral prefrontal cortex (DLPFC) is an important region for the execution of working memory tasks with reciprocal connections to other cortical structures such as the parietal, temporal and cingulate cortex and these combined participate in a cortical network related to working memory (Kubota and Niki, 1971; Petrides et al., 1993; Berman et al., 1995; Cohen et al., 1997).

Lesions or dysfunction of the caudate nucleus has been reported to produce impairment in the delayed response tasks that assesses working memory (Levy et al., 1997; White, 2009). Relevant to our own studies, Mn-exposed animals have impairments of both spatial and non-spatial working memory (Schneider et al., 2009) and they also express a significant impairment of *in vivo* dopamine release in the striatum (Guilarte et al., 2008a). Further, welders exposed to Mn express an early deficit in dopamine neuron function specific to the caudate nucleus and not the putamen (Criswell et al., 2011). These findings suggest that dopamine neuron dysfunction via impairment of dopamine release in the striatum and specifically in the caudate may be associated with the working memory deficits expressed in Mn-exposed non-human primates and in humans. Other studies have shown that the levels of NAA in the DLPFC predict the activation of cortical regions involved in the execution of working memory tasks such as the frontal, parietal and temporal cortices and this network has been found to be affected in mental disorders such as schizophrenia (Bertolino et al., 2000; Castner et al., 2004). Postmortem studies in the frontal cortex of Mn-exposed non-human primates have found a significant degree of neuronal degeneration with diffused β-amyloid plaques and α-synuclein aggregation (Guilarte et al., 2008b; Verina et al., 2013) implicating a potentially important role of this neuropathology in the working memory deficits observed in Mn-exposed non-human primates (Schneider et al., 2009). Imaging studies in welders exposed to Mn support a Mn-induced neuronal cell death or dysfunction in the frontal cortex based on decreased NAA/tCr ratio (Dydak et al., 2011), an effect that was associated with cumulative Mn exposure. Combined these studies provide evidence that several brain regions (i.e., the caudate nucleus, the frontal cortex and the parietal cortex) within the working memory network appear to have substantial neuropathology and/or dysfunction as a result of chronic exposure to Mn.

Experimental animal and human studies have shown that dopamine is a key neurotransmitter in the regulation of working memory in the frontal cortex and caudate nucleus (Levy et al., 1997; Aalto et al., 2005; Cools et al., 2008; Landau et al., 2009; Backman et al., 2011; Cools and D'Esposito, 2011). Microdialysis studies have shown that working memory tasks induce the release of dopamine in the prefrontal cortex of monkeys (Watanabe et al., 1997) and rats (Phillips et al., 2004) and there is increased blood flow to prefrontal and parietal cortex in humans performing working memory tasks (Bertolino et al., 2000; Cabeza and Nyberg, 2000). Other studies have shown that D1-dopamine receptor (D1R) antagonists can impair working memory (Sawaguchi and Goldman-Rakic, 1991) while low doses of D1R agonists can improve working memory (Arnsten et al., 1994). Contrary to using low doses of dopamine receptor agonists, high doses of D1R agonists also impair working memory performance, an effect that is abrogated by pretreatment with a D1R antagonist (Zahrt et al., 1997; Goldman-Rakic et al., 2000). These findings suggest that either low levels or excessive levels of D1R dopamine receptor stimulation can have a negative impact on working memory performance (Goldman-Rakic et al., 2000; Cools and D'Esposito, 2011). Based on this literature, it is likely that the impairment of *in vivo* dopamine release measured in the striatum of Mn-exposed animals may be responsible for their impairment in working memory (see Guilarte et al., 2008a; Schneider et al., 2009). Alternatively, it is possible that chronic Mn exposure may also alter *in vivo* dopamine release in the frontal cortex, and along with deficits of dopamine release in the caudate nucleus may precipitate deficits on working memory performance.

ANALYSIS OF *In vivo* DOPAMINE RELEASE IN THE FRONTAL CORTEX: PET IMAGING WITH [^{11}C]-FLB 457

While the displacement of D2R specific PET ligands such as [^{11}C]-raclopride by an acute amphetamine challenge has been validated and used extensively to measure *in vivo* dopamine release in the striatum (Laruelle, 2000). The use of this methodology is just emerging for the cerebral cortex (Narendran et al., 2009, 2011a,b, 2013). Since dopamine innervation to cortical structures is significantly lower than to the striatum, that is, dopamine terminals and dopamine receptor levels are much lower in the frontal cortex than in the caudate/putamen, *in vivo* dopamine release PET in cortical structures is a much more difficult task to perform. However, the development and use of high affinity D2R-PET ligands such as [^{11}C]-FLB 457 ($Kd = 0.06$ nM) and [^{18}F]-fallypride ($Kd = 0.14$ nM) have made such studies possible. Several publications have now described the reliability of using [^{11}C]-FLB 457 to measure *in vivo* dopamine release in the cerebral cortex of humans and non-human primates (Narendran et al., 2009, 2011a,b, 2013). Further, a recent study has shown that the degree of [^{11}C]-FLB 457 binding potential reduction measured by PET was directly associated with the amount of extracellular dopamine release induced by the acute amphetamine administration (Narendran et al., 2013). In summary, the ability to measure *in vivo* dopamine release in the cerebral cortex using PET is an extremely valuable approach to understand the molecular basis of the working memory impairments observed in humans and non-human primates exposed to Mn. We are currently performing these types of studies in our Mn-exposed animals in order to make associations between *in vivo* dopamine in cortical regions and working memory performance.

SUMMARY

In the last decade there has been significant progress using state-of-the-art neuroimaging and behavioral methodologies that have opened up a new understanding of Mn neurotoxicology. While historically the focus of Mn neurotoxicity has been associated with parkinsonism as a result of the high levels of exposure that occurred in the mining and processing of Mn ore and in other occupational settings, the last decade has brought about compelling experimental evidence that at lower cumulative doses of Mn that are likely to occur from occupational and environmental exposures, other non-motor neurological effects appear to be more prevalent and these seem to be associated with cognitive function deficits. The later may be the result of Mn producing brain chemistry and structural changes in cortical regions, and the frontal and parietal cortex appear to be sensitive targets. Lastly, because of its relevance to motor and cognitive domains, it is possible that dysfunction of the dopaminergic system could be a common mechanism by which Mn could have an impact on both cognitive and motor function deficits observed in humans and non-human primates.

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Biometals in rare neurodegenerative disorders of childhood

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Copper, iron, and zinc are just three of the main biometals critical for correct functioning of the central nervous system (CNS). They have diverse roles in many functional processes including but not limited to enzyme catalysis, protein stabilization, and energy production. The range of metal concentrations within the body is tightly regulated and when the balance is perturbed, debilitating effects ensue. Homeostasis of brain biometals is mainly controlled by various metal transporters and metal sequestering proteins. The biological roles of biometals are vastly reviewed in the literature with a large focus on the connection to neurological conditions associated with ageing. Biometals are also implicated in a variety of debilitating inherited childhood disorders, some of which arise soon following birth or as the child progresses into early adulthood. This review acts to highlight what we know about biometals in childhood neurological disorders such as Wilson's disease (WD), Menkes disease (MD), neuronal ceroid lipofuscinoses (NCLs), and neurodegeneration with brain iron accumulation (NBIA). Also discussed are some of the animal models available to determine the pathological mechanisms in these childhood disorders, which we hope will aid in our understanding of the role of biometals in disease and in attaining possible therapeutics in the future.

Keywords: metals, neurodegeneration, childhood, copper, iron, zinc

INTRODUCTION

The significance of biometals for the correct functioning of the human brain has long been discovered. The importance of metals such as iron, zinc, and copper results from the numerous roles that they have, such as stabilization of proteins and transcription factors, acting as co-factors for metallochaperones for cellular transport and their role in enzyme catalysis (Markossian and Kurganov, 2003; Butterworth, 2010). Deviations of metal homeostasis have been linked to neurodegenerative disorders such as Alzheimer's disease (AD), Parkinson's disease, Amyotrophic lateral sclerosis, and Huntington's disease (HD) (Dexter et al., 1993; Deibel et al., 1996; Jomova et al., 2010; Skjorringe et al., 2012). A risk factor in the development of many of these disorders appears to be an increase in age. Although the pathological characteristics of the disease are in most cases well-understood, the exact causes are often unknown.

Neurodegeneration is not restricted to an ageing population. Several debilitating neurodegenerative disorders affecting children have been identified. These include neurodegeneration with brain iron accumulation (NBIA), ATPase pathologies such as Wilson's/Menkes disease and neuronal ceroid lipofuscinoses (NCLs). These disorders share several pathological features with more common neurodegenerative diseases, including protein aggregation and oxidative stress. Aberrant biometal homeostasis has also been identified in children suffering from these diseases (**Table 1**). What is not clear is whether the distinct changes in metal levels cause neurodegeneration or occur as a result of the neurodegeneration. In some cases, the pathology associated with childhood disorders is clearly evident and often fatal at an early age. Changes in metal levels have also been identified in children with Autism spectrum disorder (ASD) which affects the behavior of children more so than hinder them physically. Dissecting the roles that biometals have in these disorders is fundamental for finding potential therapeutics to reduce or inhibit neuropathological changes associated with altered metal homeostasis (Yasuda et al., 2011).

Since biometal homeostasis plays a major role in neurodegenerative disease, the aim of this review is to highlight the effects that metals have on the developing brain. Multiple childhood disorders arise from defects in the concentrations of metals within the body and brain but little is known about how these changes can be corrected or prevented to avoid or ameliorate disease. While most of the childhood disorders are inherited and disease progression can occur not long after birth, it is critical to determine the molecular disease characteristics and metal alterations within the brain and other tissues for the development of therapeutic treatments to provide these children with a chance at life.

WILSON'S DISEASE

Wilson's disease (WD) is an autosomal recessive disease affecting copper metabolism (Bearn and Kunkel, 1954). Copper is involved in neurotransmitter synthesis, cellular respiration, scavenging of toxic free-radicals, and has an important role in maintaining homeostasis of other trace elements such as iron (Butterworth,

Table 1 | Biometal alterations in childhood disorders.

Childhood disorder	Associated gene(s)	Biometal concentrations
Wilson's disease	*ATP7B*	Copper ↑
Menkes disease	*ATP7A*	Copper ↓
Neuronal ceroid lipofuscinoses	At least 10 genes	Zinc, manganese, copper, iron, cobalt, sodium ↑ Potassium, magnesium ↓
Neurodegenerative disorders with iron accumulation	At least 7 genes	Iron ↑

2010). However, in abundance, copper is toxic and thus strict regulation of levels is critical. Free copper ions are virtually non-existent in human blood (Linder and Hazegh-Azam, 1996) and are usually bound to a multitude of proteins and chaperones for transport around the body. WD presents in patients typically between the ages of 6–20 years, and has an estimated incidence of 1 in 30,000 (Reilly et al., 1993).

The defective gene involved in WD is ATPase Cu(2+) transporting beta polypeptide (*ATP7B*), which encodes for a membrane bound copper-transporting ATPase protein primarily located within the liver cells, as part of the trans golgi network (Cox and Moore, 2002). To date, approximately 300 mutations within *ATP7B* have been associated with WD (Merle et al., 2010). Normal copper metabolism involves the transfer of copper molecules to ceruloplasmin for transport into the plasma so it can circulate to other tissues or when in excess, to the bile for excretion (Terada et al., 1998, 1999). ATP7B is involved in transporting the copper molecules to ceruloplasmin. When ATP7B is non-functional, copper accumulates to toxic levels and severely damages the liver. The liver therefore releases copper directly into the bloodstream where copper continues to cause damage to other tissues, in particular the CNS. In the brain, accumulation of copper is most evident in the basal ganglia, the region which co-ordinates movement (DeLong et al., 1984), making WD primarily a movement disorder. This neuropathology caused by the build up of copper leads to patients suffering tremors of the arms or hands, unpredictable movement, difficulty with speaking, swallowing, and walking as well as confusion, dementia, and various psychological problems (Rosencrantz and Schilsky, 2011). Some cognitive deficits have also been noted (Middleton and Strick, 2000), and could arise due to disruptions of projections of the basal ganglia to the prefrontal cortex (Bolam et al., 2000). Gliosis is also observed in the brains of patients suffering from WD (Anzil et al., 1974). The mechanism for neurological damage caused by excess copper is still not fully understood. However, it is known that copper is involved in the production toxic free radicals (Samuele et al., 2005), and excess copper has been shown to inhibit Inhibitor of apoptosis proteins (IAPs) caused by toxic deposits of intracellular copper (Mufti et al., 2006).

