THE MOLECULAR PATHOLOGY OF COGNITIVE DECLINE: FOCUS ON METALS

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THE MOLECULAR PATHOLOGY OF COGNITIVE DECLINE: FOCUS ON METALS

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In the past two decades there have been significant advances made in understanding the cellular and molecular alterations that occur with brain ageing, as well as with our understanding of age-related brain diseases. Ageing is associated with a mid-life decline in many cognitive domains (eg. Attention, working memory, episodic memory) that progresses with advancing age and which may be potentiated by a variety of diseases. However, despite the breadth of attempts to explain it, the underlying basis for age-related memory impairment remains poorly understood. Both normal and "pathological" ageing (as in age-related neurodegenerative disorders such as Alzheimer's disease) may be associated with overlapping and increased levels of "abnormal" pathology, and this may be a potential mediator of cognitive decline in both populations. An emerging hypothesis in this field is that metal ion dys/homeostasis may represent a primary unifying mechanism to explain age- and disease-associated memory impairment – either indirectly via an effect on disease pathogenesis, or by a direct effect on signaling pathways relevant to learning and memory.

There remains a concerted worldwide effort to deliver an effective therapeutic treatment for cognitive decline associated with ageing and/or disease, which is currently an unmet need. There have been numerous clinical trials conducted specifically testing drugs to prevent cognitive decline and progression to dementia, but to date the results have been less than impressive, highlighting the urgent need for a greater understanding of the neurobiological basis of memory impairment in ageing and disease which can then drive the search for effective therapeutics.

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EDITORIAL
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Editorial: The molecular pathology of cognitive decline: focus on metals

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Keywords: metals, cognition, Alzheimer's disease, amyotrophic lateral sclerosis, Parkinson's disease, TBI, down syndrome

The series of articles in this special edition reflect much of the current thinking in regards to the role of metals in aging and neurodegeneration, and highlight the deep involvement of metal ions in critical pathways that may contribute to onset/progression of neurological disease. Furthermore, metal homeostasis may impact directly on signaling cascades relevant to cognition, or may indirectly contribute to functional decline via an effect on specific pathologies in the degenerating brain. As such, the behavioral manifestations associated with normal and pathological aging may be remediated by therapeutics that intervene in metal ion dyshomeostasis.

The broader perspective on the role of metal ions in cognition is outlined in the first two articles. Opazo et al. (2014) provide an overview of the role of copper in the CNS, and more specifically review the role of copper on neurotransmission and the ubiquitin proteasome system. These areas find relevance in the apparent sensitivity of the synapse to the "metal milieu" and further, to the potential for an involvement in neurodegenerative disorders. Copper is postulated to regulate the communication between neurons by modifying the protein configuration and strength of neurotransmission within the CNS. The review by Takeda and Tamano (2014) then provides a good overview on the now established role of zinc in synaptic plasticity and cognition. They further explore the impact of the hypothalamic–pituitary–adrenal (HPA) axis on these pathways. In light of the many avenues by which the HPA axis can be activated, then this represents an interesting area for further investigation. Similarly, the precise molecular mechanisms underlying the modulation of zinc signaling across both normal and "pathological" aging remain to be clarified.

The remaining articles are split out according to disease, beginning with ALS. Lovejoy and Guillemin (2014) provide a thorough and compelling overview on the role of metals in ALS, with a specific focus on iron and copper. This review provides a great backdrop for the subsequent papers on ALS, and points toward the potential efficacy of metal-targeting compounds as an avenue for ALS therapy.

Dang et al. (2014) then specifically examine metal levels in the brain and spinal cord of TDP-43 mutant mice, and demonstrated a modulation of metals in association with an impairment in motor function. This is the first report to identify a potential link between TDP-43 and metals, with further study required to elucidate the mechanisms (which are likely to include oxidative stress) underlying the observed phenomena.

Bourassa et al. (2014) tackle the metal: ALS interaction from the perspective of the aggregation of mutant SOD1. After examining cells expressing either WT or mutant SOD1, they discovered a significant cytoplasmic deficit in copper (which was also found in the aggregates themselves) in association with all the mutant forms of the protein. Targeting this metal: SOD1 interaction may thus be a therapeutic approach to limit disease progression. These studies cumulatively add further evidence to the growing literature on the role of metals in ALS.

The next series of articles have a focus on AD, with discussions around both the mechanistic and therapeutic implications of altered metal ion homeostasis. McCord and Aizenman (2014)

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provide a thorough review on the role of zinc-dependent signaling cascades during normal brain aging and in age-related neurodegeneration, with a particular focus on the production of reactive oxygen species and subsequent oxidative stress. As highlighted in the paper, these are phenomena that are applicable across a broad range of age-related conditions, but which are particularly relevant to AD where Zn has been extensively implicated in the pathogenesis of disease.

Wong et al. (2014) explore the links between metal dyshomeostasis and brain cholesterol in AD, reviewing potential roles in APP processing, Abeta generation/aggregaton/degradation and cell toxicity. They propose a crosstalk between metals and cholesterol in AD pathogenesis. In a related review, Xu et al. (2014) examine the interaction between metals and the major genetic risk factor for AD, ApoE (which is also involved in cholesterol metabolism). The evidence demonstrates that metals bind to ApoE in an isoform-specific way, and that ApoE modulates metal homeostasis in the brain. There is also the possibility that metals may regulate ApoE levels. These data provide support for the interaction between ApoE and metals within the brain, with implications for the pathogenesis of AD. Further exploring a related notion, Flinn et al. (2014) present a research paper examining the interaction of dietary zinc in an AD mouse model, specifically in the context of different ApoE genotypes. The data demonstrate that zinc supplementation caused significant impairments in the AD mouse model containing the ApoE E4 allele. These data are very important in the context of human drug trials and dietary zinc supplementation, as clearly there are genetic confounds (that themselves may interact with or be altered by metals) that may impact on the desired effect and/or disease progressions. This study provides further support for the growing notion that ApoE genotype is a critical factor in clinical trial design.

Hancock et al. (2014) then review the evidence supporting a complex interplay between glia, zinc and synaptic function across "normal" and "pathological" aging. Brewer then presents his inorganic copper hypothesis (Brewer, 2014). This postulates that the ingestion of copper from drinking water and supplement pills, together with a high fat diet, is a primary mechanism to account for the increasing prevalence of AD in the modern world. Furthermore, he notes that zinc deficiency may also be a major contributor to the development of AD, and goes on to review some of his own work in this area of zinc supplementation as a therapeutic avenue in AD (this is a good counter view to that provided by Flinn and colleagues). The hypotheses around the role of metals in AD are many and varied, as are their potential utility as therapeutic targets. This remains an area of active investigation. The next article by Granzotto and Zatta (2014) reviews the potential role of resveratrol as a therapeutic in AD (and potentially normal aging). They focus on how this natural polyphenol may intervene in AD-related pathways, including inflammation, mitochondrial dysfunction and more specifically, those related to oxidative stress and failures in metal ion homeostasis (zinc, iron, copper, and aluminum).

Finally, Bhattacharjee et al. (2014) provide a review on the potential role of aluminum in Alzheimer's disease and go further by demonstrating (through the use of Gene Chip and miRNA array techniques) that an aluminum-enriched diet fostered an upregulation of pro-inflammatory miRNAs in the Tg2576 animal model of AD.

Finishing this section, Braidy et al. (2014) briefly review metal-protein interactions in neurodegenerative diseases and then provide an overview of the various elemental imaging technologies currently available. They then focus on the application of these methods in AD. Ultimately, as spatial resolution and detection sensitivities are improved, we will be afforded greater insight into the role of metals in disease development and progression.

The next three articles focus on the role of metals in other conditions, and utilize a variety of different models. Sun et al. (2013) examined the apoptotic response across age following a traumatic brain injury (TBI), and demonstrated a number of findings. One highlight was a proteomic analysis that identified an injury-dependent increase in Hsp27. This protein, amongst other activities, has been shown to decrease ROS and thus, an endogenous or exogenous increase could protect against metal-mediated oxidative stress and downstream consequences. Another protein, the metal (calcium) binding protein hippocalcin, was also significantly decreased following injury- and this occurred concomitantly with an increase in TUNEL-positive cells in the aged animals. The cumulative impact of apoptosis on neuronal loss post-injury is a likely contributor to the behavioral deficits that accompany TBI, and metals are likely to feature in many aspects/signaling cascades of the TBI brain.

Malakooti et al. (2014) then explore the role of metal ion homeostasis in the cognitive decline present in Down syndrome (DS). Given the overlap between AD and DS, there is good justification to support a metal-mediated aspect to the pathogenesis of DS. Whilst there are obvious candidates, such as APP, that support this link (and the subsequent manifestation of cognitive decline and/or AD), there are many unexplored candidates that are similarly affected by the trisomy on chromosome 21 that deserve further attention (including DSCR1 and ITSN1). The data reviewed demonstrate novel pathways linking DS and metals that may provide novel therapeutic avenues for the disease.

Finally, Chege and McColl (2014) provide a detailed description on the use of *C. elegans* as a model system to explore the underlying mechanisms of PD. They propose that a failure in axonal transport is fundamental, with the subsequent impairment in trafficking of metal ion homeostasis proteins (focusing on iron) resulting in downstream oxidative stress and neuronal loss. They also note the potential for the loss of synaptic connections in this model.

The last manuscript in this special edition is by Marx and Gilon (2014), and presents a different perspective on memory than neuroscientists are typically exposed to. They draw together a discussion around a model that rationalizes the phenomenon of biologic memory in physical-chemical terms and in doing so, invoke a potential role for metal ions.

The sum of these articles, which add to my own recent work in the area, present an overview of the many different

pathways through which metals may intersect in the pathogenesis of a variety of neurodegenerative diseases, and which may subsequently manifest (directly or indirectly) in behavioral deficits.

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REVIEW ARTICLE
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Copper: from neurotransmission to neuroproteostasis

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Copper is critical for the Central Nervous System (CNS) development and function. In particular, different studies have shown the effect of copper at brain synapses, where it inhibits Long Term Potentation (LTP) and receptor pharmacology. Paradoxically, according to recent studies copper is required for a normal LTP response. Copper is released at the synaptic cleft, where it blocks glutamate receptors, which explain its blocking effects on excitatory neurotransmission. Our results indicate that copper also enhances neurotransmission through the accumulation of PSD95 protein, which increase the levels of α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptors located at the plasma membrane of the post-synaptic density. Thus, our findings represent a novel mechanism for the action of copper, which may have implications for the neurophysiology and neuropathology of the CNS. These data indicate that synaptic configuration is sensitive to transient changes in transition metal homeostasis. Our results suggest that copper increases GluA1 subunit levels of the AMPA receptor through the anchorage of AMPA receptors to the plasma membrane as a result of PSD-95 accumulation. Here, we will review the role of copper on neurotransmission of CNS neurons. In addition, we will discuss the potential mechanisms by which copper could modulate neuronal proteostasis ("neuroproteostasis") in the CNS with focus in the Ubiquitin Proteasome System (UPS), which is particularly relevant to neurological disorders such as Alzheimer's disease (AD) where copper and protein dyshomeostasis may contribute to neurodegeneration. An understanding of these mechanisms may ultimately lead to the development of novel therapeutic approaches to control metal and synaptic alterations observed in AD patients.

Keywords: copper, E-ligases, neurotransmission, proteasome, synaptic activity, ubiquitination, hippocampal neurons, AMPA

INTRODUCTION

Copper has a role in different pathways on the Central Nervous System (CNS; Linder and Hazegh-Azam, 1996; Gaier et al., 2013). It is essential for brain function since its deficiency lead to brain abnormalities and defects in brain development (Everson et al., 1967; Scheiber et al., 2014). This is highlighted by Menkes disease, an inherited disorder of intestinal copper absorption that has a multitude of symptoms including severe neurological degeneration and typically results in death by the age of five (Tümer and Møller, 2010). Bioavailable copper is found in the cerebrospinal fluid (∼70 μM) as well as in the brain extracellular space (∼1 μM) (Stuerenburg, 2000).

Copper concentration varies by brain region and becomes progressively detectable during postnatal stages (Kozma and Ferke, 1979). In rat brain, copper rapidly increases between day 5–14 postnatal (Tarohda et al., 2004) and is concentrated in the neuropil, where is mainly found on presynaptic boutons that innervate postsynaptic densities of locus ceruleus neurons (Sato et al., 1994). In effect, copper seems to be concentrated in synaptosomes and synaptic vesicles relative to magnesium, zinc and iron (Colburn and Maas, 1965). In synaptic vesicles, copper can form complexes with neurotransmitters. For example, copper can form ternary complexes with Adenosine triphosphate (ATP) and norepinephrine (Colburn and Maas, 1965). Interestingly, uptake of norepinephrine is inhibited by ethylenediamine hydrochloride, indicating that copper can participate in the uptake of neurotransmitters (Colburn and Maas, 1965). It is also known that there is a reduction in dopamine associated with dietary copper deficiency in humans (Prohaska and Bailey, 1994), highlighting its role in neurotransmitter synthesis. In addition, copper might be co-ordinating with membrane constituents of synaptic vesicles and hence may play an important role in membrane structure and function. In fact, copper can form complexes with phophatidyl-L-serine and phosphatidyl inositide, which is modulated by ATP (Maas and Colburn, 1965). These early studies supported a role for copper on neurotransmission.

Abbreviations: AD, Alzheimer's disease; AMPA, α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid; ATP, Adenosine triphosphate; CNQX, 6-cyano-7-nitroquinoxaline-2,3-dione; CTR1, copper transporter 1; GABA, γ-aminobutyric acid; LTP, long term potentiation; UPS, Ubiquitin Proteasome System.

COPPER AND SYNAPTIC FUNCTION

Koefoed-Johnsen and Ussing revealed that copper converts the frog skin membrane into a structure, which becomes selectively

impermeable to chloride ions (Koefoed-Johnsen and Ussing, 1958; Palmer and Andersen, 2008), suggesting that copper could modify the permeability of plasma membrane at the presynaptic or postsynaptic levels. In agreement with a role for copper on neurotransmission, copper is released from isolated rat brain cortical synaptosomes stimulated by 50 mM KCl (Kardos et al., 1989), which was corroborated in later studies using isolated guinea-pig cerebrocortical synaptosomes (Hopt et al., 2003). Moreover, glutamate receptor activation by NMDA promotes a rapid release of copper on primary hippocampal cultures (Schlief et al., 2005).

It in this regard that it has been suggested that CNS neurons possess the machinery to uptake copper and subsequently release it at the synaptic cleft (Hartter and Barnea, 1988), where it may modulate excitatory and inhibitory neurotransmission. In agreement with this, copper blocks GABAergic and AMPAergic neurotransmission when it is applied acutely on cultured rat olfactory bulb neurons (Trombley and Shepherd, 1996). It also blocks AMPAergic neurotransmission on rat cortical neurons (Weiser and Wienrich, 1996) and GABAergic neurotransmission in acutely isolated cerebellar Purkinje cells from rat (Sharonova et al., 1998), indicating that copper modulates neurotransmission of different CNS neurons in a similar fashion. Interestingly, a recent study indicated that extra-synaptic GABA receptors are susceptible to copper modulation (McGee et al., 2013), suggesting that a spillover of copper at extrasynaptic sites, after it is released at the synaptic space, can regulate extra-synaptic receptors.

Studies performed using rat brain slices have demonstrated the acute inhibitory effect of copper on Long Term Potentation (LTP; Doreulee et al., 1997; Goldschmith et al., 2005; Leiva et al., 2009), which can be related to the effect of copper on NMDA receptor pharmacology acting as a non-competitive antagonist (Vlachová et al., 1996). Moreover, copper can inhibit LTP in the CA3 region of mouse hippocampus by a NMDA receptor-independent mechanism (Salazar-Weber and Smith, 2011). However, recent studies indicate that the role of copper on LTP regulation is more complex, because copper has shown to be required for a normal LTP response (Gaier et al., 2013, 2014a,b).

Therefore, until a few years ago, copper was considered as a negative modulator of neurotransmission. However, the effect of copper on synaptic activity has been recently evaluated in more detail (Peters et al., 2011). We have studied the synaptic activity of primary cultures of rat hippocampal neurons in the presence of copper (up to 10 μM) at different timepoints (0, 3 and 24 h). As previously described, copper blocks neurotransmission when is acutely applied to the neurons. However, after 3 h of exposure, copper promotes an increase in the AMPAergic neurotransmission, which correlates with the accumulation of PSD95 protein and with a concomitant clustering of α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptors at the plasma membrane. Therefore, copper regulates neurotransmission by a novel biphasic mechanism, which have implications for the neurophysiology and neuropathology of the CNS. This biphasic response to copper may be not limited to hippocampal cultures and AMPAergic neurotransmission, because copper can promote a similar biphasic response on NMDA currents in cultured neonatal rat cerebellum granule cells (Marchetti et al., 2014).

Primary hippocampal neurons (10–14 DIV) treated with copper ($CuCl_2$; up to 10 μM) for a short period of time (3 h) display a significant increase either in the frequency, amplitude and the time constants of synaptic events. In addition, copper increases the frequency of calcium transients, which correlated with the increase in the frequency of miniature synaptic currents, supporting the role of copper as a neurotransmission enhancer (Peters et al., 2011). Under these conditions both AMPAergic and GABAergic neurotransmission are enhanced in neurons exposed to copper. All neurotransmission parameters including amplitude, frequency and time constant of AMPA receptors were modified. However, while both the amplitude and the frequency of miniature synaptic currents were enhanced, the time constant of AMPA miniature events was decreased in copper-treated neurons (Peters et al., 2011). Interestingly, copper-treated neurons displayed changes only in the amplitude and time constant parameters of GABAergic neurotransmission. In this case, both amplitude and time constant of GABA synaptic events were increased in neurons exposed to copper. The increase in the amplitude of GABAergic currents was accompanied by an increase in $GABA_A$ receptors immunostaining. Therefore, both AMPAergic and GABAergic neurotransmission contribute to the changes in total synaptic activity induced by copper.

The fact that copper-treated neurons displayed an increase in amplitude of miniature synaptic currents may be explained by an increase in the levels of receptors located post-synaptically. In this sense, both the postsynaptic clusters of $GABA_A$ and AMPA receptors, located apparently at the plasma membrane, are increased after 3 h treatment with copper. GluA1 and GluA2 staining were significantly increased at MAP2-positive dendritic zones of copper-treated neurons. However, total levels of GluA1 and GluA2 subunits of the AMPA receptor did not change. Moreover, neurons exposed to copper for 3 h were more sensitive to AMPA compared to neurons incubated in basal conditions. Interestingly, the desensitization of AMPA receptors was slower in neurons exposed to copper as indicated by the values for peak/plateau of the AMPA evoked currents. In summary, neurons behave differently to copper under acute vs. prolonged incubation time, through mechanisms that may involve homeostatic or anti-homeostatic mechanisms (Carrasco et al., 2007).

Thus we propose that copper enhances AMPAergic neurotransmission by promoting the clustering of AMPA receptors at the plasma membrane (See **Figure 1**), in a different fashion to CTR1 (copper transporter 1), the major copper uptake protein that is endocytosed and subsequently degraded in the presence of copper (Nose et al., 2010).

The clustering of AMPA receptors to the plasma membrane was accompanied by an increase in PSD95, a critical scaffolding protein for the anchoring of AMPA receptors to the cell surface (Colledge et al., 2003). Therefore, copper-treated neurons accumulate PSD95 by a mechanism that could involve a direct interaction of PSD95 with copper that increases protein stability or decreasing its degradation by the proteasome (Colledge et al., 2003; See **Figure 2**).

Overall, these results indicate that neurons exposed to a copper-enriched media display a more efficient neurotransmission, which correlates with changes in AMPA receptor

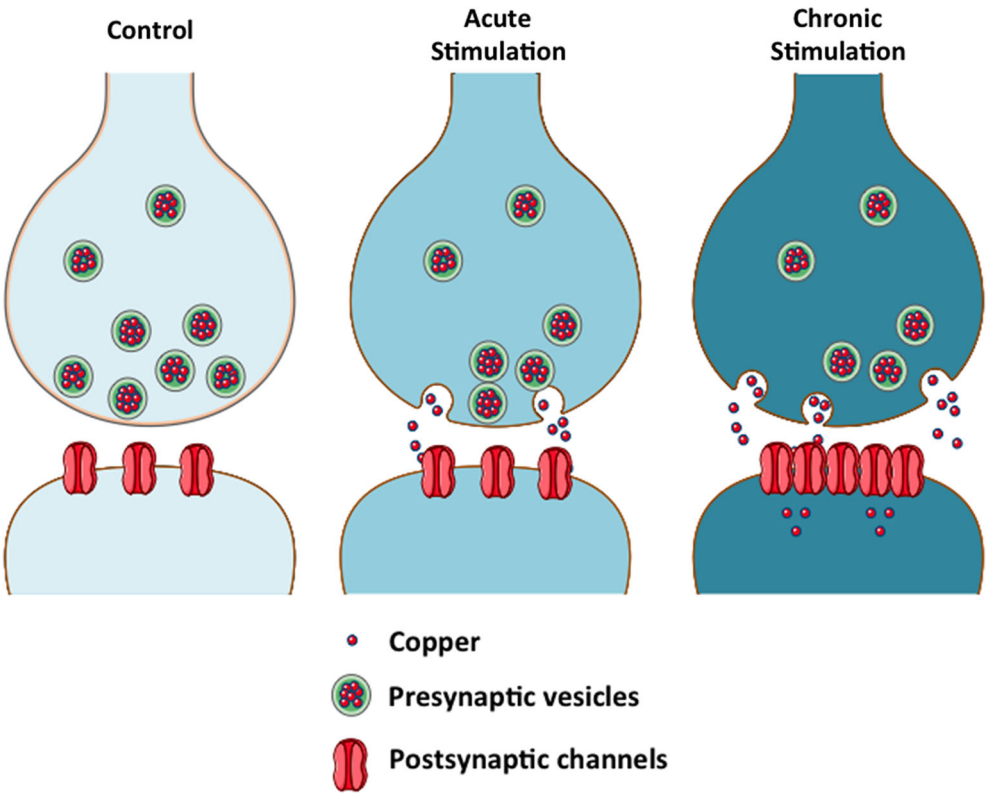

FIGURE 1 | Copper modulates neurotransmission by a biphasic mechanism. The scheme depicts the effect of copper on neurotransmission in acute and chronic conditions. Copper acts as a channel blocker under acute conditions. Sustained release of copper from the presynaptic vesicles to the synaptic cleft will lead to an increase in intracellular copper at the postsynaptic neuron, where copper might regulate the levels of scaffolding proteins that modulate the localization of channels at the plasma membrane (Peters et al., 2011).

localization/clustering and increase in the levels of PSD95. Our results indicate that copper enhances neurotransmission by changing the neuronal protein configuration and not simply due by changes in receptor pharmacology. We propose that copper might affect the neuroproteostasis of CNS neurons that lead to changes in neuronal excitability.

In support of this hypothesis, the effect of copper (3 h) on neurotransmission seemed to be unrelated to an homestotaic response resulting from the inhibition of AMPAergic neurotransmission, because after blockade of AMPA receptors for 3 h with 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX), which is a specific and potent antagonist of AMPA currents, did not change any parameter of the total miniature synaptic currents, indicating that at this time frame a compensatory mechanism is not resulting for receptor blockade. Therefore, the mechanism behind the effect of copper on this neuronal network might involve intracellular changes not related to AMPA receptor blockade. Moreover, the effect of copper on neurotransmission is a transient effect because the synaptic activity returned to the control levels after 24 h of incubation, indicating a homeostatic regulation.

These studies indicate that copper might induce biphasic effects on neurotransmission, suggesting that a fine regulation of this essential metal is probably needed by neuronal cells to maintain adequate synaptic function. A failure in this copper-dependent synaptic regulation can be relevant to brain conditions where the depletion in brain copper levels are associated to a cognitive decline such as Alzheimer's Disease (AD; Schrag et al., 2011). Therefore, further studies are required to better understand the molecular pathways that are affected by copper in living neurons. The data reviewed here indicates that copper can regulate the levels of PSD95, an intracellular scaffolding protein that modulate AMPAergic neurotransmission. Because PSD95 is degraded by the ubiquitin proteasome system (UPS; Colledge et al., 2003), copper might regulate PSD95 levels by targeting components of the UPS critical for PSD95 degradation (**Figure 2**).

COPPER AND UBIQUITIN PROTEASOME SYSTEM

Ubiquitin plays a critical role in protein degradation driven by 26S Proteasome (Hershko and Ciechanover, 1998). The UPS is a major pathway by which cells remove normal proteins and abnormally folded normal or mutant, cytoplasmic and membrane proteins (Tai and Schuman, 2008). Thus, an important number of cellular processes are regulated by ubiquitin-mediated signaling events (Hicke and Dunn, 2003) and UPS dysfunction is associated with neurodegenerative disorders (Rubinsztein, 2006) that are characterized by a metal dyshomeostasis, such as AD and Parkinson's disease (PD; Bush, 2003). The connection with ubiquitin

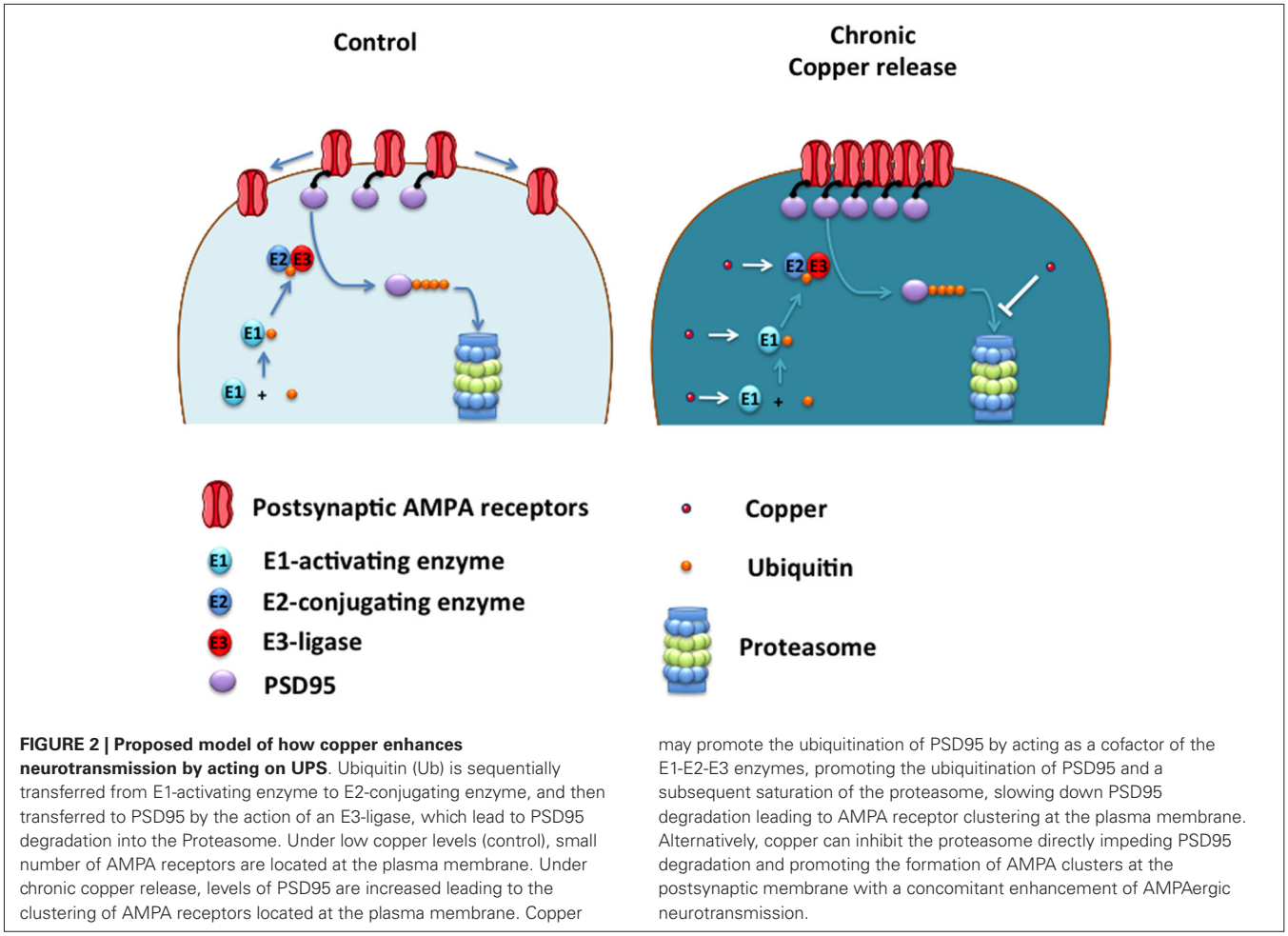

FIGURE 2 | Proposed model of how copper enhances neurotransmission by acting on UPS. Ubiquitin (Ub) is sequentially transferred from E1-activating enzyme to E2-conjugating enzyme, and then transferred to PSD95 by the action of an E3-ligase, which lead to PSD95 degradation into the Proteasome. Under low copper levels (control), small number of AMPA receptors are located at the plasma membrane. Under chronic copper release, levels of PSD95 are increased leading to the clustering of AMPA receptors located at the plasma membrane. Copper may promote the ubiquitination of PSD95 by acting as a cofactor of the E1-E2-E3 enzymes, promoting the ubiquitination of PSD95 and a subsequent saturation of the proteasome, slowing down PSD95 degradation leading to AMPA receptor clustering at the plasma membrane. Alternatively, copper can inhibit the proteasome directly impeding PSD95 degradation and promoting the formation of AMPA clusters at the postsynaptic membrane with a concomitant enhancement of AMPAergic neurotransmission.

and the proteasome is physiologically hierarchical and can be biochemically dissected in two main components (Ciehanover et al., 1978). Proteins are first ubiquitinated (Hershko et al., 1983) and then recognized by the 26S Proteasome for degradation (Deveraux et al., 1994). The key enzymes that regulate protein ubiquitination are E-activating, E-conjugating and E-ligases (Ciechanover et al., 1982; Hershko et al., 1983). Protein ubiquitination begins with the fast formation of a thiol-ester linkage between the C-terminus of ubiquitin and the active site cysteine of the ubiquitin-activating enzyme (E1) (Hershko et al., 1981; Pierce et al., 2009). This initial step requires ATP and ionic cofactors, including Mg^{2+} and an unknown metal ion (Ciechanover et al., 1982). The absence of these ionic factors stop ubiquitination. Thus, copper could act as "the unknown metal ion" in this enzymatic reaction. Further studies are needed to validate this possibility. Ubiquitin is then transferred to an ubiquitin conjugating enzyme (E2) (Hershko et al., 1983) to form a catalytically activated intermediate such as the UbcH5b Ub (Sakata et al., 2010). UbcH5b is one of the E2 enzymes that has been demonstrated to form polyubiquitin chains in cooperation with several E3 enzymes (Wu et al., 2003; Brzovic et al., 2006; Sakata et al., 2007; Windheim et al., 2008). These E3 ligases interact with UbcH5b-Ub intermediate, catalyzing the formation of an isopeptide bond between the C-terminal residue of ubiquitin (G76) and a lysine located either on a target protein or on the lysine (usually K48 for degradation) of the most peripheral ubiquitin tagged to a protein (Sakata et al., 2010), which then directs it to the 26S proteasome for degradation (Pickart, 2000).

There are several studies that connect the UPS to transition metals. For example, Kojima's group characterized the *in vitro* interaction between ubiquitin and copper by using electron paramagnetic resonance (EPR) approximation (Nomura et al., 2004). This study strongly suggested that Cu^{2+}, as a part of one metal complex, is coordinated by ubiquitin with the participation of a histidine residue (his-68). Other paramagnetic metals, such as Mn^{2+} and Gd^{3+}, did not coordinate specifically to his-68 present in ubiquitin sequence (Nomura et al., 2004). In fact, ubiquitin is retained to immobilized metal ion affinity chromatography (IMAC) resins complexed to Cu^{2+} (Hemdan et al., 1989), where his-68 is critical for this binding. Interestingly, when his-68, located at the surface of ubiquitin (Sloper-Mould et al., 2001) is replaced with another residue, the ubiquitination process (Ecker et al., 1987) or cell growth is altered (Sloper-Mould et al., 2001). All this data suggests that copper might participate upstream in the regulation of UPS. However,

copper complexed to some chelators inhibit the proteasome for unknown mechanism (Ding and Lind, 2009), suggesting the possibility that copper can regulate the UPS at different levels (**Figure 2**).

Metalloproteins are part of the UPS, acting as E3-Ring ligases or deubiquitinases (Joazeiro and Weissman, 2000; Yao and Cohen, 2002), but it is unclear if transition metals can participate upstream in the regulation of the first steps of ubiquitination. Downstream of the UPS, proteasome activity is inhibited by copper at milimolar concentration (Amici et al., 2002) and some copper-chelator complexes can also inhibit proteasome activity (Ding and Lind, 2009). Zinc is critical for the activity of E3-Ring ligases and RPN11 deubiquitinase (Joazeiro and Weissman, 2000; Yao and Cohen, 2002). Moreover, metal response to cadmium toxicity in yeast involves the inactivation of Skp1-Cullin1-F-box (SCF) ligases complexes (Yen et al., 2005) and also the activation of UPS (Jungmann et al., 1993). However, cadmium can also induce the accumulation of ubiquitinated proteins by an oxidative mechanism that leads to neurotoxicity (Figueiredo-Pereira et al., 1998). Interestingly, iron can accelerate the degradation of proteins into the proteasome by inducing oxidative modifications in the targeted protein (Iwai et al., 1998). Moreover, it can regulate the ligase activity of SKP1-CUL1-FBXL5 protein complex (Salahudeen et al., 2009; Vashisht et al., 2009). Therefore, metals such as copper may activate or inactivate early steps of ubiquitination. Interestingly, copper is specifically coordinated by ubiquitin (Hemdan et al., 1989; Nomura et al., 2004), indicating that this metal can act at early stages of ubiquitination. In fact, copper is required for Ctr1 poly-ubiquitination and subsequent degradation by a mechanism that requires the presence of the copper chaperone Atox1 (Safaei et al., 2009). Moreover, Clioquinol, a copper chelator with moderate affinity, can inhibit *in vitro* ubiquitination of Hypoxia-inducible Factor-1α (Choi et al., 2006), indicating that copper may participate as a cofactor in ubiquitination.

CONCLUSION

Inherited disorders of Cu metabolism, such as Menkes and Wilson's disease display complex neurodegenerative features, which highlight the importance of copper homeostasis (Tümer and Møller, 2010). Moreover, micromolar concentrations of copper (up to 400 μM) are present in senile plaques in AD brains (Lovell et al., 1998), which could be a source of copper for the neurons surrounding these pathological structures. Brain copper deficiency is a characteristic feature of Menkes disease, which affects brain physiology, since patients display gray matter degeneration, hippocampal neuronal loss and Purkinje cell abnormalities (Okeda et al., 1991). AD is another brain pathology characterized by neurodegeneration that produces a broad spectrum of symptoms that have been linked to copper brain depletion since cupro-proproteins such as ceruloplasmin are decreased (Connor et al., 1993; Bush, 2003) or less active as observed for superoxide dismutase 1 (Omar et al., 1999; Maynard et al., 2005). Currently, the relationship between copper and neuroproteostasis within the CNS in health and pathological conditions is poorly understood. Hence, further studies are required to determine how neuronal excitability is linked to changes in synaptic proteins promoted by copper (Gaier et al., 2013). The studies described here provide a new perspective on how copper can regulate the communication between neurons by modifying the protein configuration and strength of neurotransmission within the CNS.

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Cognitive decline due to excess synaptic Zn^{2+} signaling in the hippocampus

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Zinc is an essential component of physiological brain function. Vesicular zinc is released from glutamatergic (zincergic) neuron terminals and serves as a signal factor (Zn^{2+} signal) in both the intracellular (cytosol) compartment and the extracellular compartment. Synaptic Zn^{2+} signaling is dynamically linked to neurotransmission and is involved in processes of synaptic plasticity such as long-term potentiation and cognitive activity. On the other hand, the activity of the hypothalamic–pituitary–adrenal (HPA) axis, i.e., glucocorticoid secretion, which can potentiate glutamatergic neuron activity, is linked to cognitive function. HPA axis activity modifies synaptic Zn^{2+} dynamics at zincergic synapses. An increase in HPA axis activity, which occurs after exposure to stress, may induce excess intracellular Zn^{2+} signaling in the hippocampus, followed by hippocampus-dependent memory deficit. Excessive excitation of zincergic neurons in the hippocampus can contribute to cognitive decline under stressful and/or pathological conditions. This paper provides an overview of the "Hypothesis and Theory" of Zn^{2+}-mediated modification of cognitive activity.

Keywords: Zn^{2+} signal, hippocampus, cognition, glucocorticoid, glutamate

INTRODUCTION

Over 300 proteins require zinc to carry out their functions in microorganisms, plants, and animals. Zinc powerfully influences cell division and differentiation (Vallee and Falchuk, 1993; Maret and Sandstead, 2008; Prasad, 2008). Zinc is also essential for the growth and functioning of the brain. Zinc transport from the plasma to the brain's extracellular fluid and cerebrospinal fluid is strictly regulated by the brain-barrier system, i.e., the blood–brain and blood-CSF barrier. The brain barrier system maintains zinc homeostasis in the brain (Takeda, 2000, 2001). Zinc homeostasis is critical for brain function (Capasso et al., 2005; Mocchegiani et al., 2005) and is spatiotemporally altered in the process of neurological diseases (Barnham and Bush, 2008).

Zinc is relatively concentrated in the hippocampus and amygdala (Takeda et al., 1995). Both regions are enriched with histochemically reactive zinc, as revealed by Timm's sulfide-silver staining method (Frederickson, 1989; Frederickson and Danscher, 1990). Histochemically reactive zinc is found predominantly in the presynaptic vesicles and serves as a signal factor (Zn^{2+} signal) in both the cytosolic and extracellular compartments. Zn^{2+} is released with glutamate in a calcium-dependent and impulse-dependent manner from glutamatergic (zincergic) neuron terminals (**Figure 1**). Zn^{2+} released from these terminals modulates the activity of several important receptors, including the α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA)/kainate receptor, *N*-methyl-D-aspartate (NMDA) receptors, and γ-amino butyric acid (GABA) receptors in the extracellular compartment (Smart et al., 1994; Nakashima and Dyck, 2009), and is taken up into post-synaptic neurons to serve as an intracellular signal factor. Glutamatergic (zincergic) circuits play a key role in cognitive map building structures such as the hippocampus (Martinez-Guijarro et al., 1991; Nacher et al., 2000). It has been estimated that approximately 20% of total brain zinc is histochemically reactive, based on the finding that the removal of zinc transporter-3 (ZnT3) protein, which is responsible for the movement of zinc from the cytoplasm into synaptic vesicles (Palmiter et al., 1996), results in a 20% reduction of the total amount of zinc in the brain (Cole et al., 1999).

It is well known that the hippocampus and amygdala are involved in cognitive and emotional behavior. Synaptic plasticity such as long-term potentiation (LTP) is believed to be a key cellular mechanism involved in learning and memory and has been widely studied in relation to glutamatergic synapses in the brain, especially in the hippocampus (Bliss and Collingridge, 2013). When information is processed in memory, glutamatergic neurons form a neural circuit in the hippocampus and the amygdala. Furthermore, it has been reported that plastic changes in hippocampal synapses occur activity-dependently during the performance of associative learning tasks (Gruart et al., 2006; Clarke et al., 2010).

On the other hand, the activity of the hypothalamic–pituitary–adrenal (HPA) axis, i.e., glucocorticoid secretion, is linked to cognitive and emotional functions and can potentiate glutamatergic neuron activity (Sandi, 2011). There is some evidence that the modification of synaptic Zn^{2+} signaling by HPA axis activity, which is enhanced by stress and aging, is linked to cognitive and emotional behavior, and that abnormal modification may induce cognitive decline (Takeda and Tamano, 2009, 2010, 2012). It is well known that abnormal Zn^{2+} influx into post-synaptic neurons, which is induced by abnormal glutamatergic (zincergic) neuron activity, induces neuronal death and is involved in neurological disorders such as stroke/ischemia and temporal lobe epilepsy (Frederickson et al., 2005; Sensi et al., 2011; Takeda, 2011a; Weiss, 2011). Therefore, the homeostasis of synaptic Zn^{2+} signaling is critical in both functional and pathological aspects (Takeda,

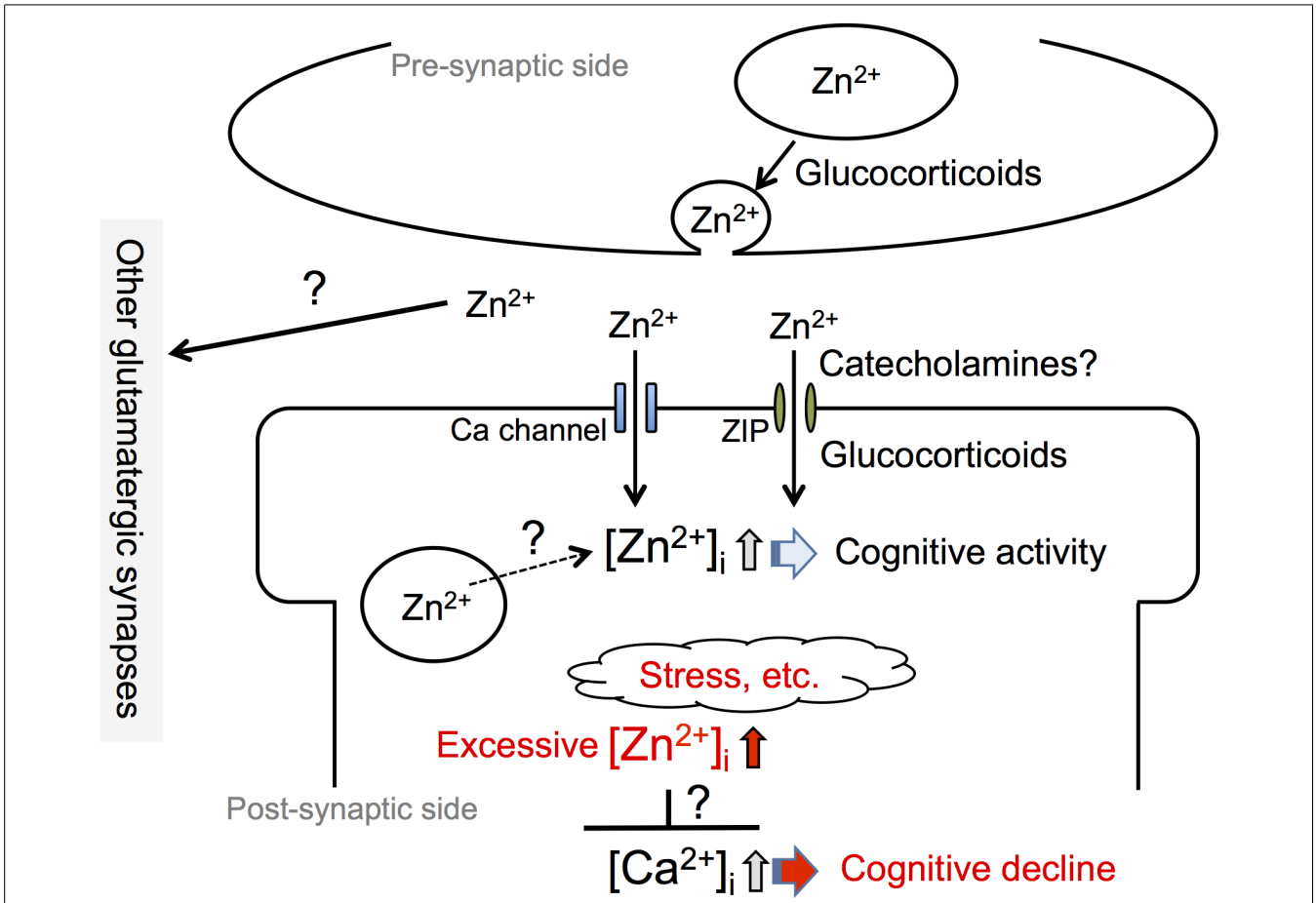

FIGURE 1 | Involvement of synaptic Zn^{2+} dynamics in cognitive activity. An increase in intracellular Zn^{2+} concentration, $[Zn^{2+}]_i$, which is induced by an influx of extracellular Zn^{2+} at zincergic synapses in the hippocampus, is involved in cognitive activity. Presynaptic glucocorticoid signaling, a non-genomic action, and post-synaptic glucocorticoid signaling, a genomic action, modify the degree of increase in intracellular Zn^{2+}. It is also possible that catecholamines modify the degree through the activity of the β-adrenergic system. The degree of increase in intracellular Zn^{2+} is linked to cognitive activity and excess intracellular Zn^{2+} signaling, which can be induced by stress, is involved in cognitive decline. The excess might affect intracellular Ca^{2+} signaling, which plays a key role for synaptic plasticity.

2011b; Takeda et al., 2013). On the basis of recent evidence that excessive excitation of zincergic neurons in the hippocampus can contribute to cognitive decline under stressful and/or pathological conditions (Takeda et al., 2009, 2011, 2012), this paper provides an overview of the "Hypothesis and Theory" of Zn^{2+}-mediated modification of cognitive activity.

SYNAPTIC Zn^{2+} HOMEOSTASIS

Total zinc concentration in the adult brain reaches around 200 μM (Markesbery et al., 1984). Extracellular zinc concentration in the adult brain is estimated to be less than 1 μM (Weiss et al., 2000). If zinc concentration in the brain's extracellular fluid is equal to that in cerebrospinal fluid (Hershey et al., 1983), it is around 150 nM – approximately one thousandth of total brain zinc concentration. In zincergic synapses, Zn^{2+} concentration in the synaptic cleft is estimated to be higher than that in the brain's (extrasynaptic) extracellular fluid, because under hippocampal-slice-experiment conditions the regions where zincergic synapses are found are intensely stained by ZnAF-2, a membrane-impermeable zinc indicator (Minami et al., 2006). The synaptic cleft is surrounded with the processes of astrocytes, which contribute to maintaining a steady concentration of zinc and neurotransmitters in the cleft. Interestingly, Zn^{2+} level in the brain's extracellular fluid, which is estimated to be approximately 20 nM (Frederickson et al., 2006), is higher than that in the plasma (<1 nM; Magneson et al., 1987). In the brain's extracellular fluid, the high ratio of Zn^{2+} concentration to total zinc concentration appears to be associated with the synaptic Zn^{2+} dynamics of the brain. There is some evidence that extracellular Zn^{2+} serves as a pool for the zinc in the synaptic vesicle and is involved in synaptic Zn^{2+} homeostasis (Takeda et al., 2006), although the chemical form of this vesicular zinc is unknown.

Basal Zn^{2+} concentration is extremely low in the intracellular (cytosol) compartment (<1 nM; Sensi et al., 1997; Colvin et al., 2008). ZnT proteins such as ZnT1, ZnT3, and ZnT10, and Zrt-Irt-like proteins (ZIP) such as ZIP4 and ZIP6 are involved in the control of Zn^{2+} levels in the cytosolic compartment, especially under static (basal) conditions (Emmetsberger et al.,

2010). Some of these transporters transport cytosolic Zn^{2+} into a variety of subcellular organelles, including mitochondria, lysosomes, endosomes, and the Golgi apparatus, probably to maintain static Zn^{2+} levels in the cytosolic compartment (Sensi et al., 2003; Danscher and Stoltenberg, 2005; Colvin et al., 2006). On the other hand, it is possible that Zn^{2+} release from subcellular organelles, which might be induced by synaptic glutamate signaling, is involved in Zn^{2+} signaling (Stork and Li, 2010). Zn^{2+} levels other than vesicular zinc serving as Zn^{2+} are estimated to be less than 5% of the total amount of Zn^{2+} in the hippocampus and cerebral cortex (Lee et al., 2011). ZnT1 is a major Zn^{2+} transporter in the plasma membrane and may be involved in cytosolic Zn^{2+} homeostasis in neurons by transporting Zn^{2+} from the somata to the extracellular space (Sekler et al., 2002). It has been reported that ZnT1 prevents excessive accumulation of Zn^{2+} in the cytosolic compartment (Nolte et al., 2004), resulting in the protection of neurons from Zn^{2+} toxicity in neurological diseases such as transient forebrain ischemia (Aguilar-Alonso et al., 2008). Tissue plasminogen activator, a secreted serine protease, is excitotoxic and increases lysosomal sequestration of increased Zn^{2+} in the cytosolic compartment through interaction with ZIP4, which may also contribute to the protection of neurons from Zn^{2+} toxicity (Emmetsberger et al., 2010). The spatiotemporal control of Zn^{2+} signaling via ZIP and ZnT maintains a steady-state environment in both the extracellular and cytosolic compartments (Fukada and Kambe, 2011).

FUNCTIONAL AND NEUROTOXIC Zn^{2+} SIGNALING

Zn^{2+} concentration is increased in the synaptic cleft during the excitation of zincergic synapses, followed by an increase in the cytosol (intracellular compartment; **Figure 1**). Released Zn^{2+} is quickly taken up into presynaptic and post-synaptic neurons and astrocytes. Calcium channels such as calcium-permeable AMPA/kainate receptors are involved in Zn^{2+} influx during neuronal excitation (Weiss et al., 2000; Jia et al., 2002; Takeda et al., 2007a). The increase in the extracellular concentration of Zn^{2+} is dependent on the frequency of depolarizing stimulation (Ueno et al., 2002). Therefore, the increase in intracellular concentration of Zn^{2+} serving as a signal factor is closely correlated to zincergic neuron excitation (Takeda et al., 2013).

Glutamate accumulates in the extracellular compartment due to excessive excitation of glutamatergic (zincergic) neurons. Excessive activation of glutamate receptors caused by excess extracellular glutamate leads to a number of deleterious consequences, including impairment of calcium buffering, generation of free radicals, activation of mitochondrial permeability transition, and secondary excitotoxicity (Danbolt, 2001; Dong et al., 2009). Glutamate excitotoxicity, a final common pathway for neuronal death, is observed in numerous pathological processes such as stroke/ischemia, temporal lobe epilepsy, Alzheimer's disease, and amyotrophic lateral sclerosis. An excess of extracellular Zn^{2+}, which is induced under glutamate excototoxicity, is harmful; excessive Zn^{2+} influx into post-synaptic neurons is involved in neurodegeneration under pathological conditions. Calcium-permeable AMPA receptors may play a key role in this Zn^{2+} influx (Liu et al., 2004; Noh et al., 2005; Weiss, 2011).