WD is a progressive disorder and was ultimately a fatal disease until the discovery of the first treatment in 1951 (Denny-Brown and Porter, 1951). With the copper-chelating agent, dimercaptopropanol, the disease is now treatable (Denny-Brown and Porter, 1951). The most common treatment to date is the use of zinc salts, which block enteral copper absorption in the stomach. Various drugs are currently available for WD and ongoing compliance with treatment is necessary.

ANIMAL MODELS OF WD

The Long Evans Cinnamon (LEC) rat is one model used for *in vivo* studies of WD. Since showing symptoms of liver failure in 1987 (Yoshida et al., 1987), the rats were first used as a model for liver cancer and hepatitis. Upon further characterization, accumulation of hepatic copper and decreased ceruloplasmin activity were found. Years later when the gene responsible for WD was discovered, the rat homolog *atp7b* was found to be mutated in the LEC rats, with a significant deletion at the 3′ end of the gene (Wu et al., 1994). Although there are similarities in liver pathogenesis between the LEC rat and humans, this rodent model shows minimal evidence of neuropathology (Terada and Sugiyama, 1999). In contrast to the LEC rat model, ATP7B knockout mice exhibit both neuro- and liver pathology. In these mice, copper levels are dramatically low at birth with a gradual increase by 2 months of age, where hepatic copper levels are approximately 50 times higher compared to wild-type controls (Buiakova et al., 1999). Another interesting model for WD is the toxic milk mouse, which also has an autosomal recessive inheritance (Theophilos et al., 1996). Female mice with the *tx* milk mutation produce copper-deficient milk, which is fatal to their pups. The *tx* mice have a single nucleotide change in the *ATP7B* gene, and studies have shown this to cause changes in the ATP7B localization in the mammary gland, therefore leading to impaired copper transport and little to no copper given to the pups. The *tx* mothers retain this copper in the liver (Rauch, 1983; Michalczyk et al., 2000).

Both the LEC rat and *tx* milk mouse models arose from spontaneous mutations. There is only one genetically engineered mouse model for WD, the *ATP7b*$^{-/-}$ mouse, which was generated by Buiakova et al. (1999). *ATP7b*$^{-/-}$ mice are born copper-deficient and display neurological symptoms such as tremors and abnormal locomotor behavior. If nursed by a homozygous *ATP7b*$^{-/-}$ mother, the pups will die at around 2 weeks of age. However, if nursed by a heterozygous mother, copper concentrations begin to rise to toxic levels in the liver by the age of 6 weeks, irrespective of initial birth concentration. Similar to human disease, these mice accumulate copper in the liver due to impaired export of copper into the bile, making these mice an invaluable model for the study of WD (Lutsenko, 2008).

MENKES DISEASE

Menkes disease (MD) is an inherited disease related to copper metabolism, but in contrast to WD, it is characterized by a significant reduction in normal copper levels in the blood, liver, and brain (Danks et al., 1973). Disease progression is also more rapid than in WD (de Bie et al., 2007). The reduction of copper is due to decreased absorption of copper from the intestine and therefore reduced transfer to copper-requiring proteins and enzymes (Danks, 1988). Symptoms present within the first year of life, generally within 3 months of birth and without treatment,

death is likely before the third year of life. MD is a rare childhood disease with an estimated worldwide incidence of 1 in 300,000 (Tonnesen et al., 1991), but can be higher in certain countries such as Australia (1 in 50,000 to 1 in 100,000) (Tumer and Moller, 2010).

MD has an X-linked mode of inheritance and is more prevalent in males than females (Kodama and Murata, 1999). At birth, babies appear normal, although premature labor has been documented (Uriu-Adams et al., 2010). An early sign in a baby with MD is the presence of sparse and abnormal hair, which looks fragile and twisted and appears to have a "kink" in it (Kaler, 2011). MD is thus also referred to as "Kinky Hair Syndrome." The baby begins to exhibit normal cognitive behavior with smiling and normal childhood dialogue; however, these behaviors do not progress further. Babies do not exhibit increases in muscle tone and over time generally become weaker and spasticity of the limbs is observed. Toward the end of the babies' short life, they succumb to blindness and respiratory failure and premature death is often due to infection or the extreme neuronal degeneration observed. A less-severe form of MD is called occipital horn syndrome (OHS) which is also an X-linked recessive inherited disorder with mutations in *ATP7A*. These patients exhibit fewer neurologic abnormalities and have a longer lifespan (Tumer and Moller, 2010). Connective tissue abnormalities tend to be the focus of this disorder with calcification of the occipital bulb, leading to horn-like structures at the base of the brain.

This detrimental disease is caused by a mutation in the gene encoding the Copper transporting ATPase-1 (ATP7A) protein (Fatemi and Sarkar, 2002), which is also a "P-type" ATPase protein similar to ATP7B. To date, there are over 200 known mutations scattered throughout the gene (His and Cox, 2004). Although both ATP7A and ATP7B are involved in the transport of copper to secreted enzymes and metalloproteins, ATP7A is also involved in the absorption of intestinal copper and can transport copper to all cell types and vital structural enzymes (Barry et al., 2011). ATP7B is predominantly involved in the exit of copper from hepatocytes. These differences in protein function are demonstrated by the fact that MD patients exhibit a variable range of symptoms throughout the body as opposed to mainly liver damage seen in patients with WD. The neurological damage caused by mutant *ATP7A* is due to the decreased activity of the copper-dependent enzymes. Decreased cytochrome *c* oxidase leads to a reduction in ATP production by the mitochondria and causes apoptotic cell death (Borm et al., 2004). Dopamine-β-hydroxylase is necessary for neurotransmitter synthesis and its activity is decreased in response to low copper levels (Kaler et al., 1995). Superoxide dismutase 1 (SOD1) is a free-radical scavenger the activity of which has been linked to several neurodegenerative disorders, particularly ALS. Copper is a co-factor imperative for SOD1 activity. Levels of SOD1 are decreased in MD patients, contributing to lower protection against toxic oxidative species within the brain (Kaler, 1994). Collectively, the reduced activity of copper-dependent enzymes is thought to be responsible for the observed neurological damage in MD.

The cellular location of ATP7A is sensitive to the concentration of copper; when copper levels are low ATP7A remains on the membrane of the Golgi but when levels are increased, ATP7A is located to the plasma membrane where it can "pump" copper to other proteins and cuproenzymes (Hasan et al., 2012). In MD, where copper levels are low, it is essentially located to the Golgi and there is a deficiency of copper transport to essential enzymes, and other tissues.

The therapeutic benefit of injection of copper histidine as a copper replacement to humans has provided mixed results and is dependent on the mutation within the ATP7A gene (Kaler et al., 1995; Christodoulou et al., 1998). Some studies have shown that early administration of copper to babies can prevent the observed neurological decline but this is likely to be in patients with mutations in *ATP7A* that still allow for some transfer of copper to copper-requiring enzymes, but not severe mutations affecting initial absorption from the intestine (Tumer et al., 1996).

ANIMAL MODELS OF MD

Spontaneous mutations in the mouse homolog of *ATP7A*, *atp7a*, have been used for modeling defective ATP7A in humans (Kuo et al., 1997). These mice are called mottled mice and different mutations in the *atp7a* gene cause different neurological and connective tissue anomalies. The two mottled mice mutations mottled brindled (Mo^{br}) and mottled macular (Mo^{ml}) show severe reductions in copper within the liver (Hunt and Clarke, 1983), and die within 1–3 weeks of birth (Lenartowicz et al., 2012). This premature death can be prevented with injected copper within the first week of birth, as well as transgenic over expression of *atp7a* (Danks, 1986).

NEURONAL CEROID LIPOFUSCINOSIS

The NCLs are a group of fatal autosomally-inherited neurodegenerative diseases occurring in childhood. The NCLs are also classified as lysosomal storage disorders since one of the main pathological characteristics is the accumulation of autofluorescent granules which contain lipids and proteins (lipopigments) in the lysosomes. Currently at least ten forms of NCLs have been described. Each form is distinguished by different defective genes (CLNs) and the age of onset (Cooper, 2003; Mitchison et al., 2004). Collectively, it is the most common neurodegenerative disorder of childhood with an estimated incidence of up to 1:12,500 (Rider and Rider, 1999). Although mutations in different genes cause different forms of the disease, all NCLs share common clinical symptoms; progressive loss of vision leading to blindness, motor and mental retardation, seizures, and finally premature death (Siintola et al., 2006). The exact function of these genes is still unknown (Tyynela et al., 2004). These genes encode soluble proteins and membrane-bound proteins, for example, CLN6 is a transmembrane protein which is localized to the endoplasmic reticulum (Heine et al., 2004). Children with defective CLN6 present with symptoms at the age of 3–5 years and tend to only live until 8–12 years of age. Currently there is no cure for any form of NCL (Sharp et al., 2003).

In NCLs, early reports have described some changes to metal levels, including iron and zinc, in blood cells of patients with NCL (Johansson et al., 1990). In a larger study of NCL patients, no association between "loosely bound" iron or copper levels in cerebrospinal fluid could be found (Heiskala et al., 1988). There is

some evidence that altered metal homeostasis may also occur in other NCL models. A recent study reported changes in expression of the metal transporter, ZnT6, in both CLN3 and CLN6 mouse cerebellar neuron precursor cell lines (Cao et al., 2011). Furthermore, reduction in expression of the gene causing CLN7 NCL disease was observed in the brains of mice fed a high-iron diet (Johnstone and Milward, 2010), suggestive of a heightened sensitivity of NCL brains to fluxes in metal concentrations.

Recently, a link between brain biometal homeostasis and CLN6 disease was discovered. Sheep harboring a natural mutation in the CLN6 gene show significant increases in manganese and more so, zinc levels in all brain regions except the cerebellum and brain stem (Kanninen et al., 2013). In the CLN6 sheep model, the accumulation of biometals was evident at the symptomatic age of 12–14 months. Early studies on the metal contents of lipopigments from CLN6 sheep revealed no obvious association between metal contents and disease progression (Palmer et al., 1988). Metal contents were variable between lipopigments from different tissues in line with the normal role of lysosomes as storage depots for metals, indicating the lysosomal origin of the lipopigments. Changes in the metal contents of retina and retinal pigment epithelial cells of CLN6 sheep have been reported, and an association with lipopigment accumulation and altered manganese levels with increasing photoreceptor cell loss suggested (Jolly et al., 1989).

ANIMAL MODELS OF NCLs

To understand the pathogenesis of NCLs, gene-specific mutant mice have been engineered which contain mutations in *Cln1, Cln2, Cln3, Cln5, Cln7,* and *Cln10* genes (Cooper et al., 2006). Naturally occurring mutations in *Cln6* and *Cln8* have also been identified (Lonka et al., 2000; Gao et al., 2002). Many of these mouse models share phenotypic features in common with human patients such as neurological and motor deficits, seizures and neuronal loss, and as such, are valuable tools to study disease mechanisms and potential therapeutic approaches. Like the human form, the mouse models display variance in timing and sequence of events toward disease progression, highlighting the genetic differences between the NCLs (Cooper et al., 2006). As the function of the majority of these genes is still unknown, using these animal models will prove invaluable in elucidating the function and mechanism behind NCL pathologies. The naturally occurring mutation in the CLN6 gene of sheep mentioned above share a similar disease progression and phenotypic features similar to that of the human CLN6 NCL form (Jolly et al., 1989; Kay et al., 2011), making them an excellent model of NCL disease due to their complex CNS. However, with large animal models it may be challenging to obtain sufficient numbers of animals for assessment due to difficulties in breeding and housing such large animals.