Zn^{2+} also plays a neuroprotective role in glutamate-induced excitotoxicity by activating pre-synaptic ATP-sensitive potassium channels and by inhibiting GABA transporter 4 (Bancila et al., 2004; Cohen-Kfir et al., 2005). It is estimated that the neuroprotective action of Zn^{2+} occurs under conditions in which zincergic neurons are not excessively excited. Zn^{2+} released from zincergic neuron terminals may also serve as a negative feedback factor against glutamate release (Minami et al., 2006; Takeda et al., 2007b). Therefore, the degree of increase in extracellular Zn^{2+} is critical in both functional and neurotoxic aspects.

Zn^{2+} SIGNALING AND COGNITION

Synaptic Zn^{2+} signaling is involved in processes of synaptic plasticity such as LTP in the hippocampus and amygdala. Enhanced plasticity in zincergic synapses is associated with cortical modification after exposure to an enriched environment (Nakashima and Dyck, 2008). The enhanced plasticity of zincergic synapses in the hippocampus underlies the acquisition of new motor and cognitive abilities (Delgado-García and Gruart, 2006; Jurado-Parras et al., 2013). These findings suggest that synaptic Zn^{2+} signaling is involved in cognitive and emotional behavior through the modulation of synaptic plasticity such as LTP (**Figure 1**).

Targeted deletion of the ZnT3 prevents vesicular Zn^{2+} uptake (Cole et al., 1999) and ablates Zn^{2+} release into the extracellular space by action potentials. There is a correlation between vesicular Zn^{2+} levels and ZnT3 protein expression (Palmiter et al., 1996). Zn^{2+} transport into the synaptic vesicle is ZnT3-dependent and is important for amassing the large pool of Zn^{2+} used in signaling (Lee et al., 2011). It has been reported that Zn^{2+} signaling is involved in cognitive and emotional behavior even in ZnT3KO (Adlard et al., 2010; Martel et al., 2010, 2011; Sindreu et al., 2011). The pool of Zn^{2+} may be located in other subcellular organelles (**Figure 1**) and/or zinc-binding proteins such as metallothionein in ZnT3KO mice. On the other hand, memory deficit and the changes in emotional (freezing) behavior have been observed in wild-type animals when acute loss or chelation of synaptic Zn^{2+} is induced by treatment with zinc chelators (Takeda et al., 2010a,b). The amount of Zn^{2+} functioning as a signal factor seems to be lower in ZnT3KO mice than in wild-type mice.

Saito et al. (2000) report that age-dependent reduction of Zn^{2+} levels in the synaptic vesicles of the mossy fibers induced by low ZnT3 expression causes both glutamatergic excitotoxicity in hippocampal neurons and the deterioration of learning and memory in senescence-accelerated mouse prone 10 (SAMP10). There are also reports of age-dependent reductions in ZnT3 expression and synaptic Zn^{2+} levels in the hippocampal mossy fibers of human amyloid precursor protein-transgenic (Tg2576) mice, suggesting that extensive modifications of the brain's Zn^{2+} pool, particularly synaptic (vesicular) Zn^{2+}, underlie the neuronal dysfunction characteristic of Alzheimer's disease (Lee et al., 2012). Furthermore, there is a significant age-related decline in cortical ZnT3 levels from age 48 to 91 in healthy people (Adlard et al., 2010) and ZnT3 levels are more markedly decreased in the cortex in Alzheimer's disease. It is likely that the increase in extracellular Zn^{2+} induced by the physiological excitation of zincergic neurons requires cognitive activity (**Figure 1**) and that

an insufficient increase is involved in the pathophysiology of Alzheimer's disease.

On the other hand, Zn^{2+} released from zincergic neurons is known to mediate parenchymal and cerebrovascular amyloid formation in Tg2576 mice (Lee et al., 2002; Friedlich et al., 2004; Stoltenberg et al., 2007). The transsynaptic movement of Zn^{2+} may be severely compromised in Alzheimer's disease, both by lack of ZnT3 expression and by sequestration in amyloid. Adlard et al. (2010) report that the genetic ablation of ZnT3 may represent a phenocopy for memory deficits in Alzheimer's disease. Deshpande et al. (2009) postulate that the sequestration of Zn^{2+} in oligomeric amyloid-β (Aβ)–Zn complexes may lead to a reduction in Zn^{2+} availability at the synapses, resulting in a loss of the modulatory activity of Zn^{2+}, and leading to the cognitive decline of Alzheimer's disease. Such changes in synaptic Zn^{2+} availability may participate in modifying cognitive activity and also in cognitive decline (**Figure 1**; Linkous et al., 2009; Bush, 2013; Bosomworth et al., 2013).

GLUCOCORTICOID SIGNALING, Zn^{2+} SIGNALING, AND COGNITION

The hippocampus is enriched with corticosteroid receptors and is the major target region of corticosteroids (Joëls, 2008). Mineralocorticoid receptors and glucocorticoid receptors are colocalized in CA1 and CA2 pyramidal cells and in dentate gyrus granule cells. In CA3 pyramidal cells, on the other hand, mineralocorticoid receptors are abundantly expressed, while glucocorticoid receptors are expressed at much lower levels (Ozawa, 2005). Mineralocorticoid receptors are extensively occupied with low levels of corticosterone, and glucocorticoid receptors are particularly activated after exposure to stress (Joëls et al., 2008; Sandi, 2011).

An increase in serum corticosterone level induces a rapid increase in hippocampal corticosterone level, in parallel with an increase in extracellular glutamate level (Venero and Borrell, 1999). Corticosterone-induced increase in extracellular glutamate levels in the hippocampus appears to be exerted through the action of membrane-associated mineralocorticoid receptors and/or glucocorticoid receptors, which increase the probability of glutamate release in synaptic activation (Karst et al., 2005; Musazzi et al., 2010). The rapid effects of corticosterone on glutamatergic transmission appear to be linked to diverse effects on synaptic plasticity and memory processes in the hippocampus (**Figure 1**). An increase in the probability of glutamate release through the action of corticosterone leads to increases both in the amount of glutamate released during learning and in the degree of activation of postsynaptic glutamate receptors. Corticosterone can contribute to an increase in the efficacy of glutamatergic transmission by AMPA receptor insertion at synaptic sites, through both the rapid and the delayed (genomic) effects. These effects are of advantage to processes of synaptic plasticity such as LTP and memory acquisition (Sandi, 2011). Therefore, it is estimated that corticosterone increases the probability of Zn^{2+} release from zincergic neuron terminals through the rapid non-genomic effect in the hippocampus (**Figure 1**; Takeda et al., 2012). Futhermore, corticosterone requires intracellular Zn^{2+} signaling for the genomic effect, possibly followed by the delayed influx of extracellular Zn^{2+} through zinc transport systems such as ZIP (**Figure 1**). Although the evidence is limited, it is likely that synaptic Zn^{2+} signaling cooperates with corticosteroid signaling in learning and memory.

In contrast, glutamate accumulates in the extracellular compartment at high levels through a corticosterone-mediated blockade of glutamate transporter activity when corticosterone is abnormally secreted under conditions of severe stress. Abnormal corticosterone secretion also contributes to abnormal glutamate release from neuron terminals (Wong et al., 2007; Howland and Wang, 2008). The extracellular spillover of glutamate impairs spatial memory retrieval. Furthermore, Wong et al. (2007) demonstrate that hippocampal long-term depression (LTD) is both necessary and sufficient to cause acute stress-induced impairment of spatial memory retrieval. Excess intracellular Zn^{2+} signaling induced by corticosterone and/or stress is also involved in the impairment of hippocampal LTP (Takeda et al., 2009, 2012), possibly followed by the impairment of learning and memory (Takeda et al., 2011; **Figure 1**). In hippocampal CA3, on the other hand, an increase in intracellular Zn^{2+} via a zinc ionophore not only decreases basal Ca^{2+} level but also suppresses increases in Ca^{2+} level via metabotropic glutamate receptors (Takeda et al., 2007a). Such excess intracellular Zn^{2+} signaling may lead to negative crosstalk in intracellular Ca^{2+} signaling, which plays a key role in LTP and LTD (**Figure 1**).

A selective increase in the nocturnal levels of corticol has been observed in aged humans (Landfield and Eldridge, 1994). Furthermore, high levels of cortisol are found in Alzheimer's disease as well as in depression. In Alzheimer's disease patients, cognitive deficits (such as in memory) and psychological symptoms (such as anxiety) are associated with an early deregulation of the HPA axis (Swanwick et al., 1998; Brureau et al., 2013). Therefore, it is possible that excess intracellular Zn^{2+} signaling through abnormal cortisol secretion is involved in cognitive deficits in both normal aging and neurological diseases such as dementia.

On the other hand, corticotrophin releasing hormone (CRH) drives the HPA axis and is considered to be the central coordinator of behavioral, autonomic, and neuroendocrine stress responses. The stress mediators activated by CRH are organized in the sympathetic nervous system, as well as in the HPA axis (de Kloet, 2008). Adrenaline, along with norepinephrine, is largely responsible for the immediate reactions that are felt under conditions of stress. Responses of adrenaline and norepinephrine, such as an increase in heart rate, occur more quickly than those of glucocorticoids. Catecholamines are released from the sympathetic nerve system and the adrenal glad. It has been reported that the enhanced memory associated with emotional experiences involves activation of the β-adrenergic system (Cahill et al., 1994; McEwen and Sapolsky, 1995). β-Adrenergic receptor activation facilitates the induction of a protein synthesis-dependent late phase in LTP in the hippocampus (Gelinas and Nguyen, 2005). Learning-facilitated LTD and LTP at mossy fiber-CA3 synapses requires activation of β-adrenergic receptors (Hagena and Manahan-Vaughan, 2012). The above evidence suggests that synaptic Zn^{2+} signaling is modified by the β-adrenergic system and is involved in cognitive activity associated with emotional experiences. The relationship between synaptic Zn^{2+} signaling and the β-adrenergic system is an issue which requires further clarification. Stress is a known precipitant

for metabolic and neurological diseases (Koenig et al., 2011) and synaptic Zn^{2+} signaling is likely to be involved in the diverse effects of stress through the stress mediators activated by CRH.

PERSPECTIVE

Synaptic Zn^{2+} homeostasis is critical for synaptic function, and seems to be controlled by two Zn^{2+} pools, one in the synaptic vesicle and the other in the extracellular compartment. Synaptic Zn^{2+} signaling is involved in cognitive activity, and both its lack and its excess are involved in cognitive decline (**Figure 1**). HPA axis activity increases with aging, and this increase is superimposed on neurological diseases such as depression and Alzheimer's disease. It is likely that synaptic Zn^{2+} signaling through the HPA axis activity is involved in cognitive decline in both normal aging and dementia, and it is possible that sympathetic nervous system activity is also involved. However, evidence related to synaptic Zn^{2+} dynamics is very limited, not only under physiological conditions, but also under stressful and pathological conditions. The molecular mechanisms of abnormal Zn^{2+} signaling in cognitive decline also remain to be clarified.

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The potential for transition metal-mediated neurodegeneration in amyotrophic lateral sclerosis

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Modulations of the potentially toxic transition metals iron (Fe) and copper (Cu) are implicated in the neurodegenerative process in a variety of human disease states including amyotrophic lateral sclerosis (ALS). However, the precise role played by these metals is still very much unclear, despite considerable clinical and experimental data suggestive of a role for these elements in the neurodegenerative process. The discovery of mutations in the antioxidant enzyme Cu/Zn superoxide dismutase 1 (SOD-1) in ALS patients established the first known cause of ALS. Recent data suggest that various mutations in SOD-1 affect metal-binding of Cu and Zn, in turn promoting toxic protein aggregation. Copper homeostasis is also disturbed in ALS, and may be relevant to ALS pathogenesis. Another set of interesting observations in ALS patients involves the key nutrient Fe. In ALS patients, Fe loading can be inferred by studies showing increased expression of serum ferritin, an Fe-storage protein, with high serum ferritin levels correlating to poor prognosis. Magnetic resonance imaging of ALS patients shows a characteristic T_2 shortening that is attributed to the presence of Fe in the motor cortex. In mutant SOD-1 mouse models, increased Fe is also detected in the spinal cord and treatment with Fe-chelating drugs lowers spinal cord Fe, preserves motor neurons, and extends lifespan. Inflammation may play a key causative role in Fe accumulation, but this is not yet conclusive. Excess transition metals may enhance induction of endoplasmic reticulum (ER) stress, a system that is already under strain in ALS. Taken together, the evidence suggests a role for transition metals in ALS progression and the potential use of metal-chelating drugs as a component of future ALS therapy.

Keywords: amyotrophic lateral sclerosis, neurodegeneration, transition metals, iron, copper, ER stress

INTRODUCTION

The burden of amyotrophic lateral sclerosis (ALS) is very significant with a world-wide disease incidence of 2 in 100,000 (Chio et al., 2013). It is a rapidly debilitating neuromuscular disorder characterized by loss of motor neurons from the cerebral cortex, brainstem, and spinal cord. Progressive muscle weakness, wasting and paralysis then follow with death typically occurring 3–5 years post-diagnosis. Approximately 95–90% of ALS cases are sporadic (sALS) while the balance is genetically linked familial disease (fALS), where mutations in superoxide dismutase 1 (SOD-1) cause 25% of cases. At least 160 mutations in SOD-1 have been associated with fALS and sALS (Sreedharan and Brown, 2013).

With the possible exception of mutant SOD-1, which forms toxic protein aggregates, the etiology of this disease is still unclear. Other postulated mechanisms of neuronal death in ALS include glutamate excitotoxicity (Rothstein, 2009), excessive generation of reactive oxygen and nitrogen species (Barber and Shaw, 2010), mitochondrial dysfunction (Rothstein, 2009), induction of endoplasmic reticulum (ER) stress (Atkin et al., 2008), axonal deterioration, and deposition of toxic ubiquitinated neuronal inclusions, where transactive response DNA binding protein 43 kDa (TDP-43) and fused in sarcoma (FUS) are major protein components (Arai et al., 2006; Rothstein, 2009). Many of these mechanisms are inter-connected and "snowballing" effects can be envisaged where events trigger, and/or exacerbate, others.

A common thread that links a variety of neurodegenerative conditions is the build-up of the transition metals iron (Fe) and copper (Cu) in the CNS (for review see Hadzhieva et al., 2013a; Jellinger, 2013). Ordinarily, these metals are incorporated into a wide range of vital enzymes, as their redox capability enables an efficient biochemical "switch" function, but this means that their homeostasis must be tightly regulated in order to prevent excessive production of reactive oxygen species that can damage cellular components. Alzheimer's diseases (AD) and Parkinson's diseases (PD), in particular, show marked brain and/or CNS increases in these metals and much attention has focused on the role of these metals in these conditions.

In AD, Fe and Cu are thought to play roles in the formation of amyloid-β plaques and tau neurofibrillary tangles (Hane et al., 2013; Savelieff et al., 2013) and recent magnetic resonance image (MRI) studies showed elevated Fe in the hippocampus in AD patients correlating to decreased structural integrity of the hippocampus, possibly caused by a process of demyelination (Raven et al., 2013). An interesting speculation by these authors was that demyelination could generate a vicious cycle of further Fe deposition as myelin contains significant Fe. However, there is still

controversy surrounding the role of Fe in AD, as others have failed to find elevated Fe in AD brain, leading to the suggestion that a citation bias exists in the literature favoring studies reporting increased brain Fe in AD (Schrag et al., 2011).

Accumulation of α-synuclein in Lewy bodies in the substantia nigra is the primary pathological hallmark of PD and results in the death of dopaminergic neurons. A variety of experimental evidences suggest that Fe and Cu not only facilitate aggregation of α-synuclein, but also potentiate the toxicity of these deposits (Santner and Uversky, 2010; Rose et al., 2011).

Despite controversy, chelation therapy targeting Fe and/or Cu has been advocated for AD and PD with encouraging results in some AD clinical trials with the chelators desferrioxamine (Crapper McLachlan et al., 1991) and clioquinol (Ritchie et al., 2003). However, the clinical value of lowering metals in these conditions still requires further validation.

A variety of other neurodegenerative disorders also show disturbances in Fe and/or Cu metabolism, leading to excess (or reduced) loading of these metals. These include the other α-synucleinopathies, a variety of disorders grouped together as "neurodegeneration with brain iron accumulation" (NBIA), multiple sclerosis, Huntington's disease, Wilson's disease, Menkes disease, Friedreich's ataxia, and the prion diseases (for reviews see Spillantini and Goedert, 2000; Hagemeier et al., 2012; Rouault, 2013).

That Fe, Cu, and zinc (Zn) may have a role in the pathophysiology of human ALS is highlighted by a diverse range of recent studies (2011–2013) showing that (a) higher concentrations of serum ferritin (an Fe-storage protein reflecting high body Fe levels) correlate to poor prognosis in ALS patients (Ikeda et al., 2012; Nadjar et al., 2012); (b) Fe accumulation in the motor cortex is responsible for frequently observed MRI abnormalities in ALS patients (Langkammer et al., 2010; Kwan et al., 2012; Ignjatovic et al., 2013); (c) inappropriately chelated Fe (i.e., capable of promoting free-radical generation) was found in the CSF of ALS patients but not normal controls (Ignjatovic et al., 2012); (d) in a group of 51 ALS patients, the levels of magnesium (Mg) ($p < 0.01$), Fe ($p < 0.05$), Cu ($p < 0.05$), and Zn ($p < 0.10$) in CSF were higher than those in controls, with some patients showing very high levels of Cu and Zn before showing critical clinical deterioration (Hozumi et al., 2011). These studies build on a multitude of others that have shown a variety of disturbances in Fe/Cu/Zn metabolism leading to metal deposition or dysfunction of key metalloenzymes in ALS (Oshiro et al., 2011).

Also of interest is the link between mutations in the hemochromatosis gene (HFE; principally the H63D polymorphism) and sALS; mutations which are thought to induce prolonged ER stress possibly through an Fe-overloading mechanism (Liu et al., 2011b). Other observations also support the possibility that excess redox-active metals may enhance ER stress in ALS by perturbing redox status, disrupting normal protein folding, and modulating ER/cytosolic calcium balance.

ALS and other neurodegenerative conditions are also accompanied by chronic neuroinflammation. Evidence shows that inflammation modulates the expression of several key proteins of Fe homeostasis, and can result in further neuronal Fe accumulation, but this potentially significant inter-play has yet to be fully explored in ALS.

Providing a therapeutic proof-of-principle argument in the role of these metals in ALS are studies in SOD-1 mutant mice where at least three different Fe (Jeong et al., 2009; Wang et al., 2011) and five Cu/Zn-chelating agents have shown significant rescue effects (Hottinger et al., 1997; Nagano et al., 2003; Petri et al., 2007; Tokuda et al., 2008). Several chelators were also able to reverse, in part, the dysregulation of Fe or Cu in SOD-1 models, reducing microglial activation that seems to occasion neuronal loss and significantly extended lifespan. Overexpression of the endogenous metal-binding protein, metallothionein (MT) (Tokuda et al., 2014), or treatment with the Cu-chelate and Cu(II)-ASTM (Soon et al., 2011) also increased survival of mutant SOD-1 mice, demonstrating a variety of rescue effects.

Taken together, these divergent data suggest that transition metals play some role in the neurodegenerative process in ALS. However, considerable gaps in our knowledge remain and in this review we seek to provide an overview of the latest research together with perspectives on future research and clinical implications.

Fe METABOLISM IN THE BRAIN AND CNS: GENERAL OVERVIEW

Iron homeostasis in the body begins by tight control of Fe release from enterocytes in the small intestine by the hormone hepcidin (Hp). Generally acknowledged as the master regulator of Fe homeostasis, Hp prevents enterocyte Fe release into the blood by binding to ferroportin-1 (Fpn). It is produced mainly by hepatocytes but is also expressed in mouse and human CNS and brain (Zechel et al., 2006; Wang et al., 2010). The major blood Fe-transport molecule is transferrin (Tf) and most cell types, including neurons, acquire Fe by a process where plasma Tf complexes with Tf-receptors (TfR) on the cell surface with subsequent internalization by endocytosis (**Figure 1**). Following Tf–TfR complex internalization to endosomes, where lower pH results in the release of Fe^{3+} from Tf, a ferric reductase, possibly six-transmembrane epithelial antigen of the prostate family member 3 (STEAP3), or duodenal cytochrome B (dcytb) then reduces Fe^{3+} to Fe^{2+} facilitating binding to divalent metal transporter 1 (DMT1) and endosomal export to the cytosol (**Figure 1**). In terms of the brain, plasma Tf and other proteins of Fe metabolism do not cross the blood–brain barrier (BBB). Hence, an additional CNS Fe-acquisition step is required, essentially an iteration of the above process, where plasma Tf binds to TfR present on the luminal membrane of cerebrovascular endothelial cells. Following cytosolic release, Fe is exported across the endothelial membrane into interstitial space where it can be acquired by neurons (by the same TfR-mediated mechanism) or bound to low-molecular weight ligands (citrate and lactoferrin, among others) for non-transferrin-bound-iron (NTBI) cell uptake (**Figure 1**). Interestingly, microglia principally take up NTBI facilitated by DMT1 and lactoferrin (Benarroch, 2009; Snyder and Connor, 2009). Typically high in ferritin, microglia are thought to play a role in the maintenance of Fe homeostasis in neurons and when activated, have been shown to release Fe from ferritin mediated by superoxide (Yoshida et al., 1995). To date, the only molecule known to efflux Fe from neurons is Fpn whose internalization in neuronal cells is regulated by Hp and ceruloplasmin (Cp) (Benarroch, 2009; Song et al., 2010; Zheng and Monnot, 2012). Iron in neuronal cells is mostly bound to ferritin or stored

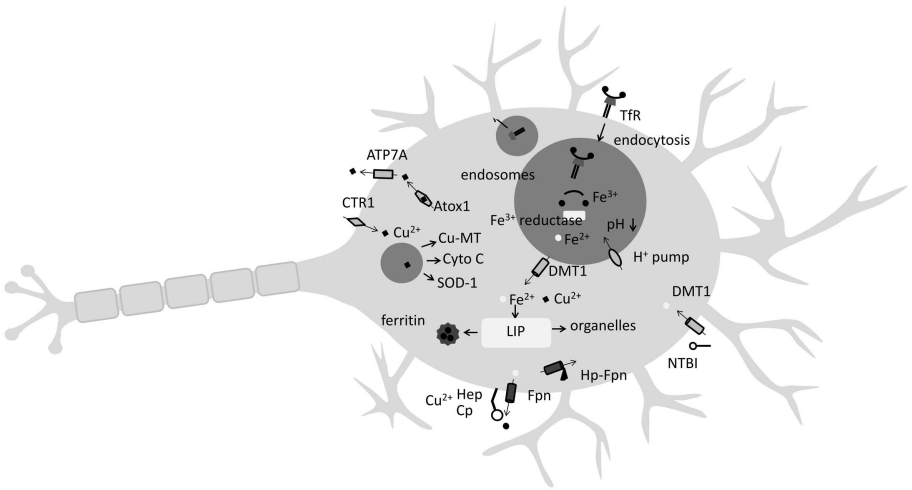

FIGURE 1 | Key features of Fe and Cu uptake and release by the neuron. Iron (Fe) is either acquired by non-transferrin bound Fe (NTBI) from low-molecular weight complexes with citrate or ascorbate or by endocytosis of the transferrin (Tf)-transferrin receptor complex (TfR). Once endocytosed, a decrease in pH and the action of a ferrioxidase enables Fe^{2+} release to the labile Fe pool (LIP) by divalent metal transporter 1 (DMT1) where it may be stored in the Fe-storage protein ferritin, or directed to organelles such as lysosomes. Copper (Cu) uptake occurs at neuron surface by copper transporter-1 (CTR1). Following endocytosis, Cu^{2+} ions are incorporated into Cu-metallothionein (Cu-MT), cytochrome *c*, or superoxide dismutase 1 (SOD-1). Ferroportin (Fpn), the only known Fe exporter from neurons, relies on the Cu-containing metalloenzymes hephaestin (Hep) or ceruloplasmin (Cp) for activity. Hepcidin (Hp) activity can internalize Fpn.

in the lysosome. Ferritin is the major Fe-storage protein and is an endogenous iron chelator (Cozzi et al., 2010). An important ferritin sub-type is mitochondrial ferritin (mitFtn), which regulates mitochondrial Fe level and Fe-related oxidization (Arosio et al., 2009). Lysosomes are also important Fe pools, acquiring Fe by turnover of Fe-containing proteins such as cytochrome *c* (Johansson et al., 2010). Due to their acidic nature, lysosomes contain Fe in the more redox-promoting Fe^{2+} state (Terman and Kurz, 2013). Controlling intracellular Fe levels are the iron regulatory protein (IRP)/iron responsive element (IRE) and the hypoxia-inducible factor (HIF)/HIF-responsive elements (HREs) (for review see Wang and Pantopoulos, 2011). Significantly, HREs are present within the TfR1 and DMT1 genes, and HIF regulates Fpn activity by inhibition of Hp (Wang and Pantopoulos, 2011). Recently, Hp was found to cause Fe overload in a rat model of cerebral ischemia (Ding et al., 2011), reflecting Hp's well-characterized ability to bind to Fpn, inducing its cellular internalization, thereby reducing Fe export (Nemeth et al., 2004).

COPPER AND IRON: PEAS IN A POD

Like Fe, Cu is a highly useful redox-active metal incorporated into many Cu-containing enzymes such as cytochrome *c* oxidase, SOD-1, monoamine oxidase, and dopamine β-monooxygenase, which play important biological roles (Zheng and Monnot, 2012). Cu is absorbed from the small intestine and delivered to the liver and kidneys where it is predominately (65–90%) bound to Cp (Sharp, 2004). Cellular Cu transport and homeostasis involve the membrane Cu transporters copper transporter-1 (CTR1), DMT1, and Cu exporter ATPases (ATP7A and ATP7B). Delivering Cu to specific intracellular targets are the Cu chaperone proteins antioxidant protein-1 (ATOX1), cytochrome oxidase enzyme complex (COX17), and Cu chaperone for SOD (CCS) (Harris, 2001). Playing a key role in Fe metabolism are the Cu-containing ferrioxidases Cp and hephaestin (Hep). Cp catalyzes the conversion of ferrous iron (Fe^{2+}) to ferric (Fe^{3+}), which is then transferred to Tf (Sharp, 2004) with Cp, and/or the multi-Cu-centered protein Hep, playing an essential role in the efflux of Fe via Fpn from enterocytes (Sharp, 2004). Significantly, Fpn is highly expressed in the epithelial cells of the choroid plexus and plays a key role in Fe efflux from the CNS (**Figure 1**). Accordingly, a decrease in Hep activity is suggested to "contribute to iron accumulation in the brain during copper deficiency" (Skjorringe et al., 2012). Additionally, Cu deficiency is known to lower GPI-anchored Cp in mouse/rat spleen and liver – and it is speculated that Cu deficiency may lower GPI-anchored Cp in astrocytes (Mostad and Prohaska, 2011). As noted above, Fe efflux from neurons is mediated chiefly by Fpn whose internalization is regulated by Hp (Nemeth et al., 2004; Song et al., 2010). Conversely, components of the Fe-transport pathway are also Cu responsive. For instance, the Fe transporter DMT1 is also a physiologically relevant Cu transporter (Sharp, 2004). Hence, both Fe and Cu homeostasis can be considered interdependent, with one affecting the other (**Figure 1**).

ALTERED Fe METABOLISM IN MUTANT SOD-1 CELL AND MOUSE MODELS

A variety of perturbations in Fe metabolism at the mRNA and protein level have been reported in both SOD-1(G37R) and SOD-1(G93A) mutant ALS mice. In late-stage disease (12 months) SOD-1(G37R) mice, a caudal-to-rostral pattern of mRNA expression of a number of proteins involved in Fe uptake and export was seen. Specifically, the mRNA levels of DMT1, TfR1, Fpn, and Cp were highest rostrally (i.e., in the cervical region) compared

to the caudal (lumbar) region (Jeong et al., 2009). Western blotting also showed the same expression pattern of these proteins. As DMT1 was expressed at twofold greater levels than either Fpn or Cp, this suggested a potential for greater Fe influx, compared to Fe efflux. Increased caudal-to-rostral spinal cord expression of the Fe-storage protein ferritin was also noted in 12-month-old SOD-1(G93A) mice. Interestingly, the mRNA expression of these proteins in younger pre-symptomatic mice (4-month-old) showed a reverse pattern of DMT1, Fpn, and Cp mRNA expression, i.e., higher lumber but lower cervical expression (**Figure 2A**), suggesting that Fe accumulation begins before significant neurodegeneration is evident.

These observations correlate to the pattern of Fe deposition and neuronal loss in the spinal cords of these mice, placing Fe "at the scene of the crime," i.e., at 12 months these mice show advanced neurodegeneration in the lumbar region (where 8 months earlier, Fe began accumulating) but less in the cervical and thoracic spinal cord (where the pattern of dysregulation now favors net Fe accumulation). However, other explanations of neuronal Fe loading are also possible. An *in vivo* model of disrupted axonal transport was also shown to lead to Fe loading in large ventral horn motor neurons (Jeong et al., 2009) and an increase in inflammatory mediators can also result in Fe accumulation (see below). While there is no direct evidence that excess Fe is responsible for neuronal loss, the injurious consequences of excess intracellular Fe are well known. That an Fe chelator was able to limit neuronal loss in this model (see below) also implies that Fe has at least some role in the neurodegenerative mechanism/s. Similar changes in the mRNA expression of these Fe metabolism proteins were also found in SOD-1(G93A) mice, but no Fe loading was detected by Perl's staining (Jeong et al., 2009).

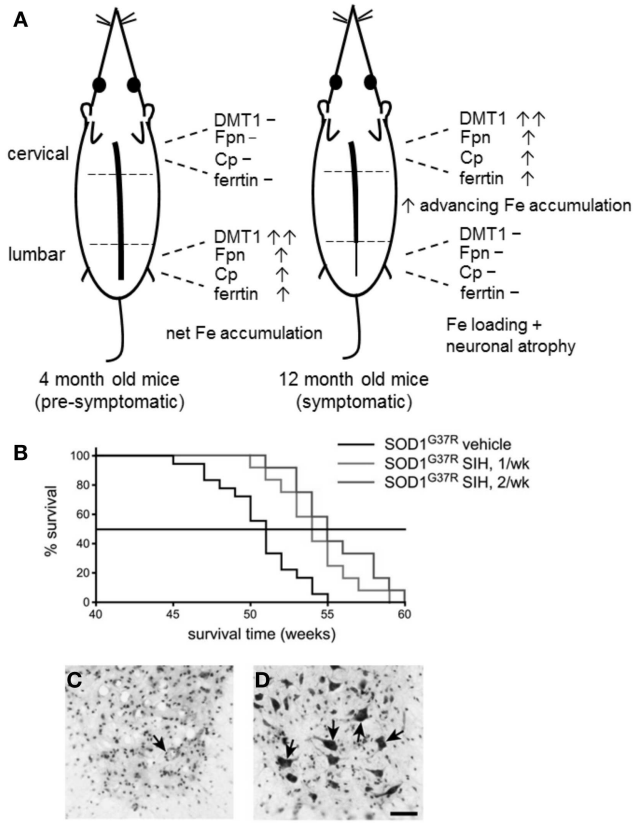

FIGURE 2 | (A) The caudal-to-rostral (tail-to-head) pattern of mRNA expression of iron metabolism proteins in young and old SOD-1(G37R) mice provides circumstantial evidence that Fe is involved in the neurodegenerative process. Advanced neurodegeneration and Fe loading is evident in the lumbar region 12-month-old mice, where 8 months earlier, the expression pattern of proteins of Fe metabolism favored net Fe accumulation. **(B)** Treatment of SOD-1(G37R) mice with the Fe chelator SIH extends lifespan in SOD-1(G37R) mice. SOD-1(G37R) mice were given SIH either once or twice a week ($n = 12$) from 8 months of age, a Kaplan–Meier graph shows the percentage of animals surviving with age. **(C,D)** Vehicle control treated 50 week-old SOD-1(G37R) mice showed loss of neurons whereas SIH treatment results in neuronal preservation (arrows) as indicated by cresyl violet-stained lumbar spinal cord tissue sections. Scale bar, 50 μM. [**(B–D)** reproduced with permission and minor modification from Jeong et al., 2009].

Considering that disturbances in mitochondrial function are also believed to play a significant role in ALS, it is interesting to note that immunofluorescence staining showed a marked increase in mitochondrial ferritin protein (mitFtn) in the ventral horn motor neurons and astrocytes in 12-month-old SOD-1(G37R) mice but not wild-type mice of the same age mice (Jeong et al., 2009). Overexpression of mitFtn has been shown to trap Fe in the mitochondria, hence Jeong et al. inferred increased mitochondrial Fe and speculated that an increased mitochondrial Fe load in neurons and glia could cause neurodegeneration as occurs in Friedreich's ataxia (Jeong et al., 2009).

Wang et al. (2011) also examined Fe metabolism in the SOD-1(G93A) ALS model and the ability of Fe chelators to mediate rescue effects (discussed below). Besides finding a 148 and 180% increase in spinal cord Fe in SOD-1 G93A mice (90- and 120-day-old, respectively) compared to age-matched WT mice, these authors also showed induction of TfR1 protein expression. Considering that a major Fe-acquisition pathway in neurons involves intracellular delivery of Fe via the TfR, upregulation of this protein may, in part, explain the increased Fe loading. Additionally, this result also confirms a general dysregulation of Fe homeostasis, as ordinarily, TfR expression decreases in response to increased intracellular Fe (Richardson and Ponka, 1997).

Fe homeostasis in SOD-1(G93A) ALS was further studied using transgenic neuroblastoma SH-SY-5Y cells stably transfected with WT SOD-1 or the G93A mutated human SOD-1 gene (Hadzhieva et al., 2013b). In agreement with studies in SOD-1(G37A) mice, increased mRNA levels of TfR1, ferritin, and DMT1 were found. Additionally, effects on Fe homeostasis within the mitochondrion, a key site for the generation of Fe–sulfur cluster precursor proteins leading to heme biosynthesis, were examined. A 2–3.7 fold increase in mitFtn1&2, iron–sulfur cluster scaffold protein (IScU), and frataxin (Fxn) mRNA was found to also correlate to increased protein expression with the exception of Fxn. Mutant SOD-1(G93A) cells were also significantly Fe loaded compared to WT cells (1.6 fold when normalized to cell number), but interestingly, isolated mitochondria were not Fe-loaded. Further experiments then sought to dissect the mechanism of TfR upregulation. As noted above, Fe deprivation induces classical TfR upregulation via IRE

sensing in IRP1 however, Fe supplementation failed to decrease TfR expression, indicating a key dysregulation in Fe homeostasis whereby TfR expression is induced in SOD-1(G93A) mutant cells by IRE/IRP1 independent mechanism/s. ROS is an alternative inducer of TfR expression and has been shown to result in liver Fe loading in alcoholic liver disease (Kohgo et al., 2008). Experiments utilizing H_2O_2 and retinoic acid to induce ROS in SOD-1(G93A) mutant cells showed increases in TfR, Ftn, mitFtn1&2, and DMT1 mRNA compared to WT cells, the authors suggesting that ROS-induced upregulation of the Fe importing system may "create a vicious cycle generating strong oxidative stress" (Hadzhieva et al., 2013b).

RESCUE EFFECTS BY Fe CHELATORS IN SOD-1 MOUSE MODELS OF ALS

Treatment of SOD-1(G37R) ALS mice with the lipophilic Fe chelator SIH increased mean lifespan by 5 weeks (**Figure 2B**). Histological staining showed that there were more surviving motor neurons in SIH-treated SOD-1(G37R) ALS mice compared with vehicle-treated SOD-1(G37R) mice of the same age (Jeong et al., 2009; **Figures 2C,D**). In untreated 12-month-old SOD-1(G37R) mice, significant accumulation of Fe was evident in motor neurons of lumbar ventral horn which also appeared atrophied. Glial cells also showed Fe loading. SIH treatment reduced fivefold to sixfold the number of Fe-containing cells detected by Perl's staining of spinal cord sections, reversed the significant weight loss observed in untreated SOD-1(G37R) mice and improved locomotor function (Jeong et al., 2009).

More recently, Wang et al. (2011) showed that the brain-permeable Fe chelators VK-28 and M30 significantly delayed ALS onset, extended lifespan and reduced spinal cord motor neuron loss in the SOD-1(G93A) transgenic mouse model of ALS. Both Fe chelators significantly attenuated the elevated Fe level and TfR expression, decreased oxygen free radicals and suppressed microglial and astrocytic activation in the spinal cords of SOD-1(G93A) mice (Wang et al., 2011).

Another mechanistic effect of M30 and structurally related chelators is induction of hypoxia-inducible factor-1α (HIF-1α), possibly through enhanced phosphorylation of the p42/44 mitogen-activated protein kinase (MAPK)/ER kinase (MEK) and protein kinase C (PKC) signaling pathways (Avramovich-Tirosh et al., 2010). As a transcriptional activator, HIF-1α is known to regulate a range of neuroprotective signaling pathways and treatment with M30 was shown to increase the gene expression of erythropoietin (EPO), vascular endothelial growth factor (VEGF), enolase 1 and inducible nitric oxide synthase (iNOS) in NSC-34 cells (Kupershmidt et al., 2009), and rat primary embryonic cortical neurons (Avramovich-Tirosh et al., 2010). *In vivo* mice studies with M30 showed upregulation of HIF-1α protein in brain (cortex, hippocampus, and striatum) and spinal cord when given over 30 days. Enhanced HIF-1α levels coincided with increased gene expression of EPO, VEGF, iNOS, glucose transporter 1 (GLUT-1), and heme oxygenase-1 (HO-1) in one or more brain regions but only VEGF showed upregulation in the spinal cord. Additionally, M30 *in vivo* also increased mRNA expression of brain-derived neurotrophic factor (BDNF) and glial cell-derived neurotrophic factor (GDNF) as well as the antioxidant enzymes catalase, SOD-1, and glutathione peroxidase (GPx) in spinal cord and some brain regions (Kupershmidt et al., 2011).

The induction of VEGF by M30 is particularly interesting in light of the many studies demonstrating a neuroprotective role of VEGF in ALS (for review see Llado et al., 2013). Indeed, genetic studies link polymorphisms in the VEGF gene to the development of sALS (Lambrechts et al., 2003; Lysogorskaia et al., 2012) and VEGF therapy has been shown to prevent motor neuron degeneration and increase survival in mutant SOD-1 mice (Storkebaum et al., 2005; Wang et al., 2007). On the basis of these and other observations, VEGF is a suggested therapeutic intervention for ALS. Besides M30, other Fe chelators including desferrioxamine (DFO; the standard clinically used Fe chelator for Fe overload) have also been shown to induce VEGF expression in non-ALS models (Beerepoot et al., 1996).

ALTERED Fe DISTRIBUTION AND METABOLISM IN ALS PATIENTS

In ALS patients, there are several indications of dysregulated iron metabolism. Increased serum ferritin levels and lower Tf (but increased Tf saturation) were recently reported by Nadjar et al. (2012) in a cohort of 694 ALS patients and 297 healthy controls. High serum ferritin was correlated to reduced survival time in ALS patients by 300 days compared to ALS patients with low level serum ferritin. Although serum Fe levels were not significantly different between ALS patients and controls, increased Tf saturation could suggest Fe loading. Previously, Ikeda et al. (2012), assessing a range of serum biomarkers in 92 Japanese ALS patients and 92 age-matched healthy controls, also found that increased serum ferritin correlated with clinical deterioration. Increased ferritin was also reported (Goodall et al., 2008; Qureshi et al., 2008) together with lower serum Tf (Mitchell et al., 2010) in ALS patients. The latter study found that, as biomarkers, higher serum ferritin and lower Tf discriminated between ALS patients and controls with 82% accuracy. These authors also quantified light-chain ferritin (L-ferritin) suggesting that increased L-ferritin might reflect activation, and potentially Fe turnover, in macrophages or brain microglia. Goodall et al. also assessed the levels of C-reactive protein, reporting that high serum ferritin was independent of expression of this inflammation marker.

High Fe in ALS patients has also been detected by MRI scanning techniques that attribute characteristic T_2 hypointensities in images of the motor cortex to Fe deposition. Most recently, assessment of 19 ALS cases and 19 healthy controls attributed 3T and 7T FLAIR hypointensities in the hand-knob region of the motor cortex to high Fe which correlated to higher upper motor neuron impairment scores (Kwan et al., 2012; **Figures 3A,B**). Subsequent postmortem investigations in an ALS patient confirmed that 7T MRI hypointensity changes in the middle and deep layers of the motor cortex correlated to the same regions as a positive Perls' DAB Fe stain (**Figure 3C**). Interestingly, higher magnification images seemed to suggest that positive Perl's DAB staining was present in cells resembling microglia, but was not evident in spinal motor neurons (Kwan et al., 2012; **Figure 3D**). Immunostaining experiments indicating that excess Fe was stored in ferritin in microglia, as co-localization was shown for ferritin and the microglial marker CD6. It was also noted that luxol fast blue stain

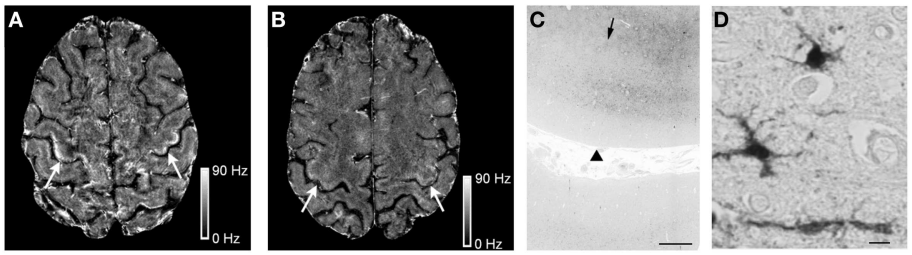

FIGURE 3 | Iron (Fe) accumulation in the motor cortex of an ALS patient. The 7T magnetic resonance imaging (MRI) scans reveal Fe accumulation in the motor cortex hand-knob region in a 51-year-old ALS patient **(A)** (arrows), compared to a healthy control. **(B)** Postmortem Fe accumulation in the middle and deeper layers of cortical gray matter and at the gray–white junction by Pearl's staining (arrowheads indicate the pial surface while arrows indicate the gray–white junction). **(C)** At higher magnification, Pearl's staining detects Fe in cells with irregular processes suggestive of microglia in the ALS motor cortex. **(D)** Scale bars: C, 1 mm; D, 10 μM. Reproduced with permission and minor modification from Kwan et al. (2012).

showed myelin pallor in the subcortical white matter of the precentral, compared to the postcentra, gyrus (Kwan et al., 2012), which is potentially interesting in light of the recent suggestion by Bartzokis et al. that demyelination may contribute to excess Fe in AD (Raven et al., 2013). Other studies have also attributed changes in T_2 MRI images to Fe build-up in ALS patients (Oba et al., 1993; Langkammer et al., 2010; Ignjatovic et al., 2013).

Increases in bulk Fe levels had previously been detected in ALS patient ventral horn (Kurlander and Patten, 1979), although not in other studies. Another indication of disturbed Fe distribution may be the presence of higher levels of reactive low-molecular weight Fe species in the CNS. Electron paramagnetic resonance (EPR) spectrometry studies showed that ALS patients had higher levels of inappropriately liganded Fe in the CNS (i.e., bidentate or tridentate ligands that leave spare Fe coordination site/s thereby increasing Fe solubility, in-turn promoting Fe redox cycling and •OH production; Ignjatovic et al., 2012).

IRON AND NEUROINFLAMMATION

Microglial activation and astrogliosis are primary hallmarks of the neuroinflammatory environment and widely present in a variety of human neurodegenerative conditions, including ALS (McGeer et al., 1993). They are also a noted feature in mutant SOD-1 mice models of ALS (Jeong et al., 2009; Wang et al., 2011).

Microglia and astrocytes have been shown to play a role in scavenging (and storing) excess Fe, thereby protecting neurons from consequences of Fe loading (Pelizzoni et al., 2013). However, it has been recognized that inflammation modifies the expression of the key Fe-regulating hormone Hp (Nicolas et al., 2002; Ganz, 2003), in turn, down-regulating the Fe export molecule Fpn, and leading to Fe accumulation (Nemeth et al., 2004). This is an element of the classical host Fe-withdrawal defense against infection and inflammation. While there is no direct evidence of a similar process occurring in ALS, interesting clues are provided by studies in dopaminergic neurons. In a study of lipopolysaccharide (LPS)-induced PD in the rat, activated microglia increased expression of the pro-inflammatory cytokines IL-1 and IL-6 and Fe loading was evident in the substantia nigra. Reduced expression of Fpn was also found. In further experiments, SH-SY5Y dopaminergic neurons incubated with media enriched in pro-inflammatory cytokines showed decreased Fpn expression and the appearance of Fe deposits (Zhang et al., 2014). Increased levels of iron regulatory protein 1 (IRP1), transferrin receptor 1 (TfR1), and Hp had previously been observed in IL-1β or tumor necrosis factor alpha (TNF-α) treated ventral mesencephalic neurons (Wang et al., 2013). Additional experiments showed LPS-induced activated microglia led to enhanced IL-1β and TNF-α release upon Fe loading.

Matrix metalloproteinases (MMPs) are also known mediators of neuroinflammation. Increased Fe loading of LPS-stimulated rat microglial (HAPI) cells was shown to enhance secretion of MMP-9 and MMP-1. Increased cellular Fe levels also impaired zymosan phagocytic activity in activated microglia (Mairuae et al., 2011). Others have hypothesized that in addition to activated microglia, Fe maybe deposited in the CNS by infiltrating circulatory monocytes. Once transformed into phagocytic macrophages, and subsequently dying back post-consumption of compromised neurons, these cells may re-release Fe into the labile Fe pool (LIP), enhancing the potential of oxidative stress damage to adjacent cells (including neurons; Andersen et al., 2013).

Collectively, these results are suggestive of the possibility of a vicious cycle whereby Fe loading of microglia and astrocytes increases mediators of neuroinflammation, but in-turn, microglial/astroglial Fe loading results from inflammation-mediated suppression of Fpn by Hp. As yet, there is no direct evidence of these processes occurring in ALS, but processes similar to these may provide a potential explanation for the characteristic T_2 hypointensities in MRI images of the motor cortex due to Fe deposition and increased serum ferritin in ALS patients. These observations also underscore the general anti-inflammatory property of Fe and Cu chelators in murine SOD-1 ALS models, as evidenced by reduced microglial activation (Jeong et al., 2009; Kupershmidt et al., 2011).

COPPER IN ALS

Most interest in Cu in ALS centers on mutations in SOD-1 and over 160 mutations have been associated with ALS (Sreedharan and Brown, 2013). Formation of toxic SOD-1 aggregates is strongly implicated (Chattopadhyay and Valentine, 2009; Sreedharan and Brown, 2013) and multiple data describe defective Cu (and Zn) binding by various SOD-1 mutants (Carri et al., 1994; Eum and Kang, 1999; Hayward et al., 2002). Early studies

showed that the Cu chelators diethyldithiocarbamate and penicillamine could reverse mutant SOD reactivity (Wiedau-Pazos et al., 1996). Undermetallation of SOD-1 seems to be a significant factor in SOD-1 aggregation *in vivo* (Hayward et al., 2002; Lelie et al., 2011). Studies in SOD-1 mutant mice showed that SOD-1 fractions isolated from spinal cords mutant SOD-1 mice that were insoluble (i.e., aggregated) were Cu and Zn depleted, whereas soluble SOD-1 fractions were highly metalated (Lelie et al., 2011). A re-distribution of Cu from gray to white matters correlated to areas of high SOD-1 and increased Zn was also found in the white matter in mutant SOD-1 mice (Lelie et al., 2011). In a study of mutant H43R SOD-1, a rapidly progressing form of fALS, metal-replete (halo) H43R SOD-1 exhibited a stable β-barrel structure, but apo-H43R SOD-1 was unstable and readily misfolded (Fujimaki et al., 2013). Spectroscopic studies showed that Cu^{2+} binding differed between the halo- and apo-forms, the authors suggesting that bound Cu^{2+} in the apo-H43R form was pro-oxidant. While SOD-1 metalation status appears important in promoting aggregation, failure of SOD-1 maturation, due to disrupted formation of a stabilizing intra-subunit disulfide bond also, appears to be significant (Seetharaman et al., 2009). Irrespective of the precise aggregation mechanism, SOD-1 aggregates are thought generally to acquire a "toxic gain of function" related to promotion of oxidative stress. Impairment of axonal transport (De Vos et al., 2007) and mitochondrial dysfunction (Li et al., 2010) could also be sequelae. More recently, SOD-1 mutants were found to interfere with protein transport between the ER–Golgi apparatus, leading to Golgi fragmentation and induction of ER stress (Atkin et al., 2013). Others have suggested that palmitoylation (a reversible post-translational modification that influences structure, function, and localization) of SOD-1 mutants could increase their targeting to cellular membranes, potentially increasing their propensity to cause mitochondrial dysfunction or ER stress (Antinone et al., 2013).

Other Cu-containing enzymes or chaperones showing modulations of expression or activity in ALS patients include Cp and MT. An early Polish study assessing the levels of Cp in 14 ALS patients found suppressed levels in eight patients (Domzal and Radzikowska, 1983) but this was not replicated in other studies (Boll et al., 2008; Goodall et al., 2008). However, a recent study of Cp isoforms in ALS showed that patients had higher abundance of non-sialylated protein forms of Cp, indicating "a Cp functional impairment" (Conti et al., 2008). Further evidence suggestive of role for Cp emerged from a proteomic 2D difference-in-gel electrophoresis analysis of CFS in ALS patients (Brettschneider et al., 2010). Compared to controls, only six proteins were found to be modulated between these groups, the ceruloplasmin precursor protein (CpPP) and the Fe delivery protein Tf were two proteins that were downregulated (Brettschneider et al., 2010).

Studies with the endogenous antioxidant chaperone MT also provide insight into the important role played by Cu in mutant SOD-1 ALS. In a cohort of 12 ALS patients, the immunohistochemical expression of the MT-1/2 isoform was significantly reduced in the spinal cord relative to the control group, although both MT-1/2 and MT-3 were found in glia (Hozumi et al., 2008). Expression of MT-3 in astrocytes in the gray matter of the lumbar spinal cord negatively correlated with ALS duration. Additionally, patients with MT-3-positive neurons also showed MT-3 positive glia. Previous studies had shown that SOD-1(G93A) mice with either MT-1/2 or MT-3 knocked-down had accelerated ALS disease course (Nagano et al., 2001) and recently SOD-1G93A mice engineered to also overexpress MT showed significantly enhanced survival together with reduced motor neuron loss and degeneration of ventral root axons and skeletal muscle atrophy. Glial activation was also reduced. Additionally, SOD-1 aggregates in the spinal cord glia were reduced in double transgenic mice (Tokuda et al., 2014).

Aside from its role in SOD-1 and other metalloenzymes or chaperones, there is some evidence of general Cu accumulation in human ALS. Most recently, 52 ALS patients showed a significant ($p < 0.05$) increase in Cu in CSF (Roos et al., 2013), but no excess of Cu was found in CSF by others (Boll et al., 2008). Slightly elevated Cu and Zn were also found in erythrocytes in SOD-1 fALS patients (Vinceti et al., 2002). In a study of spinal cord tissue from sALS patients found a significant Cu increase (Kurlander and Patten, 1979). Accumulations of Cu have also been noted in the SOD-1(G93A) mouse (Li et al., 2006; Tokuda et al., 2013), prompting assessment of various Cu chelators in this model, including D-penicilamine (Hottinger et al., 1997) and trientine (Nagano et al., 2003). Most recently, the Cu chelator tetrathiomolybdate (TM) was shown to significantly improve symptoms and survival in SOD-1(G93A) ALS mice (Tokuda et al., 2008) by removal of Cu from the Cys_{111} residue in mutant SOD-1 but not from histidyl sites essential for SOD-1 enzymatic activity, thereby preventing "aberrant copper chemistry." Subsequently, an almost threefold accumulation of Cu in spinal cords of SOD-1(G93A) mice was reversed by TM treatment and modulations in expression of a variety of Cu-containing proteins in SOD-1 mutants were reported (Tokuda et al., 2013). Specifically, in the G93A, G127X, G85R, and D90A SOD-1 mutants, western blots showed significantly increased expression of Steap2, Atox1, CTR1, and CCS (all except the G127X mutant) and significant decrease in Atp7a in spinal cord tissue. The functional significance of these modulations was described as skewing the Cu-transport system toward Cu accumulation in the spinal cord (Tokuda et al., 2013).

Additional support for a deleterious role of Cu in mutant SOD-1 ALS was provided by studies with Tg mice engineered to contain both SOD-1G86R and mottled/brindled (MoBr) mutations (Kiaei et al., 2004). The latter is a model of Menkes disease, a human genetic disorder causing copper deficiency. In SOD-1G86R/MoBr mice spinal cord, Cu was reduced by 60%, and these mice lived 9% longer than mice bearing the SOD-1G86R mutation alone, supporting a role for Cu in SOD-1-related fALS.

ENDOPLASMIC RETICULUM STRESS: AN UNDER-RECOGNIZED ROLE FOR IRON?

Triggers of ER stress include Ca^{2+} storage defects, lipid/glycolipid imbalances, oxidative stress, and a variety of environmental insults such as viral infections and drug exposure (Rutkowski and Kaufman, 2007). In ALS, major ER stress inducers are misfolded SOD-1, TDP-43, and/or FUS proteins that accumulate in the ER thereby inducing the unfolded protein response (UPR) (Atkin et al., 2008).

The UPR acts to reduce ER stress by re-establishing normal protein folding and proceeds by the activation of three distinct ER

stress transduction pathways following removal of the ER chaperon binding immunoglobulin protein (BiP) from the luminal domains of inositol-requiring enzyme-1 (IRE1), protein kinase R (PKR)-like endoplasmic reticulum kinase (PERK), and activating transcription factor 6 (ATF6). However, severe or chronic UPR triggers apoptosis (Bertolotti et al., 2000; **Figure 4**).

In human ALS patients, a variety of indicators of ER stress leading to induction of the UPR have been identified. A group of 12 sALS patients showed a 26-fold higher positive immunostaining for the ER chaperone protein GRP78 (also known as BiP) compared to controls (Sasaki, 2010) and in the spinal cords of sALS patients, UPR induction was demonstrated by upregulation of stress sensor kinases (IRE1, ATF6, and PERK), chaperones protein disulfide isomerase (PDI), ER protein 57 (Erp57), apoptotic mediators CCAAT/-enhancer-binding protein homologous protein (CHOP), and caspase 4 (Atkin et al., 2008).

It is well appreciated that Fe overload increases intracellular ROS (Galaris and Pantopoulos, 2008), a known initiator of ER stress. More specific observations of the link between Fe and ER stress include upregulation of the ER chaperons BiP and calreticulin (CRT) in Fe-loaded astrocytoma (Ye and Connor, 2000); increased BiP expression in the livers of dietary Fe-loaded mice (Petrak et al., 2007); reduced mRNA and protein expression of the major blood Fe transporter Tf was found in transfected Hep 3B hepatoma cells overexpressing CHOP (You et al., 2003; **Figure 4**). Mutations in the hemochromatosis gene (HFE) have also been associated with sALS. Both the H63D and C282Y HFE polymorphisms interfere with the ability of HFE to limit Fe uptake from Tf, which results in Fe loading (Feder et al., 1998; Lee et al., 2007). Recently, human neuronal SH-SY5Y cells with a tetracycline-controlled FLAG-tagged HFE H63D mutation showed upregulation of the UPR markers BiP, PDI, and IRE1α after tetracycline treatment (Liu et al., 2011b). Induction of the master Fe-regulating hormone Hp by ER stress has also been noted (Vecchi et al., 2009) with subsequent downstream effects on the expression of the cellular Fe exporter, Fpn, and ferritin. These studies were the first to demonstrate the reciprocal relationship between Fe homeostasis and ER stress, describing Hp induction as a marker of ER stress (Vecchi et al., 2009).

INTRACELLULAR CALCIUM SIGNALING IS ALSO RESPONSIVE TO Fe

Calcium (Ca^{2+}) storage defects are thought to be significant in ALS and another potential consequence of excess Fe in ALS may be detrimental modulation of Ca^{2+} signaling. Early observations showed that the ROS generating Fe^{2+}/H_2O_2 couple decreased Ca^{2+} uptake by skeletal sarcoplasmic reticulum (SR) (Stoyanovsky et al., 1994; Castilho et al., 1996) by inhibiting the activity of the Ca^{2+} pump Ca^{2+}-ATPase (SERCA) (Moreau et al., 1998) which transfers Ca^{2+} from cytosol into SR lumen (**Figure 4**). However, Fe^{2+} given alone enhanced Ca^{2+} uptake into rat heart SR vesicles, mimicking ruthenium red by direct inhibition of the ryanodine receptor (RyR), one of the channels by which Ca^{2+} is released from the SR (Kim et al., 1995). Interestingly, the ER stress inducer thapsigargin also inhibits SR Ca^{2+}-ATPase (thereby reducing ER lumen Ca^{2+} uptake) and was shown to increase Fe uptake in K562 leukemic cells with depletion of intracellular Ca^{2+} or chelation of

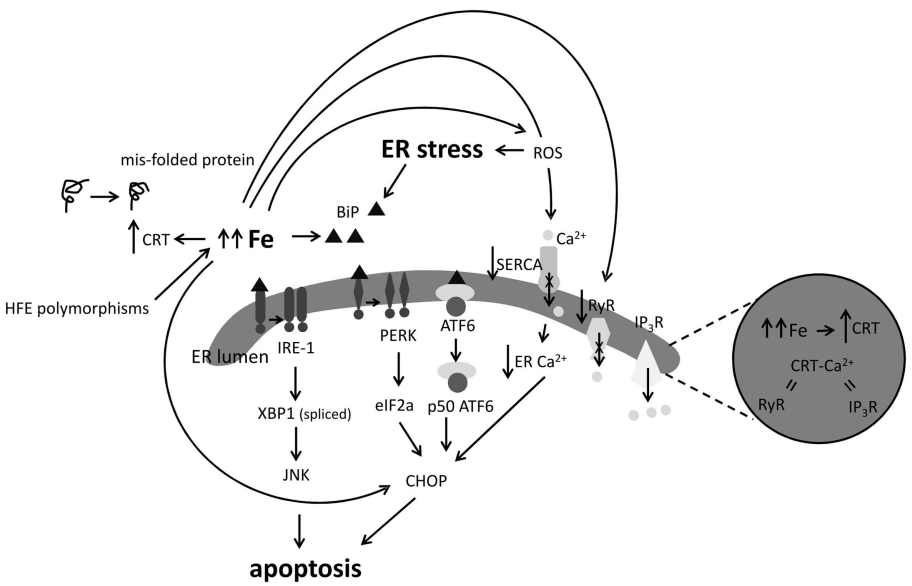

FIGURE 4 | Impact of excess iron (Fe) on indicators of ER stress and calcium (Ca^{2+}) signaling. ER stress results in the activation of the IRE1, PERK, and ATF6 apoptotic pathways. Loss of BiP induces oligomerization of IRE1 or PERK or activates ATF6 which downstream results in JNK or CHOP mediated apoptosis. High Fe in cells and animal models has been shown to induce BiP, CHOP, and calrectulin (CRT) expression. Excess Fe and resultant redox processes may inhibit function of the SERCA Ca^{2+} pump, decreasing ER Ca^{2+} levels, and leading to CHOP induction, on the other hand, Fe has been shown to inhibit the ryanodine receptor (RyR) ER Ca^{2+} exporter. Induction of CRT by Fe could suggest that Fe participates in causing protein mis-folds or results in perturbed Ca^{2+} balance in the ER.

extracellular Ca^{2+} resulting in inhibition of Fe uptake (Ci et al., 2003).

Additionally, Fe was shown to be necessary for Ca^{2+} signal generation and ERK1/2 stimulation induced by the glutamate agonist *N*-methyl-D-aspartate (NMDA), as Fe chelation reduced Ca^{2+} signal duration and prevented NMDA-induced ERK1/2 activation (Munoz et al., 2011). Fe supplementation of primary hippocampal neurons kept in Ca^{2+}-free medium also elicited Ca^{2+} signals, suggesting that hippocampal neurons require Fe to generate RyR-mediated Ca^{2+} signals after NMDA receptor stimulation (Munoz et al., 2011). Hp treatment of human osteoblasts was also shown to increase intracellular Ca^{2+}, an effect potentiated by added Fe (Li et al., 2012). Pretreatment of osteoblasts with a L-type Ca^{2+} channel blocker or a RyR antagonist inhibited Ca^{2+} release from the SR, demonstrating that the increase of intracellular Ca^{2+} induced by Hp probably reflected Ca^{2+} release from the ER, triggered by Ca^{2+} influx (Li et al., 2012). Interestingly, mRNA and protein expression of the ER chaperone CRT that plays a key role in protein folding protein in the ER, was shown to increase with Fe concentration (Liu et al., 2011a), suggesting that CRT induction may be part of the cellular response to excess Fe, which in turn could affect ER Ca^{2+} buffering. Considering that CRT functions as a misfolded protein chaperone, CRT induction could suggest that excess Fe may also negatively impact protein folding.

A ROLE FOR METALS IN TOXIC TDP-43 ACCUMULATIONS?

Neuronal cytoplasmic inclusions of TDP-43 are a major component of ubiquitinated protein aggregates found in the CNS of sporadic MND patients. Indeed, TDP-43 inclusions are a histopathological feature in the vast majority of MND patients (Da Cruz and Cleveland, 2011). Current thinking suggests these inclusions mediate neuronal damage by inducing ER stress or by affecting RNA processing (Atkin et al., 2008).

Interestingly, several lines of evidence also point to the role for metals in TDP-43 accumulation and toxicity. In SH-SY5Y neuronal-like cells expressing endogenous TDP-43, Zn treatment reduced TDP-43 expression but enhanced the formation of TDP-43 positive inclusions (Caragounis et al., 2010). However, addition of Cu or Fe did not induce similar effects, suggesting that specific Zn-associated processes might modulate TDP-43 accumulation. Additionally, treatment of SOD-1(G93A) mice with the Cu-containing chelate, Cu-ASTM, prevented the accumulation of abnormally phosphorylated and fragmented TDP-43 in the spinal cord (Soon et al., 2011). The Fe chelator M30 and a structural analog also showed reduced TDP-43 accumulation in the SOD-1(G93A) model (Wang et al., 2011). Another clue emerged from a phenotypic screen for small molecules that could reverse TDP-43 toxicity in yeast. One class of "hit" compounds were 8-hydroxyquinolines, biologically active metal chelators structurally related to clioquinol, the authors suggesting that 8-hydroxyquinolines may be able to target TDP-43 accumulation by altering metal homeostasis (Tardiff et al., 2012). It was also recently reported that mice bearing the TDP-43(A315T) mutation had increased Cu, Zn, and manganese (Mn) in the spinal cords not evident in wild-type mice (Dang et al., 2014). Increased levels of the monocyte chemoattractant protein-1 (MCP-1) in TDP-43(A315T) mouse spinal cord indicated microglial activation and inflammation. Protein oxidation was also higher in TDP-43(A315T) spinal cord and is a hallmark of metal-mediated Fenton chemistry. How and why TDP-43 inclusions alter the metallic landscape of the spinal cord in SOD-1 ALS will be an on-going research interest.

DISCUSSION

The transition elements Fe and Cu are involved in ALS progression by potentially multiple mechanisms. A clear role is evident for Cu and Zn in the key enzyme SOD-1. Mutations affecting the metal-binding sites may lead to structural instability, possibly through loss of integrity of a intra-subunit disulfide bond, and then leading to accumulation and/or to a toxic gain of function. Other Cu-containing enzymes may also be relevant in ALS progression. Early data implicated reduced Cp expression, but this was not reproduced in other studies, but higher levels of non-sialylated Cp were detected in another study in ALS patients and could indicate a Cp functional impairment. Reduced expression of MT has also been noted in ALS patients and in SOD-1(G93A) models. Conversely, MT overexpression or treatment rescues phenotype in mutant SOD-1 mice. Studies in patients and SOD-1 mice also show bulk increases in Cu in the spinal cord. That a variety of Cu chelators also extend lifespan and reverse pathophysiological effects in SOD-1 mouse models, provide evidence suggestive a role for Cu in ALS progression.

In sALS patients, high serum ferritin levels have been consistently shown to correlate with ALS progression and characteristic MRI T_2 shortening shows Fe overload in the motor cortex of ALS patients. To what extent this excess Fe drives the ALS disease process is unknown (although there are some clues provided by SOD-1 models) and it is likely that excess Fe loading represents a process secondary to primary disease mechanism/s.

As a biomarker, increased serum ferritin may infer increased body iron stores, but not necessarily so, as infection or inflammation can also induce its expression. However, increased Fe and inflammation are linked and one exacerbates the other creating a vicious cycle. Indeed, a relatively good case, albeit circumstantial, can be made that excess Fe in ALS is a consequence of inflammatory processes. That Goodall et al. reported increased serum ferritin in ALS patients in the absence of an increase of the inflammatory marker C-reactive protein, does not necessarily rule out a role for inflammation in serum ferritin induction (and Fe deposition), as more discrete localized inflammation could have occurred that did not generate a detectable serum trace. However, increases in Tf saturation, in addition to high serum ferritin reported by Nadjar et al., also point to excess Fe. Besides inflammation, another potential mechanism that could cause Fe loading in ALS could be reduced Cp expression or activity, as Cp plays a role in mediating Fe export by Fpn.

The form in which Fe is present also influences its degree of danger. Low-molecular weight labile Fe is more hazardous than Fe stored in ferritin. But Fe in ferritin is still bioavailable and can readily be released by activated microglia in a process mediated by superoxide. Increased labile Fe, or "inappropriately chelated" Fe, reported by Ignjatovic et al. in ALS patient CSF also suggests that excess Fe in ALS is present in a form that can readily contribute to redox stress.

As models of fALS, mutant SOD-1 mice reproducibly show Fe loading in spinal cord, and in SOD-1(G37R) mice, dysregulation of Fe metabolism resulting in neuronal Fe loading precedes significant neuronal loss, providing at least circumstantial evidence that Fe is involved in the neurodegenerative process. The ability of a variety of structurally diverse Fe chelators to extend lifespan, prevent neuronal loss and mediate other rescue effects provides additional evidence that Fe is probably involved in the neurodegenerative process in ALS.

Induction of ER stress due to aggregation of protein mis-folds in ALS is gaining traction as a major mechanism of neuronal loss. Various studies describe how excess Fe may increase ER stress, as Fe loading causes increases in the expression of a variety of ER chaperones. Perturbed intracellular Ca^{2+} signaling can also be an expected consequence of Fe loading due to inhibition of ER Ca^{2+} pumps. Another link to ER stress and transition metals is provided by recent studies showing enrichment of TDP-43 aggregates with Cu, Zn, and other metals. In the context of a system already under stress, it is intriguing to speculate that transition metal loading in ALS may also be enhancing ER stress. However, no direct evidence yet exists for this hypothesis.

While the collective evidence is strengthening, the insult that excess transition metals render in ALS still requires clarification. However, as a facet of a complex disease, lowering (or modulating) levels of transition metals, particularly Cu and Fe, in ALS would seem to be an avenue worth pursuing.

AUTHOR CONTRIBUTIONS

David B. Lovejoy and Gilles J. Guillemin researched and wrote the paper.

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Increased metal content in the TDP-43^{A315T} transgenic mouse model of frontotemporal lobar degeneration and amyotrophic lateral sclerosis

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Disrupted metal homeostasis is a consistent feature of neurodegenerative disease in humans and is recapitulated in mouse models of Alzheimer's disease, Parkinson's disease, amyotrophic lateral sclerosis (ALS) and neuronal ceriod lipofuscinosis. While the definitive pathogenesis of neurodegenerative disease in humans remains to be fully elucidated, disease-like symptoms in the mouse models are all driven by the presence or over-expression of a putative pathogenic protein, indicating an *in vivo* relationship between expression of these proteins, disrupted metal homeostasis and the symptoms of neuronal failure. Recently it was established that mutant TAR DNA binding protein-43 (TDP-43) is associated with the development of frontotemporal lobar degeneration and ALS. Subsequent development of transgenic mice that express human TDP-43 carrying the disease-causing A315T mutation has provided new opportunity to study the underlying mechanisms of TDP-43-related neurodegenerative disease. We assessed the cognitive and locomotive phenotype of TDP-43^{A315T} mice and their wild-type littermates and also assessed bulk metal content of brain and spinal cord tissues. Metal levels in the brain were not affected by the expression of mutant TDP-43, but zinc, copper, and manganese levels were all increased in the spinal cords of TDP-43^{A315T} mice when compared to wild-type littermates. Performance of the TDP-43^{A315T} mice in the Y-maze test for cognitive function was not significantly different to wild-type mice. By contrast, performance of the TDP-43^{A315T} in the rotarod test for locomotive function was consistently worse than wild-type mice. These preliminary *in vivo* data are the first to show that expression of a disease-causing form of TDP-43 is sufficient to disrupt metal ion homeostasis in the central nervous system. Disrupted metal ion homeostasis in the spinal cord but not the brain may explain why the TDP-43^{A315T} mice show symptoms of locomotive decline and not cognitive decline.

Keywords: amyotrophic lateral sclerosis (ALS), frontotemporal lobar degeneration (FTLD), TAR DNA binding protein-43 (TDP-43), copper (Cu), zinc (Zn), manganese (Mn), neurodegenerative disease

INTRODUCTION

Metals such as iron, copper and zinc are needed for normal cell function and alterations to the bio-availability of these metals can therefore have devastating consequences on the survival and functionality of cells in all parts of the body. This is particularly true for functionality of the central nervous system (CNS) because metal ions are also required for normal synaptic function (Tamano and Takeda, 2011). Underscoring the significance of maintaining metal homeostasis in the CNS, disrupted metal homeostasis is evident in many neurodegenerative diseases including Alzheimer's disease (AD), Parkinson's disease (PD), and amyotrophic lateral sclerosis (ALS). In AD, iron, copper, and zinc accumulate within amyloid plaques (Connor et al., 1992; Lovell et al., 1998), in PD iron is increased within the substantia nigra (Sofic et al., 1988), and in ALS copper, zinc, manganese, and several other metals are all increased in the cerebrospinal fluid (Roos et al., 2013).

In addition to human tissue affected by neurodegenerative disease, animal models of these diseases also recapitulate elements of metal dyshomeostasis; AD model mice that over-express the amyloid-β precursor protein have decreased levels of copper and zinc in the brain relative to wild-type controls (Maynard et al., 2002) copper and iron are altered in the brains of PD model mice (Matusch et al., 2010; Ayton et al., 2013) and zinc and copper levels are altered in mutant SOD1 mouse models of ALS (Kiaei et al., 2004; Tokuda et al., 2013). The presence of disrupted metal homeostasis in animal models of neurodegenerative disease as well as the human condition has encouraged development of therapeutic strategies that aim to correct this imbalance (Badrick and Jones, 2011; Crouch and Barnham, 2012). However, confirming whether the loss of metal homeostasis in neurodegenerative disease represents a cause of the disease or a consequence of neuronal dysfunction still requires further investigation.

Frontotemporal lobar degeneration (FTLD) is a collective term for neurodegenerative diseases that involve degeneration of the frontal and temporal lobes of the brain (Pickering-Brown, 2010). It can affect individuals from the age of 40, and in the aged population, is one of the most common causes of dementia (Knopman et al., 2004). There are three main sub-types of FTLD based on their histology; dementia lacking distinct histology, FTLD with tau-positive inclusions, and FTLD with tau-negative ubiquitin-positive inclusions (FTLD-U). In 2006 it was established that cytoplasmic inclusions present in the brains of people with FTLD-U and in the spinal cords of people with ALS contained aggregates of the TAR DNA binding protein-43 (TDP-43) (Neumann et al., 2006). This provided the first molecular evidence to explain the comorbidity that is common to ALS and FTLD (Lomen-Hoerth et al., 2002) and also led several groups to propose that ALS and FTLD-U represent two ends of a spectrum of TDP-43-mediated neurodegenerative diseases that can collectively be referred to as TDP-43 proteinopathies (Liscic et al., 2008; Chen-Plotkin et al., 2010).

It is currently unclear whether disrupted metal homeostasis is present in TDP-43 proteinopathies. *In vitro* studies have implicated a role for metals in the aggregation of endogenous TDP-43 (Caragounis et al., 2010), and metal-based therapeutic strategies have the potential to prevent aberrant TDP-43 metabolism (Parker et al., 2012); however, there is a current paucity of *in vivo* data. This study utilizes the recently developed TDP-43^{A315T} mouse model of FTLD/ALS (Wegorzewska et al., 2009) to assess whether the presence of a pathogenic form of TDP-43 affects metal ion homeostasis *in vivo*, and if present, to assess whether altered metal homeostasis may be associated with the symptoms of neuronal decline in an animal model of TDP-43 proteinopathy.

MATERIALS AND METHODS

MATERIALS

Rabbit antibody to TDP-43 was purchased from Proteintech (USA); rabbit antibodies to FLAG, histone H3, and GAPDH were obtained from Cell Signaling (Australia). All other chemicals were purchased from Sigma Aldrich (Australia) unless otherwise stated.

ANIMALS

TDP-43^{A315T} mice were purchased from The Jackson Laboratory Repository (Stock no. 010700; Bar Harbor, ME, USA). This mouse model was generated using the mouse prion promoter and a cDNA encoding human *TARDBP* with an A315T mutation and containing an N-terminal FLAG-tag (Wegorzewska et al., 2009). A colony of TDP-43^{A315T} mice was maintained by breeding TDP-43^{A315T} mice with non-transgenic C57/BL6 mice. Animals were group-housed under standard housing conditions with a 12 h light–dark cycle, and food and water *ad libitum*. All animals expressing the TDP-43^{A315T} were confirmed via PCR according to the distributor's protocol. Non-transgenic littermates not expressing the TDP-43^{A315T} were used as wild-type controls. All animal protocols and procedures were approved by Melbourne Research Animal Ethics at The University of Melbourne, Australia. Only male mice were included in this study and the number of animals per group is stated in the figure legends.

BEHAVIORAL TESTING

Locomotor activity was assessed using the rotarod assay (Turner et al., 2009); rod diameter 35 mm and elevation 200 mm. Mice were acclimatized to the rotarod by daily training for one week. Training involved three 5-min training runs per mouse per day. For each training run the mice were returned to the rotarod if they fell before the end of the 5-min period. Once the mice reached 5 weeks of age they were tested twice weekly. During testing, the rotarod was set to accelerate from 4 to 40 rpm over 5 min. Latency to fall was recorded in seconds. On each testing day each mouse was tested twice and only higher latency to fall score used for analysis.

Cognitive function was assessed using the Y-maze test as previously described (Hung et al., 2012) and performed at the age of 10 weeks. The floor of the maze was covered with sawdust which was replaced between each training and testing run in order to prevent residual odors that may affect performance. The walls of the maze were non-transparent and decorated internally so that each arm was visually unique. For training each mouse was allowed to explore only two arms of the maze (the novel arm was blocked with a non-transparent wall) for 10 min. At the end of training mice were removed from the maze for a period of 1 h before returning and being allowed to explore all 3 arms of the maze for 5 min. For training and testing the mice were placed into the maze at the same position in the starting arm of the maze. During testing the number of entries made to each arm of the maze was recorded and the percentage of entries into the novel third arm was calculated.

TISSUE COLLECTION AND PREPARATION

Male mice (mean age of 12 weeks) were anesthetized by intraperitoneal injection of PBS (137 mM NaCl, 8.1 mM Na_2HPO_4, 2.68 mM KCl, 1.47 mM KH_2PO_4, pH 7.4) supplemented with ketamine (20 mg/mL) and xylazine (4 mg/mL). Animals were then transcardially perfused with PBS containing 0.25% phosphatase inhibitor cocktail, 1% protease inhibitor (Roche) and 20 U/mL heparin. Perfused brain, spinal cord, liver, and quadriceps muscle were collected, frozen on dry ice and stored at −80°C until further processing.

For western blot analysis, enzyme-linked immunosorbent assay (ELISA) and protein oxidation detection, tissues were mechanically homogenized in 150–200 μL homogenizing buffer [PBS containing 1:100 protease inhibitor cocktail (Roche), 1:50 phosphatase inhibitor cocktail and 1:20 DNase (Roche)] before ultrasonication at 2 amp for 10 s. Homogenates were centrifuged at 18,000 *g* for 3 min at 4°C and the supernatant (soluble fraction) collected and the pellet (insoluble fraction) retained. Protein content was determined by the bicinchoninic acid assay (Thermo Scientific, USA). Fractions were stored at −80°C until further analysis.

WESTERN BLOT ANALYSIS

Soluble tissue fractions were prepared in 4× loading buffer [250 mM Tris, 20% (v/v) glycerol, 8% (w/v) SDS, 2% (v/v) β-mercaptoethanol, 0.01% (w/v) bromophenol blue] and heated for 5 min at 95°C. Samples containing 10–30 μg protein were then loaded onto 4–12% NuPAGE Novex Bis–Tris Midi gels (Life Technologies) electrophoresed at 200 V for 40 min. Proteins were transferred onto PVDF membranes using the iBlot

gel transfer device (Life Technologies) for 7 min according to the manufacturer's instructions. Membranes were blocked with 4% (w/v) skim milk in PBS containing 0.05% (v/v) Tween-20 (PBST) followed by incubation with primary antibody (1:1000) overnight at 4°C. After washes in PBST, membranes were incubated with secondary antibody for 1 h at room temperature. Membranes were incubated with Western Lighting Ultra ECL (Perkin-Elmer) and imaged using the Fujifilm LAS-3000 Image reader. Blots were then stripped with 1% HCl for 15 min and reprobed for GAPDH or histone H3 as loading control. The optical density (OD) of bands was quantified using the ImageJ software and standardized to loading control (GAPDH/histone H3). The relative fold change for proteins in TDP-43^{A315T} mouse tissue is expressed relative to wild-type mouse tissue.

METAL ANALYSIS

Inductively coupled plasma mass spectrometry (ICP-MS) was used to measure bulk metal concentration in tissue samples as described elsewhere (Maynard et al., 2002). Briefly, perfused brain and spinal cord tissue samples were lyophilized then digested in HNO_3 (65% Suprapur, Merck) overnight. Liver and quadriceps muscles were used as control non-CNS tissue. Tissues were heated at 90^{o}C before the addition of H_2O_2 (30% Aristar, BDH). Samples were left to stand for ∼30 min, before further heating at 70^{o}C. The average reduced volume was determined and samples were further diluted with 1% HNO_3. Measurements were made using an Agilent 7700 series ICP-MS instrument using a Helium Reaction Gas Cell and 200 ppb of Yttrium (Y89) as an internal control (ICP-MS-IS-MIX1-1, Accustandard). Results are expressed as micrograms of metal per gram of wet weight tissue (μg/g).

PROTEIN OXIDATION DETECTION AND MCP-1 ELISA

Oxidative stress and inflammation are associated with metal dyshomeostasis and neurodegeneration (Barnham et al., 2004; Molina-Holgado et al., 2007). To assess the oxidative and inflammatory status of the brain and spinal cord of the TDP-43^{A315T} we measured levels of oxidized proteins and the inflammation marker monocyte chemoattractant protein 1 (MCP-1). Brain and spinal cord soluble and insoluble fractions were analyzed for oxidative modified proteins via the OxyBlot Protein Oxidation Detection Kit (Merck Millipore, Australia) according to the manufacturer's instructions. The OxyBlot detects the carbonyl groups found on oxidized proteins. The OD of bands was quantified using the ImageJ software and values were standardized to the loading controls GAPDH and histone H3 for soluble and insoluble fractions, respectively. The relative fold change of oxidized proteins in TDP-43^{A315T} mice is expressed relative to wild-type. Levels of MCP-1 were measured in brain, spinal cord, liver and quadriceps muscle using the Mouse CCL2/JE/MCP-1 DuoSet (R&D Systems, USA) according to the manufacturer's instructions.

STATISTICAL ANALYSIS

All values are presented as mean ± SEM. All statistical analyses were performed using Graphpad Prism. Planned comparisons using two-tailed independent *t*-tests were used to analyze all data. Significance was set at $p < 0.05$.

RESULTS

TDP-43^{A315T} MICE EXHIBIT LOCOMOTOR IMPAIRMENT BUT NO COGNITIVE DEFICIT

Consistent with the original description of the TDP-43^{A315T} mice (Wegorzewska et al., 2009), the FLAG-tagged TDP-43^{A315T} was readily detected in the brain and spinal cord relative to the liver and quadriceps muscles, indicating that the mutant TDP-43 is mainly expressed within the CNS in this model (**Figure 1A**). When assessed using an antibody that detected both the FLAG-tagged mutant TDP-43 and the endogenous mouse TDP-43, expression of TDP-43 was ∼7-fold higher in the transgenic animals compared to wild-type controls (**Figure 1B**).

To investigate whether the TDP-43^{A315T} mouse model recapitulate symptoms of ALS, the rotarod was used to assess locomotor activity. Lower scores on the rotarod are indicative of impaired locomotor function. TDP-43^{A315T} mice consistently scored lower than wild-type littermates at all time-points examined (**Figure 1C**). The poor performance of TDP-43^{A315T} mice on the rotarod decreased further towards the end of the study period, dropping sharply at ∼12 weeks of age until end-stage at 14.2 ± 1.3 weeks. Unlike mutant SOD1 mouse models of ALS (Gurney et al., 1994; Wong et al., 1995) full hind-limb paralysis was not evident at end-stage in the TDP-43^{A315T} mice. Accordingly, the TDP-43^{A315T} mice maintained locomotor functionality through to end-stage (**Figure 1C**).

The Y-maze was used to examine whether the TDP-43^{A315T} mice exhibited a cognitive deficit reminiscent of FTLD. Cognitive function was examined in males at 10 weeks of age to ensure that testing was assessed before the dramatic late stage decline in locomotor function. The number of entries into the novel arm of the Y-maze, indicative of short-term memory function, did not differ significantly between the TDP-43^{A315T} mice and their age-matched wild-type littermates ($p > 0.05$; **Figure 1D**).

INCREASED METAL LEVELS IN THE SPINAL CORD OF TDP-43^{A315T} MICE

Bulk metal analysis was performed using ICP-MS and levels of Na, Mg, Al, P, K, Ca, Ti, Mn, Fe, Cu, and Zn were quantified in the brain and spinal cord of TDP-43^{A315T} and wild-type mice (**Figure 2**). Bulk levels of these metals were not significantly different in the brain between genotypes ($p > 0.05$; **Figures 2A–K**). However, Mn, Cu, and Zn were significantly increased in the spinal cord of TDP-43^{A315T} mice when compared to wild-type controls by 19.8, 16.9, and 18.8%, respectively ($p < 0.05$; **Figures 2H,J,K**). Changes to Mn, Cu, and Zn observed in the spinal cord tissue appeared to be restricted to this tissue; as per the brain, these metals were not altered in the liver or quadriceps muscle (**Figures 2H,J,K**).

INCREASED OXIDATIVE STRESS IN THE SPINAL CORD OF TDP-43^{A315T} MICE

The OxyBlot kit was used to detect the levels of oxidized proteins as a measure of oxidative stress in brain and spinal cord fractions. The level of oxidized proteins in the soluble brain and spinal cord fractions (containing mostly cytosolic proteins) of TDP-43^{A315T} mice was not different from wild-type mice (**Figure 3A**). However, when assessing the insoluble fraction, containing mainly membrane and nuclear material, there was a significant 2.2-fold increase in the level of oxidized proteins in the spinal cord of TDP-43^{A315T} mice

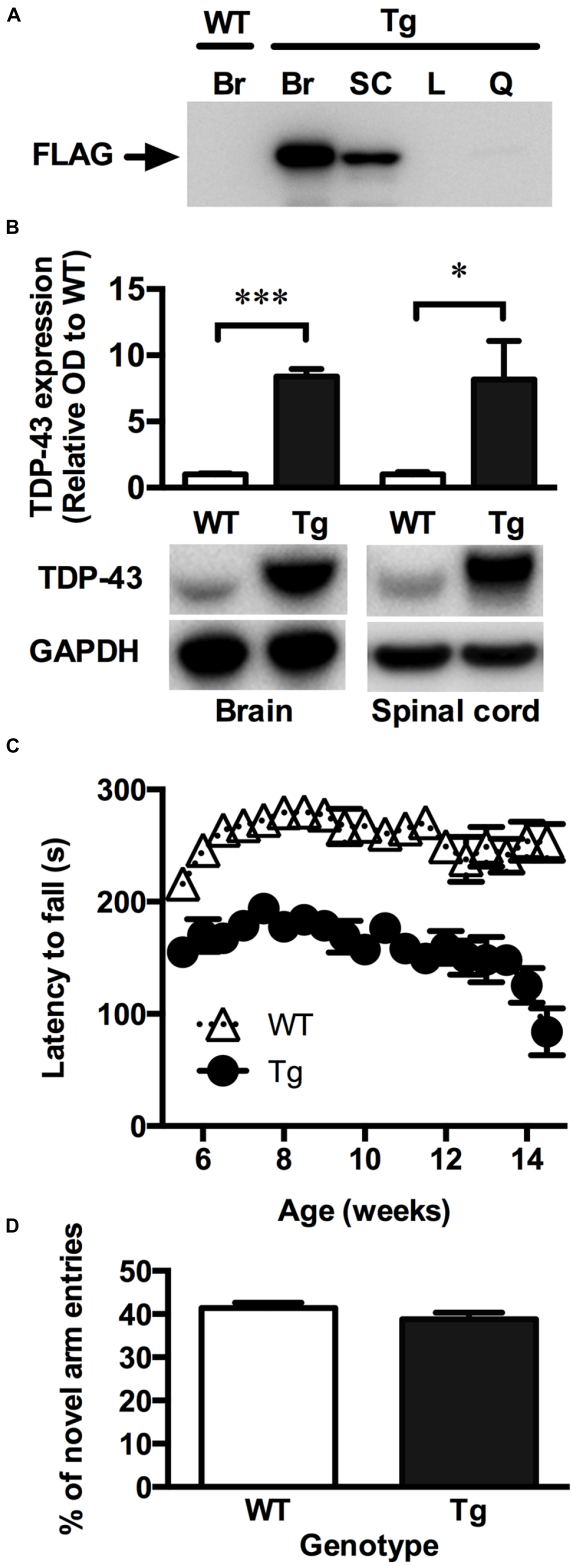

FIGURE 1 | TDP-43^{A315T} mice express mutant TDP-43 in the CNS and exhibit a locomotor impairment. (A) Representative western blot showing the absence of mutant FLAG-tagged TDP-43 (FLAG) expression *(Continued)*

FIGURE 1 | Continued
in the brain of wild-type (WT) mice and the expression of FLAG-tagged TDP-43 in the brain (Br), and spinal cord (SC) but not liver (L) and quadriceps muscle (Q) of transgenic TDP-43^{A315T} (Tg) mice. **(B)** Western blot analysis show a significant overexpression of total TDP-43 in the brain and spinal cord of Tg mice ($n = 9$) compared to WT ($n = 9$). ***$p < 0.001$; *$p < 0.05$. Blots shown are representative images. OD of bands were standardized to GAPDH and expressed relative to WT. **(C)** Tg mice exhibit a locomotor impairment compared to WT, scoring lower on the rotarod test than WT with a further decline in locomotor activity beginning at ~12 weeks. **(D)** No significant differences were detected in the number of novel arm entries of the Y-maze test between WT and Tg mice ($p > 0.05$).

when compared to wild-type ($p < 0.05$; **Figure 3B**). There were no differences in the insoluble brain fraction between genotypes (**Figure 3B**).

INCREASED MARKERS OF INFLAMMATION IN THE BRAIN AND SPINAL CORD OF TDP-43^{A315T} MICE

Monocyte chemoattractant protein-1 is involved in microglia-mediated inflammatory processes in the CNS and can be used as an indicator of increased inflammation (Deshmane et al., 2009). Levels of MCP-1, as detected by ELISA, were significantly increased in the brain and spinal cord of TDP-43^{A315T} mice by 1.7- and 1.4-fold, respectively ($p < 0.05$; **Figure 4**).

DISCUSSION

Metal dyshomeostasis is implicated in a number of neurodegenerative diseases (Sofic et al., 1988; Connor et al., 1992; Lovell et al., 1998), but whether metals play a role in the degeneration of neurons in TDP-43 associated forms of diseases such as FTLD-U and ALS is unknown. In this study, the TDP-43^{A315T} mouse model of FTLD/ALS TDP-43 proteinopathy was used to examine potential metal changes in the CNS due to expression of a pathogenic form of mutant TDP-43. Consistent with previous studies (Wegorzewska et al., 2009), FLAG-tagged mutant TDP-43 was mainly expressed within the CNS of TDP-43^{A315T} mice resulting in the overexpression of TDP-43 in the brain and spinal cord. These animals exhibited a locomotor impairment reminiscent of ALS. The TDP-43^{A315T}mice performed poorly on the rotarod test and demonstrated a progressive loss of locomotor activity towards end-stage. This observation is consistent with those reported by Esmaeili and colleagues (2013). Full hind-limb paralysis was not evident prior to the premature death of the TDP-43^{A315T} mice.

Bulk metal analysis of the spinal cord revealed a significant increase in the levels of manganese, Cu and Zn due to expression of mutant TDP-43 in the TDP-43^{A315T}mice (**Figure 2**). These changes appear specific to the spinal cord tissue as manganese, copper and zinc were not altered in the brain, liver, or quadriceps muscle (**Figure 2**). Rotarod analysis of the TDP-43^{A315T} mice revealed a clear deficit relative to wild-type mice, but the Y-maze assessment of the TDP-43^{A315T} mice indicated expression of the mutant TDP-43 did not affect the cognitive function of these animals (**Figure 1D**). This apparent demarcation between symptoms of neuronal decline in the spinal cord compared to neuronal decline in the brain is despite the higher levels of the mutant TDP-43 expression in the brain compared to spinal cord (**Figure 1B**). Thus, the effects of mutant TDP-43 expression on

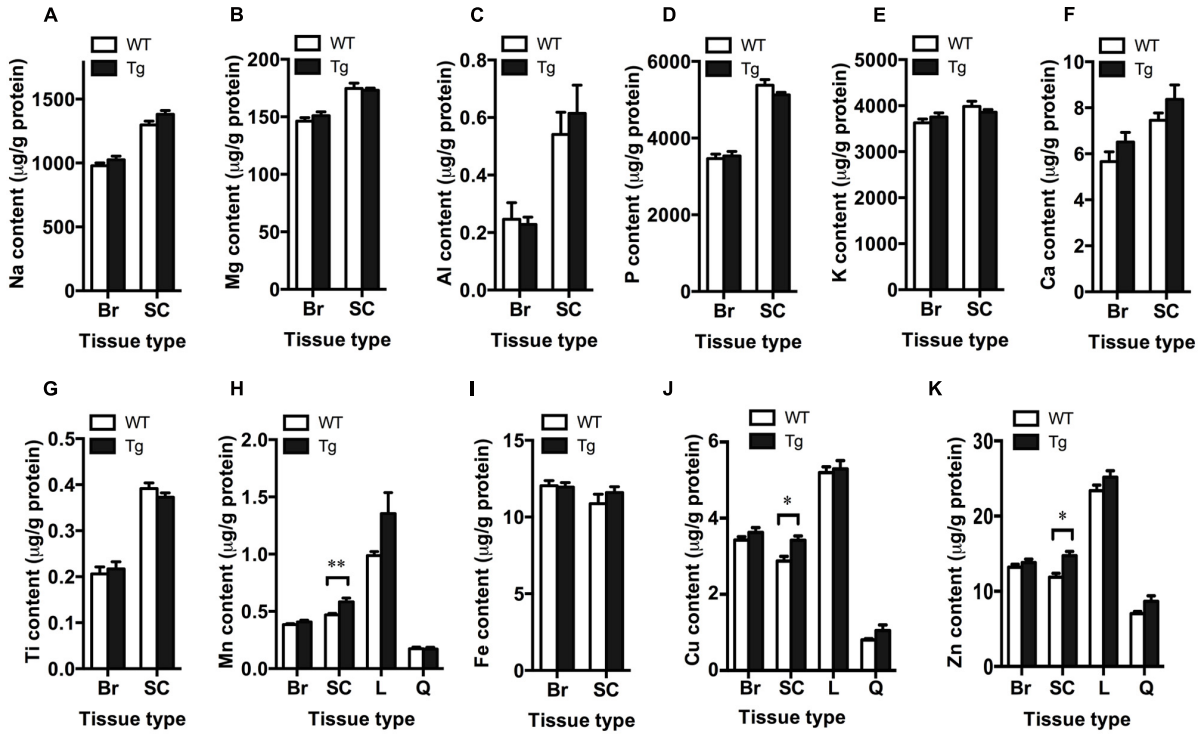

FIGURE 2 | Increased metal content in the spinal cord of TDP-43^{A315T} mice. ICP-MS analysis of bulk metal content in the brain (Br), spinal cord (SC), liver (L) and quadriceps muscle (Q) of TDP-43^{A315T} mice (Tg), and wild-type litter mates (WT). **(A–K)** Planned comparisons revealed no significant differences in bulk levels of Na, Mg, Al, P, K, Ca, Ti, Mn, Fe, Cu, and Zn in the brain between genotyptes ($n = 16$ per genotype); however, **(H)** Mn, **(J)** Cu, and **(K)** Zn were significantly increased in the spinal cord of Tg mice compared to WT controls ($n = 7$ per genotype). $^{**}p < 0.01$; $^{*}p < 0.5$.

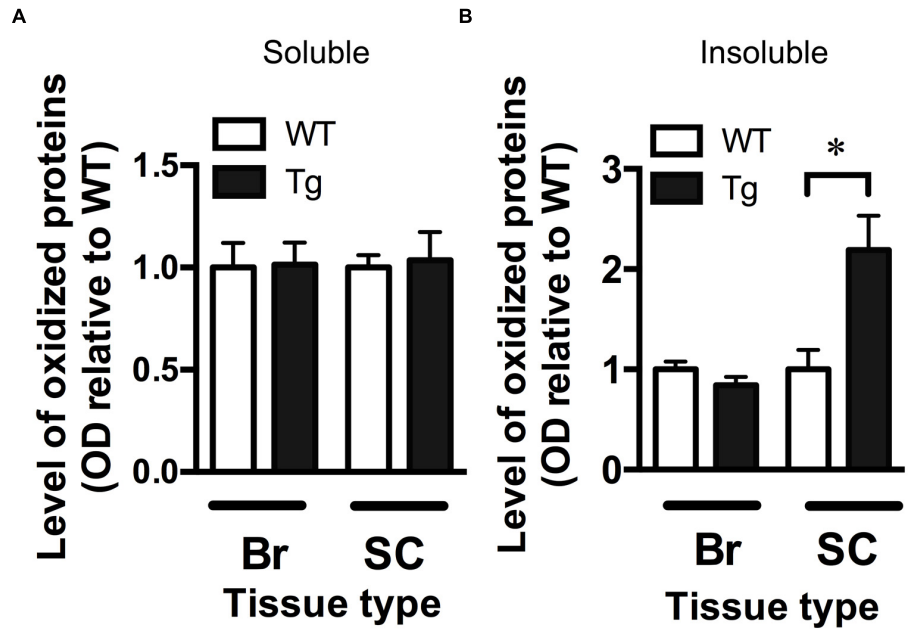

FIGURE 3 | Increased oxidative stress in the spinal cord of TDP-43^{A315T} mice. (A) The level of oxidized proteins as determined by OxyBlot is unchanged in the soluble fraction (containing cytosolic proteins) of the brain (Br) and spinal cord (SC) of TDP-43^{A315T} mice (Tg) compared to wild-type littermates (WT). **(B)** Levels of oxidized proteins were significantly increased in spinal cord insoluble fraction (containing membrane and nuclear material) of Tg compared to WT; $^{*}p < 0.05$; $n = 8$ per genotype. No changes were detected in brain insoluble factions.

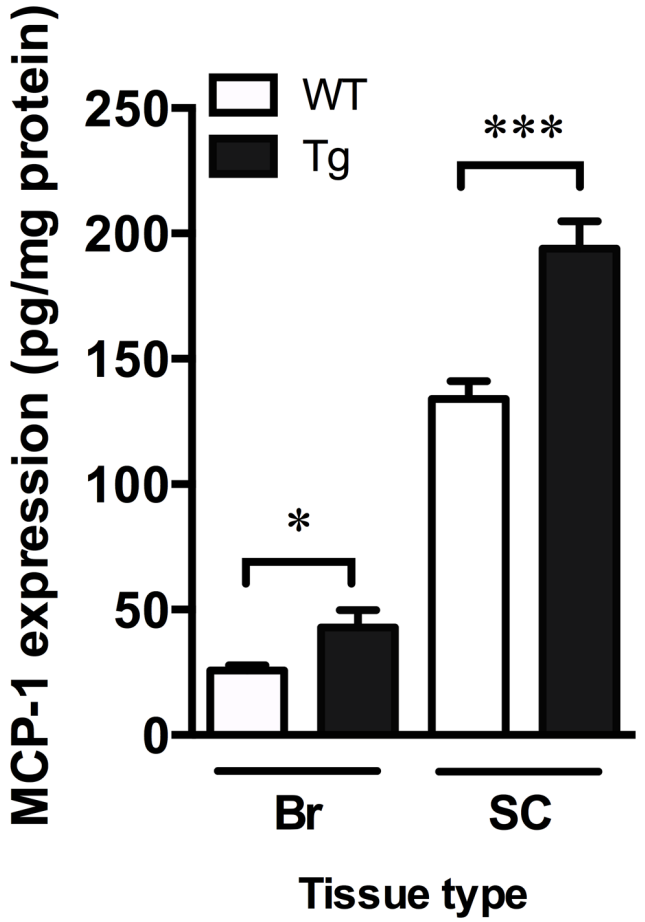

FIGURE 4 | Increased markers of inflammation in TDP-43^{A315T} mice. MCP-1 ELISA show a significant increase in MCP-1 content in the brain (Br) and spinal cord (SC) of TDP-43^{A315T} mice (Tg) compared to wild-type (WT). ***$p < 0.001$; *$p < 0.05$. $n = 9$ per genotype for brain; $n = 7$ per genotype for spinal cord.

metals in the spinal cord, but not the brain, may have contributed to the predominantly locomotor phenotype of the TDP-43^{A315T} mice.

The mechanisms by which mutant TDP-43 could contribute to elevated levels of these metals in the spinal cord remain to be fully elucidated, and it is not yet clear whether the altered metal content of the spinal cord represents a cause or consequence of neuronal dysfunction. The data generated for levels of oxidized proteins (**Figure 3**), however, are in part consistent with the altered metal content representing a causative event. Despite no change to levels of oxidized proteins in the soluble fraction, the abundance of oxidized proteins in the insoluble fraction was elevated in the spinal cords of TDP-43^{A315T} mice. Oxidative damage is evident in the spinal cords of ALS cases (Niebroj-Dobosz et al., 2004) as well as mutant SOD1 mouse models of the disease (Soon et al., 2011), and altered metal homeostasis has been associated with this oxidative damage. Most notably, the pro-oxidant toxic gain-of-function ascribed to mutant SOD1 in SOD1-associated cases of ALS is proposed to be the result of altered metallation of SOD1 (Beckman et al., 2001). The normal anti-oxidant activity of SOD1 requires equimolar binding of Zn and Cu, but disruptions to this metal stoichiometry, including disruptions caused by ALS-associated SOD1 mutations (Crow et al., 1997; Roberts et al., 2007) confer toxic pro-oxidant activity to the SOD1 (Estevez et al., 1999). Significantly, altered metallation of wild-type SOD1 also confers a pro-oxidant toxic gain-of-function (Estevez et al., 1999). It is possible therefore, that the mutant-TDP-43 induced disruption of metal homeostasis detected in the spinal cords of the TDP-43^{A315T} mice contributed to a neurotoxic oxidative mechanism already proposed as a significant pathogenic event in ALS.