NEURODEGENERATIVE DISORDERS WITH BRAIN IRON ACCUMULATION

As with copper and zinc mentioned above, levels of iron within the brain need to be tightly regulated. Iron homeostasis is regulated by proteins involved in absorption, transport, influx, and storage in tissues (Zhang and Enns, 2009). Increases in brain iron levels are typically associated with ageing and is highly prevalent in AD and HD. Neurodegenerative disorders with brain iron accumulation (NBIA) refers to a wide group of disorders affecting children, with similar outcomes: neurodegeneration and the accumulation of iron. The disease is caused by mutations in seven genes (Ke and Ming Qian, 2003). At birth, brain iron is not present, but levels rapidly increase until 20 years of age with an uneven distribution over time (Zorzi et al., 2012). The most recognized gene defects are mutations in pantothenate kinase (PANK2), which is an enzyme located within the mitochondria and regulates the formation of Coenzyme A, which is essential for deriving cellular energy via the tricarboxylic acid cycle (Leoni et al., 2012). PANK2 phosphorylates pantothenate and requires N-pantothenoyl-cysteine and pantetheine. The exact mechanism how mutations in *PANK2* lead to iron accumulation is not fully understood, but one hypothesis is that if cysteine cannot be utilized within the cell by PANK2, it accumulates over time. Cysteine can bind iron, and over time this may explain the observed accumulation of iron (Gregory and Hayflick, 2005). Although the mutation in *PANK2* directly does not cause neurodegeneration, it is the increased iron which contributes to the production of toxic and tissue-damaging reactive oxygen species (Ke and Ming Qian, 2003). Increases in iron have also been linked to several neurological disorders such as AD and PD (Batista-Nascimento et al., 2012). There are over 100 mutations in this gene and PANK2 disorders account for over half of all NBIA cases (Gregory and Hayflick, 2005). PANK2-specific NBIA, referred to as pantothenate kinase-associated neurodegeneration (PKAN) was formally called Hallervorden–Spatz syndrome before the name was changed to divert any association with the unethical euthanization of mentally ill patients during World War II by Dr. Hallervorden (Dooling et al., 1974). PKAN disease is autosomally inherited and presents in childhood as either the classical, early onset or atypical, late onset form (Hayflick et al., 2003). The extent of iron accumulation can be easily visualized and quantified using magnetic resonance imaging (MRI). The pattern exhibited by MRI-T2 weighted images shows the increased iron as "darkened spots" and provides a novel "eye of the tiger" image, making MRI an important diagnostic tool for NBIA (Schenck and Zimmerman, 2004).

The symptoms of classical PKAN disease become apparent within the first 10 years of life, with symptoms usually starting between 3 and 4 years of age. Affected children begin to have difficulty with walking and other movements, which is attributed to the build up of iron in the basal ganglia (Gregory and Hayflick, 2005), a region of the brain highly important in movement. Rigidity, spasticity, and speech difficulties soon follow (Dooling et al., 1974). Children become wheelchair bound within only a few years of diagnosis. Atypical PKAN disease begins later in life, with an average age of onset of 13–14 years and disease progression unfolds slowly over many years (Chan et al., 2008). Patients present firstly with behavioral problems such as depression and personality changes with movement and speech impediments following later. The symptoms of atypical PKAN can be highly variable from case to case.

Treatment for NBIA had been aimed at symptoms rather than the genetic defect causing the iron build up and severe neurodegeneration. Drugs aimed at reducing abnormal movement are widely prescribed, but to date, there is no preventative for NBIA disorders. Recently, reducing the accumulation of iron has been the target for treatment using iron-chelators, however this is met with varying efficacy (McNeill and Chinnery, 2011). Deferiprone is an iron chelator which acts to redistribute iron within the brain, by donating built-up iron to transferrin for transport elsewhere (Brittenham, 1992). The promising feature of this drug is the ability to cross the blood-brain barrier, necessary for targeting the build up of iron in CNS. However, no conclusive results can be drawn from these clinical studies due to the small number of patients trialed (Forni et al., 2008; Zorzi et al., 2011).

ANIMAL MODELS OF NBIA

Unlike the other childhood disorders discussed in this review, PKAN-NBIA does not have a suitable murine model of disease. A PANK2 knockout mouse was generated which showed retinal degeneration and growth retardation, but no defects on movement and gait were evident (Kuo et al., 2005; Brunetti et al., 2012). However, *Drosophila melanogaster* have been a powerful tool for understanding the biochemical basis of PANK2 mutations. Mutations in the homolog of PANK, *fumble (fbl)* exhibited a shortened lifespan, progressive locomotion defect, and degenerations in both central nervous system and retina (Gregory and Hayflick, 2005). Interestingly, mutations in PANK2 in both the *Drosophila* and mouse led to male sterility (Wu et al., 2009).

CONCLUSIONS

The importance of metals in neurological disease has been outlined in numerous reviews over many decades, however, the exact mechanisms leading to aberrant metal homeostasis and why this is so detrimental to the brain remain poorly understood. While the brain is still developing during childhood, it is highly susceptible to slight changes in metal levels as outlined above with discussion of various childhood neurodegenerative disorders. Although certain therapeutics exist for some of these disorders, they mainly target disease symptoms and prolong the disease, rather than being preventative. It is the work forthcoming from *in vitro*, animal and clinical studies, which aim to add insight into the role of metal changes and consequences so potential therapeutics can be tested and hopefully one day applied to prevent disease progression.

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Metalloproteomics: principles, challenges, and applications to neurodegeneration

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Trace elements are required for a variety of normal biological functions. As our understanding of neurodegenerative disease advances we are identifying a number of metalloenzymes involved in disease process. Thus, the future of metals in neurobiology will rely more on detailed information regarding what metalloenzymes are present and how they are involved in the pathophysiology of disease. To gain this detailed information, we will rely less on bulk measures of the amount of a trace elements in a particular tissue and turn to metalloproteomic techniques to help elucidate both metalloprotein structure *and* function. Recent advances in metalloproteomics will translate to a richer understanding of the mechanism and precise role of metalloenzymes and proteins in the brain.

Keywords: metalloproteomics, LC-ICP-MS, metals in neurodegeneration, quantitative methods, hyphenated ICP-MS techniques

INTRODUCTION

The brain is an incredibly complex organ that has an equally unique metabolic need. In humans, although the brain only composes about 2% of body mass, it consumes approximately 25% of the energy output. The excessive proportion of metabolic activity occurring in the brain, compared to the whole body is seen in numerous examples of metal-mediated cellular function. Metalloenzymes are important for all aspects of physiology, including mitochondrial function, transcriptional regulation, catabolism, and, for the brain, the production of the important secondary messenger nitric oxide (NO) by NO synthase, which depends on Fe and Zn (Mayer et al., 1991; Li et al., 1999). In line with the brain's tendency for excess, the production of NO is about 20 times greater for the central nervous system compared to the vasculature (Salter et al., 1991; Garthwaite and Boulton, 1995; Pacher et al., 2007). Further, the actual signaling pathway for NO is dependent on Fe bound to a heme in soluble guanylate cyclase (Gerzer et al., 1981; Ignarro et al., 1982). The role of metals in NO production and signaling is just one example of the vital role trace elements have in biology. Indeed, life itself would not exist without oxygen produced by chloroplasts, and transport through our body by hemoglobin, both of which require a metal ion for function.

Despite the intense amount of research into cellular mechanisms metalloenzymes have largely been overlooked, yet the "metalloproteome" dictates much of the reactivity within a cell. The targeted investigation of metalloproteins in the central nervous system will provide mechanistic insights into how reported changes in total levels of trace elements translates to specific proteins.

WHAT IS "METALLOPROTEOMICS"?

Over a decade ago, Glen Evans commented on the "omic" science revolution, stating that the advent of post-genomic sciences was brought about by a need to "analyze the components of a living organism in its entirety" (Evans, 2000). As systems biology has become more integrated into the modern laboratory the traditional streams of "omic" sciences have diversified to include specific fields of study examining the functional components of biomolecules, rather than simply their presence or structure.

Metalloproteomics is one such newly established area of study, which amalgamates proteomic and metallomic approaches to biology (Barnett et al., 2012). Proteomics is the large-scale investigation of the structural and functional properties of proteins (Anderson and Anderson, 1998), whereas metallomics encompasses the "comprehensive analysis of the entirety of metal and metalloid species within a cell or tissue type" (Szpunar, 2005). We believe the term "metalloproteomics" is more suited to the field than metallomics alone, as it recognizes the important relationship between biometals and proteins, rather than focusing solely on the presence of an individual metal species. In 2004, Hiroki Haraguchi first described metallomics as "integrated biometal science" (Haraguchi, 2004), though it is only in recent years that the potential of integrating high-end atomic spectrometry techniques into typical proteomics workflows is beginning to gain attention.

It is estimated that around one-third of all proteins in the human body require a metal cofactor for functionality (Andreini et al., 2008; Waldron et al., 2009; Barnett et al., 2012). Redox properties of metal ions mediate a plethora of cellular processes, from the electron transport mechanisms within mitochondria to the formation of myelin in developing nerve cells. Metals have

the ability to interact with multiple proteins, all with varying functions, located in every cell of the human body. Due to the abundance of proteins that are estimated to require a metal cofactor for function, this is an area that requires extensive work to be done in order to characterize the vital role metals may play in the molecular basis of disease.

Unlike glycosylation and phosphorylation, which do not always have a one-to-one relationship with protein function (Jensen, 2006), the presence of a metal cofactor is intimately linked with enzymatic function. For example the function of Cu,ZnSOD is dependent on the presence of both metals. The Zn-only containing enzyme does occur in transgenic animal models overexpressing the enzyme (Lelie et al., 2011; Rhoads et al., 2011), though it does not pose any superoxide scavenging ability without Cu. SOD can even produce superoxide rather than scavenge it in the absence of Zn (Estévez et al., 1999). Bottom-up proteomics neglect information on non-covalent cofactors, including metals. The overall goal of systems biology or proteomics is to measure how proteins change to help elucidate function, hence the interest in glycosylation and phosphorylation and other post-translational modifications. However, the functional importance of non-covalent cofactors has the promise to determine the functional output of proteins, and cannot be overlooked.

HOW BIG IS THE "METALLOPROTEOME"?

The proportion of metalloproteins in the proteome is still widely unknown. Even in relatively simple single-cellular organisms, the number of metalloproteins that have been comprehensively identified is only a fraction of the one-third of all proteins predicted to bind metals, most likely due to technical limitations. This suggests that studying the human metalloproteome will encounter significant difficulties, as the number of metalloproteins that it will encode for is larger then that of relatively simple prokaryotes.

Relatively few research groups are applying new technologies to metalloproteomics. One such group that has been actively conducting research in this field and has confirmed the lack of characterization of metalloproteins is that lead by John Tainer and Michael Adams. Their recent study of metalloproteins in *Pyrococcus furiosus* illustrates the difficulties involved in identifying metalloproteins found in even the most basic of life forms (Cvetkovic et al., 2010). It is estimated that there are around 2,000 encoded proteins in the *P. furiosus* genome (Lee et al., 2009), and if, as stated previously, one-third of them are expected to be metalloproteins, around 600 metalloproteins should be present. However, experiments conducted by the Tainer and Adams group demonstrated that only 50% of the metal peaks they analyzed contained a protein that could be linked to a known metalloprotein (Cvetkovic et al., 2010). This study demonstrates the large gap in our knowledge of what proteins even utilize a metal ion.

A conservative interpretation of these results is that for any organism yet to have it's metalloproteome mapped, 50% of these metalloproteins will have the metal association incorrectly predicted or it will not yet be known. In the human proteome, which consists of around 20,000 protein-encoding genes, an estimated number of unknown or misidentified metalloproteins can be predicted. About 6,600 protein-encoding genes will encode for metalloproteins (Waldron et al., 2009). Using *P. furiosus* as a guide, 3,300 of these will have a predicted metal association, of which a further three-quarter will exhibit a correctly predicted metal association. This leaves an estimated 4,125 metalloprotein-encoding genes that will have an incorrect metal association predicted, by current bioinformatic tools, or display no metal interactions (**Figure 1**). The complexity of a multi-cellular complex organism like the human body is expected to have a wider range of metal–protein interactions than those observed in *P. furiosus*. It should, however, serve as an indication of the complex task at hand that faces scientists embarking on the next phase of systems biology that encompasses this functional component of an organism.