In contrast to oxidative damage, the data generated for levels of the inflammatory marker MCP-1 are less clear with respect to inflammation possibly contributing to the mutant TDP-43-induced phenotype of the TDP-43^{A315T} mice. Levels of MCP-1 were elevated in the spinal cords of TDP-43^{A315T} mice, and they were also elevated in the brain (**Figure 4**). Given the mice did not display an overt cognitive phenotype indicative of neuronal dysfunction in the brain, these data may suggest the inflammation present in the brains of the TDP-43^{A315T} mice represents a relatively non-specific response to the over-expression of an exogenous protein. Alternatively, these data may represent a specific consequence of mutant TDP-43 expression, a possibility consistent with evidence for increased inflammation in ALS and FTLD (Galimberti et al., 2008; Letiembre et al., 2009; Papadimitriou et al., 2010). As discussed below, the absence of an overt cognitive phenotype in the TDP-43^{A315T} mice may be due to the premature death of these animals preventing the opportunity for the manifestation of neuronal dysfunction in the brain.

The data presented in this study indicate increased metal levels in the spinal cords of the TDP-43^{A315T} mice may have contributed to their locomotor impairment, while the lack of cognitive impairment may be due to the absence of metal dyshomeostasis in the brain. However, this study only performed bulk metal analysis whereby whole tissue homogenates were analyzed for total metal levels. Region specific changes to metals or the redistribution of metals in the brain may therefore have been undetected by this methodology. Alternate analytical techniques such as laser-ablation ICP-MS which allows for spatial resolution of metal concentrations within tissues (Hare et al., 2012) or liquid chromatography ICP-MS which allows resolution of metalloproteins prior to metal quantitation (Lothian et al., 2013) may therefore be required before excluding the possibility that expression of mutant TDP-43 caused relatively subtle changes to metals within the brains of the TDP-43^{A315T} mice. In addition, it must be noted that the TDP-43^{A315T} mouse model has recently been reported to die prematurely from gastrointestinal complications before the development of full ALS- and FTLD-like symptoms (Guo et al., 2012; Esmaeili and colleagues, 2013). Although we did not study these gastrointestinal complications, we did find that the TDP-43^{A315T} mice died suddenly before the presence of full hind-limb paralysis. This raises the possibility that if these mice had not died from their gastrointestinal complications they may have gone on to exhibit symptoms of cognitive decline. Thus, the non-CNS related premature death of the TDP-43^{A315T} mice may have been the only factor that limited the detection of potential alterations

to brain metal homeostasis and/or cognitive impairment, especially if FTLD-like symptoms develop much later than ALS-like symptoms.

Overall, this preliminary study is the first to report altered metal content in the spinal cord of the TDP-43^{A315T} mouse model of FTLD and ALS. The increase in metal content is associated with increased oxidative stress and inflammation in this tissue. The mechanism by which mutant TDP-43 can alter metal levels and the effect of these changes on the oxidative and inflammatory status of the CNS remains to be elucidated, and an analysis of these changes to spinal cord metal levels is needed to address their temporal relationship with the progressive phenotype of the TDP-43^{A315T} mice. Nonetheless, the data from this study provide evidence to support the role of metal dyshomeostasis in neurodegenerative diseases, including TDP-43 proteinopathies.

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Metal-deficient aggregates and diminished copper found in cells expressing SOD1 mutations that cause ALS

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Disruptions in metal ion homeostasis have been described in association with amyotrophic lateral sclerosis (ALS) for a number of years but the precise mechanism of involvement is poorly understood. Metal ions are especially important to familial ALS cases caused by mutations in the metalloenzyme copper-zinc superoxide dismutase (SOD1). To investigate the role of metals in aggregation of mutant SOD1, we have examined the localization of metal ions in a cell culture model of overexpression. Chinese hamster ovary cells (CHO-K1) were transfected to overexpress SOD1 fused to yellow fluorescent protein (YFP) to readily identify the transfected cells and the intracellular aggregates that develop in the cells expressing mutant or wild-type (WT) SOD1. The concentration and distribution of iron, copper, and zinc were determined for four SOD1 mutants (A4V, G37R, H80R, and D125H) as well as a WT SOD1 using X-ray fluorescence microscopy (XFM). Results demonstrated that the SOD1 aggregates were metal-deficient within the cells, which is consistent with recent *in vitro* studies. In addition, all SOD1 mutants showed significantly decreased copper content compared to the WT SOD1 cells, regardless of the mutant's ability to bind copper. These results suggest that SOD1 overexpression creates an unmet demand on the cell for copper. This is particularly true for the SOD1 mutants where copper delivery may also be impaired. Hence, the SOD1 mutants are less stable than WT SOD1 and if copper is limited, aggregate formation of the metal-deficient, mutant SOD1 protein occurs.

Keywords: amyotrophic lateral sclerosis, superoxide dismutase, X-ray fluorescence microscopy, synchrotron

INTRODUCTION

Amyotrophic lateral sclerosis (ALS) is a neurodegenerative disease that affects more than 30,000 people annually in the US, making it the most common motor neuron disease (Bruijn et al., 2004). ALS begins with muscle weakness that develops into progressive paralysis and eventually leads to death, which generally occurs within 2–5 years of the disease diagnosis. There is currently no known cure for the disease and the limited treatments available do not significantly alter the course of the disease (Miller et al., 2007). Approximately 90% of all ALS cases are sporadic in nature with no known cause. The remaining 10% have a genetic link and are known as familial ALS cases. One such form of familial ALS, which accounts for 2.5% of all ALS cases, is caused by mutations in the gene that codes for the antioxidant protein copper-zinc superoxide dismutase (SOD1).

SOD1 is a metalloenzyme that binds one copper ion and one zinc ion per monomer for the protein's activity and stability, respectively (Ellerby et al., 1996). There are over 160 known mutations in SOD1 that can cause ALS (Lill et al., 2011; Abel et al., 2013). Despite extensive research, it is currently not understood how such a large distribution of mutations and their properties can all result in the same disease. These mutations are distributed throughout the protein and result in SOD1 mutations with dramatically varied biochemical properties, such as metal binding affinity and antioxidant activity levels. Thus, mutations are classified into two distinct categories: metal binding region (MBR) mutations, which directly affect the protein's ability to bind metal causing diminished activity levels, and wild type-like (WTL) mutations, which maintain metal binding ability and have activity levels similar to that of the wild-type (WT) protein (Hayward et al., 2002; Rodriguez et al., 2002). In this study, four SOD1 mutations were evaluated, including WTL mutations (A4V and G37R) and MBR mutations (H80R and D125H). The A4V mutation occurs at the SOD1 dimer interface and has very low stability (Deng et al., 1993; Hayward et al., 2002), whereas the G37R mutation is in an electrostatic loop and has much higher stability (Borchelt et al., 1994). The H80R mutation is found in the zinc-binding region, hence zinc binding is diminished (Seetharaman et al., 2010). Since the ability of SOD1 to bind

copper is thought to be dependent on its ability to bind zinc first, this mutation is also deficient in copper. The D125H mutation occurs in a charged loop that helps coordinate the metal binding site (Hayward et al., 2002). While it does not directly affect metal-binding, this mutation severely diminishes metal binding affinity and results in a protein with low stability.

Metals have been implicated in a number of neurodegenerative diseases including ALS (Bush, 2003; Maynard et al., 2005; Vonk and Klomp, 2008). When improperly regulated, metal ions such as copper and zinc can be highly toxic to the cells. Copper is especially dangerous due to its catalytic activity and redox potential, resulting in the production of damaging reactive oxygen species (ROS), which lead to the propagation of oxidative stress found in patients with ALS (Bruijn et al., 2004; Lutsenko et al., 2010). Alterations in zinc concentrations can also cause problems such as glutamate excitotoxicity, in which excess zinc over stimulates neurons resulting in cell death (Smith and Lee, 2007). Interestingly, Riluzole, the only approved drug for the treatment of ALS, reduces excitotoxicity by inhibiting glutamate release (Doble, 1996). Thus, proper metal homeostasis is critical to maintaining a healthy cellular environment and delaying disease progression.

A common pathological feature of SOD1-ALS is the formation of misfolded SOD1 aggregates in the spinal cord motor neurons that form concomitantly with the onset of paralysis. Protein aggregates are found in several other neurological diseases, such as the amyloid-β plaques found in Alzheimer's disease (AD) and Lewy bodies composed of α-synuclein in Parkinson's disease (PD; Selkoe, 2003, 2004; Soto, 2003, 2013). The role of these aggregates in disease has been the subject of great debate for a number of years. Originally, aggregates were thought to be the source of toxicity in these diseases. However, it has also been suggested that the aggregates are a cellular response to sequester toxic soluble proteins, avoiding further damage from the misfolded and aberrant proteins (Lee et al., 2002).

The mechanisms of aggregation are not well understood, but metal ions have been implicated. For example, it has been observed that metals can aid in the precipitation and aggregation of amyloid-β (Huang et al., 1997) and α-synuclein (Bertoncini et al., 2005). It was recently shown that the SOD1 aggregates found in a mouse model of SOD1-ALS are composed of unmodified and nascent SOD1 (Shaw et al., 2008). Nascent and unmetallated SOD1 mutants are relatively unstable compared to nascent WT SOD1, suggesting that mutants are more prone to aggregation than WT SOD1 (Rodriguez et al., 2002, 2005).

In this study, we used X-ray fluorescence microscopy (XFM) to image the metal ion distribution and the metallation state of the SOD1 aggregates in Chinese hamster ovary (CHO-K1) cells overexpressing WT SOD1 or mutant SOD1 (A4V, G37R, H80R and D125H). XFM is a spectroscopic technique that can be used to determine the concentration and distribution of metal ions in biological cells and tissues. To visualize the transfected cells and the aggregates, SOD1 was fused in-frame with yellow fluorescent protein (YFP) (Prudencio et al., 2009; Qualls et al., 2013a,b). Results showed that the SOD1 aggregates were metal-deficient within the cells, which is similar to the detergent-insoluble aggregates isolated from a transgenic mouse model of SOD1-ALS (Lelie et al., 2011). In addition, we found that cells with mutant SOD1 contained a lower level of copper compared to cells expressing WT SOD1, regardless of whether the SOD1 mutant was capable of binding copper. These results were surprising and suggest that copper transport is affected regardless of the SOD1 mutation.

MATERIALS AND METHODS

Chinese hamster ovary cells (CHO-K1) (ATCC, Manassas, VA) were maintained in F12-K media with 10% fetal bovine serum, 1% penicillin/streptomycin, and 1% amphotericin- β. The cells were stored in an incubator at 37°C and 5% CO_2. Cells were seeded onto 2.5 × 2.5 mm silicon nitride windows (Silson, UK), an IR and X-ray transparent material, with a 500 nm membrane thickness. After 20 h, when the cells were approximately 80% confluent, they were transfected with plasmid cDNA (Karch et al., 2009) using lipoD293 transfection reagent (SignaGen, Rockville, MD) according to the protocol provided by SignaGen. CHO-K1 cells were transfected to express a WT or mutant (A4V, G37R, H80R, or D125H) form of SOD1 fused to a YFP tag to visualize the transfected cells and aggregates without staining. The transfections were stopped between 19–26 h, which was when YFP fluorescence was visible in most cells and punctate aggregates could be seen (Prudencio et al., 2009). The time to completion was varied so that the aggregate levels would be equivalent for each mutation used. Cells were fixed by rinsing the silicon nitride windows with PBS and then dipping in cold (∼80°C) methanol three times for 1 s each time. The windows were allowed to dry at room temperature and were stored in a desiccator until imaged. Each transfection was carried out twice, and cells from both transfections were imaged.

FTIR microspectroscopy was used to determine the protein density of the aggregates. Spectra were acquired using a Thermo Nicolet Continuum IR microscope coupled to beamline U2B at the National Synchrotron Light Source (NSLS) at Brookhaven National Laboratory (Upton, NY). A 32 × IR Schwarzchild objective produced a 7 × 7 μm beam. A 4 cm^{-1} spectral resolution and 128 scans per spectrum were used over the mid-infrared region (4000–800 cm^{-1}). To normalize the additional protein density found in the protein aggregates, 40–45 spectra were collected both on the aggregates and off the aggregates of transfected cells. For each spectrum, the protein content was determined by integrating the Amide I band from 1610–1700 cm^{-1} with a linear baseline from 1480 to 1800 cm^{-1}. The relative protein intensity was determined by calculating the intensity ratio on the aggregates versus off the aggregates for each cell.

The cells were then imaged with XFM at beamline 2-ID-E at the Advanced Photon Source at Argonne National Laboratory (Argonne, IL) to determine the distribution and concentration of metal ions. The incident beam was tuned to 10 keV using a Si(111) monochromator and the X-rays were focused to a 0.5 × 0.5 μm spot size using 20 cm zone plates (Xradia, Pleasanton, CA). The specimen was positioned at a 45° angle with respect to the incident beam and maintained in a helium-purged box. The X-ray fluorescence was detected with a four-element silicon drift detector oriented at 90° with respect to the incident

beam and a differential phase contrast detector was used for the transmitted X-ray beam. Approximately 15 cells were imaged per mutation with a 0.5-s dwell time per point. A full energy dispersive spectrum was collected at each pixel. Metal concentrations were determined based on NIST thin film standards 1832 and 1833.

To analyze the data, three regions of interest (ROI) were generated using the beamline's MAPS program: (1) the nucleus; (2) the cytoplasm; and (3) the aggregates (if present). The cytoplasm ROI was separated from the nucleus to provide a more accurate measure of metal concentrations in the cytoplasm where the SOD1 is located. The aggregates were easily located using the phase contrast images collected with the XFM data. In addition, the aggregates were small (1–3 μm) so the 500 nm beam size allowed for several pixels (approximately 10) from each aggregate to be analyzed.

After generating ROIs, the data points within each ROI were averaged and the aggregate values were normalized to the protein density as determined by FTIR. Using the average values, a ratio of the aggregate/cytoplasm was generated for copper and zinc to demonstrate any change in metal content within the aggregates. Statistics were performed on mean values from the ROIs of each cell type with SPSS software (version 14.0) using the Kruskal-Wallis non-parametric test. Significant groups were then tested with Mann-Whitney *U post-hoc* test. Significance was determined by $p < 0.05$.

RESULTS

Representative XFM and visible light images of the cells from each group (WT, A4V, G37R, H80R, and D125H) are shown in **Figure 1**. As can be seen, the iron concentration was higher in the cytosol, while the zinc content was highest in the nucleus for all cell types. The only statistically significant difference between the cell types was found in the copper content in the cytoplasm. Specifically, the WT cells contained approximately 24% more copper than all of the SOD1 mutants (A4V, G37R, H80R and D125H) as well as the untransfected cells (**Figure 2**). The mutant SOD1 cells also contained a smaller but significant ($p > 0.05$) increase in copper compared to the untransfected cells. Interestingly, there were no statistical differences between the mutant SOD1 cells, regardless of metal-binding ability. Also, no trends were observed in the zinc, iron, phosphorus, sulfur, potassium or calcium concentrations between the untransfected and transfected cells in the cytoplasm or the nucleus.

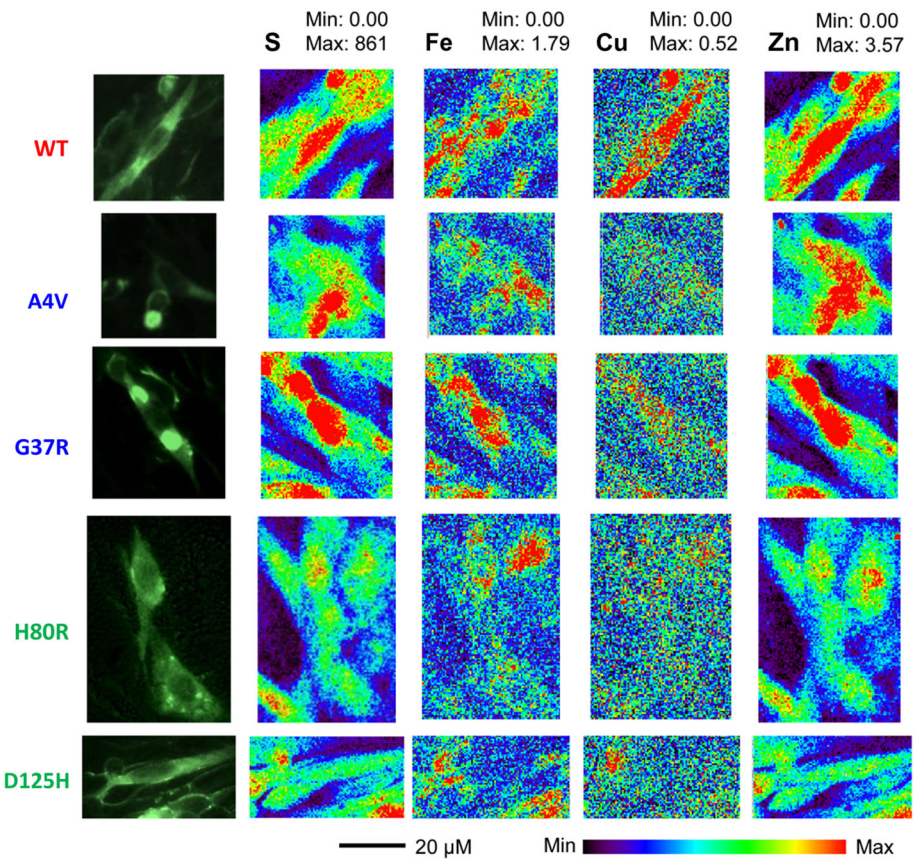

FIGURE 1 | Epifluorescence images (first column) from SOD1-YFP CHO-K1 cells with WT, A4V, G37R, H80R or D125H SOD1. XFM maps for sulfur (second column), iron (third column), copper (fourth column), and zinc (fifth column). The XFM images show the relatively large amounts of copper and zinc found in the WT cells compared to the mutant SOD1 cells. Minimum and maximum concentrations are in mM.

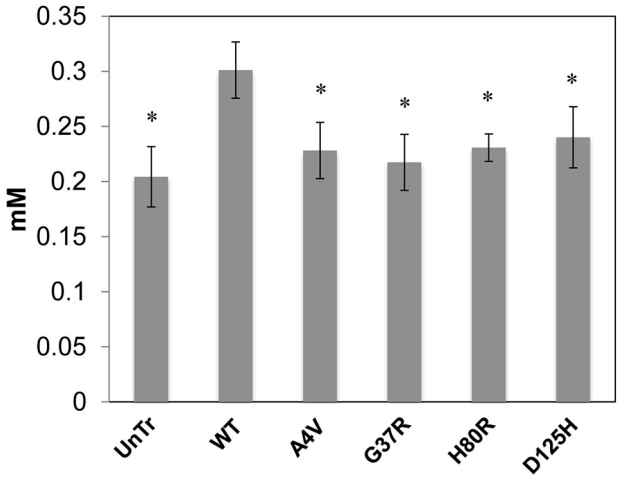

FIGURE 2 | Bar graph of intracellular copper levels (excluding the nucleus) for SOD1-YFP cells. Concentrations are in mM. The WT cells contained the highest level of copper in comparison to the cells overexpressing mutations in SOD1 and untransfected cells. All cells overexpressing mutant SOD1 had significantly higher copper content than the untransfected cells. * indicates significantly less than WT cells with $p < 0.001$.

Based on the FTIRM data, the aggregates showed a two-fold increase in protein density compared to the surrounding cytoplasm. Thus, the XFM data for the protein aggregates were normalized by this factor to account for the greater amount of material within the aggregates. **Table 1** shows the ratio of aggregate to cytoplasm concentrations for copper and zinc from cells containing mutant SOD1-YFP (A4V, G37R, and H80R) aggregates. Results showed that all of the aggregates of mutant SOD1-YFP contained lower copper and zinc concentrations compared to the surrounding area, suggesting that the aggregates are metal-deficient. This trend was seen in both the WTL (A4V and G37R) and MBR (H80R and D125H) mutations.

DISCUSSION

SOD1 AGGREGATES IN CELLS ARE METAL-DEFICIENT

The formation of SOD1 aggregates in the spinal cord is the primary pathology found in ALS patients with SOD1 mutations, but little is known about the aggregation process. The precise mechanism of aggregate formation is needed in order to gain an understanding of how aggregates impact disease progression. Cells transfected with mutant SOD1 develop intracellular aggregates, similar to those found in ALS patients. The results presented here show that the SOD1 aggregates in the transfected cells are metal-deficient. This was true regardless of the mutant's ability to bind copper (WTL and MBR mutants). These data also agree with a previous study that showed insoluble or aggregated SOD1 extracted from ALS mouse spinal cords contained no metal (Lelie et al., 2011). These data suggest that either the protein aggregated prior to being metallated or that the process of aggregation resulted in a loss of metal.

Table 1 | The ratio of aggregate/cytoplasm concentration for copper and zinc in the SOD1 mutant cells.

Mutation	Type	Copper	Zinc
A4V	WTL	0.53 ± 0.13	0.34 ± 0.21
G37R	WTL	0.45 ± 0.081	0.59 ± 0.27
H80R	MBR	0.41 ± 0.041	0.31 ± 0.14

The aggregation of nascent SOD1 prior to metallation is also supported by the very high stability of most metallated SOD1 mutations, making them less likely to aggregate once they are metallated (Hayward et al., 2002; Rodriguez et al., 2002). In contrast, apo-SOD1 is significantly less stable and contains large, intrinsically disordered regions that make the protein much more prone to aggregation (Rodriguez et al., 2005; Shaw and Valentine, 2007; Lelie et al., 2011).

In contrast to SOD1-ALS, protein aggregates found in other neurodegenerative diseases, including amyloid-β plaques in AD and Lewy bodies in PD, have been shown to have a high metal content (iron, copper or zinc) (Selkoe, 2001; Lotharius and Brundin, 2002; Leskovjan et al., 2009). For example, AD plaques, which consist primarily of aggregated amyloid-β protein and form in the brain of human patients, were found to be highly enriched in metal (calcium, iron, copper and zinc) compared to the surrounding brain tissue (Lovell et al., 1998; Miller et al., 2006). However, in a mouse model of AD, it was found that the plaques did not contain elevated metal ions (Leskovjan et al., 2009). This has led to the hypothesis that AD plaques may accumulate metal over time. For ALS, the studies presented here were limited to a cell culture model of the disease. In the future, further studies from the mouse model and/or human tissue will be needed to determine if the mechanism of protein aggregation and the role of metals is similar to other neurological diseases or unique to ALS.

COPPER CONCENTRATION IS LOWER IN CELLS OVEREXPRESSING SOD1 MUTATIONS

The WT cells overexpressing SOD1 contained significantly more copper than the untransfected cells. This increase in copper content demonstrates that there is presumably enough copper available to the cells to metallate the expressed WT SOD1.

To investigate the effects of mutant SOD1 on metal homeostasis, this study utilized two different types of SOD1 mutations including WTL mutations (A4V and G37R), which are known to bind copper and zinc, and MBR mutations (H80R and D125H), which are unable to bind copper or zinc (Rodriguez et al., 2002, 2005; Valentine et al., 2005). Results showed that all mutations contained approximately 24% less copper than the WT cells. In addition, all mutants contained similar copper concentrations regardless of metal-binding ability.

For WT SOD1, previous studies showed a reduced incorporation of copper when over-expressed in cell culture (Prudencio et al., 2012). Here we show that the copper content in the WT SOD1 cells is significantly higher than the untransfected cells, but also suggests that that there is still an insufficient supply of copper to metallate the overexpressed level of SOD1. For the mutant SOD1 in this cell culture model, a substantial fraction of the protein adopts a non-native conformation that remains soluble

and freely mobile (Prudencio and Borchelt, 2011) and decreased copper binding has also been observed (Ayers et al., 2014). Thus, it is likely that the lower copper content in all the cells transfected with mutant SOD1 is due to a combination of insufficient copper delivery to the cells and inefficient copper binding to the active site.

Delivery of copper to the cells is a highly regulated process. Due to its potential redox activity, copper can be highly toxic when not properly controlled. As a result, copper transporters and chaperone proteins tightly regulate the concentration of copper in the cell. Maintaining a strict copper balance is critical and a disruption in homeostasis is implicated in several diseases, including ALS (Gaggelli et al., 2006). For SOD1, copper is transported by the copper chaperone protein (CCS). CCS contains an SOD1-binding domain that has approximately a 50% amino acid sequence homology to SOD1, which is important for the recognition and binding of SOD1 (Schmidt et al., 1999). All four mutations examined in this study have residues that reside in the homologous binding domain of CCS, which may prevent the efficient binding of CCS to SOD1, possibly altering the transport of copper. While CCS levels were not measured in this study, future studies will address the question of copper transport directly.

Taken together, our results suggest a relationship between the metal-deficient aggregates and the decreased copper found in the cells overexpressing mutant SOD1. We suggest that SOD1 overexpression creates an added demand on the cell for Cu, which is not met for the WT or mutant SOD1. In the mutant SOD1, impaired copper transport to the protein may even further reduce the copper content in the cell. Since the mutant proteins are less stable than WT SOD1, and copper is less available, aggregate formation of the metal-deficient mutant SOD1 protein occurs.

One limitation of this work is that it utilized a cell culture model, which does not always correlate with the disease *in vivo*. That said, the data presented here agree with our previous studies on insoluble aggregates extracted from mouse spinal cords, which were found to be metal-deficient. In the future, it will be important to examine the metal content of these aggregates directly within the spinal cord tissue of mouse models of SOD1-ALS. Levels of the CCS and its ability to bring copper into SOD1 also need to be assessed. Lastly, measuring aggregates in human tissue would be the definitive experiment, but the SOD1 mutations account for a very small number of ALS cases (∼2.5%) so the amount human tissue available is very limited. However, it is clear that a combination of these studies would greatly enhance our understanding of SOD1 metallation with respect to ALS.

CONCLUSIONS

Both metal ion homeostasis and aggregate formation have been implicated in the disease progression of ALS. This study used a cell culture model of SOD1-ALS in order to examine metal ion homeostasis and aggregate formation in a systematic manner within the cellular environment. Results showed an increase in copper content when WT SOD1 was overexpressed, which decreased in cells transfected with mutant SOD1 showing a disruption in copper homeostasis. Additionally, the aggregates were found to be metal-deficient, supporting the hypothesis that they form without metal-binding that stabilizes the protein. Understanding the role that metals play in SOD1 aggregate can lead to the development of improved drug treatments to prevent aggregate formation and hopefully prevent the progression of the disease.

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The role of intracellular zinc release in aging, oxidative stress, and Alzheimer's disease

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Brain aging is marked by structural, chemical, and genetic changes leading to cognitive decline and impaired neural functioning. Further, aging itself is also a risk factor for a number of neurodegenerative disorders, most notably Alzheimer's disease (AD). Many of the pathological changes associated with aging and aging-related disorders have been attributed in part to increased and unregulated production of reactive oxygen species (ROS) in the brain. ROS are produced as a physiological byproduct of various cellular processes, and are normally detoxified by enzymes and antioxidants to help maintain neuronal homeostasis. However, cellular injury can cause excessive ROS production, triggering a state of oxidative stress that can lead to neuronal cell death. ROS and intracellular zinc are intimately related, as ROS production can lead to oxidation of proteins that normally bind the metal, thereby causing the liberation of zinc in cytoplasmic compartments. Similarly, not only can zinc impair mitochondrial function, leading to excess ROS production, but it can also activate a variety of extra-mitochondrial ROS-generating signaling cascades. As such, numerous accounts of oxidative neuronal injury by ROS-producing sources appear to also require zinc. We suggest that zinc deregulation is a common, perhaps ubiquitous component of injurious oxidative processes in neurons. This review summarizes current findings on zinc dyshomeostasis-driven signaling cascades in oxidative stress and age-related neurodegeneration, with a focus on AD, in order to highlight the critical role of the intracellular liberation of the metal during oxidative neuronal injury.

Keywords: zinc, aging, oxidative stress, Alzheimer's disease, apoptosis, autophagy

ROLE OF OXIDATIVE STRESS IN AGING

Brain aging is marked by gradual, general cellular dysfunction occurring as a result of structural, chemical, and genetic alterations that manifest themselves as cognitive decline, albeit with great variability among individuals. While these changes are a normal and unavoidable part of the life cycle of neurons, aging itself is also a risk factor for a number of late-life neurodegenerative disorders including Alzheimer's disease (AD), Parkinson's disease (PD), and amyotrophic lateral sclerosis (ALS). Although the molecular underpinnings of age-related neuropathology have not been completely resolved, one recurring explanation for the alterations observed with age, which has been revisited repeatedly for nearly 50 years, is the free radical theory of aging. This theory posits that the deleterious cellular changes that occur during aging and cognitive decline can be attributed in part to a continuous deregulation of intracellular reactive oxygen species (ROS) production over time (Harman, 1965), a phenomenon usually referred to as oxidative stress. Although this theory has been modified slightly in more recent years (Beckman and Ames, 1998), oxidative modifications caused by chronic ROS production remain recognized as a critical constituent of numerous neuropathological processes, and therefore represent a vitally important topic in the field of neurodegeneration research.

In the brain, ROS are produced as a physiological consequence of the normal oxidative processes related to cellular signaling, metabolism, and homeostasis (Lander, 1997; D'Autreaux and Toledano, 2007). Further, in addition to their presence merely as a passive byproduct of these processes, ROS also play an important and active role in a number of physiological cellular functions including gene expression, long-term potentiation, and the immune response (e.g., Sen and Packer, 1996; Knapp and Klann, 2002). As such, ROS are an integral component of a neuron's intracellular milieu. However, while ROS are important for normal cellular processing under certain circumstances, they are more widely recognized for their deleterious role in the initiation and propagation of neuronal injury (Di Carlo et al., 2012; Lizama-Manibusan and McLaughlin, 2013). Namely, unregulated, excess production of these reactive intermediates during oxidative stress can have strong toxic effects on proteins, lipids, and nucleic acids. Moreover, ROS are also capable of triggering injurious signaling cascades that ultimately result in the demise of neurons by apoptosis or other forms of cell death (Beckman and Crow, 1993). Oxidative stress-induced cellular dysfunction can also exacerbate ROS production downstream of the initial insult, thereby maintaining the oxidative stress state in a self-propagating injury cycle that can lead to neuronal death if left

unchecked (Beckman and Ames, 1998; Finkel and Holbrook, 2000).

ROS GENERATION AND MAINTENANCE OF PROPER OXIDATIVE HOMEOSTASIS

Under normal circumstances, ROS are produced primarily as a limited byproduct of oxidative phosphorylation during the formation of ATP, which occurs via a set of redox reactions in mitochondria (Chance et al., 1979). Mitochondrial dysfunction, however, is commonly associated with neural injury cascades, and thus, in addition to their role in physiological ROS generation, mitochondria are regarded as one of principal producers of oxidative intermediates in pathological conditions. ROS themselves can also contribute to mitochondrial dysfunction either indirectly through the initiation of toxic signaling cascades that target mitochondria, or through direct damage to mitochondrial DNA (Richter et al., 1988; Esposito et al., 1999; Melov et al., 1999; Wallace, 2005). In addition to mitochondria, other intracellular generators of reactive metabolites also contribute to oxidative stress during aging, including NADPH oxidases, nitric oxide synthases (NOSs), lipoxygenases (LOXs), and peroxisomes (Halliwell and Gutteridge, 2007). Extraneuronal sources of ROS such as microglia, a non-neuronal, supporting cell involved in CNS immune responses, as well as exogenous stimuli such as UV light, ionizing radiation, and environmental toxins, also contribute to age-related neuronal dysfunction.

The brain, despite representing only 2% of the body weight, receives 15% of the cardiac blood output and accounts for 20% of the body's total oxygen consumption (Lassen, 1959). The pronounced oxidative metabolism present in the brain results in a large generation of ROS during normal function. Neurons contain a system of enzymes and antioxidants to detoxify ROS after they are produced, as well as mechanisms to repair oxidant-induced damage once it has occurred; still, neurons become highly vulnerable to ROS-mediated damage when they are not able to adapt to ROS overproduction during times of stress (Lizama-Manibusan and McLaughlin, 2013). Therefore, oxidative stress occurs either from an overproduction of ROS, a deficiency in the antioxidant response, or both. Oxidative stress can thus be further defined as a condition in which the number of ROS produced surpasses a threshold over which they can no longer be adequately neutralized (Halliwell, 1992). Further, the deleterious consequences of oxidative stress tend to be exacerbated in the aged brain due to the combination of increased oxidant production (Gabbita et al., 1997), along with decreased ability to detoxify ROS and repair oxidatively stressed tissue (Barnett and King, 1995).

While it is not entirely surprising that cells become less able to combat the increased concentrations of injurious oxidative intermediates as they age, oxidative stress does not occur in isolation, and thus is not solely responsible for the toxic cellular processes observed during age-related neuropathology (i.e., Lu et al., 2014). Of course, many interrelated dysfunctional cellular processes coincide to lead to the immense cell loss observed during AD and other age-related neurodegenerative disorders. One such factor, which appears to be inextricably linked to oxidative stress damage in neurons, is zinc dyshomeostasis. Indeed, much like uncontrolled ROS production can have profoundly detrimental effects on neurons during aging, dyshomeostasis of intracellular zinc is also a crucial determinant of the fate of neurons in the aged brain.

ZINC IN THE BRAIN

Zinc is a ubiquitous trace element found throughout the body, including the brain, with particular abundance in the auditory brainstem, olfactory bulb, amygdala, hippocampus, and cortex (Frederickson et al., 1988, 2000; Weiss et al., 2000; Sekler et al., 2002). The cation plays a pivotal role in a multitude of cellular processes including neurotransmission, enzymatic activity, gene regulation, and structural maintenance and stabilization of proteins (Vallee and Falchuk, 1993; Choi and Koh, 1998; Frederickson et al., 2005). Due to its widespread function within neurons, intracellular zinc concentrations are tightly regulated, as proper homeostasis of the metal is critical in the maintenance of normal cellular processing. Indeed, zinc binds with high affinity to a very large number of proteins: roughly 3000 human genes, or 10% of the genome, have been identified as encoding for zinc-binding proteins (Andreini et al., 2006). While the majority (80–90%) of the zinc present in the brain is bound to metal-binding proteins, the remaining fraction is packaged within synaptic vesicles of a large sub-population of excitatory neurons (Cole et al., 1999; Frederickson et al., 2000). This synaptic or vesicular zinc is released in an activity-dependent manner, and can modulate the activation of several neurotransmitter receptors, including NMDA, AMPA, $GABA_A$ and glycine receptors (for review see Smart et al., 2004; Paoletti et al., 2009; Sensi et al., 2011), as well as voltage-dependent ion channels (e.g., Grauert et al., 2014). In addition, synaptically released zinc interacts with a specific postsynaptic zinc-sensing metabotropic receptor (mZnR/GPR39) to modulate synaptic activity through its effect both on the outward chloride transporter KCC2, and on the synthesis of 2-arachidonoylglycerol, an endocannabinoid that modulates probability of presynaptic neurotransmitter release (Besser et al., 2009; Chorin et al., 2011; Saadi et al., 2012; Perez-Rosello et al., 2013).

ZINC TOXICITY

It is well established that zinc exposure is toxic to neurons both *in vitro* (Yokoyama et al., 1986; Choi et al., 1988) and *in vivo* (Lees et al., 1990; Cuajungco and Lees, 1996). The overall concentration of the metal within the brain is ∼150 μM, although the vast majority of intracellular zinc is normally rendered immobile through buffering by cytosolic metal-binding proteins and sequestration into organelles (Sensi et al., 2011). However, when neurons are damaged, as occurs during oxidative stress, bound intracellular zinc can be released into the cytosol, where it then triggers a number of detrimental signaling processes including those that lead to further ROS production, marking the start of a positive feedback loop involving intracellular zinc release and ROS generation (Aizenman et al., 2000; Zhang et al., 2004a). Synaptic zinc is also associated with neuronal dysfunction by its transfer from over-active presynaptic zinc-containing neurons to postsynaptic cells via calcium-permeable channels, including, but not limited to a sub-class of AMPA receptors (Weiss et al.,

1993; Koh et al., 1996; Sensi et al., 2000). While proper zinc homeostasis is critical at all stages of life, the delicate balance required to keep zinc levels in check appears to be particularly precarious in the aged brain (Frazzini et al., 2006; Sensi et al., 2008; Cipriano et al., 2009; Takeda and Tamano, 2014). This is likely due to the fact that, as mentioned earlier, relative ROS levels increase as we age, and intracellular zinc fluxes appear to be very susceptible to perturbation by ROS. Indeed, zinc has been proposed as being a critical link between oxidative stress and aging (Frazzini et al., 2006). To begin to understand the mutual regulation between intracellular zinc release and ROS generation, it is first necessary to review what is currently known about how zinc is maintained within neurons and, more importantly, how the metal is liberated from metal-binding proteins during oxidative injury.

ZINC DYSHOMEOSTASIS AND NEURONAL INJURY

While the largest concentration of zinc in the brain is bound to intracellular metal-binding proteins, there is a second pool, localized to synaptic vesicles of glutamatergic neurons, which constitutes 10–20% of the total concentration of the metal in neurons (Frederickson, 1989). In early studies, it had been thought that cytoplasmic influx of synaptically released zinc, referred to as "translocation", was the primary source of toxic intracellular zinc increases during neuronal injury. This idea, however, was not consistent with later studies in mice that lacked the gene encoding ZnT3, the transporter responsible for loading zinc in synaptic vesicles (Cole et al., 1999). Despite the fact that these animals were devoid of vesicular zinc, increased intracellular concentrations of the metal and subsequent cell death still occurred, even in the apparent absence of zinc translocation from pre- to post-synaptic neurons (Lee et al., 2000). Thus, these findings strongly suggested that other sources of zinc release could also be contributing to the increased intracellular levels of the metal observed during neuronal injury. Since then, it has become increasingly clear that increased cytosolic zinc resulting from liberation from intracellular stores can be highly toxic during oxidative and other types of neuronal injury.

INTRACELLULAR SOURCES OF ZINC RELEASE

One of the main intracellular zinc binding proteins within neurons is metallothionein III (MT III). MT III is one member of a family of thiol-rich metal-binding proteins, and is the primary isoform found in neurons (Hidalgo et al., 2001). Close to a third of the 61–68 amino acids that constitute MT III are cysteine residues, cumulatively capable of binding up to seven zinc ions via their thiol side chains (Vašák and Meloni, 2011). Other MT isoforms have been shown to bind the seven zinc ions with varying affinities, supporting a role for MTs in the dynamic regulation of zinc levels dictated by the needs of the cell at any given time (Krezel and Maret, 2007). In this capacity, MT III acts as an intracellular regulator of zinc homeostasis via coordinated binding and release of the metal. Due to the very low redox potential of its thiols (−366 mV), MT III is readily oxidized, even by relatively mild oxidants. This oxidation results in the liberation of the bound zinc ions (Maret and Vallee, 1998); thus, while zinc itself is redox-inactive, its association with MT III makes it extremely susceptible to changes in cellular redox state. The release of zinc from MT III by oxidants causes a substantial increase in intracellular zinc concentration, and this single event has been established as a powerful inducer of neuronal injury (Aizenman et al., 2000).

Once zinc is liberated from MT III, it can have numerous adverse effects on neuronal function (Aras and Aizenman, 2011). As mentioned previously, mitochondria are the primary producers of ROS in neurons, and zinc plays a critical role in the regulation of mitochondrial dysfunction and ROS generation during neuronal injury. Following intracellular liberation of the cation, mitochondria have been shown to take up cytoplasmic free zinc through both the calcium uniporter as well as through an independent import mechanism that has yet to be identified (Sensi et al., 2003; Malaiyandi et al., 2005). Once sequestered in the organelle, zinc can inhibit the electron transport chain, thereby reducing mitochondrial membrane potential, which subsequently leads to an increase in ROS generation (Sensi et al., 1999; Dineley et al., 2005; Dietz et al., 2008; Medvedeva et al., 2009). Interestingly, MT III has also been shown to translocate to mitochondria and release zinc ions within the mitochondrial intramembranous space, suggesting a dynamic regulation of zinc homeostasis by the combined actions of MT III and mitochondria (Ye et al., 2001). In addition to the MT III-mediated zinc effects on mitochondria, the organelle itself has been shown to contain an independent store of zinc, which can be released during injury (Sensi et al., 2003). In that study, the authors observed that co-treatment with the thiol oxidant 2,2′-dithiodipyridine (DTDP) and the mitochondrial protonophore carbonyl cyanide 4-(trifluoromethoxy) phenylhydrazone (FCCP) results in a greater increase in cytosolic zinc than is observed by treatment with either drug alone, which the authors attribute to the existence of distinct stores of the metal that can each be liberated by unique injurious stimuli. Thus, it appears that mitochondria and MT III work in tandem to dynamically regulate intracellular availability of the cation. However, while it is known that both MT III and mitochondria can modulate zinc levels, additional work is still needed to reveal the specific contributions of each pool to the propagation of pro-death cascades following various forms of neuronal injury.

ZINC-MEDIATED ROS GENERATION *IN VITRO*

In addition to triggering ROS production from mitochondria, zinc has also been found to be involved in injurious oxidant generation from a number of extra-mitochondrial sources. Interestingly, ROS generation and intracellular zinc release appear to be common constituents of a number of toxic signaling pathways in neurons. One of the better-studied zinc-mediated apoptosis cascades involves exogenous ROS-triggered zinc liberation and subsequent generation of endogenous oxidative intermediates. Specifically, cytosolic accumulation of MT III-liberated zinc can be caused by exposure to the oxidant DTDP or peroxynitrite ($ONOO^-$), a physiological oxidant generated by the reaction between free radicals nitric oxide (NO) and superoxide (Beckman et al., 1990). The increase in intracellular zinc then promotes production of superoxide from the enzyme 12-lipoxygenase (12-LOX), loss of mitochondrial membrane potential, and activation of Src kinase and p38 MAPK, the latter by upstream MAP kinase kinase kinase (MAPKKK) apoptosis signal-regulating kinase 1

(ASK-1; Aizenman et al., 2000; McLaughlin et al., 2001; Zhang et al., 2004a; Aras and Aizenman, 2005). Once activated, Src and p38 directly phosphorylate the voltage-gated, delayed rectifier Kv2.1 channel at cytoplasmic residues Y124 and S800, respectively, to trigger the insertion of new channels into the plasma membrane, leading to enhanced K^+ efflux and consequent reduction in intracellular K^+, caspase activation, and finally, apoptotic cell death (Aizenman et al., 2000; McLaughlin et al., 2001; Redman et al., 2007, 2009). The oxidant-induced, zinc-initiated signaling cascade is accompanied by concomitant intracellular calcium release from the endoplasmic reticulum (ER), and downstream CaMKII activation, which is necessary for the exocytotic introduction of Kv2.1 channels into the plasma membrane (**Figure 1**; McCord and Aizenman, 2013). Exposure to NO or activated microglia-derived $ONOO^-$ can also initiate this zinc- and Kv2.1-dependent apoptosis cascade (Bossy-Wetzel et al., 2004; Knoch et al., 2008), and NO exposure has also been shown to lead to cytosolic zinc accumulation in hippocampal neurons *in vivo* (Cuajungco and Lees, 1998).

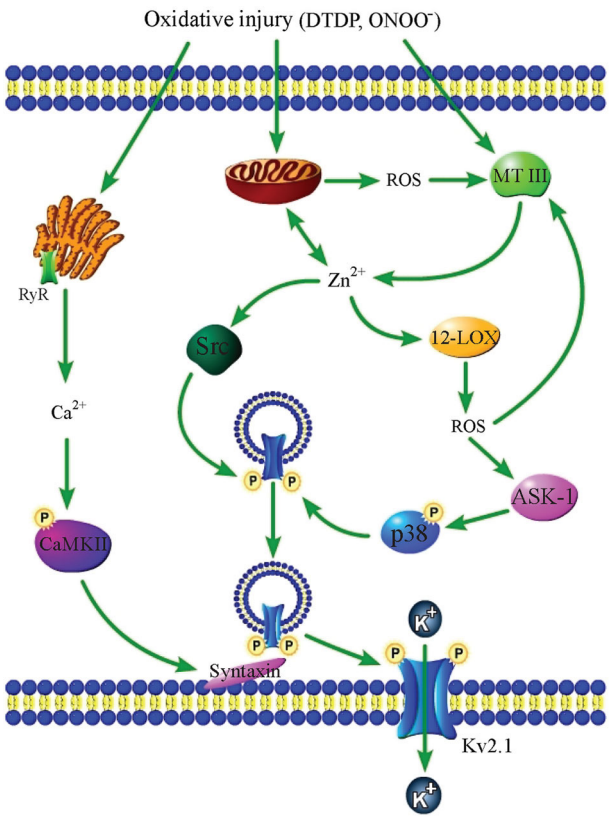

FIGURE 1 | Oxidative injury causes zinc- and calcium-dependent neuronal apoptosis. An oxidative insult such as DTDP or $ONOO^-$ exposure triggers calcium release from the ER, liberation of zinc from MT III and mitochondria, and mitochondrial ROS production. Zinc-dependent 12-LOX activation also generates ROS, which feedback on MT III to release additional zinc. Further, zinc triggers the activation of Src kinase and p38 MAPK (via upstream MAPKKK ASK-1), which then directly phosphorylate Kv2.1 channels at two amino acid residues, Y124 and S800. Calcium-activated CaMKII interacts with syntaxin to facilitate the insertion of phosphorylated Kv2.1 channels into the plasma membrane, causing the enhancement of K^+ currents that is required for apoptosis.

Independently, NO, in combination with depletion of the antioxidant glutathione, was shown to activate 12-LOX, which results in cell death of neuronal cultures (Canals et al., 2003). While this study did not investigate the role of intracellular zinc release in this process, based on the injurious stimulus used and the downstream effects observed, these findings lend further support to the notion that various stimuli could trigger a common zinc-mediated injury cascade. Additionally, a number of studies have reported cell death following zinc-dependent activation of NADPH oxidase and nitric oxide synthase (NOS), which are the enzymes responsible for generating superoxide and NO, respectively (Halliwell and Gutteridge, 2007). While these reports focused on the effect of exogenous zinc exposure, considering the close relationship between intracellular zinc release and $ONOO^-$, as well the ability of exogenously applied zinc to enter neurons, it is not unreasonable to assume that intracellular zinc stores may also play a role in NADPH oxidase and NOS co-activation (Noh and Koh, 2000; Kim and Koh, 2002). Taken together, it appears that many of the components involved in injurious mitochondrial and extra-mitochondrial ROS production, as well as the downstream processes triggered by ROS, all seem to share a common association with intracellular zinc release.

ZINC IN AD

The experimental findings summarized thus far illustrate the concept that intracellular zinc release is a common toxic event in certain forms of oxidant-induced neuronal apoptosis. As oxidative stress is a major contributor to brain aging and age-related pathology, it is feasible that zinc dyshomeostasis may also be involved in disorders associated with aging neurons (Mocchegiani et al., 2005). While the exact role of intracellular zinc in the pathophysiology of neurodegenerative disorders is not entirely clear, there is a growing body of work implicating the metal in age-related neurodegeneration. Oxidative stress-induced cell death is common between AD, PD, and ALS, as well as many other neurological disorders (Dexter et al., 1989; Olanow, 1993; Behl et al., 1994; Mecocci et al., 1994; Wiedau-Pazos et al., 1996; Smith et al., 1998). However, because the literature on the role of metals in AD is extensive, this review will focus only on zinc deregulation during AD in an attempt to paint a more cohesive picture demonstrating the fact that this metal, when unregulated, can wreak havoc on the health of neurons in an aging brain.

AD is characterized by several pathological hallmarks including amyloid plaque deposits, aggregation of neurofibrillary tangles (NFTs) composed of the protein tau in a hyperphosphorylated form, and synaptic loss and neuronal deterioration, predominantly through apoptosis (Hanger et al., 2009). Amyloid plaques are comprised primarily of β amyloid (Aβ), a ∼40 amino acid long peptide generated through cleavage of the amyloid precursor protein (APP; Kang et al., 1987). Accumulation of Aβ during AD has been shown to cause neuronal apoptosis both *in vitro* and *in vivo* (Kowall et al., 1992; Loo et al., 1993). Additionally, AD and oxidative stress appear to go hand in hand, with ROS production being both a cause and consequence of Aβ aggregation (Markesbery, 1997; Butterfield et al., 2001).

AD-INDUCED CHANGES IN CEREBRAL ZINC

The toxic role of zinc dyshomeostasis has become an important topic in the study of AD pathology (Bush and Tanzi, 2008; Greenough et al., 2013). However, the majority of this work has focused on how synaptically released zinc contributes to AD-related neuronal dysfunction and death, and studies to characterize the effects of changes in endogenous intracellular zinc levels remain sparse. Still, the small number of reports that have examined this source of zinc support a toxic role for increased intraneuronal zinc in AD. One of the first studies to consider intracellular zinc deregulation in AD brains found that not only does the metal localize to extracellular amyloid plaque deposits, but that cytosolic zinc levels are increased as well, particularly in neurons exhibiting intracellular NFTs (Suh et al., 2000). More recently, the effect of oxidative stress on intracellular zinc mobilization was determined in neurons derived from 3xTg-AD mice, a triple transgenic AD mouse model that exhibits both Aβ and tau pathology. Importantly, this study found that intracellular zinc levels are substantially higher in 3xTg-AD neurons than in control cells following exposure to DTDP (Sensi et al., 2008). Thus, while studies of cytosolic zinc changes in AD are still in their relative infancy, these results suggest that intracellular zinc liberation could be critical for the progression of AD pathology, and that these effects appear to be mediated by the metal's interaction with ROS.