WHAT ARE THE ANALYTICAL CHALLENGES TO METALLOPROTEOMICS?

The central issue that hampers the characterization of metalloproteins is difficulty in preserving their native state during analysis. Traditional proteomic approaches are generally incompatible for studying metal–protein interactions, as they tend to require denaturing conditions and enzymatic digestion, leading to disruption of the comparatively weak ionic interactions governing most metalloprotein bonds. A targeted metalloproteomics approach that acknowledges the importance of retaining native conditions is the answer to these issues, as it will provide the capability to determine the roles that metals play in the functional properties of proteins in biological systems, whilst ensuring that the detailed structural analysis of proteins is still obtainable.

The central tenet to characterizing metalloproteins is that in order to correctly identify a species the metal must still be bound to the protein. Once metalloproteins are no longer in their native state, misincorporation of metals or complete loss of metal becomes a significant problem. To accurately quantify metalloproteins it is vital that their native state is kept intact and is not altered by denaturing conditions. The use of strong acids/bases, concentrated inorganic salt, organic solvents and heat all contribute to the loss of native folded states. Thus, chromatographic separation for metalloproteomics should endeavor to use buffers that are of physiological pH, as this will help to prevent alterations to the secondary and tertiary structures that lead to the loss of metal binding. A metalloproteomics workflow must ensure that each possible source of experimental error is mitigated to a point where its influence is negligible.

Experimental error may be encountered even prior to analysis at the point of sampling. Metalloprotein integrity may be disrupted by reagents and buffers used in collection or sample preparation, and even storage conditions (Manley et al., 2009) such as repeated freeze-thaw cycles, that have an uncharacterized effect on metal–protein interactions. The post-mortem stability of SOD shows that the time in which it takes to freeze the sample post-collection does not have any significant effect on the concentration of the protein (Brooksbank and Balazs, 1984). However, the post-mortem stability of other metalloproteins is an area for further investigation. Error during this initial step of the experimentation causing loss of bound metals will impact

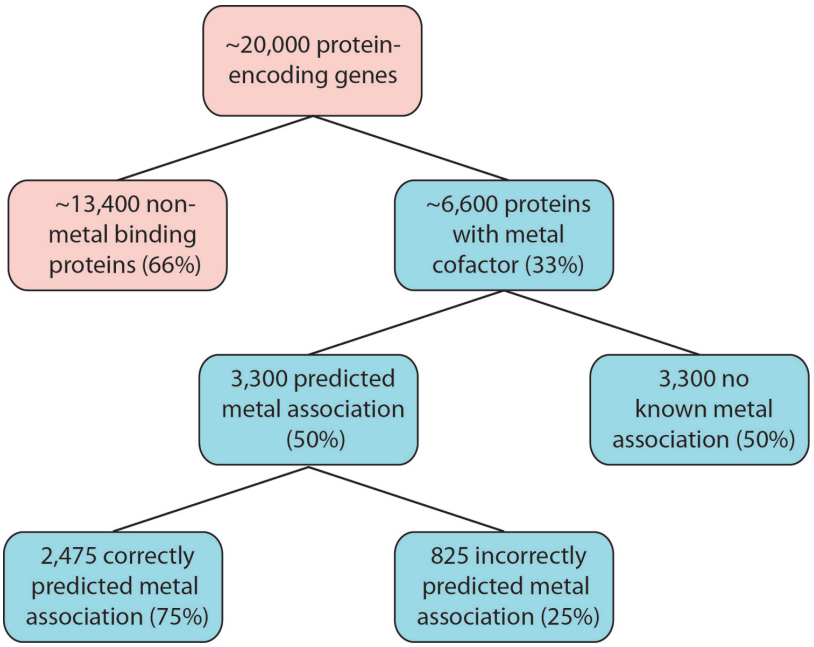

FIGURE 1 | Estimated size of the metalloproteome. Following the example of *P. furiosus*, as many as 6,600 human proteins are estimated to have metal association. Our limited knowledge of metalloproteins, combined with analytical limitations, will greatly impact on our ability to correctly predict the metal association, and therefore function, as a large number of these proteins are identified.

all of the conclusions that are drawn from the experiment. Even relatively inert chemicals, such as acetate buffers used in native size-exclusion chromatography (SEC), may impart unwanted effects on metal binding through the presence of a relatively strong complexing anion (Inagaki et al., 2000; Wang et al., 2007; El Balkhi et al., 2010).

Characterizing an unknown metalloprotein within an environment that is rich in proteins is exceptionally challenging. Metalloproteomic techniques have been used to characterize known metalloproteins, such as metallothionein isoforms (Chassaigne and Lobiński, 1998). Targeted proteomic approaches where the specific nature of the protein in question is already known does not present as many difficulties as a *de novo* approach, since these proteins can be targeted for isolation. There are multiple ways protein can be targeted and measured using a combination of chromatography techniques (del Castillo Busto et al., 2005) coupled to inductively coupled plasma-mass spectrometry (ICP-MS) and more recently with mass spectrometry. For example, mass spectrometry including top-down techniques have been used to characterize both apo- and metallated-metallothionein in both rabbit and dolphin liver as well as horse kidney (Chassaigne and Lobinski, 1998; Chassaigne and Lobiński, 1998; Ryvolova et al., 2011; Pedrero et al., 2012). These studies highlight the kind of detailed information that can be obtained by using a combination of mass spectrometry and chromatography to study a particular protein.

HOW DO WE OVERCOME THESE ANALYTICAL LIMITATIONS?

The limitations to current metalloproteomics outlined above are not insurmountable; rather they simply require some lateral thinking regarding how we integrate modern analytical technology into systems biology. A variety of individual techniques can be combined for metalloproteomic experimental procedures, and these can generally be easily integrated into traditional proteomic workflows (**Figure 2**; Lancaster et al., 2011). By not reinventing the wheel, integration of atomic mass spectrometry (specifically ICP-MS) will allow for metal quantification and direct correlation between the presence of metal species and the function of associated proteins (Lelie et al., 2011; Alvarez et al., 2012). This comprehensive approach will greatly supplement the solely sequence information normally obtained through mass spectrometry independent of any other complementary technique.

Protein peaks identified from chromatographic separation can be correlated to the metal concentration peaks identified by online ICP-MS detection, allowing for the likely position of the metalloprotein to be determined, providing not only selectivity but also sensitivity (Gercken and Barnes, 1991; del Castillo Busto et al., 2005; Lopez-Avila et al., 2006; Manley et al., 2009). 2D separation offers a powerful tool for resolving complex mixtures of metalloproteins, and the multi-elemental capacity of ICP-MS produces a hyperspectral snapshot of metal–protein interactions in a single sample. Like traditional proteomics, this approach will use the vast arsenal of protein databases available to determine which of the proteins identified is most likely to exhibit metal–protein interaction. The 20,000+ proteins predicted in human samples will produce complex fractions highlighting the large dynamic range needed and requirement for fractionation.

Although there are inherent difficulties in investigating proteins in their native state, advances in mass spectrometry and

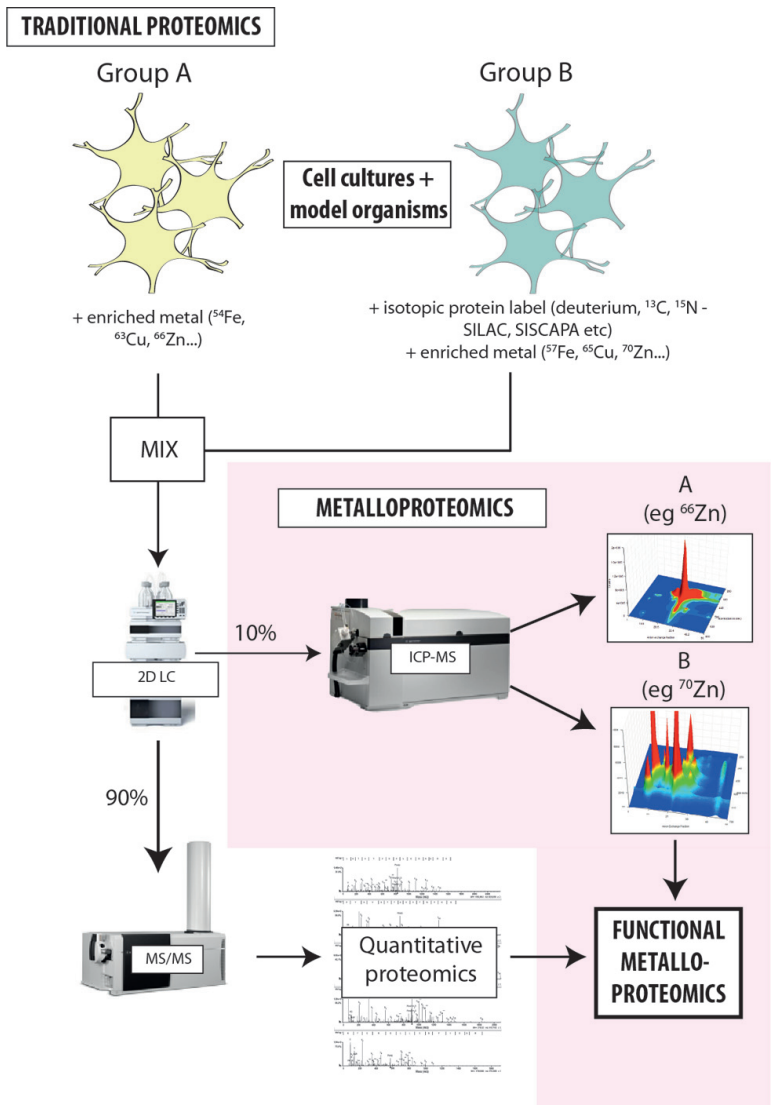

FIGURE 2 | Proposed workflow for integrated metalloproteomics. Hyphenating native separation techniques to ICP-MS is the key to unlocking the secrets of metals and protein function. Rather than relying solely on bulk measures, directly associating metals with specific proteins provides new insight into how metals carry out biochemical processes in the cell. At the current state of our knowledge about the level of metalloproteins in biology the coupling of size exclusion to ICP-MS have the promise of being quantitative thus allowing the comparison of different samples and detail about the global or metalloprotein specific changes that occur. Despite SEC being a low resolution technique it can allow researchers to make educated guesses about the ID of proteins of interests based on their MW. Continued evolution of hyphenated LC-ICP-MS will only increase the arsenal of tools at our disposal to discover, identify, and characterize metalloproteins. The workflow developed in our laboratory adapts existing isotope labeling techniques used for proteomics [such as SILAC (stable isotope labeling by amino acids in cell culture) and SISCAPA (stable isotope standards and capture by anti-peptide antibodies)] to include the addition of isotopically enriched metal salts, providing new opportunities to probe the direct relationship metal cofactors have with protein function and allowing simultaneous analysis of both metals and proteins in individual experimental groups. Highly sensitive, isotope-specific ICP-MS detection is used to align metal distribution with quantitative proteomics, directly associating the presence of a protein species with a specific, metal-mediated function. This approach is extremely cost-effective, and can be seamlessly integrated into existing workflows with minimal disruption to the standard laboratory process.

analytical techniques are now making it possible to carry out investigations on intact proteins. Groundbreaking work from Joe Beckman's laboratory has shown that using Fourier transform-ion cyclotron resonance MS it is possible to directly quantify and determine the metal status of a single protein from a specific cell type directly from tissue (Rhoads et al., 2011, 2013). This demonstrates that quantitative determination of both the amount of protein and the metal status can be achieved from biological tissue. Providing a new level of detail for a disease where it is clear that the metal status of a protein is key in the disease process (such as amyotrophic lateral sclerosis; Estévez et al., 1999), this technology is invaluable. The advent of top-down proteomics will be an increasingly valuable tool for metalloproteins identification.

HOW DOES METALLOPROTEOMICS RELATE TO NEURODEGENERATION?

The brain is the most complex organ in the human body. Some of the proteins in the brain, just like the proteins in other tissues, require metal cofactors for function. Variations of the amount of metal that is present, or defects in the way a protein associates with a metal ion may cause disease states, particularly with regard to neurodegeneration.

The level at which metals are present within the brain is generally higher then the levels of the same metals in the rest of the body, and concentrations of metals in the brain is highly compartmentalized (Harrison et al., 1968; Hare et al., 2012b; Roberts et al., 2012). When metal homeostasis in these regions is altered, brain pathologies and increased oxidative stress is observed. As we age metals accumulate in the brain (Zecca et al., 2004) and this along with any other alterations in metal homeostasis can lead to neuronal damage, death, oxidative stress and may even cause misfolding and aggregation of proteins. However, it is important to point out that the accumulation of metals in tissues is not necessarily labile metals, but also metalloproteins. For example, it is estimated that there is less than one free Cu ion per cell (Rae et al., 1999). Thus an observed accumulation in bulk levels of Cu will have concomitant accumulation of Cu-metalloproteins. This shift in thinking about the general accumulation of metals to the change in functional metalloproteins is the basis of metalloproteomics. Bulk analysis only indicates a global change and still poses the question: do all metalloproteins change or are there specific targets? Our investigations suggest the latter.

In order to fully understand the roles that these metalloproteins play in age-related diseases it is first important to understand the role they play in the aging brain. Studies of trace elements and aging have shown consistent changes in trace elements such as a decrease in Rb and K and increase in metals Fe, Cu, and Co (Ehmann et al., 1984; Takahashi et al., 2001). The identification and characterization of metalloproteins is essential to understanding their functions and elucidating the specific disease pathways they are involved in, as well as assisting with the diagnosis of specific conditions (Gercken and Barnes, 1991; Muñiz et al., 2001; Lopez-Avila et al., 2006; El Balkhi et al., 2010). Characterized metalloproteins such as hemoglobin, transferrin, SOD, and ceruloplasmin are used in clinical laboratories as markers for specific disease states (Swart, 2013), such as anemia, inflammation (Ahluwalia, 1998), Down's syndrome (Brooksbank and Balazs, 1984), and Wilson's disease (Mak et al., 2008), respectively.

Metals have long been thought to play a role in pathophysiology of Alzheimer's disease (AD) and Parkinson's disease (PD). In AD, the concentration of three of the most abundant biochemically functional metals (Cu, Zn, and Fe) are altered in respect to their locations (i.e., metal redistribution in response to plaque formation). These metals are also thought to play a role in β-amyloid aggregation, which in turn will cause plaque formation, leading to the neurodegenerative effects seen in AD (reviewed in Roberts et al., 2012). The level of Cu in AD brain tissue is decreased, as it is hypothesized that Cu is removed from the tissue and associates itself with the senile plaques that are forming (Deibel et al., 1996; Hung et al., 2010). Zn is also thought to be associated with β-amyloid plaques (Bush et al., 1994), indicating that it has also been redistributed within the brain. Fe is also thought to be associated with plaques, but the overall change of Fe levels within the brain tissue surrounding fibril inclusions has yet to be described conclusively (Lovell et al., 1998; Schrag et al., 2011).

In PD, the levels of Fe and Zn have been suggested to be elevated, while the level of Cu may be decreased. Cu and Fe have been shown to interact with the protein alpha synuclein (Davies et al., 2011; Camponeschi et al., 2013), which is involved in proteinaceous Lewy body formation. Their interaction with alpha synuclein leads to crosslinking and protein aggregation (Barnham and Bush, 2008). Initial research into post-mortem changes in metals in PD dating back to the 1920s indicated that normal Fe became diminished from cells that are present in the globus pallidus and abnormal deposits of large quantities of Fe were found in globules in the same region of the brain (Lhermitte et al., 1924). Continued pursuit of elucidating the role of metals in PD pathogenesis has still only provided limited insight into the direct metal binding proteins involved, and limitations to analytical technology still present considerable problems for accurate quantification of metal changes (Hare et al., 2012a).

CONCLUDING REMARKS

Perhaps the biggest impediment to the study of the metalloproteome has been technical limitations. However, the rapid advances in mass spectrometry and analytical chemistry in general over the past decade has helped, in some part, to overcome the shortfalls in the tools available to routinely study incredibly complex matrices. The ubiquitous nature of metal ions and metalloproteins coupled with our relatively limited knowledge about the metalloproteome presents an exciting frontier of new discoveries for the modern biochemist. As the continued growth in analytical technology finds new footholds in the life sciences, we can expect a transition from bulk analysis of trace elements to a detailed investigation of the metalloproteins. With this transition the interest in metalloproteomics will grow exponentially.

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Metal-induced neurodegeneration in *C. elegans*

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The model species, *Caenorhabditis elegans*, has been used as a tool to probe for mechanisms underlying numerous neurodegenerative diseases. This use has been exploited to study neurodegeneration induced by metals. The allure of the nematode comes from the ease of genetic manipulation, the ability to fluorescently label neuronal subtypes, and the relative simplicity of the nervous system. Notably, *C. elegans* have approximately 60–80% of human genes and contain genes involved in metal homeostasis and transport, allowing for the study of metal-induced degeneration in the nematode. This review discusses methods to assess degeneration as well as outlines techniques for genetic manipulation and presents a comprehensive survey of the existing literature on metal-induced degeneration studies in the worm.

Keywords: neurodegeneration, behavior, metals, *C. elegans*, neurotransmitters

INTRODUCTION

The simplicity of the small nematode *Caenorhabditis elegans* (*C. elegans*) makes it a suitable model organism for biomedical research. Compared with mammalian models, it has a number of advantages that makes it a powerful tool for studies on human disease. First, 60–80% of human genes have corresponding genes in *C. elegans*, depending on the specific bioinformatic methods used (Titus and Michael, 2006). Many genetic factors causing human diseases have corresponding worm homologs, allowing for mechanism-based studies. Second, the small size and the fast life-cycle of the worm allow for easy maintenance. The adult worm is only 1.3 mm long and it takes 3 days for an embryo to reach adulthood and reproduce. A 100 mm petri-dish can accommodate thousands of self-fertilizing nematodes within couple of days. This is particularly important for pharmaceutical drug screens. Third, *C. elegans* are transparent, which allows *in vivo* study with fluorescent reporters, such as green and red fluorescent proteins. This has been widely used to study axon guidance, neurodegeneration, endocytosis and fat metabolism in living worms. Fourth, the simple, but complete nervous system makes it easy to investigate neurological function in *C. elegans*. Although a worm has only 302 neurons, it shares similar neurotransmitters with humans, including dopamine (DA), acetylcholine (ACh), serotonin (5-HT), *gamma*-aminobutyric acid (GABA), glutamate, and others. Fifth, a variety of tools are available in *C. elegans.* The genome and cell line lineage have been completely mapped, RNA interference (RNAi) libraries are able to knock down 90% of genes, a large pool of knockout mutants are available from the *C. elegans* genetic center (CGC) and commercial worm-specific microarray probes have been developed.

A big advantage afforded by *C. elegans* as a model for studies on human neurological diseases is the simplicity of its nervous system: the worm contains 302 neurons and about 5,000 synapses (White et al., 1986). Despite this simplicity, *C. elegans* has a complete nervous system with four functional categories of neurons based on their circuitry: (1) motor neurons, which pass synaptic signals to muscle cells; (2) sensory neurons, which convert environmental signals into internal stimuli; (3) interneurons, which receive and deliver signals between neurons; and (4) polymodal neurons, which have two or more of the functions described above. Most commonly, studies are performed on different types of neurons based on their neurotransmitter profile.

The biosynthesis and transport of neurotransmitters are conserved in the nematode and human nervous system. Among different types of neurons, dopaminergic (DAergic) and gamma-aminobutyric acid (GABA-ergic) neurodegeneration are the two best characterized. DAergic neuron death is a well-known feature of Parkinson's disease (PD). *C. elegans* hermaphrodites have eight DAergic neurons; two pairs of cephalic (CEP) neurons, a pair of anterior deirid (ADE) neurons, and a pair of postdeirid (PDE) neurons (WormAtlas, 2002, 2003, 2004, 2005, 2006, 2007, 2008, 2009; Toth et al., 2012; WormBase, 2013). Since their genome has been fully characterized it is also known that they contain all genes responsible for DA biosynthesis, packaging and reuptake (Sulston et al., 1975; Jayanthi et al., 1998). Male *C. elegans* have additional DAergic neurons (Sulston et al., 1975). DA signaling is important in mediating learning and locomotion behavior (Sawin et al., 2000). DA is also a requirement for olfactory adaptation (Colbert and Bargmann, 1995). The role of DA in modulation of locomotor activity has been exploited to study DAergic degeneration. Using green fluorescent protein (GFP) (Nass et al., 2002; Cao et al., 2005) or mCherry protein (Harrington et al., 2012) to visualize DAergic neurons in *C. elegans*, researchers are able to monitor neurodegenerative processes in living animals. Many known causes of PD also result in DAergic neurodegeneration in *C. elegans*, including aberrant proteins [alpha-synuclein (Cao

et al., 2005; Harrington et al., 2012)], heavy metals [i.e., manganese (Mn) (Benedetto et al., 2010), methylmercury (MeHg) (VanDuyn et al., 2010), aluminum (VanDuyn et al., 2013)], and environmental contaminants [i.e., fungicides (Harrison Brody et al., 2013), pesticides (Negga et al., 2012), insecticides (Mocko et al., 2010), 1-methyl-4-phenylpyridinium (MPP^+) (Pu and Weidong, 2008; Mocko et al., 2010), and 6-hydroxydopamine (6-OHDA) (Cao et al., 2005; Ruan et al., 2010)]. The DAergic neurons are mechanosensory and defects or loss of these neurons results in behavioral changes in response to environmental stimuli (i.e., food sensing).

In vertebrates, GABA is the most abundant neurotransmitter in inhibitory synapses in the central nervous system (CNS), while in nematodes, GABA is used as both an inhibitory and excitatory neurotransmitter primarily at neuromuscular synapses, as worms do not have a CNS. GABAergic neurons in the nematode consist of 26 neurons that are categorized under six classes, DD, VD, RME, RIS, AVL, and DVB, based on their synaptic outputs. DD and VD innervate the dorsal and ventral body muscles, RME innervate the head and AVL and DVB innervate the enteric muscles and RIS are interneurons (White et al., 1986). Worms defective in GABAergic function exhibit "shrinking" behavior, whereby the nematode simultaneously hypercontracts both ventral and dorsal body muscles, as well as abnormal defecation and foraging. *unc-25* encodes the GABA enzyme glutamic acid decarboxylase and *unc-47* encodes the GABA vesicular transporter (McIntire et al., 1997; Jin et al., 1999). *unc-30* encodes a homeodomain protein of the Pitx family and regulates the differentiation of the D-type GABAergic neurons (Jin et al., 1994). Overexpression of genetic risk factors associated with Alzheimer's disease (AD) [tau (Kraemer et al., 2003; Miyasaka et al., 2005; Brandt et al., 2009)] and Amyotrophic lateral sclerosis (ALS) [TDP-43 and FUS (Vaccaro et al., 2012)], as well as exposure to several heavy metals [lead (Pb), mercury (Hg), copper (Cu), chromium (Cr), and Mn (Du and Wang, 2009; Xing et al., 2009b)] results in GABAergic neurodegeneration in nematodes. The loss or injury of GABAergic neurons alters locomotion (McLntire et al., 1993), foraging (White et al., 1986) and defecation (McLntire et al., 1993) behaviors, which may be used to monitor the integrity of GABAergic neurons.

In addition to DAergic and GABAergic neurons, neurodegeneration has also been studied in cholinergic neurons. In *C. elegans*, ACh is the major excitatory neurotransmitter, which directly or indirectly regulates locomotion (crawling and swimming) (Mullen et al., 2007), egg laying (Bany et al., 2003), pharyngeal pumping (McKay et al., 2004), defecation cycling (Thomas, 1990), and male mating (Liu and Sternberg, 1995). Gain-of-function nicotinic ACh receptor *acr-2* (Barbagallo et al., 2010) and selenium (Se) (Estevez et al., 2012) are both able to induce cholinergic motor neuron degeneration and paralysis in worms.