ZINC-MEDIATED β AMYLOID AGGREGATION AND TAU PHOSPHORYLATION

Zinc plays an important role in Aβ aggregation, as the peptide has been shown to bind to the metal (Bush et al., 1994a). Additionally, not only does zinc exposure induce the aggregation of amyloid plaques (Bush et al., 1994b; Esler et al., 1996), but the plaques themselves are also rich in zinc, as well as copper. It is thought that Aβ is primarily responsible for inducing a state of oxidative stress during AD through direct production of oxidants (Markesbery, 1997; Huang et al., 1999; Butterfield et al., 2001), as well as through activation of microglia and subsequent generation of $ONOO^-$ (Goodwin et al., 1995; Meda et al., 1995; McDonald et al., 1997; Sturchler-Pierrat et al., 1997; Weldon et al., 1998). As mentioned earlier, $ONOO^-$ production originating from both neurons and microglia appears to be a key trigger of zinc-dependent neuronal apoptosis (Zhang et al., 2004a; Knoch et al., 2008). Further, hydrogen peroxide, an oxidant produced by Aβ directly (Huang et al., 1999), can cause the release of zinc from MT III, subsequently causing aggregation of Aβ. Although it was not specified if MT III is localized intra- or extracellularly, Aβ aggregates induced by endogenous zinc release are morphologically distinct from those induced by exogenous zinc application, suggesting a unique role for different zinc pools during AD (Durand et al., 2010). Interestingly, chelation of the cation facilitates the dissolution of these toxic deposits both *in vitro* (Huang et al., 1997) and in post-mortem AD brain tissue (Cherny et al., 1999). Unlike humans, aged mice and rats do not express Aβ in an aggregated form, and as such, do not exhibit the related neuropathology. Intriguingly, one notable difference between human and rodent Aβ is the peptide's ability to bind zinc, with human Aβ exhibiting a much higher affinity for the metal (Huang et al., 2004). Thus, it is tempting to speculate that zinc could be directly responsible for the characteristic toxic aggregation of Aβ observed in AD patients, although more work is needed to definitively confirm this.

While it is known that both exogenous and synaptically released zinc induce Aβ aggregation (Bush et al., 1994b; Deshpande et al., 2009), it appears that increased intracellular zinc may also play a role in this process, although conclusive work demonstrating this has yet to be undertaken. Aβ was originally identified as solely localizing extracellularly; however, other studies have shown that Aβ is also found in the cytoplasm of neurons (Turner et al., 1996; Wild-Bode et al., 1997; Gouras et al., 2000). Interestingly, a number of studies have shown that intracellular Aβ ($Aβ_i$) formation precedes the appearance of extracellular Aβ, lending support to the hypothesis that an intracellular pool of the peptide is a prerequisite for extracellular plaque formation (Walsh et al., 2000; Wirths et al., 2001). However, further work is required to clarify if $Aβ_i$ plays a causative role in the formation of extracellular plaques, and if intracellular zinc release promotes the induction of $Aβ_i$. Nonetheless, in support of a role for cytosolic zinc, $Aβ_i$ accumulation has been shown to correlate with microglial activation, increased NO production, and p38 activation (Rodrigo et al., 2004; Takuma et al., 2009), all of which are events that have previously been linked to pro-apoptotic intracellular zinc release (McLaughlin et al., 2001; Bossy-Wetzel et al., 2004; Knoch et al., 2008). Further, $Aβ_i$ accumulation can be triggered by exposure to oxidants (Ohyagi et al., 2000), and its accumulation has been shown to be localized to mitochondria, which also contain zinc (Rodrigo et al., 2004), and to the ER (Hartmann et al., 1997), an organelle recently shown to be involved in zinc- and Kv2.1-dependent neuronal apoptosis (McCord and Aizenman, 2013). Zinc has been shown to be localized to the ER (Stork and Li, 2010; Taylor et al., 2012), and is released following $OONO^-$ exposure (Lin et al., 2013), further bolstering a potential role for the cation in $Aβ_i$ accumulation within the ER. Taken together, it is reasonable to hypothesize that intracellular zinc release could be an important factor in the accumulation of Aβ within neurons observed during AD (**Figure 2**).

Despite the presence of zinc in Aβ and evidence for its critical role in the aggregation of this toxic peptide, not all findings support a deleterious role for zinc in AD. In fact, exposure to low micromolar concentrations of the metal has been shown to destabilize Aβ aggregation and be protective against Aβ-induced toxicity (Garai et al., 2007). However, while low levels of zinc may protect neurons from Aβ-mediated damage, exposure to higher concentrations of the metal are toxic under otherwise equivalent conditions (Lovell et al., 1999). Like the effect of exogenous zinc on AD-related toxicity, the concentration of endogenous zinc within AD brains is also a contentious issue, although discrepancies in these studies could very well be a result of differences in the conditions under which the measurements were taken. Nonetheless, it appears that although some studies report decreased zinc levels in certain regions of AD brains (Danscher et al., 1997; Panayi et al., 2002), the overall trend supports an increase in cerebral zinc during AD (Thompson et al., 1988; Deibel et al., 1996; Danscher et al., 1997; Religa et al., 2006). Indeed, zinc chelation has proven to be neuroprotective against Aβ-mediated

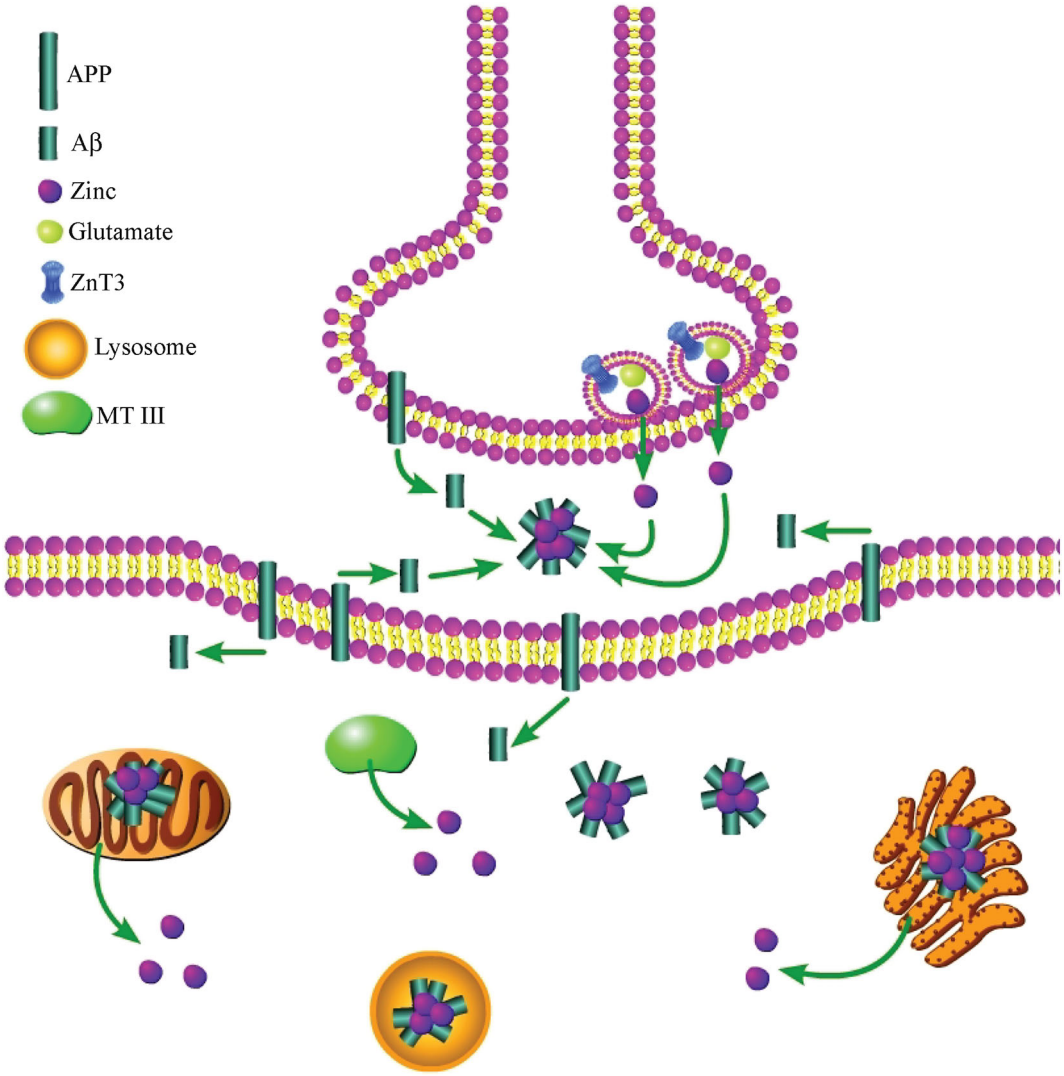

FIGURE 2 | Both extra- and intracellular zinc contribute to the toxic aggregation of Aβ during AD. Synaptically released zinc, following secretion of zinc-containing glutamatergic vesicles, facilitates Aβ aggregation after the peptide has been cleaved from membrane-bound APP. An increase in intracellular zinc, which can be liberated from numerous sources, also enhances Aβ accumulation in the cytosol of neurons. Further, both Aβ aggregates and zinc have been found within mitochondria, lysosomes, and the ER.

toxicity (Lee et al., 2004). Moreover, high concentrations of the metal have been localized to amyloid plaques and neuropil derived from AD brains (Constantinidis, 1990; Lovell et al., 1998; Suh et al., 2000), further supporting a role for increased zinc in brain areas relevant to AD pathology. Aβ-localized zinc has also been shown to contribute to AD-related damage via its effect on toxic iron accumulation (Duce et al., 2010). During AD, increases in intracellular iron can exacerbate oxidative stress and contribute to tau aggregation (Bartzokis et al., 1994; Smith et al., 1997; Yamamoto et al., 2002). Recently, APP was shown to possess ferroxidase activity that contributes to iron export and a reduction in oxidative stress in a mouse model of AD. APP ferroxidase activity is inhibited by zinc, and is negatively correlated with increased Aβ accumulation, suggesting that the zinc originated from within amyloid plaques (Duce et al., 2010). Aβ pathology can also influence the activity of certain kinases that have been closely associated with zinc deregulation and neuronal injury. Namely, numerous studies report increased phosphorylation of p38 in AD brains (Hensley et al., 1999; Zhu et al., 2000, 2001; Pei et al., 2001; Sun et al., 2003), which is also required for zinc-mediated neuronal apoptosis (McLaughlin et al., 2001). Additionally, ASK-1, the upstream MAPKKK of p38, has been linked to AD-related toxicity. Specifically, Aβ-mediated ROS production leads to activation of ASK-1 and downstream cell death in PC12 cells and cortical neurons (Kadowaki et al., 2005). Thus, many of the pathological changes that take place during AD appear to be very similar to those observed in injury related to oxidant-induced zinc liberation and downstream apoptosis.

Similar to Aβ, zinc can also directly bind to tau to facilitate aggregation of the protein into NFTs (Mo et al., 2009). Like the bimodal regulation of Aβ-induced toxicity, modulation of tau by exogenous zinc also appears to be concentration-dependent, with lower concentrations of the metal causing a decrease in phospho-tau, while higher levels cause an increase (Boom et al., 2009). Although these findings are based on exogenously applied zinc, the authors conclude that because tau accumulates intracellularly, the observed effect is likely due to translocation of exogenous zinc into the cytosol. Thus, while the effect of endogenous intracellular zinc release was not investigated, these studies nonetheless support a potential role for intracellular liberation of the cation in the regulation of tau during AD. Further support for this idea comes from evidence for accumulation of zinc predominantly within neurons that display NFTs (Suh et al., 2000). As mentioned previously, hyperphosphorylation of tau is required for its aggregation into NFTs, and many of the kinases involved in the zinc- and Kv2.1-mediated apoptosis cascade described previously also phosphorylate tau. Specifically, tau can be directly phosphorylated by both p38 (Reynolds et al., 1997) and CaMKII (Litersky et al., 1996). Further, exogenous zinc application can trigger Src kinase-dependent inactivation of PP2A, the primary phosphatase responsible for dephosphorylating tau (Liu et al., 2005, 2008). As mentioned earlier, zinc-dependent Src activity is also responsible for phosphorylating Kv2.1 channels prior to their insertion into the plasma membrane during to apoptosis (Redman et al., 2009).

EFFECT OF ZINC ON AUTOPHAGIC DYSFUNCTION DURING AD

Autophagy is a catabolic system used within cells to clear dysfunctional or unused proteins and macromolecules before they cause damage to neurons. Degradation of malfunctioning cellular components during autophagy occurs in lysosomes, which are acidic organelles containing hydrolase enzymes that facilitate the decomposition process. Autophagy is important in the clearance of protein aggregates (Johansen and Lamark, 2011), and autophagic deregulation has come to be regarded as a key occurrence in AD-related pathology (Cuervo, 2008; Nixon and Yang, 2011). It has been suggested that cell death can result from oxidative stress-induced accumulation of Aβ within lysosomes, leading to lysosomal membrane permeabilization (LMP) and subsequent release of Aβ and other toxic molecules into the cytosol (Zheng et al., 2009). In addition to toxicity caused by LMP, reduced autophagy, which has been observed in AD, can also lead to cell death due to a buildup of damaged molecules that would otherwise be degraded. In this scenario, decreased autophagy can be toxic to neurons independently of the effects of LMP. While this area of AD research is still relatively new, zinc also appears to play an important role in lysosomal dysfunction triggered by oxidative stress. Namely, oxidant exposure has been shown to cause an accumulation of zinc within lysosome-derived vesicles, as well as within the cytosol itself, leading to apoptosis of hippocampal neurons. Further, apoptosis is prevented by the zinc chelator TPEN, demonstrating that the increase in cytosolic zinc is responsible for the observed toxicity (Hwang et al., 2008).

Zinc deregulation during autophagic dysfunction has also been shown to have clinical significance. Administration of the prototype AD drug clioquinol can cause a reduction of tau and Aβ, as well as an improvement in cognitive performance (Regland et al., 2001; Ritchie et al., 2003). The effect of clioquinol on Aβ aggregation was initially thought to occur because of the drug's ability to chelate zinc (Cherny et al., 2001). However, more recent work revealed that clioquinol actually functions by acting as an ionophore-like compound to increase influx of zinc into cells from the extracellular space, which can then induce autophagy in neurons and astrocytes (Park et al., 2011). Specifically, this study found that zinc localizes to autophagic machinery (autophagic vacuoles, autolysosomes, and lysosomes), and is necessary for the clioquinol-induced clearance of accumulated huntingtin protein, which aggregates in Huntington's disease. Thus, it appears that clioquinol may be important in degrading the Aβ and tau aggregates observed in AD, in part through its effect on extra- and intracellular zinc levels (**Figure 3**). However, this same study also showed that clioquinol causes zinc-dependent cell death in cortical neurons, although this experiment was not performed in the presence of Aβ or under other conditions that would mimic zinc levels or the overall cellular environment manifested in AD, and it is thus difficult to deduce the effect of clioquinol on neuronal viability during AD from this study alone.

Both abnormally increased and decreased autophagy can be detrimental to neurons depending on the circumstances in which it takes place. The studies summarized here underscore the complex nature of the role of zinc in regulating autophagic dysfunction. Indeed, it appears that changes in intracellular zinc levels can dictate if autophagy will adopt a pro-death or pro-survival function (Lee and Koh, 2010). As zinc dyshomeostasis can be observed both extracellularly and intracellularly during AD, much work remains to acquire a better understanding of the delicate balance of zinc that underlies normal cellular function, and at exactly what point metal-regulatory processes go awry to propagate the pathological effects observed during neurodegeneration.

MT III AND AD

As mentioned previously, MT III is one of the primary zinc-binding proteins within neurons, and therefore plays an integral role in maintaining homeostasis of the metal. In contrast to MT I and MT II, which are normally induced by increased levels of free metals within cells, MT III is constitutively expressed. Knockdown of MT III can lead to increased oxidant-triggered intracellular zinc levels, while overexpression of the protein substantially reduces the amount of zinc detected within neurons following oxidant exposure (Aras et al., 2009). Interestingly, numerous independent studies have reported downregulation of MT III during AD (Uchida et al., 1991; Tsuji et al., 1992; Yu et al., 2001). While these studies did not directly measure the effect of reduced MT III expression on intracellular zinc levels, they suggest that increased intracellular zinc concentration due to a reduction in MT III expression could be relevant to the pathological effects observed during the progression of the disease. Others, looking at the molecular consequences of changes in MT III during AD have shown that exogenous MT III exposure prevents the

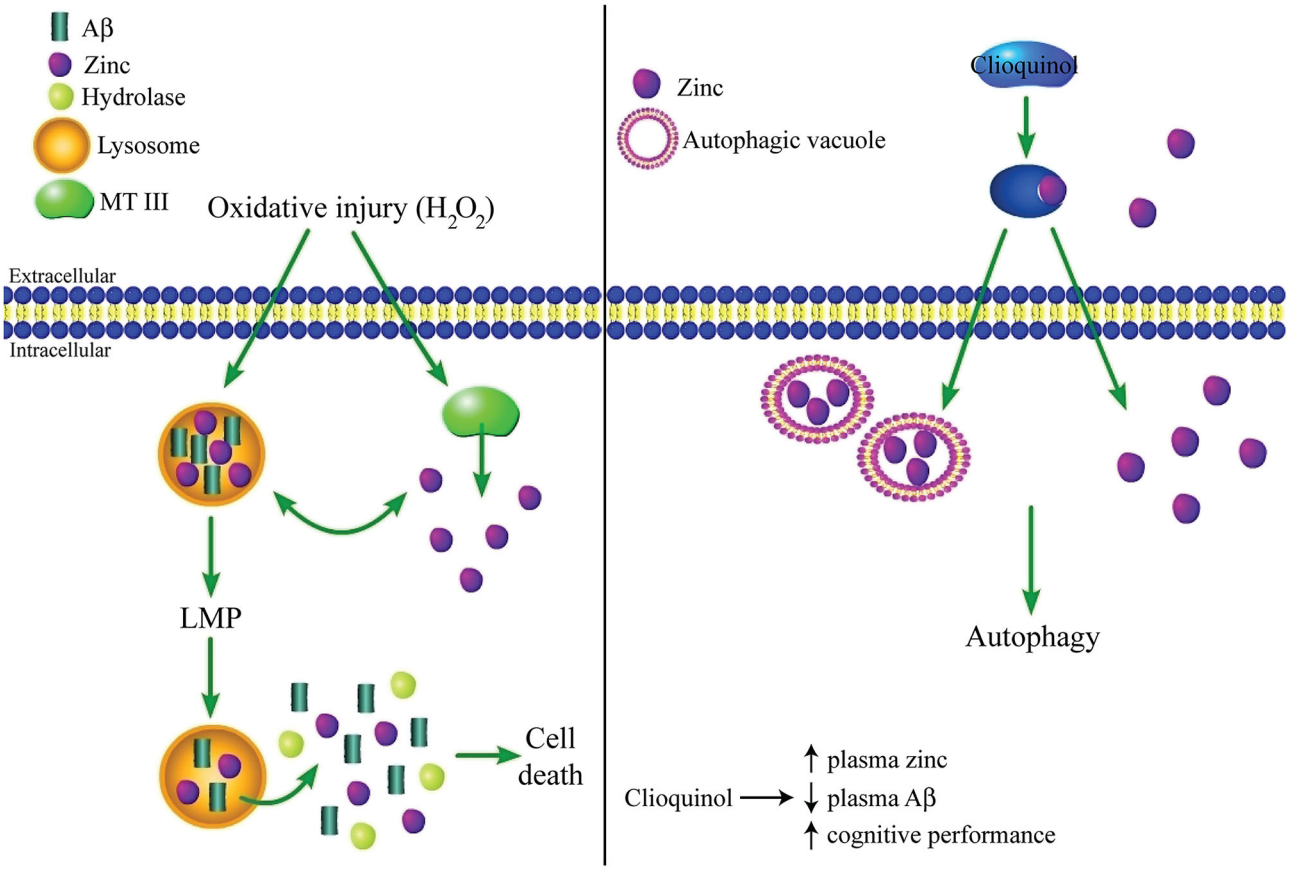

FIGURE 3 | Complex regulation of lysosomal function and autophagy by zinc. (Left panel) Oxidative injury can lead to accumulation of zinc and Aβ within lysosomes, presumably following zinc liberation from internal stores such as MT III. This accumulation triggers LMP, which causes cell death due to the release of zinc, Aβ, and other toxic molecules like hydrolases into the cytosol. Alternatively, under other conditions zinc may have a protective effect by restoring autophagy, which is reduced in AD. **(Right panel)** Clioquinol, a clinically effective AD drug, increases intracellular zinc concentration by acting as an ionophore-like compound for the cation. Zinc accumulates in autophagic vesicles, which is necessary for the induction of autophagy and clearance of protein aggregates. However, while clioquinol has been shown to cause a reduction in Aβ and an improvement in cognitive performance in clinical trials, it can also cause zinc-mediated toxicity in otherwise healthy neurons *in vitro*, demonstrating the need for future work to clarify the role of zinc in AD-related autophagic dysfunction.

accumulation of Aβ and rescues neurons from Aβ-induced cell death (Irie and Keung, 2001), supporting a beneficial role of MT III through the sequestration of extracellular zinc. Along these lines, MT III has been suggested to be secreted by cells in the brain, albeit by not well-defined pathways (Chung and West, 2004; Manso et al., 2011). Seemingly paradoxically, a small number of studies have reported increased MT III expression in AD (Zambenedetti et al., 1998; Carrasco et al., 1999), although investigations describing this phenomenon are much less common than those reporting decreased MT III. Further, MT III can act as both an acceptor and a donor of zinc, and thus changes in MT III expression could have different effects depending on the cellular environment and redox state within neurons. Knockdown of MT III in astrocytes has also been shown to lead to a decrease in the degradative capacity of lysosomes (Lee et al., 2010), consistent with the reduced autophagy observed during AD. However, the lack of MT III also corresponds with decreased oxidant-induced zinc release, revealing the need for further studies to fully understand the role of MT III and its association with zinc in autophagic dysfunction. Nonetheless, the fact that changes in MT III expression are almost universally observed in AD suggests that deregulation of MT III is likely contributing to the zinc dyshomeostasis observed.

ZINC AND ITS RELATION TO CALCIUM DEREGULATION DURING AD

Like zinc, intracellular calcium dyshomeostasis also appears to play a crucial role in AD-related pathology (LaFerla, 2002; Berridge, 2010, 2013). Simultaneous increases in intracellular zinc and calcium have been observed in a number of injurious signaling cascades related to ischemic, excitotoxic, and oxidative injury (Sensi et al., 2002; Medvedeva et al., 2009; Vander Jagt et al., 2009). However, details regarding the downstream signaling pathways activated by these two metals during oxidative injury are just beginning to arise (McCord and Aizenman, 2013). Similarly, although evidence has implicated calcium dyshomeostasis

as a trigger for AD pathology (Khachaturian, 1989), if and how calcium and zinc cooperate to regulate pathological signaling during AD is still unclear. While there is some indication of a correlation between intracellular zinc and calcium levels in AD brains (Ishihara et al., 2002), direct evidence linking the two metals to pathological processes specifically associated with this disorder does not yet exist. Still, despite the lack of definitive proof, intracellular calcium and zinc release do appear to mediate many of the same processes during AD.

Just as Aβ can induce zinc liberation from internal, metal-binding stores via ROS production, the peptide can also trigger calcium release from the ER. This increase in calcium leads to ROS generation, mitochondrial dysfunction, caspase activation, and apoptosis (Ferreiro et al., 2006). As discussed earlier, all of these cellular changes have also been reported during oxidant-induced, zinc- and calcium-mediated neuronal apoptosis. Additionally, like zinc, cellular alterations induced by increased intracellular calcium contribute to intraneuronal Aβ accumulation and neurotoxicity (Pierrot et al., 2006; Demuro and Parker, 2013). Specifically, a depolarization-induced increase in cytosolic calcium can trigger phosphorylation of APP and tau, leading to subsequent $A\beta_i$ accumulation and cell death (Pierrot et al., 2006). These calcium-dependent phosphorylation events are mediated by GSK-3β, a kinase known for its role in the phosphorylation of both tau and APP (Aplin et al., 1996) that has previously been shown to be activated downstream of ER calcium release (Hartigan and Johnson, 1999). Zinc can also induce GSK-3β phosphorylation, an event that corresponds to zinc-mediated activation of p38 MAPK (An et al., 2005). In another study, accumulation of $A\beta_i$ was shown to trigger IP3-mediated calcium release from the ER that is necessary for $A\beta_i$-induced toxicity (Demuro and Parker, 2013).

Like zinc, changes in intracellular calcium levels are extremely sensitive to oxidative stress. DTDP, a common inducer of intracellular zinc release (Aizenman et al., 2000; McLaughlin et al., 2001), has also been shown to trigger calcium release from the sarcoplasmic reticulum following oxidation of ryanodine receptors (RyRs) in cardiomyocytes (Zaidi et al., 1989). Further, DTDP-induced ER calcium release has recently been observed during zinc-dependent apoptosis in cortical neurons (McCord and Aizenman, 2013). Additionally, RyRs can also be nitrosylated by nitric oxide, leading to calcium release (Xu et al., 1998; Kakizawa et al., 2013). Deregulation of both the ER and mitochondria and the resultant effects on intraneuronal calcium levels appear to be important determinants of the progression of AD-related pathological processes (Green et al., 2008; Adam-Vizi and Starkov, 2010; Mattson, 2010). Both oxidative stress and mitochondrial dysfunction have been shown to occur early in the pathogenesis of AD (Nunomura et al., 2001; Moreira et al., 2006), and mitochondria and the ER can physically interact to regulate intracellular calcium levels in response to changes in redox state (Csordas and Hajnoczky, 2009; Hayashi et al., 2009). As mentioned previously, intracellular zinc can also regulate mitochondria and ROS production (Dineley et al., 2003; Sensi et al., 2003). Further, increased intracellular calcium triggered by glutamate exposure has been shown to contribute to mitochondrial ROS production and subsequent release of zinc from intracellular stores (Dineley et al., 2008). While these studies were not specific to any one disease, it is conceivable that similar injurious parallel processes are taking place during AD. In fact, a signaling cascade has been proposed to account for the seemingly coordinated pathways activated by zinc and calcium during AD (Corona et al., 2011). Taken together, and considering the interrelated roles of calcium and zinc in other injury models, work to reveal potential parallel processing by the metals awaits as an exciting new opportunity to enhance our understanding of the cellular signaling events underlying AD neuropathology.

ZINC AND NORMAL BRAIN AGING

Although proper regulation of cytoplasmic zinc is crucial in determining the fate of neurons during AD and other neuropathological conditions, little is known regarding the status of intracellular release of the metal during normal, healthy aging. It is known, however, that the concentration of vesicular zinc, and the expression of ZnT3, the transporter responsible for packaging zinc into synaptic vesicles, are reduced with increasing age. Specifically, synaptic zinc levels have been shown to be decreased in the hippocampus of aged rats (Ricci et al., 1989; Amenta et al., 1990; Mocchegiani et al., 2004), and this reduction correlates with age-induced memory impairments (Guidolin et al., 1992). Further, Adlard et al. (2010) observed age-dependent memory deficits in mice lacking the gene encoding ZnT3, and also found reduced cortical ZnT3 levels in aged wild-type mice, as well as in healthy, older humans between the ages of 48–91 years. Low hippocampal ZnT3 expression and concomitant decrease in vesicular zinc concentration has also been observed in the senescence-accelerated mouse prone 10 (SAMP10) model of aging (Saito et al., 2000). While vesicular zinc levels decrease as a function of age, the total concentration of the metal in the brain appears to be unaffected by increasing age in both rodents and humans (Takahashi et al., 2001; Rahil-Khazen et al., 2002).

Though the role of cytoplasmic zinc in normal aging remains unclear, increased intracellular zinc concentration due to influx of the metal from extracellular space was recently shown to restore age-associated cognitive deficits in mice. Namely, administration of PBT2, a second-generation 8-hydroxy quinolone analog of the zinc ionophore-like AD drug clioquinol, improves cognitive ability in aged mice through a mechanism involving redistribution of zinc, resulting in an overall increase of the metal within hippocampal neurons, as well as concurrent cellular changes indicative of neurogenesis and enhanced synaptic plasticity. The status of intracellularly stored zinc was not monitored in this study, although overall brain zinc levels were unchanged by administration of PBT2 (Adlard et al., 2014). In addition to diminishing cognitive decline during normal aging, PBT2 also improves cognitive performance in mouse models of AD (Adlard et al., 2008). Thus, it seems that age-related cognitive impairment could occur through similar mechanisms under both physiological and pathological circumstances, and that cerebral zinc dynamics play an important part of the cellular processes underlying these cognitive changes. As such, determining how zinc localized to intracellular stores contributes to the cognitive deficits that accompany aging could greatly enhance our understanding of the function of the metal during normal aging.

Dietary zinc deficiency is common among the elderly, and has been attributed to dysfunction in the body's immune response, including changes in the antioxidant defense system, which manifest in part as an increase in the incidence of infection and inflammatory processes (Kelly et al., 1996; Mocchegiani et al., 2000, 2008; Haase and Rink, 2009; Wong et al., 2013). Unfortunately, although zinc deficiency is frequently observed in the aged population, results regarding the effect of zinc supplementation on healthy aging have been somewhat inconsistent, and a consensus has yet to be reached on the benefits of such treatment (Mocchegiani et al., 2008). Most studies on the effect of zinc deficiency on aging have been focused on areas of the body outside of the brain, and thus, at present, the effects of dietary zinc deficiency on brain aging are not well understood. While it has been shown that activities of many proteins known to be modulated by zinc are altered with increasing age, there is a dearth of reports explicitly studying if and how dietary zinc deficiency contributes to these changes (for review see Mocchegiani et al., 2005). As such, additional research is needed to clarify the role of dietary zinc in the aging brain.

Kv2.1-MEDIATED K^+ EFFLUX AND AD

A necessary downstream event in zinc-mediated apoptosis is enhanced K^+ efflux through the delayed-rectifier Kv2.1 channel. Low intracellular K^+ is a requisite step in many apoptosis pathways, as it facilitates protease and nuclease activation, cytochrome c release from mitochondria, and apoptosis-related cellular volume decrease (Bortner et al., 1997; Hughes et al., 1997; Yu et al., 1997; Maeno et al., 2000; Cain et al., 2001). Intracellular zinc release and downstream Kv2.1-mediated K^+ efflux can both be triggered by exposure to DTDP, NO, and activated microglia (Pal et al., 2003; Bossy-Wetzel et al., 2004; Knoch et al., 2008). Further, oxidant-induced K^+ current enhancement is prevented by the zinc chelator TPEN, demonstrating the dependence of apoptotic Kv2.1 activity on zinc (McLaughlin et al., 2001). In addition to its role in zinc-mediated apoptosis, Kv2.1 also appears to be involved in the toxic cellular processes related to AD. For a number of years, studies have demonstrated an enhancement of voltage-gated, delayed rectifier K^+ currents following exposure of neurons and astrocytes to Aβ (Jalonen et al., 1997; Colom et al., 1998; Yu et al., 1998). More recently, though, changes specifically within the Kv2.1 channel have been identified in animal models of AD. Namely, upregulation of Kv2.1 mRNA and protein has been reported in rats injected with Aβ; this change in Kv2.1 expression is accompanied by impaired performance on spatial memory tasks (Pan et al., 2004). It is well known that deficits in acetylcholine are intimately related to AD-associated cognitive decline, thus forming the basis of acetylcholinesterase (AChE)-based therapy in the disease (Bartus et al., 1982; Francis et al., 1999). Interestingly, the AChE inhibitor galantamine, used in the treatment of AD, has been shown to cause a reduction in basal delayed rectifier K^+ currents in hippocampal neurons (Pan et al., 2003; Vicente et al., 2010), likely arising from Kv2.1 channels (Zhang et al., 2004b).

Hydrogen peroxide exposure has been shown to directly oxidize Kv2.1 channels, leading to channel oligomerization and downstream apoptosis. Importantly, an oxidation-resistant Kv2.1 channel cysteine mutant (C73A) that prevents oligomerization also attenuates toxicity induced by Aβ exposure (Cotella et al., 2012). Interestingly, enhanced oligomerization of Kv2.1 is also observed in a mouse model of AD, although how this change in Kv2.1 structure contributes to AD-related cognition decline was not determined (Cotella et al., 2012). In contrast to reports of enhanced Kv2.1-mediated K^+ currents during apoptosis, this study found that oxidant exposure actually decreases K^+ currents, and the Kv2.1C73A mutation blocks this effect. However, this discrepancy could be explained by the fact that it takes ∼3 h to observe the Kv2.1-mediated K^+ current enhancement after oxidant exposure (McLaughlin et al., 2001), and this group only examined currents immediately following the oxidative insult. Further, the toxic effects of Kv2.1 channel oligomerization may occur independently of the change in K^+ currents, as a second oligomerization-impaired channel mutant (C73S) was found to be non-conducting, yet still rescues cells from oxidant-induced apoptosis (Cotella et al., 2012). Thus, it is not yet clear how alterations in Kv2.1-mediated K^+ currents may influence Aβ-mediated toxicity.

CONCLUSIONS

Oxidative stress can generally be considered both a cause and an effect of the neuropathological changes seen in AD and other age-related neurodegenerative disorders. In addition, links between increased intracellular zinc, oxidative stress, and age-related neurodegeneration have been established in numerous studies, and zinc dyshomeostasis appears to be a common constituent of a multitude of pathological neuronal processes. Nonetheless, a direct causative role of intracellular zinc release in human neurodegenerative disorders has yet to be firmly established. It is known that zinc homeostasis is critical for proper brain function, and even minor disturbances to this delicate balance can trigger an accumulation of zinc, which can have extremely adverse effects on the fate of neurons in AD and related disorders, in part through an apoptotic enhancement of Kv2.1-mediated K^+ currents. As such, modulation of intracellular zinc levels could be a particularly important target in order to protect against AD-related injurious cellular processes. However, while chelation of zinc may be an effective neuroprotective strategy *in vitro*, the potential therapeutic benefits of zinc chelation become much more complex when studying changes in zinc levels *in vivo*, in particular as zinc can also act as a neuromodulator or neurotransmitter, and it has a wide range of additional essential functions in neurons, as well as throughout the organism, including, but not limited to regulation of gene expression. This matter is thus complicated by the fact that both increased and decreased intracellular zinc can be neurotoxic, presumably depending on specific cellular conditions. Still, the fact that drugs like the zinc ionophore-like compound clioquinol have been effective in abrogating some of the pathological consequences of AD in preliminary clinical trials (Regland et al., 2001; Ritchie et al., 2003), presumably through its effect on modulating zinc levels in order to restore autophagy, indicates that the metal plays a pivotal role in the progression of AD, and that strategies targeting zinc could hold the key to finding better treatments for this currently incurable disease. Still, future work is required to determine exactly how clioquinol and its association with zinc

improve the outcome of AD progression. Further, investigation into the potential off-target effects of this drug is crucial, as increased intracellular zinc, as illustrated throughout this review, is lethal to neurons in a variety of settings.

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Metals and cholesterol: two sides of the same coin in Alzheimer's disease pathology

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Alzheimer's disease (AD) is a multifactorial neurodegenerative disease. It begins years prior to the onset of clinical symptoms, such as memory loss and cognitive decline. Pathological hallmarks of AD include the accumulation of β-amyloid in plaques and hyperphosphorylated tau in neurofibrillary tangles. Copper, iron, and zinc are abnormally accumulated and distributed in the aging brain. These metal ions can adversely contribute to the progression of AD. Dysregulation of cholesterol metabolism has also been implicated in the development of AD pathology. To date, large bodies of research have been carried out independently to elucidate the role of metals or cholesterol on AD pathology. Interestingly, metals and cholesterol affect parallel molecular and biochemical pathways involved in AD pathology. The possible links between metal dyshomeostasis and altered brain cholesterol metabolism in AD are reviewed.

Keywords: Alzheimer's disease, amyloid precursor protein, Aβ, cholesterol, metals, iron, copper, zinc

INTRODUCTION

Alzheimer's disease (AD) is a multifactorial neurodegenerative disease characterized by pathological hallmarks of extracellular β-amyloid (Aβ) plaques (Glenner and Wong, 1984a,b; Masters et al., 1985) and intracellular neurofibrillary tangles (Delacourte and Defossez, 1986; Kosik et al., 1986; Lee et al., 1991) in the brain. The rate of AD progression is variable, but on average, patients may live up to 10 years after diagnosis (Whitehouse, 1997). Approximately 8–10% of the population over the age of 65 have AD, and its prevalence doubles every 5 years thereafter (Cummings, 2004; Bertram and Tanzi, 2005). These data, coupled with ever increasing life expectancy, marks AD as one of the most significant health and socio-economic problems, particularly in industrialized nations.

As with most diseases, genetic and environmental factors can contribute to its development. AD can be broadly characterized as either familial or sporadic. Early-onset familial AD (FAD) are caused by mutations within three genes, which encode the amyloid precursor protein (*APP*) and presenilins 1 and 2 (*PSEN1* and *PSEN2*; Holmes, 2002; Tanzi and Bertram, 2005; Bertram et al., 2007). These mutations are autosomal dominant, and symptoms of AD manifest prior to 65 years of age. FAD accounts for less than 5% of AD cases (Janssen et al., 2003; Raux et al., 2005). The disease etiology for late-onset sporadic AD is complex and multifactorial, which may involve age-related alterations in metabolism, repair mechanisms, immune response, and environmental factors such as life style, prior brain trauma, and oxidative stress (Muller-Spahn and Hock, 1999; Chen et al., 2009). Genome-wide association studies (GWAS) have identified candidate genes that significantly increase the risk of late-onset AD. By far, the strongest risk factor found is the ε4 allele of the apolipoprotein E (*APOE*) gene (Farrer et al., 1997). Possessing just a single ε4 allele increases the risk of developing AD by two- to fivefold, while having two alleles increases the risk to more than fivefold (Poirier et al., 1993; Strittmatter et al., 1993; Holmes, 2002; Poirier, 2003; Bertram et al., 2007; Coon et al., 2007).

Neuritic plaques are multi-cellular lesions containing Aβ peptides (especially the neurotoxic $A\beta_{42}$ species), reactive astrocytes, activated microglia, and dystrophic neurites (Maulik et al., 2013). Aβ peptide is produced by the proteolytic cleavage of APP by β- and γ-secretases (see APP Processing and Aβ Generation). Interestingly, these plaques also have an enrichment of cholesterol (Panchal et al., 2010) and metals such as copper, iron, and zinc (Goodman, 1953; Connor et al., 1992; Bush et al., 1994c; Lovell et al., 1998; Suh et al., 2000; Collingwood et al., 2005; Stoltenberg et al., 2005; Miller et al., 2006; Baltes et al., 2011), which indicate a failure of cholesterol and metal regulatory systems in the brain. While the underlying etiology of AD is yet to be clearly established, mounting evidence derived from epidemiological, clinical and biochemical studies have independently implicated roles for metals and cholesterol in the pathogenesis of AD. This review presents an overview of the roles of metals and cholesterol in APP/Aβ metabolism and their relationship in the development of AD.

APP PROCESSING AND Aβ GENERATION

Amyloid precursor protein is a type I trans-membrane protein that can be post-translationally modified by *N*- and *O*-glycosylation, tyrosine sulfation, and phosphorylation (Weidemann et al., 1989; Walter et al., 2000). Full-length APP is sequentially processed via two pathways: the non-amyloidogenic and amyloidogenic. The cleavage by α- or β-secretases at the N-terminus of the Aβ domain generates soluble APP derivatives: sAPPα and sAPPβ, respectively, leaving behind membrane tethered C-terminal fragments (CTFα and CTFβ, respectively). Subsequent cleavage of these CTFs by the

γ-secretase generates either p3 (from CTFα) or Aβ (from CTFβ), and liberation of the APP intracellular domain (AICD; reviewed in Zheng and Koo, 2011; **Figure 1B**).

The trans-membrane aspartyl protease β-site APP cleaving enzyme 1 (BACE1) is the major β-secretase in neurons (Sinha et al., 1999). This is the rate-limiting enzyme involved in the generation of Aβ (Vassar et al., 1999; Yan et al., 1999). In contrast, α-secretase cleavage of APP can be stimulated by a disintegrin and metalloproteinase (ADAM) family of proteases (reviewed in Lichtenthaler, 2011) and a number of other molecules (e.g., phorbol ester) or via protein kinase C activation, in which case the cleavage is regulated by tumor necrosis factor α-converting enzyme (TACE; Buxbaum et al., 1998; Blacker et al., 2002). Studies have indicated that in neurons, α-secretase activity is likely to be primarily mediated by ADAM10 (Kuhn et al., 2010). The mature γ-secretase is a polytopic complex consisting of four individual components: presenilin (PS); nicastrin (Nct); anterior pharynx defective 1 (Aph1); and presenilin enhancer 2 (Pen-2; reviewed in Edbauer et al., 2003; Iwatsubo, 2004). Presenilin, an aspartyl protease, is the main catalytic unit of the complex. Aβ of varying length is the result of hierarchical and site-specific cleavage of APP by β- and γ-secretase (**Figure 1B**).

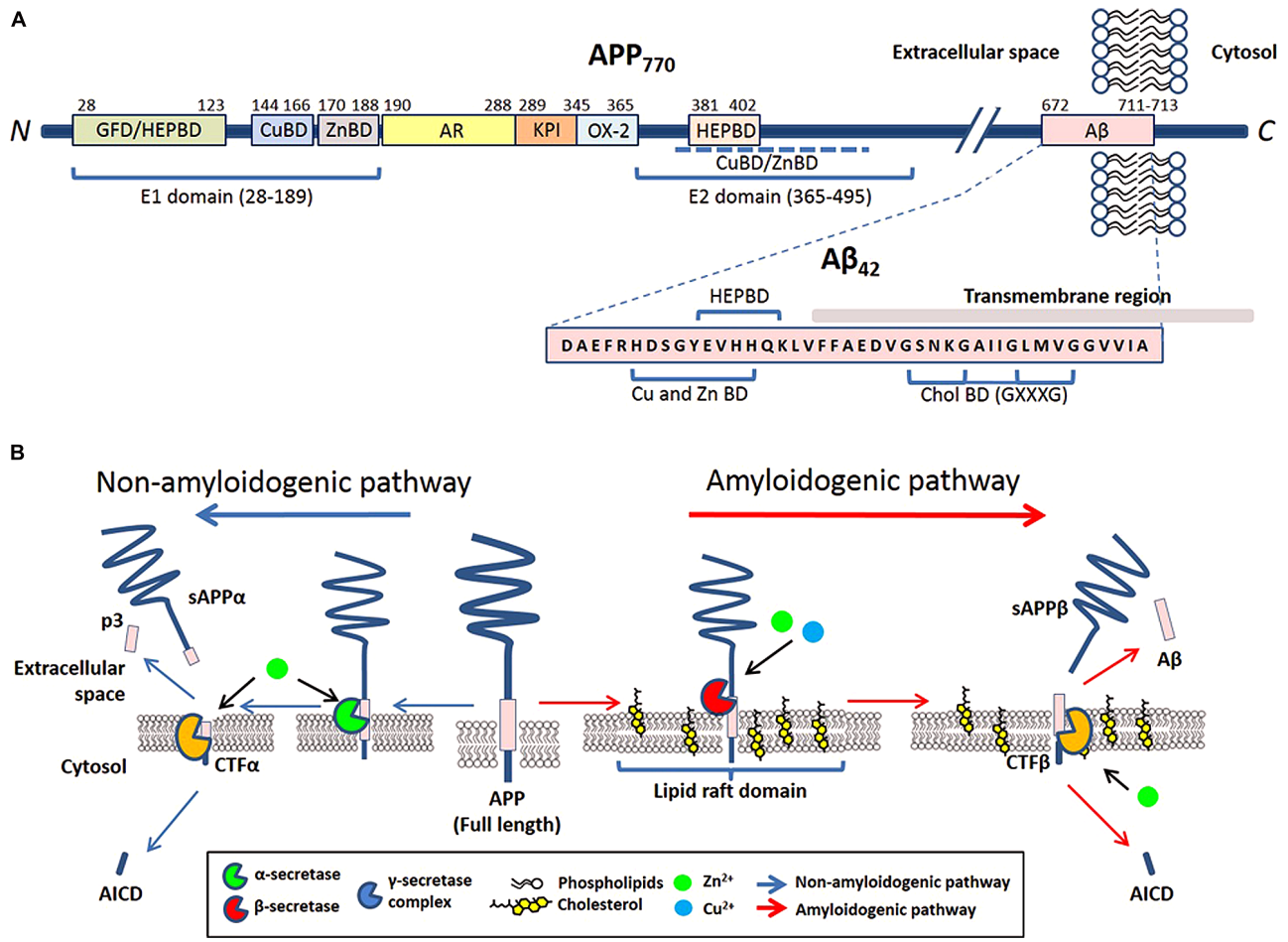

FIGURE 1 | The involvement of metals and cholesterol in post-translational modification of APP. (A) Schematic of reported metal and cholesterol binding domains in APP_{770} and Aβ in relation to other recognized motifs. APP_{770} is the longest isoform of APP with the APP_{751} isoform lacking the OX-2 domain and the neuron prevalent isoform APP_{695} lacking both OX-2 and Kunitz-type protease inhibitor (KPI). Within the extracellular presented ectodomain of APP, the E1 region at the N-terminal contains a copper binding domain (CuBD) and zinc binding domain (ZnBD) that is C-terminally orientated compared to the growth factor domain (GFD) which incorporates a heparin binding domain (HEPBD). The E1 domain is followed by the acidic region (AR), KPI and OX-2 before the E2 domain of APP, containing a HEPBD and CuBD/ZnBD that is yet to be exactly mapped (Dahms et al., 2012). The E2 domain is followed by the Aβ peptide that is partially embedded into the transmembrane region. Aβ also has a recognized CuBD/ZnBD as well as a Cholesterol binding region (CholBD) that incorporates the GXXXG motifs. **(B)** Proteolytic processing of APP predominantly follows two pathways that are initiated by separate secretases. The non-amyloidogenic pathway (*blue arrows*) initiates with the cleavage of full-length APP by α-secretase within the Aβ sequence. Following further cleavage by the γ-secretase complex, this pathway results in the generation of soluble N-terminal APP fragment (sAPPα) and C-terminal fragments (p3 and AICD). The alternative amyloidogenic pathway (*red arrows*) involves sequential cleavage of APP by β-secretase followed by the γ-secretase complex, which results in the liberation of a soluble N-terminal sAPPβ fragment, Aβ peptide, and AICD. Copper and zinc affect the processing of APP and Aβ generation on neuronal membranes through their direct influence on the enzymatic activity of β-, α-, and γ-secretases. The influence of cholesterol is through its requirement in lipid raft domains, the location for amyloidogenic processing of APP.

Other than APP, all three APP-cleaving secretases can digest other biological substrates required for multiple biological functions such as regulation of development, differentiation, and proliferation.

METAL AND CHOLESTEROL MODULATION OF APP AND Aβ METABOLISM

The dynamics of biological metal ions (e.g., copper, zinc, and iron) is critical for many physiological functions. Metal ions are key components in many enzymatic functions, which include catalysis, structural stability, transportation of oxygen, and cellular signaling. The passive flux of metals between the circulation and the brain is tightly regulated by the blood–brain barrier (BBB; Duce and Bush, 2010). The impact of metals on the brain causing neurodegeneration may be caused by increased toxic exposure, as well as a breakdown in the mechanisms that compartmentalize and regulate metal homeostasis.

The brain is the most cholesterol-rich organ in the body. Functionally, cholesterol plays a critical role in neuronal development and maintenance of synaptic plasticity. As a component of the plasma membrane, it regulates ion homeostasis, endocytosis, and intracellular signaling pathways. It also serves as a precursor for the production of steroid hormones, vitamin D, and oxysterols. Like metals, experimental work has shown compartmentalization between levels of cholesterol in the serum and brain that is regulated by BBB (Hung et al., 2013). Substantial evidence correlates cholesterol homeostasis dysregulation with AD. In cell culture systems, production of Aβ is linked to cholesterol levels. However, the exact influence of cholesterol in Aβ generation is still unclear.

METALS AND APP

The APP sequence contains putative binding sites for copper (Hesse et al., 1994; Atwood et al., 2000; Simons et al., 2002; Barnham et al., 2003; Valensin et al., 2004) and zinc (Bush et al., 1993, 1994a,b,c). Copper binds to APP between residues 142 and 166 (White et al., 1999a; Barnham et al., 2003), a site where it can also catalytically reduce copper (Multhaup et al., 1996). Recently, two copper binding residues at histidine 149 and 151 have been identified as crucial for APP metabolism, protein folding and stability (Spoerri et al., 2012). The Aβ segment of APP is another region that directly interacts with copper and is explained in more detail in Section "Metal Modulation of Aβ Generation, Aggregation, and Cell Toxicity." The N-terminal copper binding domain of APP has been found to play crucial roles in homodimerization (Hesse et al., 1994; Kaden et al., 2008), and an elevation in copper levels increases APP homodimerization (Noda et al., 2013). Zinc binds to a conserved region of amino acids between position 170 and 188 of APP (Bush et al., 1993, 1994a). The coordination binding involves two key cysteines at positions 186 and 187, as well as other potential ligands (e.g., C174, M170, D177, and E184). Similar to copper, the binding of zinc may also play an important functional role in homodimerization of APP (Scheuermann et al., 2001; Ciuculescu et al., 2005; **Figure 1A**).