Another neurotransmitter, serotonin (5-HT), is synthesized in eight types of neurons and regulates locomotion, defecation, egg laying, male mating and pharyngeal pumping in worms (Mendel et al., 1995; Segalat et al., 1995; Weinshenker et al., 1995; Niacaris and Avery, 2003). The basal slowing response and egg laying assay have been well-characterized in studies on 5-HT signaling. Mutants for 5-HT enzymes, *bas-1* and *cat-4* (both involved in 5-HT and DA biosynthesis), have been used to analyze serotonin-mediated behaviors (Loer and Kenyon, 1993; Waggoner et al., 1998; Sawin et al., 2000). The locomotor behaviors have been used most often for analysis of neurodegeneration and application of exogenous 5-HT inhibits locomotion, but stimulates egg-laying and pharyngeal pumping (Horvitz et al., 1982; Segalat et al., 1995; Weinshenker et al., 1995; Sawin et al., 2000; Rogers et al., 2001; Niacaris and Avery, 2003). In many of the worm behavioral assays it is difficult to determine the effects of DA vs. 5-HT. Degeneration of other types of neurons induced by metal exposure is less studied in worms, thus not discussed here.

Finally, glutamate is important in synaptic transmission, plasticity and disease, with excitotoxicity, inflammation, oxidative stress, and mitochondrial dysfunction implicated in ALS. Excitatory and inhibitory ionotropic glutamate receptors (iGluRs) mediate some behavior and approximately 10 putative iGluR subunits are expressed in *C. elegans (glr 1-8 and nmr 1-2)* (Brockie et al., 2001). Six of the subunits, including *glr-1*, *glr-2*, *glr-4*, *glr-5*, *nmr-1*, and *nmr-2*, are expressed in many of the *C. elegans* interneurons. GLR-1 in particular has been linked to mediating the avoidance of hyperosmotic stimuli (Mellem et al., 2002), control of forward and backward movement (Brockie et al., 2001), foraging behavior (Hills et al., 2004), and long-term memory (Rose et al., 2003). There is also conserved sequence and function of the vesicular glutamate transporter (*eat-4*) (Lee et al., 1999), and other glutamate transporters (*glt-1; glt-3-7*) (Mano et al., 2007).

In summary, *C. elegans* provides a valuable research tool to study the mechanisms of metal induced human neurological disorders by permitting the visualization of different types of neurons and assessment of their function by florescence labeled cell bodies and functional behavior assays, respectively.

TECHNIQUES FOR STUDYING NEURODEGENERATION IN *C. elegans*

Conserved neurotransmitter biology and high homology with mammalian systems make *C. elegans* a unique system for studies on neurodegenerative disease. The processes of synaptic release, trafficking and formation of neurotransmitters are also conserved. Methods to study neurotoxicologic endpoints have included examining the morphology, behaviors, and changes in gene expression and neurotransmitters.

TOOLS FOR ASSESSING NEURONAL STRUCTURE

Fluorescently tagged neurons and microscopy

Contributing to the investigative value of this species is the transparency of the worm, allowing for *in vivo* visualization of fluorescently labeled neurons. Cell bodies and individual processes can be visualized *in vivo* in the worm. Morphological changes, characterized by puncta, blebs, neuronal absence or shrinkage, presence of vacuoles, dorsal or ventral cord gaps, loss of cell bodies or strand breaks in neuronal processes can be used as indicators of neurodegeneration (Nass et al., 2002; Martinez-Finley and Aschner, 2011). GFP has frequently been employed as a reporter construct to visualize specific neurons and synapses in *C. elegans*. DAergic neurons have been tagged for visualization most frequently through the *dat-1*::GFP reporter (Helmcke et al., 2010;

VanDuyn et al., 2010), serotonergic neurons through *tph-1*::GFP (Sze et al., 2000; Nass et al., 2002), GABAergic neurons through *unc-25*::GFP (Cinar et al., 2005), and cholinergic neurons through *unc-1*::GFP (Winnier et al., 1999; Nass et al., 2002), and glutamatergic neuron through *eat-4*::GFP (Lee et al., 1999; Earls et al., 2010). Reporter gene fusions also allow for visualization of neuronal morphology and protein expression patterns. Visualization of the neurons is useful, but it is important to correlate the structural damage with cellular and molecular changes as quenching of GFP fluorescence is a possibility and can result in false data interpretation. Both confocal and standard fluorescence microscopy have been used to visualize fluorescently tagged neurons. Electron microscopy has also been used historically to measure structural integrity and fluorescence-activated cell sorting (FACS) has been utilized for purification.

TOOLS FOR ASSESSING NEURONAL FUNCTION

Behavioral assays

C. elegans behavior is used as a reliable and sensitive measure of the function of neurons as the behaviors are sensitive to underlying cellular and molecular alterations. There are a large variety of behavioral tests in *C. elegans* to assay neurodegeneration. There are nematode equivalents of mammalian DAergic, serotonergic, cholinergic, GABAergic and glutamatergic neurons (see above) and high homology exists between the two systems. A number of behaviors can be assayed to determine the function of the cholinergic and glutamatergic neurons, but these behaviors have not been extensively studied in regards to metals and neurodegeneration, and therefore are not included in this review [for a review see (WormBase, 2013)]. Many of the assays listed below have been designed to assess DA, 5-HT and GABA subtypes.

Basal slowing response

Bacterial mechanosensation induces the DA-mediated slowing of locomotion in the presence of food (bacteria), and can be measured by counting the number of body bends per 20-s interval (Sawin et al., 2000). Locomotor rates are compared between the well-fed worms placed on plates of food vs. those placed on plates without food and the measurement is referred to as the change (Δ) in body bends over a set period of time. A lower value represents less slowing on food and therefore deficits in DAergic function as worms deficient in DA do not slow their locomotion in the presence of food. The *cat-2* strain is tyrosine hydroxylase (TH)-deficient and therefore defective in bacterial mechanosensation, making it a positive control (Sawin et al., 2000). Proper controls for the assay include well-fed nematodes and consistency in the size and thickness of the bacterial lawn in all plates used. *Bas-1* and *cat-4* mutants also do not slow in response to the bacterial lawn (Sawin et al., 2000). Application of exogenous serotonin can rescue the behavior in *bas-1* and *cat-4* mutants. In this assay the key to parsing out 5-HT effects from DA effects is the state of the worms; the 5-HT version of this task requires starved worms, while the DA version requires that the worms be well-fed.

Ethanol preference

Ethanol preference in *C. elegans* requires DA and serotonin. This has been shown using *cat2* and *tph-1* mutants as these worms do not show a normal wildtype response in the ethanol preference task (Lee et al., 2009). In this assay young adult worms are preincubated on a seeded control or ethanol plate. After an incubation period the worms are removed from the preincubation plates and placed on assay plates. Assay plates are divided into quadrants, with two quadrants laced with ethanol and the other without. Worms are placed at an origin point of an assay plate and allowed to move for 30 min at which point they are scored for their quadrant of preference. A preference index (PI) is calculated as [(number of worms in ethanol quadrants) − (number of worms in control quadrants)]/Total number of worms tested (Lee et al., 2009). Worms with functional DA and 5-HT develop ethanol preference whereas mutant worms do not.

Area-restricted searching (ARS)

This task capitalizes on the fact that worms must have functional DA in order to search for food. Wildtype worms that have consumed and exhausted the immediate food source will expand their search to surrounding areas. This behavior is measured by time-dependent reduction in turning frequency after the last food encounter. After a short time if the worm cannot find more food in the immediate area, the frequency of high-angled turns is reduced and the animal begins to explore more distant areas. Functional DA is required for ARS as ablation of dopaminergic neurons eliminates ARS behavior and preincubation with DA restores the ARS behavior in worms with defective DA (Hills et al., 2004). The glutamatergic neurons also participate in this behavior as glutamate receptor mutants exhibit problems with the ARS behavior (Hills et al., 2004).

Habituation task/tap withdrawal response

Functional DA allows worms to alter their behavior in the tapping task. Nematodes respond to a mechanical stimulus (plate tapping) by accelerating their forward locomotion rate or by swimming backwards. Repeatedly tapping of the plate results in worms habituating to the stimulus and decreasing the frequency of their reversals. What is measured is the time that it takes to habituate to the tapping rather than whether or not the worm responds to the tapping. All worm strains tested in this task are able to reduce their reversal rates but the distinguishing factor of whether DAergic neurons are functional or dysfunctional is the lag time in response. Intertapping interval is also an important consideration in this assay. *cat-2* mutant worms habituate to tap faster than wildtype worms and this response can be rescued by pre-exposing mutants to exogenous DA (Groves and Thompson, 1970; Broster and Rankin, 1994; Rose and Rankin, 2001; Sanyal et al., 2004).

Pharyngeal pumping and thrashing behaviors

Pharyngeal pumping and thrashing rates are controlled by various mechanisms in the nematode. The pharynx is responsible for feeding via rhythmic contractions. Age-related decline in pharyngeal pumping rate has been described in *C. elegans* and is correlated with a decline in survival probability and body movement decline. Pumps per minute are manually counted using a dissecting microscope to assess pharyngeal pumping rate. The maximum pumping rate of the pharynx is ∼300 pumps per

minute (in adults that are at least 2 days old). Thrashing behavior is a common test in *C. elegans* for measuring motility. Thrashing behavior is assessed by videotaping nematodes that are placed in ∼10 μL of water on a transparent plate with shallow, concave wells. A thrash is a change in direction in midbody bending. A body bend is counted as a change in the direction of the posterior bulb of the pharynx. There are multiple programs used to assess this behavior including Worm Tracker, Nemo, Parallel Worm Tracker, OptoTracker, Multimodal illumination, and tracking system, CoLBeRT, many of which use MATLAB, JAVA, or LabVIEW software to quantify movement (WormBase, 2013). This assay, along with visualization of the neurons, has been used to assess GABAergic function after lead or mercury exposure (Helmcke et al., 2009; Xing et al., 2009b).

MEASUREMENT OF NEUROTRANSMITTERS

The neurotransmitters, DA and serotonin, and neuromodulators, octopamine and tyramine, can all be detected in *C. elegans* extracts by high-performance liquid chromatography (HPLC) (Sulston et al., 1975; Horvitz et al., 1982; Sanyal et al., 2004; Alkema et al., 2005). Thousands of worms (over 30 ul of packed worms) must be used for HPLC analysis and the worms must be washed several times to avoid bacterial contamination (Sulston et al., 1975; Horvitz et al., 1982; Sanyal et al., 2004; Alkema et al., 2005).

USING *C. elegans* AS A MODEL OF NEURODEGENERATION: MODIFYING GENETICS

The *C. elegans* model system has become a popular and innovative tool in advancing mechanistic studies concerning various neurodegenerative processes. The generation of genetic knockouts and knockdowns can be achieved rapidly in nematodes due to their quick life cycle and short lifespan. Classical forward genetics studies involve treating organisms with mutagens to induce DNA mutations followed by isolation of individuals with atypical phenotypes of interest. Reverse genetics allows the opposite strategy by altering a specific gene in order to study its function and the role it may play in various processes and pathways. RNA interference (RNAi) and transgenesis, which can be accomplished using microinjection and gene bombardment techniques are also tools for achieving gene knockdown.

Microinjection

This approach uses a microinjection needle to introduce a plasmid carrying the gene of interest into the cytoplasmic core of distal gonad arms in a hermaphrodite worm, allowing the delivery of the plasmid to several germ cell nuclei and the production of extrachromosomal DNA arrays (Ahringer, 2006). The simplest approach to transgenesis typically involves microinjecting a plasmid that contains a cloned 5′ regulatory sequence of the gene of interest fused to a reporter gene whose activity can then be assayed; a 3′ UTR should also be included (Praitis and Maduro, 2011). As the worm is fully transparent, it is usually desired to fuse genes of interest to a fluorescent reporter whose intensity can then be imaged, such as GFP, yellow fluorescent protein (YFP), or m-cherry (Miller et al., 1999). There are also sequences specific for subcellular targeting that can be included in the plasmids, such as a nuclear localization signal (NLS) for nuclear targeting or a mitochondrial targeting sequence (MTS) for mitochondrial localization. Similarly, tissue or cell-specific expression can also be studied using transgenes that are directed by tissue or cell-specific promoters (Praitis and Maduro, 2011). Additionally, it is typical to include a coinjection marker (antibiotic resistance, tissue-specific fluorescent promoter, etc.) to follow the transgene through the transformation process in larvae and subsequent crossing. However, this is not always necessary if the transgene containing the gene of interest includes a marker itself that can be conveniently distinguished from non-transgenic worms (e.g., a bright GFP reporter).

Following microinjection of transgenes, the DNA arrays produced are extrachromsomal and are not fully integrated into the genome (Ahringer, 2006). Without integration, the arrays remain unstable and can be lost through successive generations. Gamma or UV irradiation or exposure to chemical mutagens can induce integration of the arrays into chromosomes. A small population of the transformed nematodes is mutagenized, resulting in several F1 animals that produce the F2 generation that can then be assayed for 100% transmission of the transgene. Integration of the original transgene is vital for any further genetic manipulation of the transformed lines, eventually allowing multiple transgenes to be incorporated into the same strain (Praitis and Maduro, 2011). As with most genetic manipulations, integrated strains should be backcrossed several times in order to remove any mutations introduced into the genome from the integration process.

However, high copies of the transgene in the extrachromosomal array can lead to undesirable expression of the transgene, ranging from increased transgene expression relative to the targeted endogenous gene to gene silencing from tandem repeat sequences present in the array. For this reason, another method of transgenesis uses microparticle bombardment. Biolistic transformation allows for direct insertion of transgenes into the chromosome, especially with a low-copy number that becomes fully integrated. Bombardment is usually done with the DNA-coated gold beads that are then bombarded onto L4 and adult hermaphrodites. This technique revolves around the ability of DNA to form a complex with gold particles in the presence of $CaCl_2$, where the DNA itself is protected from degradation by using cationic polyamines (e.g., spermidine) (Isik and Berezikov, 2013). Researchers have also found that this "gene gun" technique not only reproducibly allows for invariable expression levels and patterns that are difficult to obtain with extrachromosomal arrays, but that low-copy transgene expression does not get silenced in the germline (Praitis et al., 2001). Newer techniques to allow single- or low-copy transgene expression without bombardment are in the process of development, including the use of ultraviolent trimethylpsoralen (Kage-Nakadai et al., 2012).

RNA interference (RNAi)

This methodology involves the exogenous introduction of double-stranded RNA (dsRNA) that is complementary to a specific sequence of the gene of interest into the model organism, resulting in the activation of an endogenous cellular pathway that causes significantly decreased expression of that gene. This technique is advantageous in the *C. elegans* system and is dependent

on RNA-dependent RNA polymerases (RdRPs) (Simmer et al., 2002); unlike mammals, RNAi is actually heritable in nematodes, as the systemic gene knockdown can persist in the progeny.

RNAi effect can be achieved in three ways: (1) by microinjection of a dsRNA sequence complementary to the gene of interest into the body cavity, including the gonad and intestine, (2) by soaking worms in a dsRNA-containing solution, and (3) by feeding worms with *E. coli* expressing the dsRNA of interest (Tabara et al., 1998).

The unique, highly systemic nature of RNAi in *C. elegans* is thought to occur due to a rapid transport of dsRNA from cell to cell through the SID-1 channel. This transmembrane protein allows for passive, bidirectional transport to cells that do not initially receive the dsRNA delivery, and is selectively gated by the presence of dsRNA (Shih and Hunter, 2011). Wildtype N2 strains are sufficient in producing knockdowns using RNAi; however, some RNAi-hypersensitive strains can be necessary to increase the knockdown strength and phenotype penetrance. These strains include *rrf-3*, *eri-1*, and *lin-15B*. *rrf-3* is a *C. elegans* RNA-dependent RNA polymerase (Simmer et al., 2002), while *eri-1* (enhanced RNAi) is a 3′-5′ exoribonuclease that negatively regulates RNAi (Kennedy et al., 2004) and *lin-15B* is important in cell differentiation and negatively regulates vulval development, as well as RNAi (Wang et al., 2005). There are currently two commercially available RNAi feeding libraries. The Ahringer lab library contains about 16,757 clones that were constructed from cloning gene-specific genomic sequences between two T7 promoters. The Vidal lab library contains 11,511 clones and was made by the Gateway cloning system that clones full-length open reading frames (ORF) templates into double T7 vectors. Together, these libraries cover approximately 94% of the *C. elegans* genome (Ahringer, 2006; Antoshechkin and Sternberg, 2007).

Gene knockout

In comparison to RNAi-mediated gene knockdown, knockout in *C. elegans* was made simple by the development of the MosDel system, or Mos-mediated deletion. A plasmid containing Mos1 is delivered to a DNA strand that is next to the target gene, causing a break in that strand. Upon induction of cellular DNA damage repair mechanisms, a DNA template that lacks the targeted gene of interest is introduced and matches the sequence of the broken DNA strand (Frokjaer-Jensen et al., 2010). Thus, this technique tricks the cell into knocking out the gene by repairing the original double-strand break with a DNA template that does not contain the gene of interest. Large-scale projects have aimed to utilize the MosDel system to create large libraries of transposon-tagged alleles (Bazopoulou and Tavernarakis, 2009). Prior to the development of this method, knockouts were primarily generated using random chemical mutagenesis (i.e., EMS mutagenesis) to create loss-of-function mutants that were then screened using gene-specific primers for random deleted regions in targeted genes. However, with this technique there is the possibility of random background mutations and it is not as specific as the MosDEL system. The commercial availability of deletion mutants is possible through the *C. elegans* Gene Knockout Consortium (GKC) and the National BioResource Project of Japan (NBRP), both of whom send their isolated deletion mutants to the *Caenorhabditis* Genetics Center (CGC) for distribution (Antoshechkin and Sternberg, 2007).

MECHANISMS OF NEURODEGENERATION INDUCED BY METAL

Aluminum (Al)

Al is one of the most abundant metal elements in the Earth's crust and exposure to bioavailable Al may be significant owing to its ubiquitous presence in soil and fertilizers, cookware, and pharmaceutical and cosmetic preparations (Verstraeten et al., 2008). Although the physiological requirement for Al has yet to be defined, it has been posited that Al may have an etiopathogenic role in neurodegenerative diseases (Halatek et al., 2005). Studies with Al in the form of $AlCl_3$, $Al(NO_3)_3$, or $Al_2(SO_4)_3$ identified phenotypic abnormalities, including toxic effects on lifespan, body size, development, reproduction, locomotion, behavioral plasticity, and memory in the worm (Wang et al., 2009; Page et al., 2012). Additionally, Page et al. showed changes in elemental composition of whole worms exposed to Al, noting a significant increase in multiple metals and ensuing oxidative stress (Ba, Fe, Cr, and Cu), hypothesizing that altered levels of these elements contributed to the aforementioned phenotypes seen in chronic Al toxicity (Page et al., 2012).

VanDuyn et al. addressed molecular attributes of Al-induced DA neurodegeneration in *C. elegans* (VanDuyn et al., 2013). A brief exposure to Al decreased mitochondrial membrane potential and cellular ATP levels and led to DAergic neurodegeneration, which was dependent upon SMF-3, a homolog to the human divalent metal transporter (DMT1). Al exposure significantly decreased SMF-3 protein levels and exacerbated DAergic neurodegeneration in the presence of human PD-associated protein α-synuclein, Nrf2/SKN-1 and Apaf1/CED-4. Deletion of SMF-3 conferred resistance to Al due to sequestration of Al in an intracellular compartment (VanDuyn et al., 2013). Neuroprotection was also reported by Ye et al. showing that a treatment with trace vitamin E could ameliorate memory deficits in Al exposed worms (Ye et al., 2008).

The acute and chronic effects of Al_2O_3 nanoparticles (NPs) in *C. elegans* have been recently addressed (Yu et al., 2011; Li et al., 2012). Acute toxicity was associated with increased lethality and altered growth, reproduction and stress responses, whereas chronic toxicity led to increased oxidative stress. Li et al. noted decreased locomotion behaviors in nematodes chronically exposed to Al_2O_3-nanoparticles (NPs) concomitant with increased reactive oxygen species (ROS) generation and disruption of ROS defense mechanisms, secondary to the suppression of Mn-SODs (Li et al., 2012).

Copper (Cu)

The essential trace element Cu serves as a cofactor in many critical biological processes such as in iron (Fe) homeostasis (ceruloplasmin), catecholamine biosynthesis (tyrosinase, dopamine-b-hydroxylase), oxidative phosphorylation (cytochrome c oxidase), and oxidative stress protection (superoxide dismutase) (Arredondo and Nunez, 2005). Therefore, Cu deficiency or overload may result in multiple pathologies, including irreversible CNS damage (Prohaska,

2000; Taly et al., 2007; Diaz-Veliz et al., 2008). In *C. elegans*, Cu deficiency led to decreased Cu/Zn superoxide dismutase (SOD), reducing defenses against oxidative stress (Yang et al., 1998). Exposure to excess $CuSO_4$ induced detrimental effects on brood size and life span, an increase in generation time and impaired development (Harada et al., 2007; Calafato et al., 2008). PD worms expressing α-synuclein or lacking parkin do not show increased sensitivity to Cu toxicity (Ved et al., 2005). In the context of AD, Cu has been shown to increases amyloid deposits and Aß oligomer aggregation, and decrease the amount of soluble Aß oligomer (Rebolledo et al., 2011). The increased Aß aggregation is associated with improvement in behavioral deficits and synaptic function. This protective effect is not attributable to the activation of the SKN-1/NRF2 phase II detoxification pathway (Rebolledo et al., 2011). Luo et al. noted that high Cu concentrations significantly increased the paralysis rate of the Aß(1–42) transgenic worms, whereas lower Cu concentrations decreased paralysis rate. ROS were identified to be responsible for the paralysis and the ROS induced by Aß(1–42) and Cu was mediated through *sod-1*, *prdx-2*, *skn-1*, *hsp-60*, and *hsp-16.2* genes (Luo et al., 2011). The amyloid precursor protein (APP) of AD has a copper-binding domain (CuBD) located in the N-terminal cysteine-rich region that can strongly bind Cu (II) and reduce it to Cu (I). The CuBD of *C. elegans*, APL-1, protected against Cu-mediated lipid peroxidation and neurotoxicity, therefore the CuBD of APP is predicted to play a role in detoxification of neuronal Cu (White et al., 2002; Cerpa et al., 2004).

Cadmium (Cd)

Cd is an environmental pollutant that has been classified as a category 1 human carcinogen (IARC, 1993). Cd exposure is associated with teratogenic and mutagenic responses (WHO, 1996). The major routes of human exposure include diet and cigarette smoke (EFSA, 2009). In *C. elegans*, Cd has been shown to alter behavior. It also resulted in decreased growth, life span and reproduction and affected feeding and movement (Popham and Webster, 1979; Boyd et al., 2010; Hoss et al., 2011; Hsu et al., 2012). Cd exposure was also shown to cause GABAergic neurodegeneration in worms. At low Cd concentration neuronal loss was observed, while at high Cd concentration axonal degeneration and neuronal loss, as well as reduced size of AVL, RMEs and RIS neurons was noted in fluorescently labeled GABAergic neurons (Du and Wang, 2009).

Iron (Fe)

The essential trace element Fe exists abundantly in the environment and is present in various enzymes and proteins. It has a central role in many essential cellular processes such as DNA synthesis, mitochondrial respiration, oxygen transport, and neurotransmitter synthesis (Cairo et al., 2002). Fe homeostasis has to be maintained within a small range because a dysregulation caused by Fe deficiency or overload leads to hematological, neurodegenerative and metabolic diseases (Dusek et al., 2012; Tandara and Salamunic, 2012). Genes involved in Fe and energy homeostasis in vertebrates are conserved in the nematode. These include aconitase, ferritin, divalent metal transporter-1 (DMT-1), frataxin, and Fe sulfur cluster assembly proteins. The Fe regulating protein-1 (IRP-1) homolog (ACO-1) of *C. elegans* has aconitase activity and is post-translationally regulated by Fe. Although ACO-1 is predicted to be the IRP-1 homolog in *C. elegans*, it fails to bind to the ferritin mRNAs and no conserved Fe responsive elements have been found on ferritin mRNAs in *C. elegans* (Gourley et al., 2003).