A number of *in vivo* and *in vitro* studies highlight the reciprocal regulation between APP and metal ions. The regulation of *APP* gene expression is linked to altered cellular copper levels. Studies in the *in vitro* cell culture show that copper depletion by overexpressing copper transporter ATP7A result in down-regulation of *APP* gene expression and APP protein level; conversely, *APP* gene expression level is up-regulated under conditions of copper overload due to ATP7A-deficiency (Armendariz et al., 2004; Bellingham et al., 2004b). On the other hand, copper concentration is increased in brain and liver tissue as well as primary neuronal and skin fibroblast cells from APP and amyloid precursor-like protein 2 (APLP2) knockdown mice (White et al., 1999b; Bellingham et al., 2004a; Hung et al., 2009; Acevedo et al., 2011). The difference in copper level is even more pronounced in aged mice (Needham et al., 2014). In contrast, APP over-expressing transgenic mice have decreased copper in the brain (Maynard et al., 2002; Bayer et al., 2003; Phinney et al., 2003). Copper treatment stimulates the movement of APP from the *trans*-Golgi network to the plasma membrane and attenuates internalization of APP to BACE1-rich endosomes. However, copper treatment does not result in any detectable change in APP processing (Hung et al., 2009; Acevedo et al., 2011). In humans, low copper diet is associated with a significant decrease in APP expression in platelets from healthy postmenopausal women (Davis et al., 2000).

Iron regulates APP translation, which involves an iron response element (IRE) RNA stem loop in its 5′-untranslated region (UTR). The APP IRE is homologous with the canonical IRE RNA stem-loop that binds iron regulatory proteins (IRP1 and IRP2) to control intracellular iron homoeostasis by modulating ferritin mRNA translation and transferrin receptor mRNA stability (Rogers et al., 2002). IRP1, but not IRP2, selectively binds to the APP IRE in human neural cells (Cho et al., 2010). Intracellular metal chelation selectively down-regulates APP 5′-UTR translation, which is reversed by cytoplasmic labile iron (Venti et al., 2004). The regulation of APP by iron through the 5′-UTR indicates that iron has a role in APP metabolism.

In the brain, ferroportin (Fpn) is required for excess iron to exit the cell (Donovan et al., 2005; Ganz, 2005). Fpn channels transport iron through the plasma membrane where it is required to be converted to its ferric form before being released and loaded onto transferrin, the extracellular iron-transporting protein that transfers iron between cells (Swaiman and Machen, 1984). APP may play a role in the iron export mechanism of cells through the stabilization of Fpn (Duce et al., 2010). APP knockout mice exposed to dietary iron results in ferrous iron accumulation and oxidative stress in cortical neurons. Ablation of APP in HEK293T cells and primary neurons negates iron export, which can be restored by the addition of exogenous APP (Duce et al., 2010). This iron-export capability of APP requires tau to traffic endogenous APP to the cell surface (Lei et al., 2012).

CHOLESTEROL AND APP

Cholesterol is not symmetrically distributed laterally and between the two leaflets of the lipid membrane bilayer. The significance of this asymmetry is not yet known, although cholesterol has been implicated in cell membrane fluidity, integrity, and function (Wood et al., 1999; Hayashi et al., 2002). Patches

of the membrane highly enriched with cholesterol and sphingolipid are termed lipid rafts (also known as detergent-resistant microdomains). Cholesterol provides structural stability in rafts by serving as a molecular spacer, filling in voids between raft proteins and other raft lipids such as sphingolipids and gangliosides (Xu and London, 2000; Ramstedt and Slotte, 2006). Therefore, modulation of cholesterol can result in dissociation, dysregulation, and/or inactivation of raft proteins. Indeed, APP processing and activity is influenced by its membrane domain localization.

Binding of cholesterol to APP occurs in the trans-membrane carboxyl-terminal region between amino acids 672 and 770 (or CTFβ) through interactions with membrane-buried GXXXG motifs (G, glycine; X, any amino acid; Barrett et al., 2012; **Figure 1A**). The GXXXG motif is involved with APP homodimerization (Kim et al., 2005; Munter et al., 2007; Kienlen-Campard et al., 2008; Miyashita et al., 2009; Sato et al., 2009). Competitive studies of C99 with cholesterol suggest that complexing of cholesterol:C99 at a 1:1 ratio is preferred over C99 homodimers under most physiological conditions (Song et al., 2013). The binding of cholesterol directly to APP and CTFβ may promote amyloidogenic processing by increasing the localization of APP/CTFβ to cholesterol-rich membrane domains and organelles, where γ- and β-secretases preferentially reside (Beel et al., 2010).

In cultured rat neuronal cells, up-regulation of *APP* gene expression reduces cholesterol biosynthesis while down-regulation of *APP* gene expression has the opposite effect (Pierrot et al., 2013). Membrane cholesterol content, however, is not affected. Sterol receptor element binding protein (SREBP) and rate limiting enzyme HMG-CoA reductase (HMGCR) control biosynthesis of cholesterol. The site-2 zinc metalloprotease (S2P) cleaves SREBP at Site-2 within the membrane-spanning domain (Brown and Goldstein, 1999). Interaction of APP with SREBP1 prevents S2P-mediated processing of mSREBP1 nuclear translation of its target genes including HMGCR (Pierrot et al., 2013). Interestingly, the APP/Aβ GXXXG motif is critical in the regulation of HMGCR. In contrast to neuronal cells, APP interaction with SREBP1 and resulting cholesterol biosynthesis is not detectable in astrocytes (Pierrot et al., 2013). APP expression associated reduction of cholesterol and oxysterol production is mediated via down-regulation of both HMGCR and 24-hydroxylase [required to convert cholesterol to 24S-hydroxycholesterol (24OHC)] activities, respectively. Since membrane cholesterol remains the same, it is suggested that APP controls cholesterol turnover (Pierrot et al., 2013).

METAL MODULATION OF APP PROCESSING ENZYMES

Metals can indirectly affect Aβ generation by altering secretase-dependent processing of APP. To date, all three secretases involved in APP cleavage are known to have interactions with different metal species. The α-secretase TACE contains a zinc ion in its catalytic domain (Cross et al., 2002). TACE enzymatic activity is controlled by a "cysteine-switch" motif mediated by an intramolecular bond between cysteine and a zinc atom in its catalytic site. Subsequently, it has been shown that other regions of the TACE prodomain are able to circumvent the "cysteine-switch" and inhibit enzymatic activity (Buckley et al., 2005). Correspondingly, the metalloprotease ADAM10 can be inhibited by its dominant-negative form that has a point mutation in its zinc-binding site (Lammich et al., 1999).

The major β-secretase BACE1, binds copper in its C-terminal domain, the same region that interacts with domain I of copper chaperone for superoxide dismutase-1 (CCS; Angeletti et al., 2005). The expression of BACE1 reduces superoxidase 1 (SOD1) activity. In contrast, in cells overexpressing both BACE1 and CCS, SOD1 activity is restored by CCS (Angeletti et al., 2005). An interaction between BACE1 and CCS has been demonstrated by co-immunoprecipitation from brain homogenates and their co-transport through the axon (Angeletti et al., 2005).

Presenilin, the active subunit of the γ-secretase, is also sensitive to metal levels. Neonatal cortical cultures exposed to zinc increases C-terminal fragmentation of PS1 by enhancing synthesis of the protein (Park et al., 2001). However, zinc induces oligomerization of an APP γ-secretase substrate and inhibits its processing, which supports a role for zinc dysregulation in Aβ processing (Hoke et al., 2005; Greenough et al., 2011).

Taken together, these results suggest a direct influence of metals on secretase enzymatic activity to process APP and therefore may have detrimental implications in AD pathology when metal homeostasis is altered.

CHOLESTEROL MODULATION OF APP PROCESSING

Previous studies show that full-length APP, Aβ, APP-CTFs, and PS1 are associated with lipid rafts (Lee et al., 1998; Simons et al., 2001; Hur et al., 2008). Studies with cultured cells demonstrate cholesterol depletion by β-cyclodextrin extraction or inhibition of cholesterol biosynthesis by statins (Simons et al., 1998; Wahrle et al., 2002), result in decreased Aβ production. Conversely, increasing cellular cholesterol levels enhance Aβ production and reduce α-secretase cleavage of APP (Bodovitz and Klein, 1996; Frears et al., 1999).

Since APP, β- and γ-secretases are associated with lipid raft domains, it is not surprising that altered cellular cholesterol content affects Aβ generation, aggregation, and clearance. The presence of lipid raft domains has been found in plasma membranes and endosomes. More recently, a study uncovered lipid raft-like domains in mitochondria-associated endoplasmic reticulum (ER) membranes (MAMs), a sub-compartment of the ER connected to mitochondria (Area-Gomez et al., 2012). Lipid rafts are sensitive to altered cholesterol metabolism, and cholesterol depletion results in lipid raft destabilization (Eckert et al., 2010). As previously mentioned, cholesterol enriched in lipid rafts can influence dynamics of proteins within these rafts. Altered cholesterol levels affect lipid raft localization of APP and its derivatives together with secretases required for APP processing. Biochemical isolation of lipid rafts indicates that BACE1 and γ-secretase protein are localized within these lipid domains (Wahrle et al., 2002; Vetrivel et al., 2004; Kalvodova et al., 2005; Osenkowski et al., 2008), while the α-secretase ADAM10 is predominantly localized outside the lipid rafts (Kojro et al., 2001). Consistent with other lipid raft domains, MAMs have a high concentration of APP, PS1, and PS2 (catalytic subunits of γ-secretase) and γ-secretase activity. APP is believed to exist in either pool within

plasma membranes (Ehehalt et al., 2003). Experimental evidence suggests that amyloidogenic processing of APP occurs in lipid rafts while the non-amyloidogenic processing occurs mainly in the non-raft regions. If this is the case, then cholesterol levels contribute to regulation of APP processing through these two pathways. The non-amyloidogenic pathway predominates, because only small amounts of APP appear to be present in lipid rafts under physiological conditions (Bouillot et al., 1996; Parkin et al., 1999). Increasing membrane cholesterol levels may increase overall percentage of lipid rafts, which favors APP and BACE1 interaction and increases Aβ generation. Several studies support this idea. Firstly, imaging of fluorescently tagged APP and BACE1 demonstrates that cholesterol loading does not increase Aβ production through BACE1 catalytic activity but rather by altering the accessibility of BACE1 to its substrate APP in lipid rafts (Marquer et al., 2011). Secondly, APP and BACE1 copatch at the plasma membrane upon antibody cross-linking, which increases Aβ production in a cholesterol-dependent manner (Ehehalt et al., 2003). Lastly, inhibition of γ-secretase activity leads to an accumulation of APP-CTFs in lipid rafts (Vetrivel et al., 2004).

Niemann–Pick type C disease (NP-C) is a lysosomal lipid storage disorder, characterized by accumulation of cholesterol and sphingolipids within the endosomal–lysosomal system. The majority of NP-C cases are caused by functional loss of NPC1 protein activity, due to genetic mutation. Neuronal degeneration underlies neurological symptoms in NP-C patients, which include cerebellar ataxia, dysphagia, dysarthria, and dementia. Altered cholesterol distribution within subcellular compartments has been implicated in the aberrant trafficking and processing of APP similar to that observed in AD (Runz et al., 2002; Vanier and Millat, 2003; Jin et al., 2004; Walkley and Suzuki, 2004; Vance, 2006; Kodam et al., 2010; Kosicek et al., 2010; Malnar et al., 2010, 2012). In cell models of NP-C, cholesterol overload due to NPC1 deficiency leads to increased APP lipid raft localization and internalization from the plasma membrane to BACE1-rich endosomes, where amyloidogenic processing occurs (Kosicek et al., 2010; Malnar et al., 2010). This can be corrected by cholesterol depletion in cultured cells using lipid-deficient serum, lovastatin treatment, or methyl-β-cyclodextrin treatment (Malnar et al., 2012). The cholesterol-dependent change in APP trafficking and lipid raft localization parallels previous studies of APP's response to changes in cellular copper levels (Hung et al., 2009; Acevedo et al., 2011). Furthermore, cholesterol-dependent APP trafficking and metabolism may explain some of the metal changes observed in NP-C tissue samples (Hung et al., 2014). Taken together, these evidences suggest a synergistic interaction between copper and cholesterol pathways in the regulation of APP metabolism that may contribute to AD pathogenesis.

Altered intracellular cholesterol metabolism can also affect APP processing. Cultured cells exposed to a cholesterol transport inhibitor, U18666A, accumulate cholesterol in late endosomes and lysosomes, and results in a dose-dependent decrease in Aβ production (Runz et al., 2002; Davis, 2008). However, the inhibitor also increases accumulation of γ-secretase, CTFβ, and Aβ-related peptides in vesicular organelles (Runz et al., 2002; Jin et al., 2004). From these studies, it can be inferred that cholesterol is able to influence APP processing through re-internalization of surface APP, as well as redistribution of APP and its processing enzymes within subcellular compartments.

METAL MODULATION OF Aβ GENERATION, AGGREGATION, AND CELL TOXICITY

Aβ binds to zinc, copper, and iron to form various precipitous complexes, which are dependent on pH, buffer conditions, and initial peptide aggregation rate (Bush et al., 1994b; Huang et al., 1997; Garai et al., 2006; Tougu et al., 2008). Human Aβ binding of zinc, and both oxidized and reduced copper (Bush et al., 1994c; Atwood et al., 2000; Syme et al., 2004; Syme and Viles, 2006; Danielsson et al., 2007; Himes et al., 2008; Karr and Szalai, 2008; Shearer and Szalai, 2008; Hureau and Faller, 2009) is mediated by nitrogen ligands from histidine at positions 6, 13, and 14 together with an oxygen ligand (Curtain et al., 2001). Interestingly, rat and mouse have different amino acids at the metal ion coordination site, which could explain why these animals resist developing amyloid pathology compared to other mammals (Gaggelli et al., 2008). More details regarding the biophysical and biochemical binding of Aβ and the above mentioned metals have been reviewed (Faller and Hureau, 2009; Rozga et al., 2009).

Neurotoxic effects of Aβ depend on peptide aggregation, metal ion interaction, and generation of reactive oxygen species (ROS) with the subsequent formation of soluble covalently cross-linked oligomers. Both Cu:Aβ and Fe:Aβ complexes have been shown to exhibit cytotoxic effects (Schubert and Chevion, 1995; Liu et al., 2011; You et al., 2012), which can be rescued by chelation or competitive binding (Huang et al., 2004; Wu et al., 2008; Perrone et al., 2010). Interestingly, it has been shown that modifying copper binding histidine 6 or 13 to alanine induces significant cell toxicity in primary cortical cell cultures at levels similar to the wild-type peptide (Smith et al., 2010). However, modifying histidine 14 (a known ligand for copper and the cell plasma membrane), did not induce any measurable toxicity that correlates with the ability of the modified peptide to bind to cell membranes (Smith et al., 2010).

Under normal physiological conditions, non-toxic monomeric forms of Aβ are the predominant species (Haass et al., 1992; Vigo-Pelfrey et al., 1993; Shoji, 2002). However, pathological stimuli are thought to trigger complex conformational changes and assembly of Aβ peptides to form a heterogeneous mixture of oligomers and fibrils. This aggregation of Aβ is a critical event for neurotoxicity to occur. Soluble Aβ oligomers, and not fibrils, are currently considered the proximate neurotoxin in AD pathology (Dahlgren et al., 2002; Kayed et al., 2003; Cleary et al., 2005; Haass and Selkoe, 2007; Lesne et al., 2008; Roychaudhuri et al., 2009; Shankar and Walsh, 2009). However, as both Aβ oligomers and fibrils can interact synergistically with tau and cause mitochondrial function impairment in the P301L tau transgenic mouse model (Eckert et al., 2008), the distinction in all forms of neurotoxicity between Aβ species is not clear. Both copper and iron have been shown to modify Aβ and accelerate its aggregation *in vitro* (Mantyh et al., 1993; Atwood et al., 2000; Ali et al., 2005). Oxidation of the Aβ side-chain by copper leads to covalent oligomerization (Ciccotosto et al., 2004; Ali et al., 2005). Tyrosine at position 10 of Aβ is

particularly susceptible to free radical attack. When complexed to Cu^{2+} or Fe^{3+} and in the presence of H_2O_2, Aβ forms dityrosine cross-linked oligomers, which are suggested to seed accelerated Aβ aggregation (Atwood et al., 1998, 2004; Barnham et al., 2004). Unlike zinc, copper mediates Aβ oligomer formation rather than amyloid fibrils, and thus Aβ:Cu oligomers are not recognized by the β-sheet marker, thioflavin T (Jiao and Yang, 2007; Tougu et al., 2009).

Investigations on metal-mediated modulation of Aβ have been carried out in APP transgenic models supplemented with either dietary copper or zinc. Administration of copper to APP23 mice, overexpressing human APP with the AD-related Swedish mutation, elevated copper levels in the brain compared to wild-type littermate controls, resulting in a lowering of soluble and insoluble Aβ (Bayer et al., 2003). Dietary zinc supplementation also reduced Aβ plaques in brains of Tg2576 (another transgenic mouse model carrying the Swedish-APP mutation) and TgCRND8 (a triple transgenic mouse model carrying APP with Swedish and Indiana mutations). However, AD-like spatial memory impairments are increased in the zinc-fed transgenic mice (Linkous et al., 2009). Conversely, decreased dietary zinc in a APP/PS1 transgenic mouse model of AD elevated plaque volume (Stoltenberg et al., 2007). Elevation of brain copper by crossing TgCRND8 with a transgenic mouse model deficient in the copper transporter, ATP7B, reduces plaque load as well as soluble and insoluble Aβ levels (Phinney et al., 2003). These evidences suggest that an intracellular shift in copper reduces Aβ aggregation.

Intracellular zinc export takes place through the zinc transporters (ZnT) protein family. Currently eight ZnTs are known, of which, ZnT-1 is the only member that exports zinc across the plasma membrane within the brain (Lovell et al., 2005). ZnT-3 transports zinc to glutamatergic vesicles in hippocampal granule, pyramidal, and interneuron cells (Cole et al., 1999; Linkous et al., 2008), ZnT-4 sequesters cytosolic zinc into acidic vesicles (Kelleher and Lonnerdal, 2002) and ZnT-6 sequesters zinc in the *trans*-Golgi network and vesicular compartments (Huang et al., 2002). The highest concentration of labile zinc is present in synaptic vesicles that are released during synaptic transmission of neocortical glutamatergic fibers. As mentioned, the activity of ZnT-3 is required for the passage and pooling of zinc within these presynaptic vesicles, making it available for an interaction with the Aβ that is predominantly located within the synapse. Crossing of ZnT-3 knockout mice with Tg2576 mice, reduces both cerebral plaque load (Lee et al., 2002) and amyloid angiopathy (Friedlich et al., 2004). This supports the theory that high concentrations of zinc in the synaptic cleft play a role in amyloid formation in AD.

Oxidative stress-induced damage of brain tissues is a major hallmark of AD. The redox chemistry involved in the production of toxic ROS from metal enriched Aβ complexes and general metal dyshomeostasis is implicated in this process. Binding of oxidized copper or iron to Aβ results in reduction of the metal valency state and subsequent production of H_2O_2 (Huang et al., 1999; Opazo et al., 2002; Tabner et al., 2002; Nelson and Alkon, 2005). This can be further exacerbated by the reaction of hydrogen peroxide with reduced metal to produce hydroxyl radicals through Fenton and Haber–Weiss reactions (Fenton, 1894; Haber and Weiss, 1934). Hydroxyl radicals are highly chemically reactive and contribute to generation of lipid peroxidation products, protein carbonyl modifications, and nucleic acid adducts such as 8-hydroxy guanosine, all of which feature strongly in AD neuropathology (Smith et al., 1996, 1997). Of note, evidence suggests that the biological reductants involved in Aβ redox cycling are most likely cholesterol and long chain fatty acids (Opazo et al., 2002; Barnham et al., 2004; Haeffner et al., 2005; Nelson and Alkon, 2005; Puglielli et al., 2005; Smith et al., 2006). This is consistent with experimental evidence demonstrating that toxicity associated with Aβ occurs on the plasma membrane (Ciccotosto et al., 2004). Additionally, the products of lipid oxidation such as oxysterols, 7β-hydroxycholesterol and 4-hydroxy-2-nonenal, which in turn increases Aβ cross-linking (Murray et al., 2005), are elevated in AD tissues and mouse models of the disease (Opazo et al., 2002; Haeffner et al., 2005; Nelson and Alkon, 2005; Puglielli et al., 2005; Smith et al., 2006).

CHOLESTEROL MODULATION OF Aβ GENERATION, AGGREGATION, AND CELL TOXICITY

The majority of *in vivo* data provide support for an involvement of cholesterol in Aβ generation (Sparks et al., 1994; Bodovitz and Klein, 1996; Bouillot et al., 1996; Lee et al., 1998; Simons et al., 1998, 2001; Frears et al., 1999; Kojro et al., 2001; Wahrle et al., 2002; Ehehalt et al., 2003; Vetrivel et al., 2004; Kalvodova et al., 2005; Osenkowski et al., 2008). However, the impact of altering plasma cholesterol on brain Aβ generation remains unclear. Animal studies report no correlation (Parkin et al., 1999) or inverse correlation (Jin et al., 2004; Davis, 2008; Marquer et al., 2011) between dietary or peripheral cholesterol and Aβ. Several reasons can account for this disparity between studies, which include genetic background, the transgenes present, age, gender, and/or treatment conditions and environment. Another significant reason may be associated with the inherent selectivity of the BBB. Cholesterol in the brain is synthesized *de novo* and it is unclear to what extent peripheral or dietary cholesterol influences brain cholesterol levels due to limited BBB penetration. Moreover, most studies that examine the effects of high dietary cholesterol on Aβ levels fail to measure brain cholesterol levels in the same experimental settings. It is therefore uncertain if alteration of brain Aβ levels is due to cholesterol changes in the brain or some other indirect mechanism that is caused by the modulation of peripheral cholesterol. Effects of Aβ generation under *in vivo* paradigms and of cholesterol modulating genes on APP processing/Aβ generation have been reviewed recently in detail (Maulik et al., 2013). Results from these studies have shown strong evidence that modulating cholesterol synthesis (Crameri et al., 2006), intracellular trafficking (Burns et al., 2003; Bryleva et al., 2010; Kodam et al., 2010; Borbon and Erickson, 2011), uptake (Bales et al., 1997, 1999; Holtzman et al., 2000; Irizarry et al., 2000; Cao et al., 2006; Kim et al., 2009), and removal (Koldamova et al., 2005; Wahrle et al., 2005, 2008) causally influence APP processing and Aβ generation.

Cholesterol-rich lipid rafts may play a role in catalyzing the aggregation of Aβ to its neurotoxic oligomeric state. Aβ isolated from AD patients is associated with lipid rafts in a cholesterol-dependent manner and reducing cholesterol levels results in less

aggregated Aβ peptides (Schneider et al., 2006). Cholesterol is likely to modulate Aβ aggregation through modifying raft composition. The ganglioside GM1, which is predominantly found in the central nervous system, can bind Aβ peptides in lipid rafts to form a complex that acts as an endogenous seed to promote amyloid oligomerization, aggregation, and subsequent fibril formation (Choo-Smith et al., 1997; Kakio et al., 2002; Kim et al., 2006; Okada et al., 2008; Matsuzaki et al., 2010). This has been shown to be a primary mediator of oxidative stress on plasma membrane (Zampagni et al., 2010). Some studies examining effects of cholesterol on Aβ toxicity *in vitro* provide evidence that decreasing cholesterol, sialic acid, and ganglioside synthesis is protective to PC12 cells, while increasing cholesterol levels lead to increased Aβ neurotoxicity (Wang et al., 2001; Lin et al., 2008). Interestingly, it has been observed that sustained ROS production is associated with Aβ toxicity when exogenous cholesterol is increased (Ferrera et al., 2008). Other studies disagree with these results. PC12 cells and cultured neurons with high cholesterol levels in the membrane are resistant to Aβ toxicity, while low cholesterol levels increase their susceptibility (Zhou and Richardson, 1996; Yip et al., 2001; Arispe and Doh, 2002; Sponne et al., 2004). These divergent results suggest a dynamic yet intricate correlation between cholesterol and Aβ peptide, such that cholesterol's influence on physical properties of lipid rafts can modulate Aβ binding and aggregation to affect cell viability.

METAL MODULATION OF Aβ DEGRADATION

The over-production of toxic Aβ is only one side of the equation that contributes to senile plaque production and AD pathology, with the other possible side, less frequently studied but equally important, involving a fault in the degradation and clearance regulatory pathways of Aβ (reviewed in Carson and Turner, 2002; Ling et al., 2003). Three proteases in the brain most frequently studied in Aβ degradation, are insulin-degrading enzyme (IDE), neprilysin (NEP), and plasmin. Of these three proteases, IDE and NEP are members of the zinc metallopeptidase family of proteins that have a zinc binding domain with common sequence homology that can be potentially altered with aberrant zinc metabolism (Vekrellis et al., 2000; Fan et al., 2009). Additionally, metal binding ligands of both enzymes are oxidatively modified in the AD brain by various ROS, such as hydroxyl radicals and products of ROS, such as 4-hydroxy-2-nonenal (Wang et al., 2003; Caccamo et al., 2005; Shinall et al., 2005). These data suggest that the generation of ROS, perhaps as a product of metal:Aβ redox cycling, may serve to inactivate proteases involved in Aβ degradation. Conversion of plasminogen to plasmin involves cleavage from either tissue-type (tPA) or urokinase-type plasminogen activator (Ledesma et al., 2003). Inhibition by tPA cleavage of plasminogen is again caused by increased redox cycling and production of ROS in the presence of copper/ascorbate (Lind et al., 1993). Plasmin itself may also be regulated by site-specific oxidation; in particular, modification of the histidine molecule that resides in its active site (Lind et al., 1993). Lastly, Aβ is a substrate for matrix metalloproteinase (MMP), and plasmin has been shown to activate MMP2 degradation of Aβ, a process that is inhibited in the presence of zinc but not copper (Crouch et al., 2009).

CHOLESTEROL MODULATION OF Aβ DEGRADATION

A number of recent studies have shown that cholesterol may be involved in Aβ clearance by regulating Aβ degrading enzymes. After synthesis, IDE is transported via the secretory pathway to the cell membrane where it either remains or is secreted. Given that a subset of IDE is localized in lipid rafts (Bulloj et al., 2008), it is possible that cholesterol levels or distribution can regulate the transport and release of this protease to influence Aβ degradation. Similar to IDE, the mature form of NEP also associates with lipid rafts (Sato et al., 2012). Contradictorily, targeting NEP chimeric proteins to lipid rafts fails to efficiently degrade Aβ in this fraction (Hama et al., 2004). It is of note that plasmin is also a raft protein (Ledesma et al., 2003). Mice deficient in seladin-1, which is required for cholesterol synthesis, present disorganized rafts and impaired plasmin function (Crameri et al., 2006; Stefani and Liguri, 2009). These evidence supports the notion that cholesterol, possibly through raft maintenance, is required for plasmin degradation of Aβ.

APOE ASSOCIATION WITH METAL AND CHOLESTEROL ON ITS ROLE OF Aβ CLEARANCE

There is strong evidence that ApoE plays a central, if not direct, role in the pathogenesis of AD. The human *APOE* gene exists as three polymorphic alleles (ε2, ε3, and ε4), and individuals possessing the ε4 allele are at highest risk of developing AD (Bales et al., 2009; Reiman et al., 2009; Castellano et al., 2011). ApoE is well known for its involvement in the transportation of cholesterol. Together with a multitude of other apolipoproteins, lipoprotein receptors, and lipid transporters, ApoE controls cholesterol homeostasis in the brain (brain cholesterol homeostasis reviewed in Hung et al., 2013). Studies in human and transgenic mice demonstrate an isoform dependent (ε4 > ε3 > ε2) accumulation of Aβ levels and amyloid plaque load.

Currently, there is no clear evidence that ApoE affects APP processing and Aβ production *in vitro* and *in vivo* (Biere et al., 1995; Cedazo-Minguez et al., 2001; Irizarry et al., 2004). However, ApoE appears to play an important role in Aβ clearance through several possible mechanisms. *In vitro* studies with neuronal cells have shown that lipidated ApoE binds to soluble Aβ in an isoform-dependent manner (ε2 > ε3 > ε4) and is internalized into various brain cells for degradation by receptor-mediated endocytosis (Beffert et al., 1998, 1999; Yang et al., 1999; Cole and Ard, 2000; Yamauchi et al., 2000, 2002). ApoE may also facilitate removal of Aβ from the brain through the BBB (Cirrito et al., 2005; Zlokovic, 2008). ApoE may be able to facilitate the cellular degradation of Aβ *in vitro*, however, the mechanism and whether it is isoform-specific still requires clarification (Crameri et al., 2006; Bryleva et al., 2010).

There are very limited studies investigating metal interaction with ApoE and its relationship with APP or Aβ. ApoE protein binds copper, iron, and zinc, suggesting that ApoE has the ability to sequester metals. This may underlie its isoform-dependent antioxidant activity (ε2 > ε3 > ε4; Miyata and Smith, 1996). Interestingly, ApoE4 contains a cysteine to arginine substitution at positions 112 and 158. Since cysteine is believed to be involved in transition metal

binding, reduced affinity of ApoE4 to metal may therefore relate to diminished antioxidant effects of the ApoE4 allele (Moir et al., 1999).

ATP binding cassette transporter A1 (ABCA1) is a cell surface membrane protein that promotes efflux of cellular cholesterol to acceptor molecules, including ApoE and ApoA1. The ApoE4 isoform has been found to reduce ABCA1-mediated cholesterol efflux in astrocytes and neurons *in vitro* (Michikawa et al., 2000; Gong et al., 2007). Studies involving ABCA1-deficient mice show poor lipidation with decreased levels of ApoE (70–80% reduction) and a concurrent increase in amyloid plaque burden (Wahrle et al., 2004, 2005; Hirsch-Reinshagen et al., 2005; Koldamova et al., 2005). Conversely, ABCA1 overexpression in mice result in increased lipidation and ApoE levels, and decreased amyloid plaque formation (Wahrle et al., 2008). Interestingly, both ApoE and ABCA1 can be modulated by transcription factor liver-X-receptors (LXRs), which may be a key regulator in brain lipid homeostasis. Indeed, deficiencies in LXRα and/or β augment AD pathology (Zelcer et al., 2007), whereas treating AD mice with LXR agonists, including Bexarotene, result in reduced amyloid plaque burden and improved cognitive function (Eckert et al., 2007; Riddell et al., 2007; Vanmierlo et al., 2011; Cramer et al., 2012).

TWO SIDES OF THE SAME COIN: POSSIBLE CROSSTALK BETWEEN METALS AND CHOLESTEROL IN APP/Aβ METABOLISM

Independently, there are large bodies of research detailing the influence of metals or cholesterol on the development, progression, and pathogenesis of AD. However, there are limited studies on the relationship between metals and cholesterol in AD pathology. The review thus far gives an overview of the impact of either metals or cholesterol on the amyloidogenic and non-amyloidogenic processing pathways of APP. Interestingly, there are many overlaps between these two factors impacting various processes in both of these pathways. The review will now examine interactions between metals and cholesterol with APP and Aβ.

Epidemiological studies have shown that dietary intake of trans- and saturated fats lead to an unfavorable cholesterol profile in AD patients and may associate with cognitive decline (Morris et al., 2003, 2004). A follow-up study indicates that higher copper intake is associated with an accelerated rate of cognitive decline and that the copper-dependent acceleration is lost in individuals who did not consume a high fat diet (Morris et al., 2006). In animal studies, cholesterol-fed rabbits have exacerbated neurodegeneration following consumption of trace amounts of copper (Sparks and Schreurs, 2003). Lowering cholesterol levels in patients by atorvastatin (a cholesterol lowering drug which inhibits HMGCR) saw an increase in circulating ceruloplasmin levels (Sparks et al., 2005), a ferroxidase involved in iron homeostasis and due to its role as a plasma copper transporter, a surrogate marker of plasma copper status. Interestingly, like copper, increased dietary cholesterol leads to dysregulation of iron regulatory proteins in rabbits and also iron accumulation in Aβ plaques (Ghribi et al., 2006). An epidemiological study in a large cohort of adults found that abnormally high dietary cholesterol and iron intakes increase the risk of AD (Mainous et al., 2005).

Based on evidence from the studies reviewed in Sections "Metals and APP" and "Cholesterol and APP," it may be inferred that both metals and cholesterol are able to modulate APP metabolism/expression through a process that remains to be elucidated. Conversely, APP itself can regulate metal and cholesterol homeostasis. Therefore, APP may be envisioned as a key regulator linking both metal and cholesterol homeostasis, whereby unregulated metal or cholesterol leads to a downstream effect on APP that may ultimately cause an erroneous outcome in collateral systems.

One way in which copper, iron, and zinc can impact on the pathology of AD stems from their relative ease in switching oxidation states. This property makes it particularly useful for enzymatic reactions requiring electron transfer (Waldron et al., 2009). Metals can affect APP processing (**Figure 1B**) and Aβ degradation (**Figure 2**) by altering catalytic properties of secretases, which are metalloproteins (reviewed in Sections "Metal Modulation of APP Processing Enzymes" and "Metal Modulation of Aβ Degradation"). Cholesterol, on the other hand influences APP processing (**Figure 1B**) and Aβ degradation (**Figure 2**) through lipid raft association of substrates (APP and Aβ) and enzymes (APP processing secretases and Aβ degrading proteases; reviewed in sections "Cholesterol Modulation of APP Processing" and "Cholesterol Modulation of Aβ Degradation"). Cholesterol influences both the quantity and quality of the lipid raft domains. For example, cholesterol can modulate the order of raft components to provide the right environment for protein binding or function. In other words, metals modulate APP processing and Aβ degradation through the mechanistic action of the enzyme, whereas cholesterol does so through manipulation of the environment and presentation of the substrate and/or enzyme on the plasma membrane. Although this relationship between metals and cholesterol may not be mutually exclusive, a fault in either system may still lead to similar pathological outcomes in AD (**Figure 2**).

More recently, copper has been observed to directly influence the lipid raft protein, flotillin-2 (Hung et al., 2009). Flotillin-2 interacts with APP at the cell surface (Schneider et al., 2008). The endocytosis of APP to BACE1-rich endosomes, required for β-cleavage of APP, is sensitive to flotillin-2 depletion (Ehehalt et al., 2003; Schneider et al., 2008). Analogous to cholesterol depletion, elevated copper reduces flotillin-2 association with lipid rafts, thereby reducing endocytosis of APP and attenuating Aβ production (Hung et al., 2009).

Interestingly, both metals and cholesterol are able to catalyze the oligomeric aggregation of Aβ required for its cytotoxic effect (see "Metal Modulation of Aβ Generation, Aggregation, and Cell Toxicity" and "Cholesterol Modulation of Aβ Generation, Aggregation, and Cell Toxicity"; **Figure 2**). The mechanism of aggregated Aβ toxicity is still a matter of debate. However, elevation of ROS in both metal:Aβ or GM1:Aβ complexes suggests an involvement of metal as a mechanistic partner to redox cycle and generating harmful ROS products from both metal- and cholesterol-based aggregation of Aβ *in vitro*.

Oxysterols also play an important role in the regulation of cholesterol in the brain and the body. In the brain, oxysterols are produced by conversion of cholesterol to the oxidized species, 24OHC, by the enzyme 24S-hydroxylase. 24OHC represents

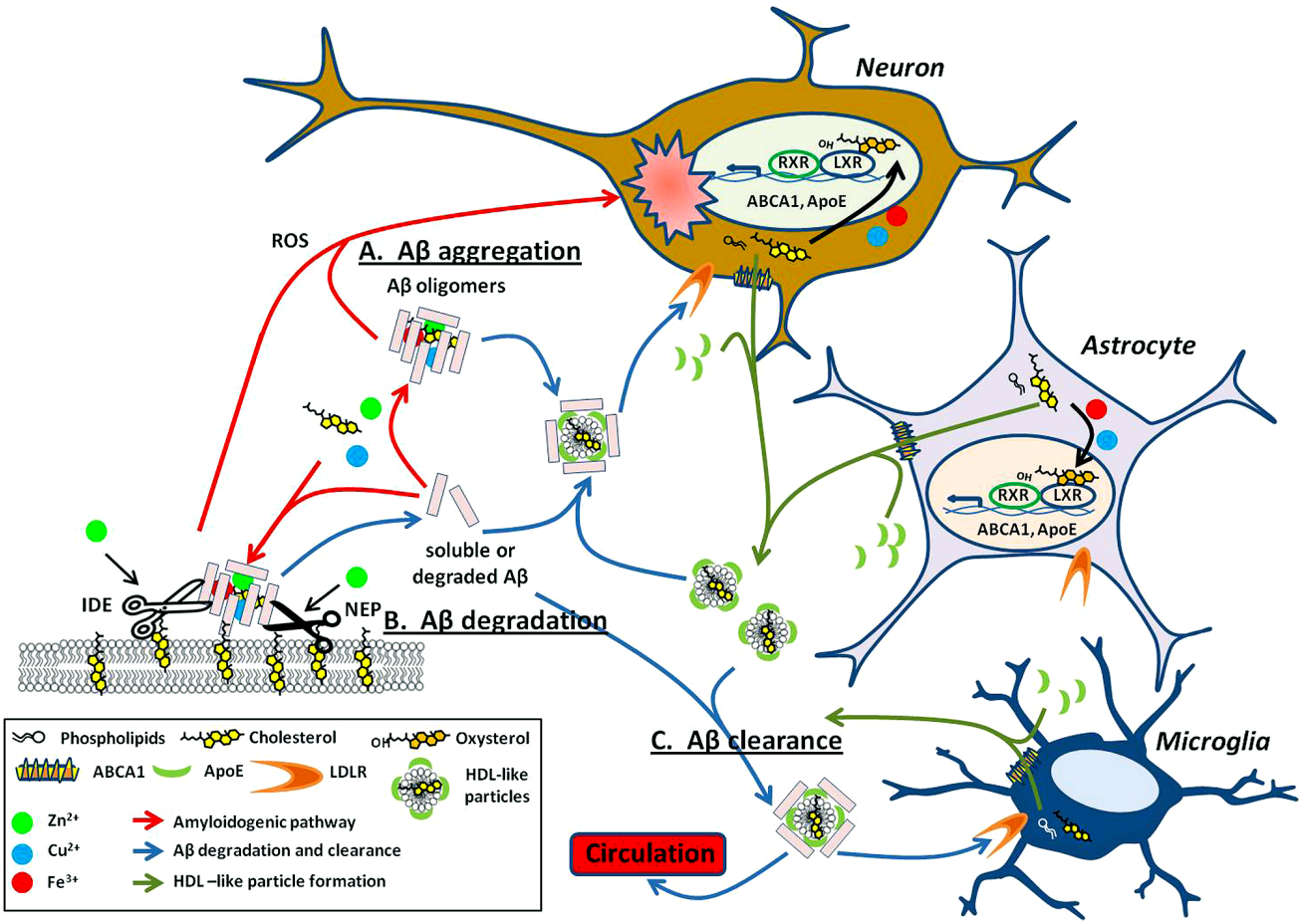

FIGURE 2 | Metals and cholesterol implicated in Aβ processing and neurotoxicity. (A) Upon cleavage from APP, both metal and cholesterol bind to Aβ monomers promoting oligomerization of the peptide into multiple types of Aβ aggregates. These aggregates are either present within the extracellular space or bound to the surface of the plasma membrane (*red arrows*). Select Aβ aggregates are neurotoxic through multiple mechanisms such as their ability to generate reactive oxygen species (ROS) and may have implications in AD associated neuropathology. The ability of copper, iron, and cholesterol to promote redox cycling are acutely involved in the cytotoxicity caused by aggregated Aβ. **(B)** As with APP processing enzymes (**Figure 1B**), proteases that degrade Aβ, such as neprilysin (NEP) and insulin degrading enzyme (IDE) are dependent on metals for their catalytic activity. These proteases are also lipid raft associated and modulated by cholesterol levels in these domains. **(C)** Lipidated ApoE, produced mainly by astrocytes and microglia, binds soluble Aβ and facilitates its degradation through receptor-mediated endocytosis within neurons and microglial or clearance from the brain through the blood–brain barrier (BBB; *blue arrows*). Lipidated ApoE regulation is modulated by nuclear transcription factors LXRs. LXR heterodimerizes with RXR to transcriptionally regulate ABCA1 and ApoE. ABCA1 exports cellular cholesterol and phospholipids that in turn lipidate ApoE to form HDL-like particles (*green arrows*). These HDL-like particles are required for the clearance of Aβ. Intriguingly, the LXR agonist, oxysterols is elevated in the AD brain and may result from cholesterol oxidation by metals and 24S-hydroxylase. Evidence implies that the involvement of metal and cholesterol in the Aβ processing pathway is not just deleterious (as in **A**) but may also have importance in degradation and clearance of this potentially harmful peptide **(B,C)**.

one of the main forms of cholesterol that can be trafficked out of the brain to the circulatory system by its permeability across the BBB (Bjorkhem et al., 1998; Lund et al., 1999; Ehehalt et al., 2003; Lutjohann and von Bergmann, 2003; Schneider et al., 2008). Interestingly, APP has been shown recently to regulate 24S-hydroxylase levels (Pierrot et al., 2013). Oxysterols are agonists of LXRs, the latter of which form heterodimer complexes with retinoid x receptor (RXR) to transcriptionally regulate the production of a number of genes involved in the cholesterol regulatory pathway including ApoE, ABCA1, ABCG1, and SREBP1 (Bjorkhem, 2013). Through this pathway, oxysterols are able to regulate cholesterol efflux from cells via LXRs (**Figure 2**). In the progression of AD, levels of oxysterols are elevated, possibly due to effects of 24S-hydroxylase and non-enzymatic oxidation of cholesterol caused by elevated metal levels (Iuliano, 2011). This may be a way in which the brain is utilizing a feedback mechanism to clear excess cholesterol and Aβ peptides. Therefore, it is not surprising that elevated LXR-induced expression of ApoE4 (with defective Aβ and cholesterol clearance) compared to ApoE2, results in continued accumulation of neuritic plaques. The pathology of the disease continues to progress in a positive feedback loop of increased cholesterol, ROS, and Aβ generation (**Figure 2**).

CONCLUSION

Metal and cholesterol are intrinsically linked to the pathogenesis of AD. Despite large bodies of research examining the abnormalities of metals and cholesterol in AD, the reciprocal influence of these two factors in the cause and progression of the disease remains to be elucidated. This review presents an overview of how metals and cholesterol independently impact upon the amyloidogenic and non-amyloidogenic processing of APP. It highlights the close and complex relationship between metals and cholesterol in the maintenance of normal brain physiology and the progression of AD pathology, with respect to interactions with AD-related proteins APP and Aβ. In the scheme of APP processing and Aβ metabolism, a disturbance to one homeostatic system may likely lead to a direct or indirect dysregulation of the other, although some of its molecular actions are mutually exclusive, the eventual deleterious outcome is the same. Continued research into metal or cholesterol influences on AD pathology must take careful consideration of the other factor, given that they are intrinsically linked. For example, the study of an influential effect metals has on ApoE should always take into account its lipidation status, which affects its conformation and activity. Conversely, the study of Aβ aggregation in lipid rafts should consider the role metals play in the generation of ROS products and subsequent cell toxicity. Further research is needed to investigate molecular mechanisms that link metals and cholesterol with various players involved in AD pathogenesis. This knowledge is critical for future design and implementation of effective therapeutic strategies to treat AD.

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Interactions of metals and Apolipoprotein E in Alzheimer's disease

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Alzheimer's disease (AD) is the most common form of dementia, which is characterized by the neuropathological accumulation of extracellular amyloid plaques and intracellular neurofibrillary tangles (NFTs). Clinically, patients will endure a gradual erosion of memory and other higher order cognitive functions. Whilst the underlying etiology of the disease remains to be definitively identified, a body of work has developed over the last two decades demonstrating that AD plasma/serum and brain are characterized by a dyshomeostasis in a number of metal ions. Furthermore, these metals (such as zinc, copper and iron) play roles in the regulation of the levels of AD-related proteins, including the amyloid precursor protein (APP) and tau. It is becoming apparent that metals also interact with other proteins, including apolipoprotein E (ApoE). The Apolipoprotein E gene (APOE) is critically associated with AD, with APOE4 representing the strongest genetic risk factor for the development of late-onset AD. In this review we will summarize the evidence supporting a role for metals in the function of ApoE and its consequent role in the pathogenesis of AD.

Keywords: Apolipoprotein E, Alzheimer's disease, zinc, copper, metals

INTRODUCTION

Apolipoprotein E (ApoE) is the predominant apolipoprotein in the brain where it is synthesized and secreted primarily by astrocytes in high-density lipoprotein (HDL)-like particle (Bu, 2009). A primary function of ApoE is to serve as a ligand for the low-density lipoprotein (LDL) receptor family of proteins, which mediate delivery of cholesterol to neurons. That function is essential for axonal growth, synaptic formation and remodeling and all of those events are important for learning, memory formation and neuronal repair (Mauch et al., 2001; Pfrieger, 2003). Decreases in the levels of ApoE or LDL receptors lead to synaptic remodeling impairment and a progressive loss of synapses in the cortex and hippocampus (Mulder et al., 2004; Liu et al., 2010).

ApoE is also a polymorphic protein with three common allele variants: APOE2, APOE3 and APOE4. The APOE4 gene is the strongest and only confirmed genetic risk factor for the development of late onset Alzheimer's disease (LOAD), which enhances the risk level by three times in heterozygous individuals and by twelve times in homozygous individuals (Bertram, 2009). The least frequent APOE2 allele (found in 5–10% of individuals) seems to have a protective effect against the development of AD while the most frequent APOE3 allele (found in 70–80% of the population) represents intermediate risk (Corder et al., 1994; Mahley and Huang, 2006). The structural differences between the three ApoE isoforms is limited to amino acid residues 112 and 158, where either cysteine or arginine is present: ApoE2 (Cys112, Cys158), ApoE3 (Cys112, Arg158) and ApoE4 (Arg112, Arg158; Mahley and Rall, 2000). The single amino acid difference at these two positions affects the structure of ApoE isoforms and their ability to bind lipids, receptors and amyloid beta (Aβ), the latter which is the main constituent of the extracellular plaques found in the AD brain (Zhong and Weisgraber, 2009; Chen et al., 2011; Frieden and Garai, 2012).

The connection between metals, Aβ and abnormal forms of tau (as found in the neurofibrillary tangles (NFT) present in the AD brain) has been investigated extensively in the pathogenesis of AD (Grasso et al., 2012; Greenough et al., 2013; Wärmländer et al., 2013). However, the effects of metallation on ApoE are less well known. In this review, evidence supporting the hypothesis that zinc and copper play a role in the function of ApoE will be covered, along with the key points on the current understanding of the influence of ApoE and metals on the pathogenesis of AD.

APOE AND ITS ROLE IN AD PATHOGENESIS

APOE4 has been found to be associated with an increased prevalence of AD and a lower age of onset. Clinical data shows the frequency of AD and mean onset age are 91% and 68 years old in APOE4 homozygous carriers, 47% and 76 years old in APOE4 heterozygous carriers, and 20% and 84 years old in APOE4 non-carriers (Corder et al., 1993; Rebeck et al., 1993); suggesting that APOE4 genotype confers a significantly higher risk of development of AD with an earlier age of onset in a gene dose-dependent manner. It has also been reported that the prevalence of an E4 allele is considerably higher in mild cognitive impairment (MCI) than in control individuals (Pa et al., 2009); with APOE4 MCI individuals showing poorer memory performance at an

earlier stage in AD compared with non-carriers (Smith et al., 1998). APOE4 can also influence cognition in healthy people. Healthy APOE4 carriers show an accelerated longitudinal decline in memory tests (Caselli et al., 2004, 2007). In the brain, ApoE mediates delivery of cholesterol to neurons, which is essential for axonal growth, synaptic formation and remodeling and all of those events are important for learning, memory formation and neuronal repair (Mauch et al., 2001; Pfrieger, 2003). Astrocytes preferentially degrade ApoE4, leading to reduced ApoE4 secretion and ultimately to reduced brain ApoE levels (Riddell et al., 2008). Taken together therefore, the lack of functional ApoE present in AD is likely to directly contribute to the cognitive impairment seen in this disease.

One of the first pieces of evidence linking ApoE to AD pathology was the observation of ApoE immunoreactivity in extracellular amyloid plaques and NFTs (Namba et al., 1991). It has since been shown that ApoE forms complexes with Aβ and these complexes are thought to influence Aβ deposition and clearance (Wildsmith et al., 2013). Aβ deposition detected by Pittsburgh Compound B positron emission tomography (PIB-PET) follows a strong APOE allele-dependent pattern (E4 > E3 > E2) (Kok et al., 2009; Morris et al., 2010; Castellano et al., 2011). An ApoE isoform-specific effect on the amount of Aβ accumulation as well as in the number of amyloid plaques was also found in amyloid precursor protein (APP) transgenic mice expressing different human ApoE isoforms (E4 > E3 > E2; Fagan et al., 2000, 2002; Fryer et al., 2005b). The mechanisms underlying isoform-specific influences on Aβ aggregation and accumulation in the brains are not fully understood, but it's likely due to their different abilities to clear Aβ (Wildsmith et al., 2013). *In vitro* and *in vivo* studies show that many ApoE receptors are involved in ApoE-mediated Aβ clearance from the brain (Bu, 2009; Kim et al., 2009; Holtzman et al., 2012). A recent study demonstrated that ApoE loses its ability to clear Aβ when ApoE is cleaved at the hinge region of ApoE (Jones et al., 2011). After cleavage, the ApoE-Aβ complex cannot bind to ApoE receptors due to the lack of N-terminal ApoE which contains the binding sites of ApoE receptors. It also has been demonstrated that ApoE4 is more susceptible to the cleavage at hinge region (Jones et al., 2011), increasing the likelihood of the ApoE receptor binding region (N-terminal ApoE) being cleaved, which supports the idea that ApoE4 has the least ability to clear Aβ compared to ApoE2 and ApoE3. Another supportive finding is that ApoE4 clears Aβ at blood brain barrier (BBB) via the very low-density lipoprotein receptor (VLDLR) whereas Aβ is cleared at a higher rate in the presence of ApoE2 and ApoE3 by both VLDLR and lipoprotein related protein 1 (LRP1; Deane and Zlokovic, 2007).

It has also been proposed that the poor stability, clearance and poor lipidation status of ApoE4 accounts for its contribution to an elevated risk for the development of AD. ApoE4 is the least stable of all three ApoE isoforms (Morrow et al., 2002) and has been reported to be preferentially susceptible to proteolytic degradation into cytotoxic fragments (Huang et al., 2001). Much higher levels of ApoE fragments are detected in the brains of AD patients (Huang et al., 2001; Harris et al., 2003; Jones et al., 2011) and these fragments have been shown to damage hippocampal neurons and result in memory impairment (Harris et al., 2003; Andrews-Zwilling et al., 2010).