In contrast, Kim et al. reported that *aco-1* and *ftn-1* expression levels are regulated by Fe. Both aco-1 and *ftn-1* gene expression is inversely correlated. *ftn-1* and *ftn-2* are encoding ferritins in *C. elegans* (Kim et al., 2004). In mutant animals lacking ACO-1 and FTN-1, reduced lifespan has been observed, indicating that *aco-1* and *ftn-1* are important for regulating Fe homeostasis. *daf-16* mutants show decreased lifespan upon Fe treatment, suggesting that DAF-16 signaling might be also involved in Fe homeostasis (Kim et al., 2004). HIF-1 (hypoxia-inducible factor-1) is a negative regulator of ferritin transcription, inhibiting *ftn-1* and *ftn-2* transcription during Fe deficiency. Furthermore, the activation of the Fe uptake gene smf-3 (a homolog of DMT-1) by HIF-1 during Fe-deficiency (Romney et al., 2011) provides a mechanism to maintain sufficient Fe stock for growth and survival when Fe is limited in *C. elegans*.

Fe overload in worms causes phenotypic and behavioral defects as well as alteration the resistance to oxidative stress, characterized by reduced lifespan, body size, generation time, brood size, head thrash and body bend frequencies, as well as chemotaxis plasticity (Hu et al., 2008; Valentini et al., 2012). Several of these defects (body bend frequency and life span) were transferred from Fe-exposed *C. elegans* to their progeny (Hu et al., 2008). The adverse effects of Fe on locomotive behavior suggest that Fe might be involved in disruption of synaptic function between neurons and muscle cells. In *C. elegans* models of Aß toxicity, Fe was shown to possess high affinity for Aß. Aß accumulation in the Aß-expressing strain CL2006 resulted in Fe homeostasis disruption. In addition to increasing Fe content, Aß has also been shown to increase ROS generation (Wan et al., 2011).

Lead (Pb)

Lead is a pervasive environmental neurotoxicant; it is particularly toxic to developmental brains, causing long-term detrimental effects on learning, memory and behavior in children (Neal and Guilarte, 2010). Pb exposed *C. elegans* show severe abnormalities in their lifespan, development, locomotion, learning and memory behaviors (Ye et al., 2008; Zhang et al., 2010). Younger larva (L1, L2, L3) nematodes exhibited increased lethality and reproductive toxicity (brood size, generation time) compared to L4 stage or adult nematodes (Guo et al., 2009; Xing et al., 2009a). L3 larvae showed higher sensitivity in transgenerational behavioral and growth effects than older life stages (Yu et al., 2013). Neurodegeneration, as shown by neuronal loss and dorsal/ventral cord gaps is more pronounced in L1 larvae compared to older nematodes (Du and Wang, 2009; Xing et al., 2009b).

Pb exposure decreases thermotaxis behaviors in adult *C. elegans*. This behavior is mainly controlled by the AFD sensory neurons and it has been shown that Pb caused severe deficits in the structural properties of these neurons. Pre-treatment with antioxidants inhibited ROS elevation and mitochondrial dysfunction caused by Pb and prevented the induction of oxidative damage, severe deficits in thermotaxis and damage to the AFD

sensory neurons (Wu et al., 2012). Vitamin E and mild UV radiation or a pre-treatment with heat shock increase the resistance of nematodes to Pb toxicity, ameliorating the effects on locomotion behaviors, stress response and memory deficits (Ye et al., 2008; Wang et al., 2010; Ye et al., 2010).

Methylmercury (MeHg)

Mercury (Hg) and methylmercury (MeHg) are of greatest concern as MeHg is an ubiquitous environmental contaminant (Sanfeliu et al., 2003) and a major source of human exposure is associated with the consumption of seafood (Fitzgerald and Clarkson, 1991). McElwee et al. compared the toxicity of $HgCl_2$ and MeHgCl, noting the latter is significantly more toxic than $HgCl_2$ when assessing feeding, movement and reproduction, and in its ability to increase the steady-state levels of stress response genes (McElwee and Freedman, 2011). Helmcke et al. showed that while lethality, pharyngeal, pumping, growth and development were affected in *C. elegans*, brood size, lifespan, trashing rate, and nervous system morphology were largely unaffected in response to MeHg exposure. Concerning Hg uptake in *C. elegans*, its concentrations are as high as those found in mammalian systems (Helmcke et al., 2009; Helmcke and Aschner, 2010). It is also confirmed that the involvement of oxidative stress in MeHg toxicity is through alterations in GSH levels, increasing expression of HSP and glutathione-S-transferase (Helmcke and Aschner, 2010). In mammals, Nrf-2 regulates oxidative stress response. The worm Nrf-2 homolog, SKN-1, is also expressed in *C. elegans* DA neurons and a reduction in *skn-1* gene expression increases MeHg-induced DAergic neurodegeneration (VanDuyn et al., 2010).

Manganese (Mn)

The naturally abundant micronutrient Mn is an essential trace element and is of crucial importance as co-factor for a wide variety of enzymes, including arginase, pyruvate carboxylase, the antioxidant enzyme SOD, as well as enzymes involved in neurotransmitter synthesis and metabolism (Santamaria and Sulsky, 2010). An imbalance in Mn homeostasis caused by either Mn deficiency or overload is well known to cause severe CNS dysfunction, whereas Mn deficiency is because of its ubiquitous presence in food extremely rare. Therefore, several mechanisms are involved in maintaining the Mn homeostasis. The NRAMPs/divalent metal transporters play distinct roles in Mn transport and the identified *C. elegans* homologs (SMF-1, SMF-2, and SMF-3) have been characterized with corresponding roles in Mn homeostasis and sensitivity (Au et al., 2009; Bandyopadhyay et al., 2009; Settivari et al., 2009). The three SMFs differ in localization and function, and SFM-3 has to be shown as the major Mn transporter. A deletion of the *smf-1* or *smf-3* increased Mn tolerance against Mn-induced DAergic neurodegeneration with *smf-3* as the most resistant mutant, whereas the deletion of *smf-2* increases Mn sensitivity indicating the protective role of *smf-2* against Mn (Au et al., 2009). The DA reuptake transporter, DAT-1, has also been associated in Mn toxicity and dysfunction sensitizes the worm to Mn neurotoxicity (Chen et al., 2006; Benedetto et al., 2010). In *C. elegans* PMR1, a P-type Ca^{2+}/Mn^{2+} ATPase is involved in the regulation of Ca and Mn ions and PMR-1 knockdowns can render resistance to stresses, such as oxidative stress (Cho et al., 2005; Bandyopadhyay et al., 2009). McColl et al. provide the first quantitative subcellular visualization of endogenous Mn concentrations in *C. elegans* identifying Mn enriched within specific cell types, especially intestinal cells (McColl et al., 2012).

Concerning Mn toxicity in *C. elegans*, it has been investigated that the anti- or pro-oxidative role for Mn is dependent on the Mn-concentration worms are exposed to (Lin et al., 2006). In case of Mn overload accelerated development, an increase in total fertility, reduced body and brood size and life span was observed (Lin et al., 2006; Xiao et al., 2009). Mn toxicity in *C. elegans* has been associated with increased ROS formation and glutathione production, head mitochondria membrane potential and DA neuronal death (Settivari et al., 2009; Benedetto et al., 2010). Benedetto et al. found that Mn-induced oxidative stress was dependent on the extracellular levels of DA, which was confirmed by using strains lacking functional DA receptors (DOP-1, 2, 3) and the DA transporter (DAT-1) that regulate extracellular DA, and strains lacking vesicular monoamine transporter 2 (CAT-1 in worms) and tyrosine hydroxylase (CAT-2 in worms) that regulate intracellular DA levels. Decreased extracellular DA levels are protective against Mn toxicity (Benedetto et al., 2010). They identified NADPH dual-oxidase, BLI-3, potentiates the formation of RONS from DA-derived species obtained by the Mn (II) and extracellular DA exposure, while the bli-3 mutant a hyper-resistant phenotype of Mn toxicity showed which could be a potential therapeutic target against Mn toxicity (Benedetto et al., 2010). Consistent with the oxidative stress associated with Mn exposure, SKN-1 (antioxidant transcription factor contributing to protection against ROS; homolog of mammalian Nrf2) overexpression or nuclear relocalization of SKN-1 protects from Mn neurotoxicity indicating a beneficial strategies in limiting Mn toxicity (Benedetto et al., 2010). Prominent theories on neurodegeneration implicate further protein aggregation, including α-synuclein. Recently suppression of α-synuclein-induced toxicity has been demonstrated by expression of PARK9 encoding a putative P-type transmembrane ATPase (ATP13A2) protein (Gitler et al., 2009).

Zinc (Zn)

Zn is one of the most prevalent essential elements. It is a cofactor in many enzymes and transcription factors involved in several cellular processes and cellular signaling pathways (Shuttleworth and Weiss, 2011). Zinc deficiency as well as excess causes a wide spectrum of defects in multiple organ systems. Zn's role in memory, learning, neurogenesis, processes related to brain aging and neurological diseases have been extensively reviewed (Szewczyk et al., 2011).

In *C. elegans*, families of the CDFs (cation diffusion facilitators), ZIPs (Zrt- and Irt-like proteins) and MTs (metallothioneins) are involved in Zn metabolism. Deletion of MT-1 and MT-2 results in increased Zn accumulation, whilst *mtl-1* knockout worms show heightened sensitivity to increased Zn level (Leszczyszyn et al., 2011). *cdf-1* and *sur-7* are both members of the CDF family, and mutations in either gene result in increased sensitivity to Zn, indicating that both genes are necessary for Zn tolerance. However, *sur-7* mutants were more tolerant than a *cdf-1* mutant to increased Zn concentrations, suggesting functional

differences between these two proteins (Yoder et al., 2004). In addition, mutations of haly-1, which encodes an enzyme converting histidine to uric acid, cause elevated levels of histidine and protect against Zn toxicity (Murphy et al., 2011). A loss-of-function study of *cdf-1*, *cdf-2*, *sur-7*, and *haly-1* showed that *haly-1* promotes Zn resistance in the genetic backgrounds characterized by Zn sensitivity and functions in parallel to *cdf* genes in modulating Zn sensitivity. Zn exposure in *C. elegans* results in multiple biological defects affecting life span, reproduction, locomotion behavior (head trash, body bend) and chemotaxis plasticity (Wang et al., 2007). The phenotypic and behavioral toxicities could be transferred from Zn-exposed nematodes to their progeny (Yoder et al., 2004; Wang et al., 2007). While defects such as life span, generation time, brood size, and chemotaxis plasticity could be partially rescued in the progeny, no rescue phenomena could be observed for body sizes and the locomotion behaviors. In addition, it has been reported that the pesticide Mn/Zn-ethylene-bis-dithiocarbamate promotes neurodegeneration in DAergic and GABAergic neurons (Guven et al., 1999; Negga et al., 2011, 2012; Harrison Brody et al., 2013).

CONCLUSION

C. elegans is a valuable model to study human neurological diseases due to conserved genome, low culture cost, short generation cycle, transparent tissue and simple nervous system, together with well-developed tools. A solid number of nematode models related to human neurological disorders have already been developed, including AD (Miyasaka et al., 2005; Brandt et al., 2009), Parkinson's disease (Cao et al., 2005; Hamamichi et al., 2008), Huntington's disease (Parker et al., 2001; Caldwell et al., 2003), ALS (Vaccaro et al., 2012), and early-onset torsion dystonia (Cao et al., 2005; Chen et al., 2010). These disease models facilitate the study of metal-induced neurological toxicity and many novel discoveries have already been made in *C. elegans* to uncover the mechanisms of metals related human neurological disorders. Despite of these advantages, limitations of the nematode system need to be considered. For example, some genes and signaling pathways inherent to mammals (such as α-synuclein and Aβ discussed above) are missing in this model. In sum, *C. elegans* provide a valuable platform for exploratory study of human neurological diseases by providing mechanistic information on processes inherent to neurodegenerative disorders.

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