ApoE4 is a less effective lipid carrier under physiological conditions than ApoE3 or ApoE2 (Michikawa et al., 2000; Hara et al., 2003). Lipidation of ApoE is mediated primarily by ATP-binding cassette A1 (ABCA1) and the lipidation status of ApoE is related to its Aβ-binding properties (Tokuda et al., 2000). Reducing ApoE lipidation status by ablating ABCA1 in APP transgenic mice markedly enhances brain amyloid plaque levels, and conversely, enhancing ApoE lipidation status by the up-regulation of ABCA1 significantly reduced amyloid load (Kim et al., 2009). These results are consistent with the hypothesis that non-lipidated ApoE in the brain can stimulate Aβ aggregation and deposition (Hatters et al., 2006) while lipidated ApoE facilitates the clearance of Aβ and it is much less susceptible to proteolysis than lipid-free ApoE (Weisgraber et al., 1994; Narayanaswami et al., 2004). Some ApoE receptors and ABCA1 appear to influence ApoE expression and lipidation (Hirsch-Reinshagen et al., 2004; Wahrle et al., 2004; Fryer et al., 2005a; Liu et al., 2007; Wahrle et al., 2008).

Based on the pathological definition of the disease, AD is associated not only with the abnormal accumulation of amyloid plaques, but also with the accumulation of NFTs which form intracellularly and are composed primarily of aggregated phosphorylated and acetylated tau (Iqbal et al., 2010). Tau primarily stabilizes microtubules, and its aggregation in AD causes deficits through a loss-of-function mechanism. Recently, evidence has also shown that when it is abnormally modified, tau becomes enriched in dendritic spines where it can interfere with neurotransmission (Morris et al., 2011). Evidence from *in vivo* and *in vitro* studies indicates that ApoE3 and ApoE4 function differently with respect to the phosphorylation and aggregation of tau. ApoE3 binds to the microtubule-binding repeat regions of tau with its N-terminal domain (Strittmatter et al., 1994), however, this interaction can be impeded by the phosphorylation of tau. On the other hand, whilst ApoE4 has been shown to not significantly interact with tau (Strittmatter et al., 1994), it does increase tau phosphorylation and accumulation in the neuronal soma and dendrites, facilitating the formation of NFTs during aging and AD progression (Harris et al., 2003; Brecht et al., 2004; Andrews-Zwilling et al., 2010). One proposed mechanism is that ApoE4 can activate the extracellular signal-regulated kinases (ERK) pathway in AD brains and lead to tau phosphorylation, which is likely modulated by zinc (Harris et al., 2004).

THE INVOLVEMENT OF METALS IN AD PATHOGENESIS

The formation of the classical neuropathological features of AD are not only influenced by APOE genotype, but also mediated or triggered by an imbalance of metal ions. Altered metal homeostasis has been demonstrated in the brain and plasma/serum in AD patients. Compared with age-matched control, AD patients show elevated zinc and copper in cerebrospinal fluid (CSF; Hozumi et al., 2011), whereas plasma and serum zinc was found to be lower (Vural et al., 2010). Free copper in the blood of AD patients is substantially higher than controls (Squitti et al., 2014). In addition, the concentration of zinc, copper, and iron in brain parenchyma (350 μM, 70μM, and 340 μM, respectively) are further elevated in AD patients (800 μM, 300 μM, and 700 μM,

respectively; Lovell et al., 1998). These metals are also enriched in both senile plaques and NFTs (Ayton et al., 2013). Zinc, along with copper and iron (released during neural transmission), directly bind to Aβ and accelerate its aggregation and accumulation into amyloid plaques (Morante, 2008; Altamura and Muckenthaler, 2009). Therefore, the zinc, copper and iron sequestration into amyloid deposits is thought to result in a loss of cellular and synaptic metals. The loss of synaptic zinc is particularly relevant to the maintenance of normal cognition. An important regulator of synaptic zinc is the zinc transporter-3 (ZnT3) protein which is essential for loading zinc into synaptic vesicles (Linkous et al., 2008). It has been shown that ZnT3 levels decrease with aging in the brains of both mice and humans and are reduced even further in the brains of AD patients (Adlard et al., 2010). ZnT3 KO mice display defects in learning and memory at 6 months of age, and the authors suggest that these mice provide a phenocopy for the synaptic and memory deficits of AD (Adlard et al., 2010). In addition, copper is another important metal involved in the cognitive decline in AD. Free copper in blood is potentially toxic, particularly if the free copper pool expands, as it does in Wilson's disease (Brewer et al., 1998). More importantly, there's a strong positive correlation between the level of free copper and the severity of cognitive loss in AD (Squitti et al., 2006), which can be observed over a given period of time (Squitti et al., 2009).

Although we still do not know if the metal ion dyshomeostasis present in AD is a cause or consequence of the disease, there is a growing body of evidence showing a direct correlation between metal ions and key AD-related key proteins. Both zinc and copper facilitate Aβ aggregation. Aβ tends to form fibrils in the presence of zinc, whereas in the presence of copper it prefers to form oligomers (Tõugu et al., 2009). The copper-Aβ oligomer complex has been shown to be more toxic than the zinc Aβ fibrils, which in some conditions actually confer protection (Rosenblum, 2014). Metal dyshomeostasis is also involved in the regulation of other AD-related proteins, like APP and tau. Zinc, for example, regulates the activity of some of the secretases involved in the processing and function of APP, with α-secretase activity up-regulated by zinc indirectly through a disintegrin and metalloproteinase (ADAM; Lammich et al., 1999); however, the activity of the γ-secretase complex is inhibited by zinc (Hoke et al., 2005). The copper binding domain of APP (histidine residues 149 and 151) is crucial for APP stability and metabolism (Spoerri et al., 2012) and copper enhances APP dimerization and promotes Aβ production (Noda et al., 2013). Consistent with this concept, APP knockout mice have elevated copper levels in the cerebral corte (White et al., 1999). These studies show that APP may directly influence copper homeostasis, and its interactions with copper may be also neurotoxic.

Metals are also involved in tau pathology, and are enriched in tangle-bearing neurons (Sayre et al., 2000). Synaptically released zinc induces tau hyper-phosphorylation through pathways including Src-dependent, glycogen synthase kinase 3β (GSK3β) and ERK pathways (Lei et al., 2011; Xiong et al., 2013). Copper directly binds to tau (Martic et al., 2013) and regulates its aggregation and phosphorylation (Squitti et al., 2006; Zhou et al., 2007). Aberrant activation of cyclin-dependent kinase 5 (CDK5) was found to be correlated with the tau pathology after chronic copper exposure in a mouse model of AD (Kitazawa et al., 2009). Iron binding to the hyper-phosphorylated tau protein also facilitates the formation of the NFTs (Altamura and Muckenthaler, 2009) and the iron chelator, deferoxamine (DFO), decreases iron-induced activities of CDK5 and GSK3β and tau phosphorylation (Guo et al., 2013). Thus, the development of the two most prominent pathological features of the AD brain, plaques and tangles, are likely to be mediated by metal ions. This area has been extensively reviewed in the past (Adlard and Bush, 2006; Bush and Tanzi, 2008; Duce and Bush, 2010; Hung et al., 2010; Ayton et al., 2013), and supports the notion of the regulation of metal homeostasis as a promising area of investigation for future AD therapeutics.

EVIDENCE SUPPORTING THE LINK BETWEEN METALS AND ApoE

The mechanism by which ApoE4 is associated with AD is still unknown; however, an emerging linkage between metals and ApoE might give a clue. Evidence shows that ApoE isoforms bind to metals such as zinc, copper and iron (that are also involved in the pathogenesis of AD), with the affinity for copper being greater than for iron and zinc (Miyata and Smith, 1996). The precise binding sites for metals on ApoE have yet to be determined, but the four-helix bundle of the N-terminus may allow a coordination of metals (Miyata and Smith, 1996). The metal sequestration properties of ApoE might present metals to Aβ peptides, leading to amyloid deposition or it might account for the antioxidant function of ApoE in AD development. Furthermore, studies support the notion that ApoE2 has the highest affinity for zinc and ApoE4 has the lowest. This is likely a result of structural differences amongst the three isoforms. Cysteine is a strong ligand for zinc, arginine is not (Karlin and Zhu, 1997), so the affinity for zinc is predicted to be greatest for ApoE2 which has cysteine residues at amino acid position 112 and 158 and weakest for ApoE4 which lacks cysteine residues. This likelihood is supported by the results showing that ApoE protects Aβ from zinc-induced precipitation in the order of ApoE2 > ApoE3 > ApoE4 (Moir et al., 1999). It is also speculated that ApoE4 has a reduced copper binding capacity because of its lack of cysteine residues (Hung et al., 2013). Although direct evidence for the metal:ApoE interaction needs to be demonstrated, these data clearly provide a potentially important avenue of investigation for understanding the mechanism underlying the higher risk of AD in APOE4 carriers. Metal ions, such as zinc, play an essential role in stabilizing protein structures and contributing to protein function (Wang et al., 2010). We further speculate that metal binding might help to stabilize ApoE in an order of E2 > E3 > E4 in the proteolytic process, which leads to less ApoE4 and more ApoE4 fragments. This is consistent with the previous findings that APOE4 carriers have less full-length ApoE but more ApoE fragments in brain parenchyma and plasma than APOE2 carriers (Riddell et al., 2008; Gupta et al., 2011); with decreased ApoE levels in APOE4 carriers considered an important factor for AD onset/development (Verghese et al., 2011; Holtzman et al., 2012). Therefore, the stability of ApoE may be affected by metals, and this may help account for the

differential effect of the three ApoE alleles on the development of AD.

There are also a number of studies that have investigated the effect of metals on the expression levels of apolipoproteins. The gene expression of apolipoprotein A and apolipoprotein B has been found to be regulated by zinc and copper (Zhang et al., 1995; Reaves et al., 2000; Cui et al., 2002) and more importantly, another AD-related apolipoprotein, clusterin (apolipoprotein J; Jones, 2010), is increased after zinc exposure (Trougakos et al., 2006). It is possible that altered metal levels in AD patients might affect the expression/transportation of apolipoproteins, including ApoE. Alternatively, metals might have different effects on the expression/transportation of the three ApoE isoforms, accounting for the different risk levels for AD among the three allele carriers.

Finally, studies have shown that ApoE can regulate synaptic zinc and glutamate levels in the hippocampus. The depletion of ApoE leads to a reduced expression of ZnT3, in parallel with a reduction in synaptic zinc content in APOE knockout mice, suggesting the ApoE modulates zinc homeostasis in the brain (Lee et al., 2010). The synaptic zinc is required for long-time potentiation (LTP) and is critical for the proper functioning of

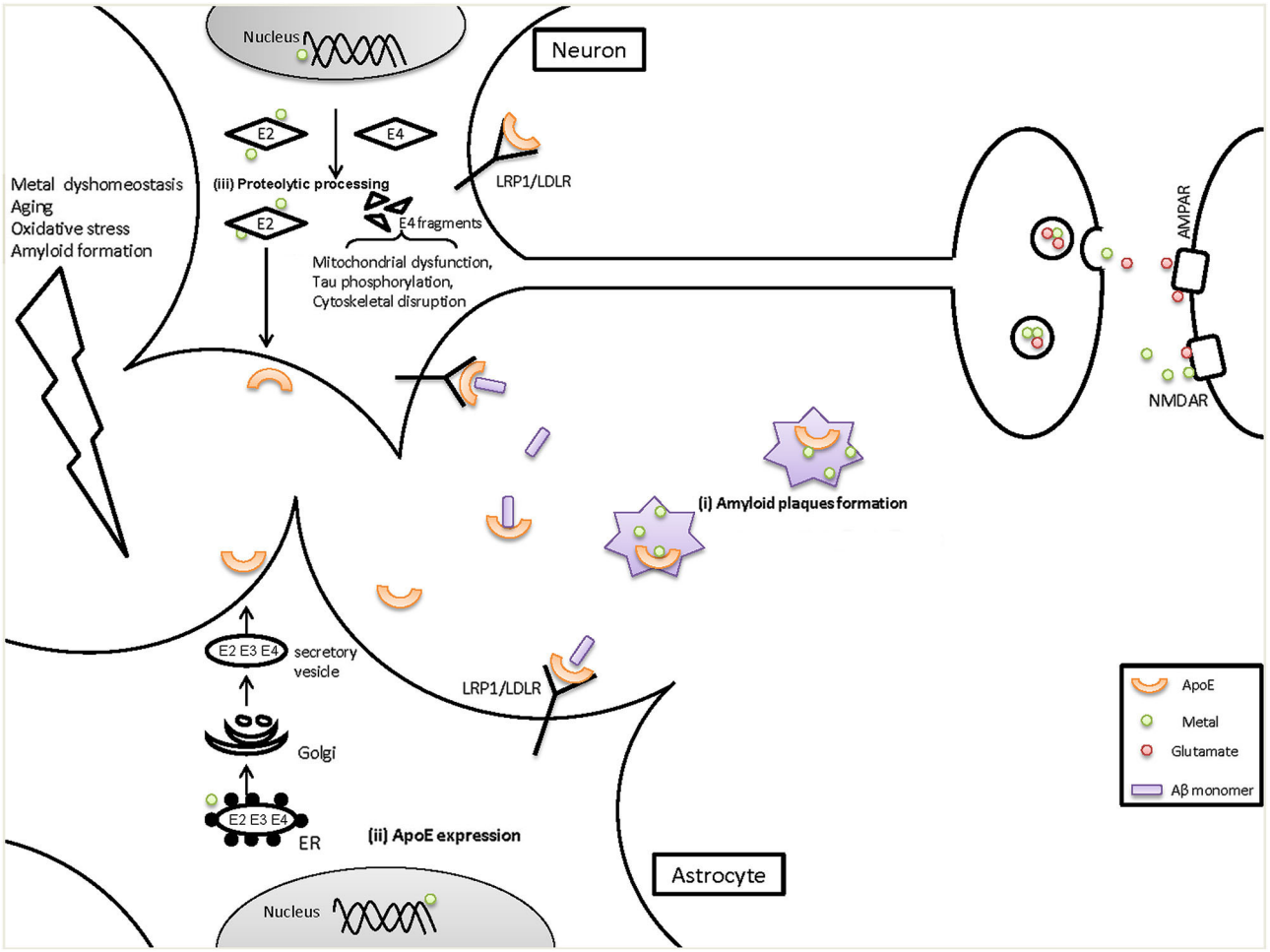

FIGURE 1 | The potential role of metals on ApoE in the pathogenesis of AD. ApoE4 is a risk factor for the onset and development of AD, but the mechanisms are not fully understood. We have provided evidence for several points of interaction between metals and ApoE in AD, as shown here. (i) It has been demonstrated that both metals (zinc, copper and iron) and ApoE accumulate in the amyloid plaques, which could cause metal dyshomeostasis (less functional metals available) and decreased ApoE levels in the brain. (ii) Metal dyshomeostasis in AD patients might influence the expression of ApoE in astrocytes which are the main source of brain ApoE, resulting in decreased ApoE levels. ApoE levels can be affected by transcriptional level in nucleus and/or within rough endoplasmic reticulum (ER) where ApoE is synthesized. Reduced ApoE levels would contribute to AD pathogenesis as ApoE can mediate Aβ clearance through LRP1/LDLR and helps to maintain the vesicular zinc and glutamate levels at synapse. (iii) In response to aging, Oxidative stress and amyloid formation, neurons turn on or increase their expression of ApoE. However, neuron-ApoE is cleaved and generate C-terminal truncated fragments. ApoE4 is much more susceptible than ApoE2 and ApoE3. In the proteolysis of ApoE, metals bind to ApoE2 and stabilize its intact structure whereas ApoE4, which has a decreased affinity for metals, tends to be degraded to fragments. ApoE4 fragments can induce severe impairments to mitochondrial function and to the cytoskeleton, leading to neurodegeneration. Additionally, more ApoE2 is secreted in the brain compared with ApoE4, which will then impact various brain functions such as maintaining the normal levels of synaptic zinc and glutamate. In contrast, decreased levels of ApoE4 would reduce their levels and impair hippocampal LTP and then cause cognitive damage.

hippocampal circuitry in health and disease (Pan et al., 2011). So decreased ApoE levels would lead to synaptic zinc deficiency and cognitive impairments. Studies with human APOE Targeted Replacement (TR) mice demonstrated that compared to APOE2 and APOE3 TR mice, APOE4 TR mice have decreased levels of glutamate (Dumanis et al., 2013), which is an excitatory neurotransmitter co-released with zinc at the synapse during neuronal activity, and which is important to maintain normal hippocampal LTP and cognitive function (Paoletti et al., 2009). Thus, this may contribute to the increased risk of neurodegeneration associated with APOE4 carriers.

Taken together, these findings support an interaction between metals and ApoE that may be important in the pathogenesis of AD (**Figure 1**). In this review we summarized the evidence showing that metals bind to ApoE in an isoform-specific way, and that ApoE modulates metal homeostasis in the brain. There is also the possibility that metals may regulate ApoE levels. However, some key issues need to be directly addressed to provide definitive evidence for a metal:ApoE interaction, including the following: (1) Do metals (apart from zinc) have differential affinities for the three ApoE isoforms?; (2) Is the stability or degradation of ApoE isoforms affected by metal binding?; (3) Are changes in neuron- and astrocyte-specific ApoE expression/transportation caused by altered metal levels in AD? If so, what's the underlying mechanism? Thus, further study is required for an integrated understanding of the interactions between metals and ApoE, and how they act together in the development and progression of AD.

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Spatial memory deficits in a mouse model of late-onset Alzheimer's disease are caused by zinc supplementation and correlate with amyloid-beta levels

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Much of the research in Alzheimer's disease (AD) that uses mouse models focuses on the early-onset form of the disease, which accounts for less than 5% of cases. In contrast, this study used a late-onset AD model to examine the interaction between increased dietary zinc (Zn) and the apolipoprotein E (ApoE) gene. ApoE ε4 is overrepresented in late-onset AD and enhances Zn binding to amyloid-β (Aβ). This study sought to determine if elevated dietary Zn would impair spatial memory in CRND8 mice (CRND8), as well as mice who carry both the mutated human amyloid precursor protein (APP) and ApoE ε4 genes (CRND8/E4). Mice were provided with either lab tap water or water enhanced with 10 ppm Zn ($ZnCO_3$) for 4 months. At 6 months of age, spatial memory was measured by the Barnes maze. CRND8 mice exhibited significant memory deficits compared to WT mice, as shown by an increased latency to reach the escape box. For the CRND8/E4, but not the CRND8 mice, those given Zn water made significantly more errors than those on lab water. During the probe trial for the WT group, those on Zn water spent significantly less time in the target quadrant than those on lab water. These data suggest that increased dietary Zn can significantly impair spatial memory in CRND8/E4. WT mice given Zn water were also impaired on the 24-h probe trial when compared to lab water WTs. Within the CRND8/E4 group only, levels of soluble Aβ were significantly correlated with average primary latencies. Within the Zn-treated CRND8/E4 group, there was a significant correlation between insoluble Aβ and average primary errors. Levels of the zinc transporter 3, ZnT3, were negatively correlated with soluble Aβ ($p < 0.01$). These findings are particularly relevant because increased intake of dietary supplements, such as Zn, are common in the elderly—a population already at risk for AD. Given the effects observed in the CRND8/E4 mice, ApoE status should be taken into consideration when evaluating the efficacy of therapies targeting metals.

Keywords: apolipoprotein E, hAPP, CRND8, transgenic mice, Barnes maze, metals, ZnT3, copper

INTRODUCTION

Alzheimer's disease (AD) is the primary cause of dementia in the elderly and currently affects more than five million Americans (Alzheimer's Association, 2013). The present research focuses on how the biometals, such as zinc (Zn), copper (Cu), and iron (Fe), interact with amyloid beta (Aβ)—the key constituent of the plaques that are characteristic of the disease, which mediates plaque formation and consequent oxidative damage.

Zn, Cu, and Fe are found in high concentrations in and around amyloid plaques (Lovell et al., 1998; Maynard et al., 2005), consistent with the notion that Aβ is a metalloprotein that possesses binding sites for both Zn and Cu (Bush et al., 1993; Hesse et al., 1994). Zn has been shown to be particularly effective in promoting Aβ aggregation (Bush et al., 1993), and the sequestration of Zn by Aβ leads to an intracellular deficiency of this metal (Grabrucker et al., 2011). Cu, on the other hand, causes a shift in the processing of the amyloid precursor protein (APP) towards the non-amyloidogenic pathway (Borchard et al., 1999) but once Aβ is present, it binds both Cu and Fe. Therefore, Cu and Fe binding is problematic as these metals are redox-active and Aβ reduces both metals, resulting in the production of hydrogen peroxide (H_2O_2; Huang et al., 1999). H_2O_2 further reacts with the reduced metal to produce hydroxyl radicals that can cause oxidative damage (Bush, 2003). Both aging and plaque-related inflammation may promote a more acidic brain environment, which is conducive to Fe and/or Cu binding (Atwood et al., 1998).

Evidence suggests that Zn is crucial for Aβ deposition, and the presence of Zn *in vitro* prevents the proteolytic degradation of $Aβ_{1-42}$ by matrix metalloprotease 2 (Crouch et al., 2011). Zn also inhibits the activity of alpha-secretase and promotes gamma- and beta-secretase activity (Capasso et al., 2005). The zinc transporter, ZnT3, is responsible for loading zinc into the synaptic vesicles

(Palmiter et al., 1996; Cole et al., 1999; Linkous et al., 2008). Eliminating synaptic Zn by genetic ablation of zinc transporter 3 (ZnT3) in an AD mouse model resulted in a 50% reduction in amyloid (Lee et al., 2002). ZnT3 levels, however, have been shown to decrease as a function of age and AD (Adlard et al., 2010). Interestingly, Bjorklund et al. (2012) found that in brains exhibiting AD neuropathology, ZnT3 levels were lowest in those patients who received a diagnosis of AD, while those who did not receive a diagnosis had ZnT3 levels similar to those of healthy controls.

Given the complexity of biometals in AD, the behavioral outcome due to changes in metal levels is difficult to predict, but this has been studied in various mouse models. Long-term dietary Zn enhancement in transgenic (Tg) mouse models of AD (TgCRND8, Tg2576, and APP/presenilin 1 (PS1) mouse models) has been shown to result in cognitive deficits (Linkous et al., 2009; Railey et al., 2011), as well as an increase in both brain Zn levels and the number and size of Zn-positive plaques in the cortex and hippocampus (Wang et al., 2010). Other findings have found that a severe Zn deficiency (<10 ppm Zn) enlarged amyloid plaques (Stoltenberg et al., 2007); thus either an overabundance or deficiency in Zn can potentiate amyloid deposition.

The observations derived from these early-onset mouse models may be of diminished utility for the majority of AD cases that are classified as late-onset (manifest after age 65 years) (Harvey et al., 2003). The emphasis on early-onset genetics dominating AD research is due to the strong relationship between those genes and development of the disease. Genetic linkages to the late-onset form of AD are less specific, but the ε4 allele of the apolipoprotein E (ApoE) gene has been identified as a major risk factor (Saunders et al., 1993; Blacker et al., 1998; Huang and Mucke, 2012). The ApoE gene has three alleles (ε2, ε3, and ε4) resulting in three isoforms of the protein (Zannis et al., 1981), with ApoE ε4 overrepresented in patients with late-onset AD (Weisgraber and Mahley, 1996). While ApoE ε4 is considered a susceptibility factor it does not guarantee the development of disease (Weisgraber and Mahley, 1996). The exact mode of action of ApoE ε4 in AD remains elusive. One proposed mechanism is the interaction between ApoE ε4 and Aβ, which may increase plaques and/or impede Aβ clearance (Mahley et al., 2006). Amongst those with AD, ApoE ε4 carriers typically have a larger number of plaques than ApoE ε3 carriers (Schmechel et al., 1993). Similarly, ApoE ε4 mice that overexpress human APP have an elevation in Aβ plaque deposition, reduced numbers of presynaptic terminals, and an impairment in learning and memory tasks as compared to their ε3 counterparts (Huang, 2006).

The ApoE alleles differ at two residues: ε2 (Cys^{112}, Cys^{158}), ε3 (Cys^{112}, Arg^{158}), and ε4 (Arg^{112}, Arg^{158}) (Rebeck et al., 2002). Cysteine binds to Zn and Cu with high affinity and the Zn binding site on APP is located in a cysteine-rich area (Bush and Tanzi, 2002). The increased number of cysteine residues in ε2 and ε3 correlates with their ability to bind zinc in the synaptic cleft and may contribute to the protective effect of these alleles by preventing zinc binding to Aβ (Lee et al., 2010). ApoE ε4 is also implicated in the formation of neurofibrillary tangles (NFTs). It may increase the phosphorylation of the tau protein (Harris et al., 2004) and Zn may be a key modulator of this process (Craddock et al., 2012). Through their relatively high-binding affinity for zinc, Aβ plaques will cause a reduction in intracellular levels of zinc that will in turn lead to a destabilization of microtubules and subsequent liberation of tau (Craddock et al., 2012). These findings suggest that Zn and ApoE ε4 may act together to affect multiple processes in AD through the promotion of NFT formation and increased Aβ deposition, which may be due to increased Zn binding in the presence of ApoE ε4.

In order to examine the effect of excess zinc in the more prevalent form of AD we have developed a late-onset model, examining two types of Tg mice with the (1) humanized ApoE ε4 and mutated hAPP cross (CRND8/E4); or (2) those only with mutated hAPP (CRND8), and investigated the effect of Zn supplementation on spatial memory in these mice. Protein levels of soluble and insoluble Aβ, and ZnT3 were measured to elucidate molecular mechanisms that may be associated with excess dietary zinc in AD.

MATERIALS AND METHODS

SUBJECTS

To address the primary research questions of the study, the creation of a mouse strain modeling late-onset AD was necessary. The CRND8/E4 experimental animals were obtained by breeding CRND8 males (University of Toronto) with female mice who were homozygous for the human ApoE ε4 knock-in and the knock-out of murine ApoE (Jackson Labs). Resultant CRND8/E4 were therefore heterozygous for human ApoE ε4 and murine ApoE in addition to being hemizygous for the hAPP mutation. Tg CRND8 mice carry a mutant form of APP 695 containing both the Swedish (KM670/671NL) and Indiana (V717F) mutations on a hybrid C3H/B6 genetic background and exhibit extensive amyloid deposition by 3 months of age. There is a potent increase in $A\beta_{42}$ at around 10 weeks of age in this model making the $A\beta_{42}$ to $A\beta_{40}$ ratio 5:1 (Chishti et al., 2001). The Tg1HolApoe^{tm1unc}/J strain expresses human ApoE ε4 under the direction of human glial fibrillary acidic protein (GFAP). Experimental WT mice were those littermates who did not inherit the hAPP transgene from the cross between CRND8 males and WT females (C3H/B6). CRND8 mice were those offspring who inherited the hAPP transgene from the CRND8 males that were bred with WT females.

Mice were bred in three groups in order to facilitate behavioral testing. The offspring were group housed with same-sex littermates with 2–4 animals per cage. Each cage contained an igloo, nylabone, and running wheel (Bio-serv). The mouse colony was maintained with a 12-h light/dark cycle. Food (Harlan diet 7012) and water were provided *ad libidum*.

Behavioral group numbers are shown in **Table 1**. This study used both male and female mice, and analyses were conducted to determine possible sex differences. Zn supplementation began at 6 weeks of age and continued throughout behavioral testing. Behavioral testing began at 5 months of age and mice were sacrificed within 10 days following the conclusion of all behavioral testing (approximately 6 months of age). Mice were euthanized by CO_2 asphyxiation and brains were removed and stored in a −80° freezer for later analysis. There were no differences in body weight on the day of euthanasia when evaluated by water condition. All experiments and procedures performed on these mice were

Table 1 | Group distributions and male to female ratio for Barnes maze (BM) and Western blot (WB) analyses.

Group	BM *N* =	BM m:f	WB *N* =	WB m:f
WT + Lab Water	12	7:5	0	n/a
WT + Zn	12	6:6	0	n/a
CRND8/E4 + Lab Water	11	6:5	10	5:5
CRND8/E4 + Zn	11	5:6	11	5:6
CRND8 + Lab Water	12	8:4	10	6:4
CRND8 + Zn	11	9:2	9	7:2

approved by the George Mason University Institutional Animal Care and Use Committee.

WATER PREPARATION

The 10 ppm $ZnCO_3$ was supplemented to laboratory tap water (herein referred to as Zn water) and was prepared using a starting solution of 10,000 mg/L of zinc dissolved in 5% HNO_3. The final solution was buffered with Na_2CO_3 to bring it to a pH of 7.0 (Linkous et al., 2009). All waters were made and stored in separate polycarbonate carboys and dispensed to animals in 500 mL glass bottles. Water was analyzed regularly using inductively coupled plasma-optical emission spectroscopy and ion chromatography at the United States Geological Survey (USGS, Reston, VA) to confirm metal content. Laboratory tap water without any supplement, herein referred to as lab water, was used for the control group. Water consumption was measured every week and there were no significant differences when evaluated by water condition.

BARNES MAZE

The Barnes maze (BM) is designed to assess spatial memory. The maze (Med Associates) consisted of an elevated platform 122 cm in diameter (91 cm above the ground) with 40 equidistant holes located around the edges. The maze's surface color was white. An escape box was located under one of the holes and four distinctive extra-maze reference cues were placed around the maze, with one on each side (large black letters on a white background). Testing took place in a brightly illuminated room with a 300-watt light situated directly over the maze in order to make the maze surface undesirable, given the natural tendency of mice to prefer dark enclosed areas over bright/open areas. All animals started each trial from a dark enclosed start box positioned in the center of the maze. The orientation of the start chamber was varied for each trial. The maze was cleaned with 70% ethanol solution between all trials to eliminate scent trails.

Procedure

BM testing took place over seven consecutive days with the 24-h probe trial occurring on the seventh day.

Habituation: On the first day of testing all mice were given two habituation trials. The mice were placed in a start chamber in the center of the maze for 10 s after which time the start box was lifted and they were gently guided to the escape box and allowed to enter on their own. Once inside, mice stayed in the escape box for 30 s before being returned to their home cage for a 2 min inter-trial interval.

Acquisition: Days 2–6 were acquisition days. The mice received three trials per day with a 15 min inter-trial interval. The trials on the acquisition days ended when the animal entered the escape box or when the maximum trial length of 3 min was reached. Mice were allowed 30 s in the escape box after locating it. If the animal did not find the escape box in the allotted 3 min trial period they were gently guided to the escape box and allowed 30 s inside. Primary latency (amount of time elapsed prior to first reaching target hole) and primary errors (number of errors made prior to reaching target hole) were measured.

Probe Trial: On day 7 the animals received one probe trial 24 h after the last training trial. The escape box was removed from the target hole that had been previously learned and the mice were given 3 min to explore the maze. Percent of time in the correct quadrant was measured.

TISSUE PREPARATION

Frozen brain samples were minced into small pieces and homogenized in 1 mL of a mixture of PBS and protease/phosphatase inhibitor cocktail (Sigma-Aldrich). The homogenate was then centrifuged at 4°C at 24,000 RPM for 1 h. Soluble and insoluble fractions were separated. The insoluble fractions were additionally reconstituted in 1 mL of a mixture of PBS and protease/phosphatase inhibitors. Both soluble and insoluble fractions were then lyophilized for approximately 12 h until fully dehydrated.

WESTERN BLOT ANALYSES

For Western blot (WB) analysis, lyophilized insoluble fractions were reconstituted in 250 μL of PBS and protease/phosphatase inhibitor cocktail. Soluble fractions were reconstituted in 50 μL of PBS and cocktail. A BCA protein assay was done to determine protein concentration for each sample. Fifteen microliters of samples were heated at 90°C for 10 min and then spun down and loaded onto Criterion XT precast 4–12% gels. After the gels were run and transferred to a PVDF membrane they were blocked in 5% tris buffered saline with Tween20 (TBST) skim milk for 1 h. The membranes were then briefly rinsed with TBST and incubated overnight at 4°C with the following primary antibodies: WO2 (1:2000; in-house antibody prepared at The Florey Institute of Neuroscience and Mental Health); ZnT3 (1:10,000, in-house antibody prepared at The Florey Institute of Neuroscience and Mental Health); and GAPDH (1:10,000; Millipore Cat: MAB 374). After three washes of TBST for 5–10 min, secondary antibody incubation was performed (polyclonal rabbit anti-mouse IgG conjugated to horseradish peroxidase (HRP); 1:10,000; Dako P0260) at room temperature for 1 h. Three additional washes of TBST were done before enhanced chemiluminescent substrate was added for imaging using Multigauge software (Fujifilm). After imaging, the membranes were briefly rinsed with TBST and then the membranes were stripped using Re-blot Plus Strong solution (Millipore). The bands were scanned and the intensities of the bands were measured using the Fuji Reader LAS-4000. Protein levels for WO2 and ZnT3 were normalized to GAPDH (**Figure 5**).

STATISTICAL ANALYSES

Statistical differences among different genotypes and water conditions against measured behavioral parameters were determined by repeated-measures analysis of variance (RMANOVAs). When necessary, Greenhouse-Geisser estimates of sphericity were used to correct for degrees of freedom. Bonferroni *post-hoc* tests were used to evaluate group differences. Correlations between protein levels and measured behavioral parameters were calculated using the Pearson correlation coefficient (r). Data are expressed as mean ± SEM. A significance level of $p < 0.05$ was used for all analyses. Trends were reported at a level of $p < 0.10$.

RESULTS

BARNES MAZE BEHAVIOR

Three genotypes (WT, CRND8/E4, CRND8) × two water conditions (lab water and zinc water) mixed design RMANOVAs were performed for measures of primary latency, primary errors, and percent of time in the target quadrant for the 24-h probe trial. Between group variables were genotype and water type. The within subjects variable was testing day (five levels). Sex was entered as a covariate for all statistical analyses.

On days 1–5, there was a significant within-subjects effect of day for primary latency ($F_{(3.03,172.43)} = 44.29$, $p < 0.001$) indicating overall improvement across testing days for all groups. There was a significant effect on primary latency between genotypes ($F_{(2,57)} = 4.124$, $p < 0.05$). A Bonferroni *post-hoc* test revealed that primary latencies across days 1–5 were significantly shorter for WT mice as compared to CRND8 mice ($p < 0.05$), but not compared to CRND8/E4. The CRND8/E4 were also not significantly different from CRND8 mice (**Figure 1A**). On average CRND8/E4 animals had latencies that were shorter than CRND8 animals, but longer than WT animals. For primary latency, there were no main effects of water condition.

On days 1–5, there was a significant within-subjects effect of day for primary errors ($F_{(3.39,200.0)} = 23.32$, $p < 0.001$). For primary errors there were no significant main effects of water or genotype across all groups on days 1–5 or during probe trials (**Figure 1B**). Further analyses within genotypes revealed no significant differences between Zn water and lab water in WT and CRND8 mice (**Figures 2A,B**). However, within the CRND8/E4 genotype there was a significant main effect of water. CRND8/E4 mice on Zn water made significantly more primary errors than those CRND8/E4 on lab water on days 2–3 ($F_{(1,17)} = 5.64$, $p < 0.05$; **Figure 2C**).

There were no significant main effects of genotype on the 24-h probe trial. There was, however, a significant main effect of water condition within the WT group. Zn water WT mice spent significantly less time than lab water WT mice in the target (correct) quadrant (23% vs. 41% respectively) ($F_{(1,21)} = 3.28$, $p < 0.05$; **Figure 3**). Both CRND8/E4 and CRND8 groups raised on either Zn or lab water scored close to chance during the probe trial.

In summary, there were significant main effects of water condition within the CRND8/E4 and WT genotypes, but not in the CRND8 group. As predicted, Zn water CRND8/E4 mice had higher primary errors in the BM compared to those on lab water. Significant genotype differences did exist between CRND8 mice compared to WT mice on measures of primary latency, as was expected. Mice carrying both the ApoE ε4 and mutated hAPP genes were not more impaired than the CRND8 transgene. However, on the primary error measure, CRND8/E4 mice on Zn water had the highest number of primary errors. WT mice on Zn water were spent significantly less time in the target quadrant when compared to those WTs on lab water.

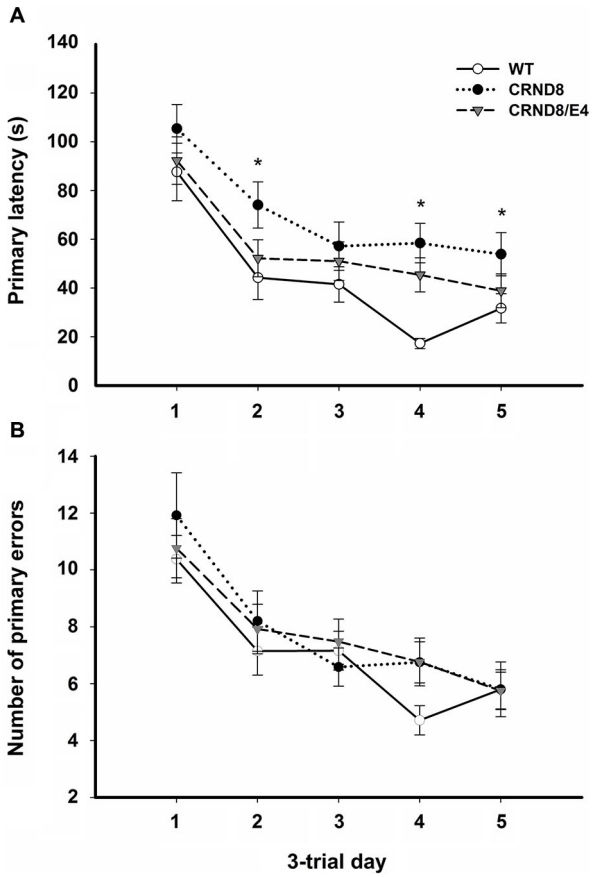

FIGURE 1 | Average primary latency and primary errors across genotypes in the Barnes maze, regardless of water condition. **(A)** Average primary latencies were significantly shorter for WT mice as compared to CRND8 mice ($p < 0.05$), but not CRND8/E4s. Overall, there was a significant decrease across days in primary latency for all genotypes ($p < 0.001$). **(B)** Average primary errors in the Barnes maze. Primary errors were not significantly different between any of the genotypes. All groups did, however, improve significantly over time ($p < 0.001$). Points are mean ± SEM. * denotes $p < 0.05$.

PROTEIN ANALYSIS

Relative levels of soluble Aβ, insoluble Aβ, and ZnT3 were obtained from WB analysis. This analysis was conducted only on Tg mice (CRND8 and CRND8/E4). The control for both Tg groups was a CRND8/E4 in the lab water group which may explain why all significant correlations with behavior were seen only in the CRND8/E4 group; all protein correlations presented here are within that genotype only. There were no significant main effects for water and genotype. There were no significant main effects on levels of ZnT3. There were also no

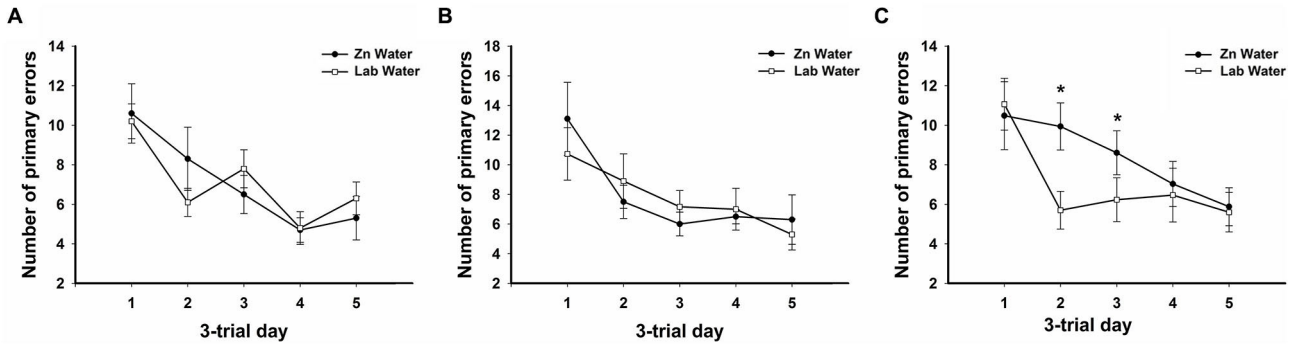

FIGURE 2 | Average primary errors for all genotypes. There were no significant differences between Zn and Lab water **(A)** WT and **(B)** CRND8 mice. **(C)** Within the CRND8/E4 mice, however, those on Zn water had significantly more primary errors than the lab water group on days 2 and 3 ($p < 0.05$). Points are mean ± SEM. * denotes $p < 0.05$.

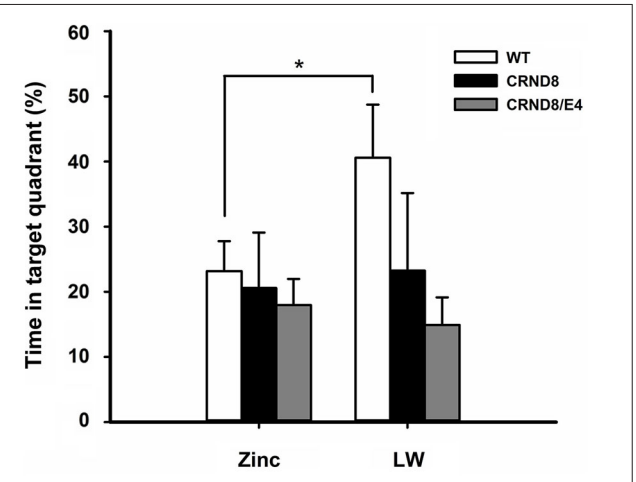

FIGURE 3 | Percent of time spent in the target quadrant in the Barnes maze. WT mice on Zn water spent significantly less time in the target quadrant during the 24-h probe trial when compared to lab water WT mice ($p < 0.05$). Tg mice consistently scored at or below chance levels. Points are mean ± SEM. * denotes $p < 0.05$.

significant main effects for soluble Aβ or insoluble Aβ. Regardless of water condition, however, levels of soluble Aβ were significantly correlated with the average primary latencies for day 3 ($r = 0.657$, $p < 0.01$) and day 4 ($r = 0.463$, $p < 0.05$; **Figure 4A**). Those mice on Zn water had levels of insoluble Aβ that were significantly correlated with average primary errors for day 3 ($r = 0.754$, $p < 0.01$) and day 5 ($r = 0.658$, $p < 0.05$; **Figure 4B**). Those on control lab water had soluble Aβ levels which significantly correlated with the average primary latency on day 3 ($r = 0.712$, $p < 0.05$; **Figure 4C**). Mice on Zn water had levels of soluble Aβ that were negatively correlated with levels of ZnT3 ($r = -0.791$, $p < 0.01$; **Figure 4D**).

DISCUSSION

These data show that the performance of the mice modeling late-onset AD depended significantly upon the presence or absence of Zn in the drinking water. Overall, CRND8/E4 mice did not perform worse than the CRND8 mice, as had initially been predicted. However, CRND8/E4 mice raised on Zn water made the most errors in the BM of all six groups, and also had significantly increased errors compared to those raised on lab water. On the primary latency measure, the CRND8 mice performed significantly worse than the WT mice. The CRND8/E4 group's performance was intermediate between the other groups, and did not differ significantly from either. In contrast to the effect on primary errors, there was no significant effect of Zn water on primary latency. The observation that primary errors, and not primary latencies, detected a Zn effect in the late-onset mouse model is consistent with O'Leary and Brown (2013) who have suggested that errors are a more sensitive measure of memory than latency in the Barnes maze.

The effect on errors may be due to enhanced Zn binding to Aβ when in the presence of ApoE ε4. ApoE ε4 has the weakest affinity for binding Zn, due to the amino acid substitution of arginine which is not a ligand for Zn (Lee et al., 2010). In contrast, the ApoE ε2 and ApoE ε3 isoforms with cysteine residues strongly bind Zn, and may prevent it from binding with Aβ, which could in turn prevent oligomerization and deposition (Lee et al., 2010). While there are numerous *in vitro* studies investigating the association between Zn and ApoE ε4, this is a novel behavioral study examining the relationship between the two in a mouse model of late-onset AD.

Zn water did not result in significant latency deficits in the CRND8 and WT groups. For the WT group, Zn significantly reduced the percent time spent in the target quadrant for the 24-h probe trial; thus the WT mice on Zn water were impaired on the most difficult memory task. Neither the CRND8/E4 or CRND8 groups performed above chance on either water condition (**Figure 4**); thus, additional impairments caused by Zn were difficult to assess. Given the difficulty of the 24-h probe task for the Tg mice, running probe tests as the last run on alternate days, as is done in the Morris water maze (MWM), could be a useful strategy.

We have previously demonstrated significant latency impairments in the MWM in both Zn water rats and Tg (CRND8 and

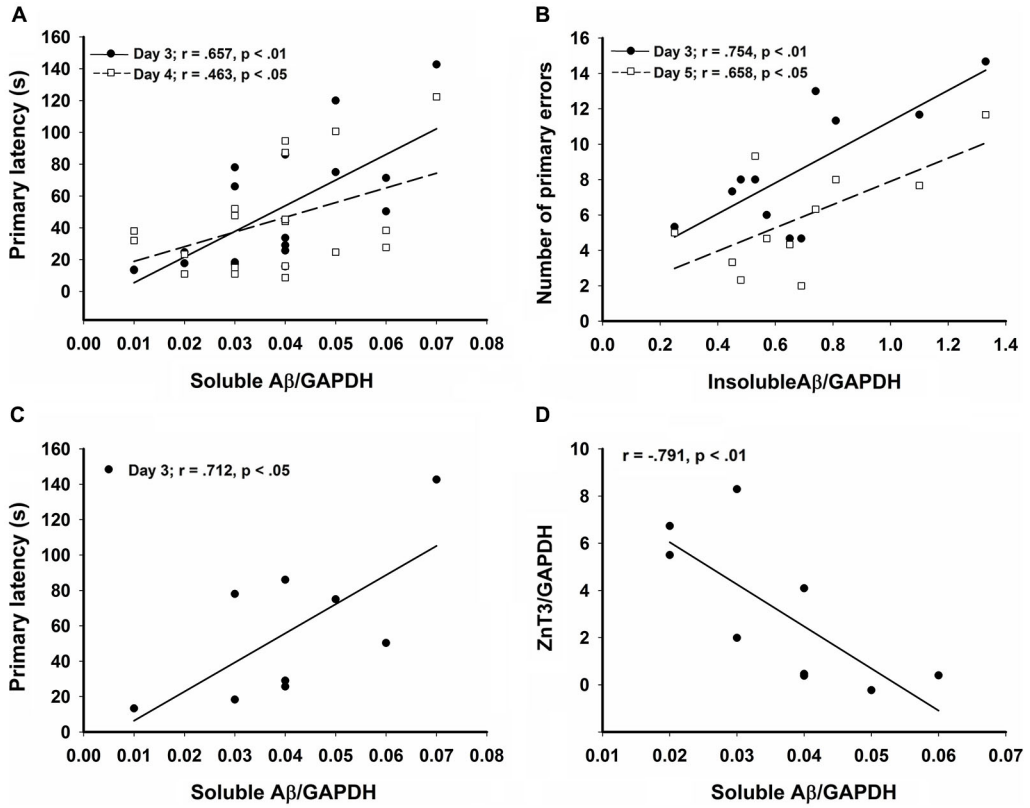

FIGURE 4 | Significant correlations in the CRND8/E4 group showing (A) soluble Aβ vs. average primary latencies for days 3 ($r = 0.657$, $p < 0.01$) and 4 ($r = 0.463$, $p < 0.05$); (B) In Zn water mice, insoluble Aβ vs. average primary errors for days 3 ($r = 0.754$, $p < 0.01$) and 5 ($r = 0.658$, $p < 0.05$); (C) In lab water mice, soluble Aβ vs. average primary latency on day 3 ($r = 0.712$, $p < 0.05$); and (D) In Zn water mice, soluble Aβ negatively correlates with ZnT3 ($r = -0.791$, $p < 0.01$). * denotes $p < 0.05$.

Tg2576) mice (Linkous et al., 2009; Railey et al., 2010, 2011). The MWM is a more stressful test than the BM (Hölscher, 1999) and this may account for the different effects of zinc on latency seen in this experiment. Overall, data from our lab indicate that Zn water has adverse behavioral effects on WT and CRND8 mice but that additional mechanisms are at work for CRND8/E4 mice. Although Zn supplementation exacerbated behavioral deficits in the Tg2576 and CRND8 mice, this led to a paradoxical decrease in amyloid deposits in the Tg2576 mice (Linkous et al., 2009), which was also observed by Harris et al. (2014); however they found no significant behavioral differences.

The deficits in spatial memory that were observed in the Barnes maze, in connection with dietary Zn supplementation, may be attributable to the effect of Zn on other metals in the brain, specifically Cu. Excess dietary Zn prevents adequate absorption of Cu through the intestinal wall (Milne et al., 2001). Therefore, Zn supplementation may potentiate a Cu deficiency that is inherent in Tg mice overexpressing mutant hAPP (Phinney et al., 2003). Brewer (2014), however, found a non-significant delay in cognitive impairment in AD patients who received Zn supplements, and suggests that AD is the result of a Zn deficiency. He also noted that these subjects had relatively low Zn serum levels and suggested that this would lead to an overload of copper. Brewer (2014) considers zinc deficiency a risk factor for AD, due to its interaction with copper (Brewer, 2014).

In contrast, Klevay (2008) has suggested that AD is due to a Cu deficiency instead of Cu toxicity and spatial memory deficits in Zn-treated Tg mice can be remediated by the concurrent administration of small amounts of Cu (Railey et al., 2011). APP-overexpressing mice have lower levels of Cu in the brain (Bayer et al., 2003) which would support increasing dietary Cu to treat AD. This is consistent with another study where APP23 mice were given Cu supplementation which resulted in a reduction of Aβ and an increase in superoxide dismutase 1 (SOD1) activity (Bayer et al., 2003). These conflicting findings from different labs suggest that metal dyshomeostasis in AD can be due to either a deficiency or overabundance of metal ions, and that alterations to the levels of one metal can impact levels of another. The initial metal status of the patient should be an important factor in considering any form of dietary metal-based therapy.

In contrast to our observations, Zn supplementation in the 3xTg mouse actually improved performance on behavioral tasks, and decreased both Aβ and tau pathology (Corona et al., 2010), while decreased dietary Zn elevated plaque volume in APP/PS1 mice (Stoltenberg et al., 2007). Carrying mutations in PS1, or

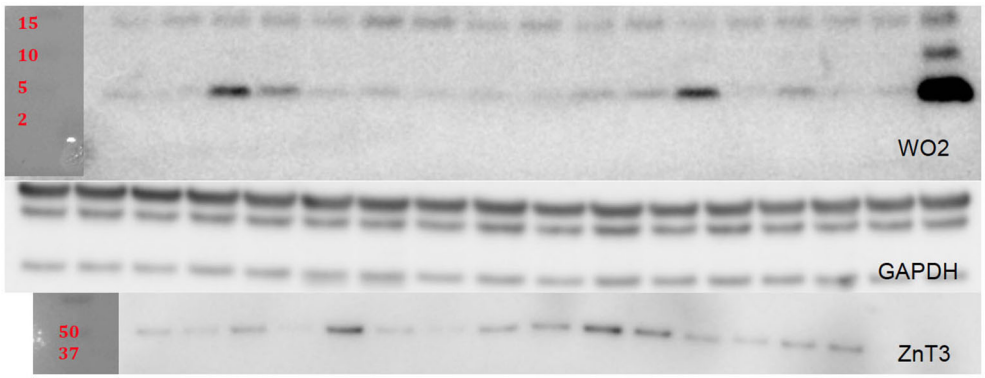

FIGURE 5 | Representative blots are shown for WO2 and ZnT3 which were normalized to GAPDH.

in the case of the 3xTg, a mutation in tau, can alter the ability of these proteins to interact with metals. The divergent results may stem from differences in the mouse model used, chemical composition of zinc solutions, and age of onset and duration of Zn supplementation. Our mice were given a standard rodent chow which contains adequate levels of Zn, in addition to the Zn supplementation, which would be in contrast to the Zn deficiency that is reported in the elderly population. While Zn therapy may be beneficial for those with a Zn deficiency, excess Zn, and Zn in the presence of ApoE ε4, may actually be harmful.

In a mouse model carrying the three different human ApoE alleles in the 5xTg mouse, the ApoE transgenes delayed pathology typically observed in the 5xTg strain (Youmans et al., 2012). Although having an expanded window of time to study the progression of AD pathology could be useful, using either the 3xTg or 5xTg strains may be contraindicated for studying the effects of Zn or other metals in late-onset AD. These multiple transgene models have mutations in PS1, which affects cellular turnover of Zn and Cu, since PS1 has Zn binding sites, which will affect SOD1 activity (Sensi et al., 2008; Greenough et al., 2011; Southon et al., 2013). Therefore, studies looking at the relationship between metals and pathobiology of late-onset AD should avoid incorporating mutated PS1 transgenes. Also, enzymes that modulate tau are Zn-dependent.

The current data did not support the predicted outcome of more behavioral impairment in CRND8/E4 mice as compared to CRND8 mice. On most measures the CRND8/E4 mice performed slightly, but not significantly, better than CRND8 mice. This is consistent with other recent behavioral data in younger Tg mice modeling late-onset AD (Moreau et al., 2013). Similar observations of intermediacy were reported in circadian rhythm and nest building behavior, as well as in markers for inflammatory cytokines (Graybeal et al., 2014). Recent studies have shown that any isoform of human ApoE delays AD pathology in early-onset Tg mice (Youmans et al., 2012; Tai et al., 2013).

One possible explanation for the greater impairment associated with the ApoE ε4 allele in old age is due to its interaction with metals and their dysregulation with increased age. Zn and Cu have an interdependent relationship and they exist in a delicate homeostasis whereby a shift towards either excess or deficiency may influence Aβ neurotoxicity. Lee et al. (2012) have shown that Zn accumulates around dystrophic neurons in aged Tg2576 mice and suggest that this excess Zn "crowded around amyloid plaques" and could directly cause the death of neurons. Such an effect would likely be greater in the case of ApoE ε4 which does not bind Zn and Cu as efficiently as the other forms of ApoE.

Another interesting finding was that ZnT3, which is the transporter responsible for loading zinc into synaptic vesicles, was negatively correlated with soluble Aβ levels, which is the more toxic form of Aβ. Although a general decline in levels of ZnT3 has been associated with cognitive decline (Bjorklund et al., 2012), it has also been demonstrated that the regions of brain that express ZnT3 may change so that the overall level of expression remains unaltered, but the localization of expression may vary and be centered more around areas of pathology in the Tg CNS (Lee et al., 2012). This may explain why no overall differences for ZnT3 expression were detected. It is notable that within the Zn water group we observed a correlation between insoluble Aβ and primary errors, as this may be due to the ability of Zn to aggregate Aβ. The relationship between amyloid load and learning and memory remains controversial; for example, Gruart et al. (2008) found no relationship between hippocampal-dependent learning and amyloid load, suggesting that other mechanisms may be involved.

ApoE ablation reduces the amount of ZnT3 and results in significantly lower levels of synaptic Zn compared to WTs (Lee et al., 2010). Zn is required for both presynaptic and postsynaptic mossy fiber long-term potentiation, and dysregulation of synaptic Zn alters the excitatory input of the mossy fiber-CA3 synapse in the hippocampus (Pan et al., 2011). Furthermore, ApoE-ε4 targeted replacement mice have altered levels of glutaminase and vesicular glutamate transporter 1 (Dumanis et al., 2013). ApoE-ε4 is correlated with decreased dendritic spines (Dumanis et al., 2009), and treatment of neurons with ApoE-ε4 recombinant protein reduces expression of glutamate receptors (Chen et al., 2010). Future research should examine the effects of Aβ, Zn supplementation, and ApoE status on glutamatergic pathways.

Since this study demonstrates that CRND8/E4 mice are sensitive to dietary Zn-enhancement, this suggests that therapies targeting metals should consider ApoE allele status in their analyses. Currently, the only FDA-approved drugs for treating AD work by alleviating symptoms instead of targeting the underlying etiology of the disease. A newer class of drugs currently under evaluation, the 8-hydroxyquinolines (8-OHQ), are selective Zn/Cu chaperones (Greenough et al., 2013). Two drugs in this class, Clioquinol (CQ) and PBT2 have a higher affinity for binding Zn and Cu when compared to Aβ. Instead of chelating these metals from the system, they are able to return metals back inside the cell, thereby restoring the bioavailability of metal ions necessary for cellular processes (Adlard and Bush, 2012). This restoration of intracellular metal concentrations has been shown to increase synaptic proteins, reduce Aβ accumulation, and improve cognitive functioning in Tg mice (Adlard et al., 2008, 2011; Crouch et al., 2011).

CONCLUSIONS

This experiment showed that Zn water caused significant impairments in a mouse model of late-onset AD, containing the ApoE ε4 allele; consequently, studies on the role of metals in AD need to take into account the ApoE status of either the human subjects or the mouse models. Furthermore, due to the interaction between different metals such as Cu and Zn, care needs to be taken when conducting both *in vitro* and *in vivo* studies, where the outcomes and interpretations of studies may be confounded by this interrelationship. Future studies examining the effects of Zn and/or Cu deficiencies and excesses in the different human ApoE alleles of a late-onset model of AD is warranted.

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Glia and zinc in ageing and Alzheimer's disease: a mechanism for cognitive decline?

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Normal ageing is characterized by cognitive decline across a range of neurological functions, which are further impaired in Alzheimer's disease (AD). Recently, alterations in zinc (Zn) concentrations, particularly at the synapse, have emerged as a potential mechanism underlying the cognitive changes that occur in both ageing and AD. Zn is now accepted as a potent neuromodulator, affecting a variety of signaling pathways at the synapse that are critical to normal cognition. While the focus has principally been on the neuron: Zn interaction, there is a growing literature suggesting that glia may also play a modulatory role in maintaining both Zn ion homeostasis and the normal function of the synapse. Indeed, zinc transporters (ZnT's) have been demonstrated in glial cells where Zn has also been shown to have a role in signaling. Furthermore, there is increasing evidence that the pathogenesis of AD critically involves glial cells (such as astrocytes), which have been reported to contribute to amyloid-beta (Aβ) neurotoxicity. This review discusses the current evidence supporting a complex interplay of glia, Zn dyshomeostasis and synaptic function in ageing and AD.

Keywords: ageing (aging), microglia, zinc, Alzheimer disease, synapse regulation, astrocyte-neuron interactions

INTRODUCTION

Ageing is an inevitable biological process wherein physical and mental capabilities are diminished over time, often resulting from a variety of factors such as cumulative oxidative stress and altered cell metabolism. This functional decline then ultimately results in a loss of synaptic plasticity. Ageing in itself does not require a treatment *per se*, but maintaining cognitive function into old age is a concept many aspire to. Currently, normal ageing is considered to be associated with an overall decline in cognition occurring via structural and functional brain changes over a period of time (Meunier et al., 2014). While we have a strong understanding of the physical decline that occurs in peripheral organs and systems (e.g., muscle and bone); the particular molecular and cellular changes that occur within the brain and which ultimately underlie the progression of normal ageing are yet to be fully determined. Despite the lack of consensus on the precise neural alterations that occur, it is clear that there is a fine line between healthy and pathological ageing.

HEALTHY AGEING VS. ALZHEIMER'S DISEASE

Currently, the mechanisms underlying ageing within the brain remain poorly understood, and indeed one of the hallmarks of ageing is its variability (Meunier et al., 2014), with the preservation or loss of cognitive functions differing between individuals. The functional memory decline that does occur, however, is actually well characterized, with executive functioning, processing speed and reasoning ability declining from middle age (Deary et al., 2009). While the molecular and cellular mechanisms underlying this are yet to be fully elucidated, it is important to note the potential intersection with pathological ageing, as seen in conditions such as Alzheimer's disease (AD). Ageing is the greatest risk factor for the development of AD, which is the most common form of age-related dementia (Mosconi et al., 2010; Reitz et al., 2011), and it has been suggested that AD may simply be an acceleration of the normal ageing process. Indeed, many of the cognitive impairments seen in normal ageing are further exacerbated in AD. Symptomatically, AD is characterized by marked deficiencies in episodic memory, attention, perception and speech (Mesulam, 1999) as well as altered mood (Lopez et al., 2001). Pathologically it is defined by the accumulation of intracellular neurofibrillary tangles (comprised of abnormally phosphorylated tau protein) and extracellular plaques (comprised of misfolded forms of the amyloid-β (Aβ) peptide) within the brain. With regards to the potential for a mechanistic overlap between ageing and AD, recent evidence points to zinc (Zn) homeostasis as key player in both normal and pathological ageing. Specifically, it has been demonstrated that there is a modulation in brain Zn homeostasis in both ageing and AD (Religa et al., 2006; Haase and Rink, 2009) that results in a neuronal Zn deficiency that may ultimately underlie the onset and progression of cognitive deficits seen in both.

ZINC

Zn; an essential trace element and second in abundance in mammalian tissues (Wang et al., 2005; Paoletti et al., 2009), is critical for immunity, growth and development (Nolte et al., 2004), is a cofactor for more than 300 enzymes and is essential for the

correct functioning of over 2000 transcription factors (Takeda, 2000; Levenson and Tassabehji, 2007; Jeong and Eide, 2013). The brain has the largest Zn content (Vasto et al., 2008), the levels of which are tightly controlled by three main families of proteins that have a distinct tissue and cell level pattern of localization and expression (Hennigar and Kelleher, 2012). These are; the metallothioneins (MT's; that also coordinate a variety of other metal ions), zinc transporters (ZnT's) and Zn-regulated and iron-regulated transporter proteins (ZIP's; recent evidence has also implicated the presenilin family as capable of influencing Zn concentrations (Greenough et al., 2013)). Currently there are four MT isoforms, 10 ZnT's, 15 ZIP's and two presenilins. The ZnT's coordinate intracellular Zn homeostasis while the ZIP's primary function is to regulate Zn uptake (Guerinot, 2000). The role of the MT's is to control cytosolic concentrations through the binding and distribution of Zn (Mocchegiani et al., 2001). A number of studies have examined the effect of altered MT on brain metal levels, with mice deficient in both MT-I/II (Manso et al., 2011) and MT-III (Erickson et al., 1997), for example, shown to have altered brain Zn levels. Cumulatively, these proteins are responsible for the influx and efflux of Zn^{2+} in a variety of cellular compartments, including vesicles, Golgi apparatus, and mitochondria (**Figure 1**).

As Zn is essential for normal brain function, Zn dyshomeostasis has been linked to a range of neurological abnormalities including (but not limited to) depression and schizophrenia (Levenson and Tassabehji, 2007), AD, amyotrophic lateral sclerosis (ALS), Down's syndrome, multiple sclerosis (Grabrucker et al., 2011) and normal age-related cognitive decline (Adlard et al., 2014). Neurological events such as ischemia, seizures and traumatic brain injury have also been linked to altered Zn levels (Nolte et al., 2004; Grabrucker et al., 2011). In addition to this, excess Zn has been implicated in the processes that lead to cellular damage through excitotoxicity or oxidative stress (Morris and Levenson, 2012). This is particularly pertinent at the synapse where the presence or absence of Zn can be crucial.

ZINC AT THE SYNAPSE (NEURON-NEURON)

Though it is well-known that Zn is tightly bound to macromolecules in the brain, a small number of Zn ions (approx. 10–15% total brain Zn) exist as chelatable Zn (Levenson and Tassabehji, 2007) primarily within synaptic vesicles at glutamatergic synapses (Paoletti et al., 2009; Sensi et al., 2009; Karol et al., 2010). During neuronal activation Zn is released into the synaptic cleft alongside glutamate (Lee et al., 2012) where it interacts with synaptic receptors, ion channels and transporters to modulate synaptic activity (Takeda and Tamano, 2012). Most importantly, this Zn acts on neuronal receptors and voltage-gated calcium channels (VGCC) to regulate downstream signaling pathways and neuronal processes such as; normal neuronal firing, long-term potentiation (LTP) and long-term depression (LTD) by acting on N-methyl-D-aspartate (NMDA) and α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptors, particularly in the hippocampus. This is further evidenced by the age-dependent cognitive phenotype recently reported in zinc transporter 3 (ZnT3) knockout mice (Adlard et al., 2010), which lack synaptic Zn at the glutamatergic synapse. Interestingly ZnT3 mRNA and protein levels are decreased in AD (Beyer et al., 2009; Adlard et al., 2010). Research has also demonstrated that Zn is capable of inducing functional and conformational changes in NMDA receptors (Sirrieh et al., 2013) and is therefore considered essential for the modulation of synaptic neurotransmission (Roberts et al., 2012).

Likewise, Salazar et al. (2005) further demonstrated an intimate connection between Zn and vesicular glutamate transporter 1 (VGlut1) which is expressed in synaptic terminals that co-release Zn and glutamate. Glutamate, the major excitatory neurotransmitter in the central nervous system (CNS),

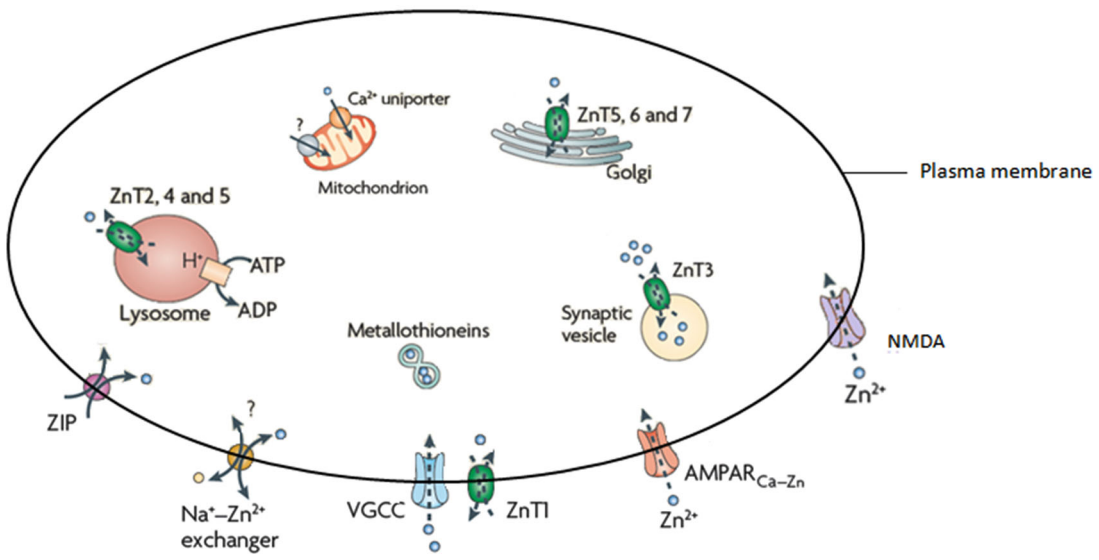

FIGURE 1 | Illustration of the proteins responsible for the homeostasis of Zn. Adapted from Sensi et al. (2009).

is essential to synaptic plasticity underlying cognition and memory. Vglut1 and ZnT3 coexist in nerve terminals and are also co-targeted to the same vesicle population and are reciprocally regulated; Zn uptake is increased by Vglut1 expression while glutamate uptake is increased by production of ZnT3. This becomes exceedingly relevant as evidence is emerging for a role of astrocytes in Zn homeostasis and gliotransmission.

ZINC AND ASTROCYTES

Astrocytes have a variety of roles in regulating volume and composition of extracellular space, forming and controlling the blood-brain barrier (BBB) and also maintaining the architecture of gray matter (Kettenmann and Verkhratsky, 2008). Astrocytes are capable of rapidly accumulating Zn (Nolte et al., 2004) and they also express a range of ZnT's including ZIP14 (Bishop et al., 2010) and ZnT3 (which is intimately involved in cognition) under stress conditions (Sun et al., 2012). It is known that glial cells in the cerebellum also express ZnT1, ZnT3, ZnT4 and ZnT6 (Wang et al., 2005). The biological implications, and indeed the cognitive effect, of this Zn accumulation and Zn transporter expression are currently unknown and remain an under-researched area. That being said, astrocytes take up glutamate from the synapse and convert it to glutamine which is subsequently released and retrieved by neurons for conversion back to glutamate and into the main inhibitory transmitter gamma-aminobutyric acid (GABA; Yeh et al., 2013). This glutamate-glutamine cycle is essential to maintaining glutamatergic and GABA-ergic neurotransmission, disruption of which could cause synaptic dysfunction (Yeh et al., 2013) and cognitive deficits. Moreover, activation of astrocytic G-protein coupled receptors stimulates the release of glutamate as well as potentiates NMDA receptor functions (Lee et al., 2007). Thus, the close association/co-release of Zn and glutamate at the synapse may, therefore, extend to a role for astrocytes/glia in the synaptic regulation of Zn that may be critical for the prevention of excitotoxicity or various other post-synaptic or cognitive processes.

Additionally, astrocytes increase intracellular Ca^{2+} levels in response to synaptic transmission (Morris et al., 2013). This may be important in LTP which is induced by high-frequency stimulation causing a sustained increase in transmission that usually depends on Ca^{2+} influx through NMDA receptors. A recent study by Han et al. (2013), which grafted human astrocytes into mice, showed enhanced LTP and improved performance on cognitive tests. It should be noted here that oxidative stress (previously mentioned as a major contributor to cellular damage) is known to affect metabolic pathways of astrocytes that are important for the delivery of metabolites to neurons (Theusen et al., 2010). This demonstrates a common cause of functional disruption across cell types essential to Zn homeostasis that may be especially detrimental at the synapse.

TRIPARTITE SYNAPSE

The role of Zn at synapses is particularly germane to both normal and pathological ageing. Neuroplasticity deteriorates in normal ageing and loss of synapses is a key indicator and currently the best correlate of cognitive decline in AD (Takahashi et al., 2010). However, the current study of synapses falls well-short of that necessary to fully comprehend the complex processes that occur. The model of a synapse over the past decades of research has been that of neuron-neuron electrical impulses, but recent advances in both scientific thinking and techniques has brought us to a more accurate representation. Currently the most accepted model is that of the "tripartite" synapse where the pre- and post-synaptic neuron terminals are physically enveloped by astrocytic processes which actively participate in synaptic neurotransmission. These astrocytic processes release a variety of neuroactive molecules including adenosine, GABA, prostaglandins and ATP which can influence both neuronal and synaptic physiology (Volterra and Bezzi, 2002).

Though the link has previously not been made, the evidence presented here suggests that there may be an interaction between neurons, astrocytes, Zn and glutamate that is critical to normal synaptic health and function, potentially affecting neurotransmission and other cellular cascades involved in cognition, as discussed earlier. This is an area of research that clearly needs further investigation, as the diminished learning and memory abilities seen in ageing may be caused by a failure in neuron-glia communications at the synapse that disrupts Zn homeostasis to result in downstream modifications to, among other things, synaptic ion channels.

MICROGLIA AT THE SYNAPSE

In addition to the role of astrocytes at the synapse, recent studies investigating microglia suggest this tripartite system may be just as constraining to synaptic research as the previous model. Currently, the literature is dominated by what is thought to be the primary function of microglia; surveying the environment for damage and protecting neural material (Stollg and Jander, 1999). Resting microglia have small cell bodies and elongated processes, in response to a traumatic or pathogenic event they alter their morphology, withdraw their processes and become globular. These activated microglia are able to migrate to the damage site, phagocytose any cellular debris and release various neuroactive compounds (Wake et al., 2009). However, it is almost certain that microglia have a much larger role in normal brain function and cognition than previously thought. Resting microglia have been shown to be highly dynamic, extending and retracting processes with brief static periods, apparently at random (Nimmerjahn et al., 2005). Microglia express most of the major classes and subtypes of both excitatory and inhibitory neurotransmitter receptors and ion channels which have classically been found at neuronal synapses, though little is known about their role in inactivated microglia. Indeed, research by Wake et al. (2009) showed microglial processes making direct transient contact with synapses. Moreover, microglia appear to contact neighboring astrocytes, neuronal cell bodies and blood vessels (Morris et al., 2013). These findings have vast implications for our current understanding of both synaptic and cognitive functioning in that such findings require scientists to completely alter their perceptions and re-consider the current models of synapses as well as the dominant focus on neurons throughout past and present research and literature.

Figure 2 illustrates the potential structure of this new synaptic model.

Microglia also release cytokines, which have central physiological roles in synaptic plasticity, neurogenesis and learning and memory in the normal brain (Morris et al., 2013) possibly through their influence on MT expression and hence Zn concentration (Vasto et al., 2008). Microglia may, however, have a more direct role in Zn homeostasis as Higashi et al. (2011) recently learned. Microglia can directly uptake Zn via ZIP1, which is also a trigger for sequential microglial activation. In a study by Knoch et al. (2008) the release of intraneuronal Zn^{2+} and a subsequent increase in neuronal voltage-gated K^{+} currents as caused by the release of ROS from activated microglia lead to neuronal cell death. This suggests the primary mechanism of neuronal apoptosis may in fact, be the earlier damage to glial cells. This is accordance with findings by Kaindl et al. (2008, 2012) that activation of microglial NMDA receptors results in an increase in oxidative stress *in vitro* (Kaindl et al., 2008, 2012). Glial senescence during ageing can also impact normal synapse function (Wong, 2013) and result in aberrant connectivity between neurons.

ZINC, GLIA AND PATHOLOGICAL AGEING

Both Zn and glia appear to have multifarious roles within the brain, especially within synapses. Indeed, synaptic loss is the fundamental feature of the ageing brain that links neuropathology to cognitive decline in AD (Talantova et al., 2013). This is largely applicable to pathological ageing and neurodegenerative disorders such as AD wherein the abnormal deposition of misfolded Aβ peptide into plaques, which bind Zn, results in a significant decrease in intracellular Zn (Bush et al., 1994). Moreover, the plaques are in abundance in brain areas with high densities of

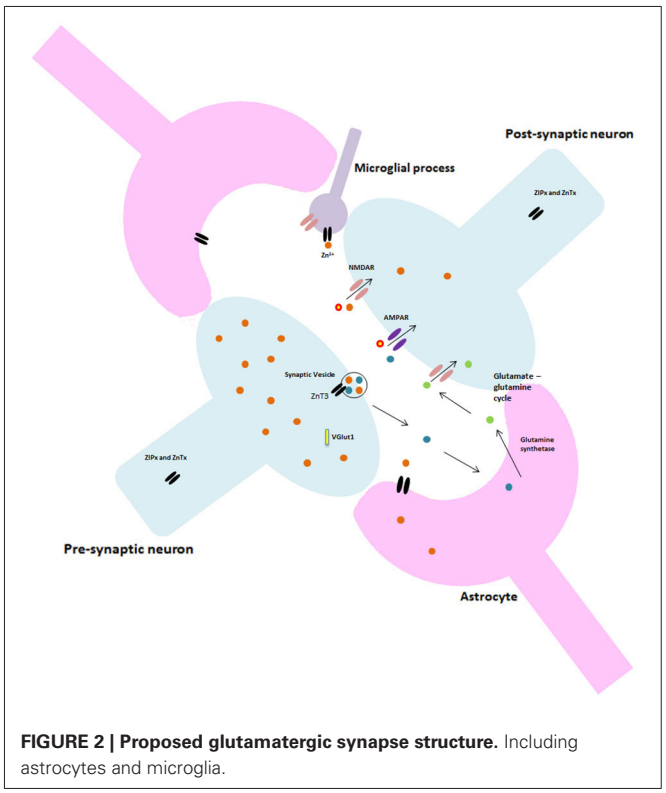

FIGURE 2 | Proposed glutamatergic synapse structure. Including astrocytes and microglia.

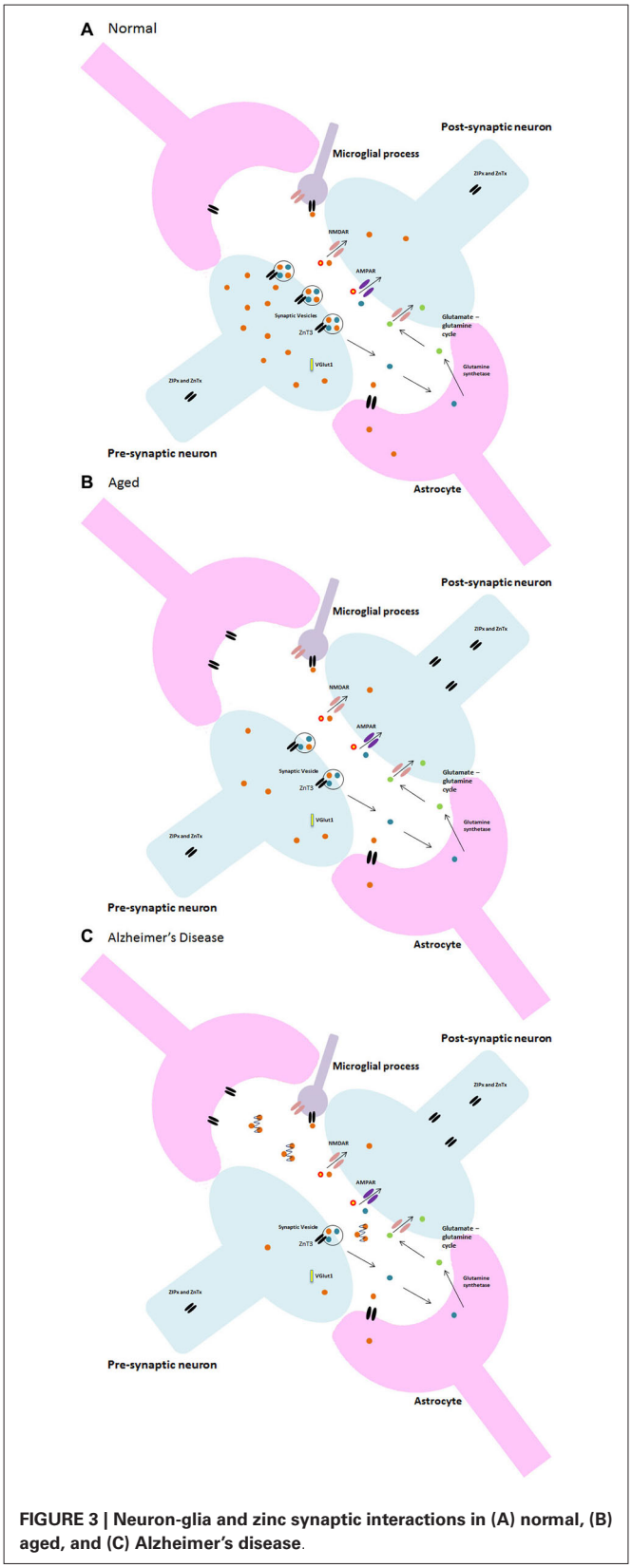

FIGURE 3 | Neuron-glia and zinc synaptic interactions in (A) normal, (B) aged, and (C) Alzheimer's disease.

glutamatergic synapses such as the hippocampus, exhibiting a similar distribution to that of Zn with glutamatergic neurons. Additionally, microglia have been suggested to play a role in plaque formation (Stollg and Jander, 1999). Further research by Desphande et al. (2009) clearly demonstrated oligomers of Aβ interfering with synaptic function, suggesting that Zn at the NMDA receptor attracts the Aβ in addition to its high binding affinity to synapses. Keeping the aforementioned information in mind, it is reasonable to suggest that AD may be the result of synaptic dysfunction caused by a disruption of the fine and complex interplay between Zn, neuronal, glial and microglial communication that occurs within the synapse. Due to the high binding affinity of Aβ to both Zn and synapses, upon activation of the pre-synaptic neuron and the subsequent release of Zn into the synapse, the Zn can be captured by the Aβ and lodged within the plaque to ultimately disrupt synaptic transmission. A decrease in available Zn for neurotransmission and calcium signaling then causes downstream errors that may result in further Zn dyshomeostasis in a negative feedback loop eventually leading to glial damage and apoptosis through microglial activation. Disruption of cytokine signaling and failure of the signaling mechanisms maintaining the phenotype of microglia in the normal brain may contribute to learning and memory dysfunction and synaptic pathologies such as AD or dementia which, in some of its forms, is at its onset a result of a failure to maintain microglia in their ramified state (Morris et al., 2013). A diagrammatic representation of the change in Zn^{2+} in the progression from normal to pathological ageing is illustrated in **Figure 3**.

CONCLUSION

Herein evidence supporting a link between Zn, glia and cognitive decline has been presented and discussed. The research thus far suggests the possibility of a feedback loop between Zn homeostasis, synaptic excitation, neurons, astrocytes and microglia. Perhaps the most appropriate definition is that of Morris et al. (2013) that a synapse is "a complex, dynamic and often transient structure involving several cells interacting with a sophisticated extracellular matrix and milieu". The contribution of microglia and astrocytes to synaptic plasticity mechanisms relevant to learning and memory must be included in studies. Only by including these cell types in future research will we come to truly understand the intricate molecular mechanisms underlying the ageing processes; thereby discovering potential avenues for intervention to ensure that we are able to enjoy our twilight years to the best of our cognitive ability.

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Alzheimer's disease causation by copper toxicity and treatment with zinc

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Evidence will be presented that the Alzheimer's disease (AD) epidemic is new, the disease being very rare in the 1900s. The incidence is increasing rapidly, but only in developed countries. We postulate that the new emerging environmental factor partially causal of the AD epidemic is ingestion of inorganic copper from drinking water and taking supplement pills, along with a high fat diet. Inorganic copper can be partially directly absorbed and elevate the serum free copper pool. The Squitti group has shown that serum free copper is elevated in AD, correlates with cognition, and predicts cognition loss. Thus, our inorganic copper hypothesis fits well with the Squitti group data. We have also shown that AD patients are zinc deficient compared to age-matched controls. Because zinc is a neuronal protective factor, we postulate that zinc deficiency may also be partially causative of AD. We carried out a small 6 month double blind study of a new zinc formulation and found that in patients age 70 and over, it protected against cognition loss. Zinc therapy also significantly reduced serum free copper in AD patients, so efficacy may come from restoring normal zinc levels, or from lowering serum free copper, or from both.

Keywords: Alzheimer's disease, inorganic copper, serum free copper, zinc deficiency, cognition

INTRODUCTION

Cancer, heart disease, and stroke have increasingly become major killers in our Western societies. We have looked for environmental factors behind the increased mortality from these diseases, and we have found them. They include cigarette smoking, air pollution, diets high in fats and sugars and lacking in fruits and vegetables, and lack of exercise, to name but a few. Considerable effort has gone into identifying and mitigating the environmental factors causing so much mortality from these diseases.

In stark contrast, a new disease epidemic has crept into our midst, clearly also strongly caused by environmental factors, but almost no effort has been made to find the environmental culprits and mitigate them. Perhaps this is because this new disease does not quickly kill, rather it only robs a large segment of our elderly of their ability to function effectively. This new disease epidemic is Alzheimer's disease (AD), and its close cousin, mild cognitive impairment (MCI).

Why do we say it's a new epidemic? First, it is an epidemic, with 10% of those aged 60, 20% of those aged 70% and 30% of those aged 80 in the U.S. affected with the disease (Alzheimer's Association, 2010). There are over 5 million Americans with AD, with an equal number with MCI, 80% of whom develop AD at the rate of 15% per year. Second, it is new, because it didn't exist or was very rare prior to 1900, and developed a rapidly increasing prevalence after about 1950.

The evidence that AD didn't exist, or was rare, prior to 1900 is quite good. In their book, Dying for a Hamburger, Waldman and Lamb (Waldman and Lamb, 2005) examined this question. They point out that Osler, a clinician who edited a textbook pulling together all known diseases in the late 1800s, did not mention an AD like disease, although one entire volume was devoted to diseases of the brain (Osler, 1910). Gowers, who wrote a textbook of neurology during this period, also did not describe an AD like disease (Gowers, 1888). Most important, Boyd, who wrote a textbook of pathology during the late 1800s, updated in the early 1900s, did not describe amyloid plaques neurofibrillary tangles, hallmarks of AD brain pathology, in brains at autopsy (Boyd, 1938).

Some say that since AD is a disease of aging, there just were not enough old people around in the period of the 1800s, so the disease was not observed, or at least not noticed. It is not true that elderly people weren't around back then. Waldman and Lamb showed that in 1911, half the French population were living to age 60, the age of onset of AD (Waldman and Lamb, 2005). I checked the US census for 1900, and there were 3.2 million people over age 60, at today's rate providing 36.3 thousand AD cases, more than enough to have been frequently seen in the clinics of Osler and Gower, and to have been frequently encountered at autopsy by Boyd.

Others say that the disease does not represent a "new" epidemic because now our ability to recognize it as a disease is much better, in other words our "diagnostic ability" is now greatly improved. While this could conceivably explain the failure of clinicians, such as Osler and Gowers, to recognize the disease, although this seems unlikely given their thoroughness, it can't explain why pathologists such as Boyd didn't observe amyloid plaques and neurofibrillary tangles in brains at autopsy.

So one fact is we have a new epidemic. The second fact is that the epidemic is primarily occurring in developed countries (except for Japan), and not in undeveloped countries (Ferri et al., 2005).

These two facts make it clear that something or some things, newly present in the last 100 years in the environment of developed, but not undeveloped, countries is causing this epidemic of AD. Waldman and Lamb come to this same conclusion, and because they believed the new factor was beef eating and AD was a prion disease acquired from beef, they named their book, Dying for a Hamburger (Waldman and Lamb, 2005). Certainly beef eating fits the criteria of being associated with development over the last 100 years, but we find no evidence that AD is a prion disease. Nevertheless, we believe Waldman and Lamb were on the right track, because we believe a high fat diet is one causal factor for the AD epidemic, and beef eating is associated with a high fat diet. Grant has shown that AD prevalence is positively correlated with dietary fat intake across many countries (Grant, 1997). However, we believe an additional factor plays a major causal role for the AD epidemic. For our view on this additional factor, read on.

OUR HYPOTHESIS THAT INTAKE OF INORGANIC COPPER IS A MAJOR CAUSAL FACTOR IN THE AD EPIDEMIC

The major stimulus to our awareness that ingestion of inorganic copper could be a risk factor for AD resulted from the studies of Sparks and Schreurs, reported in 2003 (Sparks and Schreurs, 2003). In studies of a cholesterol fed rabbit model of AD, they found that addition of as little as 0.12 ppm copper to the distilled drinking water of the rabbits greatly enhanced both AD type brain pathology, and cognition loss by the animals. By reference, the Environmental Protection Agency in the U.S. allows up to 1.3 ppm copper in human drinking water, over 10 times that causing toxicity in the rabbit AD model. Allowances for copper in drinking water are similar, or higher, around the world. The work of Sparks and Schreurs has been confirmed by them in other AD models, including the mouse model (Sparks et al., 2006), and have been confirmed by another group (Singh et al., 2013).

We emphasize that we are talking about ingestion of inorganic copper as being toxic, not organic copper. Organic copper is copper in food, safely bound to proteins. Organic copper is absorbed from the intestine into the blood, and processed by the liver, and put into safe channels. In contrast copper in drinking water (or in pills containing copper) is a simple inorganic salt of copper, not bound to anything. We have evidence that at least a portion of inorganic copper is absorbed into the blood, bypasses the liver, and contributes immediately to the "free copper" pool in the blood (Hill et al., 1986). A small increase in copper in food, equivalent to 0.12 ppm, would be trivial and completely non-toxic, whereas it is exquisitely toxic in drinking water, as shown in the rabbit model.

The size of the "free copper" pool in the blood is very important in AD, as shown by Squitti et al. (2005). Depending on how it is measured, about 65–85% of the copper in blood is covalently bound in ceruloplasmin (Cp), a copper containing protein secreted into the blood by the liver, and is safe copper. The remainder is loosely bound to albumin and some other molecules, and is called "free copper". The Squitti group has shown that blood free copper levels in AD are significantly elevated compared to age matched controls (Squitti et al., 2005), that free copper levels are inversely correlated with cognition measure in AD (the higher the free copper, the worse the cognition measures) (Squitti et al., 2006), and are positively correlated with loss of cognition over time (the higher the free copper, the greater the loss of cognition) (Squitti et al., 2009). Thus, it is very rational that addition to the free copper pool by ingestion of inorganic copper, because some of it immediately increases the free copper pool, could be a risk factor for AD causation.

The level of total plasma copper in AD patients versus age matched controls should not be confused with the levels of blood free copper we first discussed. Some authors have found total serum copper elevated in AD, while others have not. But the relevant finding is that blood free copper is elevated in AD.

So ingestion of inorganic copper in drinking water could be a risk factor in AD, but are humans ingesting inorganic copper in their drinking water? The answer is yes, it is being leached from their copper plumbing! Looking back at the time course of the AD epidemic it is remarkable how closely it parallels the explosive use of copper plumbing in developed countries. Copper plumbing began to be used in the early 20th century, but was curtailed by World War I and then II. After 1950 copper plumbing took off, and now 90% of U.S. homes have copper plumbing. Similarly the AD epidemic took off in the latter half of the 20th century, but only in developed countries. Copper plumbing is not used very much in undeveloped countries, because of its expense. Japan is an interesting exception that supports the copper in drinking water causation hypothesis. It is a developed country, but with a lower rate of AD (Ueda et al., 1992), and has shunned copper plumbing for fear of toxicity. Yet, when Japanese migrate to Hawaii, where copper plumbing is used, they developed the higher rate of AD seen in other developed countries (White et al., 1996).

We stated above that leaching of copper from copper plumbing into drinking water is a risk factor of AD, but what is our evidence that copper is actually leached into drinking water in significant amounts? Our evidence is that we have measured it. We wanted to make sure our patients with Wilson's disease, a disease of copper toxicity, weren't ingesting excessive copper in their drinking water. These patients came from all over N. America, attracted to our clinic because of new Wilson's disease treatments we were developing. In a sample of drinking water from 280 homes, we found about a third were higher than the 0.1 ppm causing toxicity in the rabbit AD model, about one third were 0.01 ppm or lower, a level we view as safe, and about one third were between 0.01 and 0.1 ppm, an area of unknown safety (Brewer, 2011). So people are ingesting plenty of inorganic copper from their drinking water, which according to the rabbit AD model could account for the high and increasing prevalence of AD.

There is another source of inorganic copper leading to high ingestion in the developed world, and that is use of supplement pills containing copper. As with copper in drinking water, the copper in pills is a simple salt, such as copper sulfate, and is thus inorganic. Morris et al. (2006) did a study in Chicago of nutrient intake, and cognition, over a period of years. They found that

those in the highest quintile of copper intake, if they also ate a high fat diet, lost cognition at six times the rate of other groups. People were in the highest quintile of copper intake because they took supplement pills containing copper.

One can also build a case for the other risk factors for AD tying into the copper hypothesis. We believe another major risk factor for AD is a high fat diet (Grant, 1997). Copper oxidizes certain fat molecules into derivatives that are toxic to neurons. Elevated homocysteine levels are a risk factor for AD (as they are for atherosclerosis) (Seshadri et al., 2002). Homocysteine interacts with copper to oxidize cholesterol to an intermediate damaging to neurons. The apoliprotein E4 (apoE4) allele is a risk factor for AD, while apoE2 is protective and apoE3 neutral (Miyata and Smith, 1996). Apoliprotein may help remove copper from the brain, and apoE4 has no copper binding cysteines, apoE3 one, and apoE2 two. Certain hemochromatosis (Moalem et al., 2000) and transferrin (Zambenedetti et al., 2003) alleles increase risk for AD. These genes affect iron levels, and iron, like copper, is a transition element that produces oxidant radicals. Oxidant damage is a predominant type of damage in the AD brain. Recently it has been shown that certain alleles of the ATP7B gene, also known as the Wilson's disease gene, increase risk for AD (Bucossi et al., 2011, 2012). This is further direct support of our hypothesis, because this gene controls free copper levels.

Recently there has been some direct studies that strongly support a critical role for copper toxicity in the AD brain. It has been shown that "labile copper" is elevated in the AD brain, and that this elevation is associated with oxidant pathology in the AD brain (James et al., 2012).

In summary, we believe our hypothesis that ingestion of inorganic copper is a major risk factor for AD is well supported. Recounting the network of supporting data around this hypothesis that constitutes good support, we have the observation that trace amounts of inorganic copper in drinking water greatly enhances AD pathology and cognition loss in AD animal models, we have the concordance of the AD epidemic and use of copper plumbing, the data showing toxic levels of copper in drinking water all over N. America, the data of Morris et al. (2006) showing cognition loss in those using copper supplements, the data of Squitti et al. (2005) showing a high serum free copper in AD patients, the data showing that gene mutations potentially influencing copper levels increase AD risk, and the direct data showing that "labile copper" is elevated in the AD brain.

Some authors have concluded the opposite, that copper deficiency is a causal factor in AD. An example of this is Klevay (2008), who hypothesize just that, that AD is copper deficiency. However, this hypothesis is not well thought out, since AD patients display none of the manifestations of copper deficiency, which are a very low serum copper, anemia and bone marrow depression, and myelopolyneuropathy neurologic syndrome. Another example is Kessler et al. (2008) who gave copper to AD patients, and claimed to see no worsening. They saw no improvement either, which disproved their hypothesis that patients were suffering from copper deficiency. We believe that ingestion of a high fat diet is another important risk factor for AD, and that these two, ingestion of inorganic copper plus a higher fat diet, set the stage on which the other risk factors act.

It is sometimes said that a hypothesis is only useful if it can be tested. It is a little hard to test this one definitively. The AD animal model studies have probably gone as far as one can go with animal studies. It is unethical to give humans potentially toxic inorganic copper. Epidemiologic studies could be designed, but these end up showing associations, which don't prove causation. Inorganic copper ingestion could be prevented in a large sample of elderly (people age 60 or over), and AD outcomes compared with an equally large sample of controls who continued ingestion of inorganic copper at their normal rates, but this would take years to complete, and be quite expensive. In the meantime, those who believe this hypothesis is likely to be true, would be well-advised to limit ingestion of inorganic copper.

OUR HYPOTHEIS THAT ZINC DEFICIENCY IS A RISK FACTOR FOR AD AND COGNITION CAN BE STABLIZED BY ZINC SUPPLEMENTATION

As people grow older, they become relatively zinc deficient compared to younger people, as measured by serum zinc levels. To evaluate the zinc status of AD patients we did a study on AD patients and age-matched controls with Earl Zimmerman and his group at Albany, NY. Because elderly people take many mineral supplements, to make sure supplements weren't affecting the results, we stopped all supplements use one month prior to the study. In 29 age-matched controls, mean serum zinc was 82.7 μg/dl, as expected well below that of younger people, which runs around 100 μg/dl. But the mean serum zinc of 29 AD patients was 76.2 μg/dl, significantly less than the mean of age-matched controls (Brewer et al., 2010). Baum et al. (2010) have also found zinc deficiency in serum of AD patients.

The fact that zinc levels are elevated in the AD brain led to early speculation that zinc excess and zinc toxicity was a factor in AD. However, now it is realized that amyloid plaques bind zinc avidly, and this causes an increase brain zinc in AD. The uptake of zinc by plaques is another cause of diminished zinc availability to neurons.

Zinc has many important protective roles in neurons. For example, it helps quench glutamate-stimulated neuronal firing, preventing damage from excessive firing (Takeda, 2010). It also inhibits calcineurin, which if too active, has downstream adverse effects (Crouch et al., 2011). Serum zinc, when it is low, is a reliable indicator of systemic zinc deficiency. But the brain in AD may suffer even more from zinc deficiency than indicated by the serum levels. That is because the amyloid plaques in the AD brain are avid binders of zinc, and may make zinc even less available for neurons.

Additional insight and credibility for the hypothesis that the AD neuron is zinc deficient comes from the studies of the zinc transporter-3 (ZnT3). This transporter is the zinc pump that loads neuronal vesicles with zinc, the vesicles that are discharged into the synapse with neuronal firing. ZnT3 knockout mice exhibit deficits in memory and learning at 6 months of age, and are said to be "a phenocopy of the synaptic and memory deficits of AD" (Adlard et al., 2010). These same authors also found that ZnT3 levels decreased with aging in the brains of mice and

humans, and decreased significantly more with aging in the brains of AD patients compared to age-matched controls.

For all these reasons, we hypothesize that zinc deficiency is a risk factor in AD, that is, a factor which can precipitate, or at least assist, cognitive decline. Given this hypothesis, it seemed reasonable to evaluate the possible beneficial effect of zinc supplementation.

In reviewing the literature, it turned out zinc supplementation, both oral and parenteral, was tried in open label studies in 1991 by Constantinidis (1992). He reported excellent efficacy, although the trials were uncontrolled. Corona et al. (2010) have also reported beneficial effects of zinc supplementation in a rodent AD model.

In our trial, we used a new zinc formulation developed by myself and a company, Adeona Pharmaceuticals. I had previously developed zinc acetate capsules, called Galzin, and had them FDA approved for treating Wilson's disease. The two problems with Galzin were, first, it had to be taken away from food and second, it had to be taken multiple times a day if one wanted to achieve around the clock plasma zinc elevations. The new zinc formulation employed a zinc binding agent that released the zinc slowly, so it wasn't irritating to the stomach, and also achieved around the clock plasma zinc elevation.

The study was a placebo controlled 6 month trial in mild to moderate AD patients (Brewer, 2012). Thirty AD patients received 150 mg once daily of the new zinc formulation, and 30 age-matched controls received matching placebo. When giving zinc in high dose like this, one has to be concerned about inducing copper deficiency, which if allowed to become severe, can lead to a severe neurologic syndrome. For that reason we regularly monitored serum Cp, a good measure of copper status, as well as hemoglobin levels, anemia being one of the first manifestations of copper deficiency. During the 6 month study only one patient had zinc dose reduced because of a decreasing Cp. Endpoints were increased serum zinc, decreased serum free copper, and better cognitive scores compared to controls. Cognition was measured by Alzheimer's Disease Assessment Scale for Cognition (ADAS-cog), Clinical Dementia Rating Scale, Sum of Boxes (CDR-SOB), and Mini Mental State Examination (MMSE).

We anticipated a possible decrease in serum free copper with zinc therapy because zinc blocks the absorption of copper in the intestine. This is the basis of using zinc as a therapy for Wilson's disease, an inherited disease of copper accumulation and copper toxicity.

We found serum zinc significantly increased and serum free copper significantly decreased in zinc treated patients versus controls. All three cognition scores were in a favorable direction in the zinc treated group versus controls, but none were significant, although CDR-SOB was close at $p = 0.1$. Looking at the data *post hoc*, we realized cognition was relatively stable in controls until age 70, when it began deteriorating much more rapidly, while zinc treated patients remained relatively stable at age 70 and over. We reanalyzed the data on those age 70 and over, and the 15 age matched controls showed significant cognition loss compared to the 14 AD patients, with ADAS-Cog at $p = 0.037$, CDR-SOB at $p = 0.032$, and MMSE close to significant at $p = 0.067$ (Brewer, 2012).

Thus, we conclude from this trial that zinc supplementation will significantly stabilize cognition in AD patients age 70 and over. We believe it will also stabilize cognition in younger patients, but it is harder to show it statistically because controls are deteriorating so slowly before age 70. This study should be confirmed by a larger study, and carried out longer, to evaluate whether the stabilization by zinc therapy is maintained long term.

Assuming zinc therapy has efficacy in this study, the mechanism could be restoration, or partial restoration, of protective zinc in the neuron, or lowering of toxic tree copper levels. Or perhaps both mechanisms are operative since we achieved both. Thus, at this point we can't be certain that zinc deficiency is a risk factor in AD, because zinc supplementation may be acting through lowering serum free copper.

SUMMARY

We point out that we have a serious new epidemic of AD, particularly in developed countries, that is robbing a large portion of our elderly of their golden years. We hypothesize that ingestion of inorganic copper from drinking water and supplement pills, together with a high fat diet, are major causal factors in the epidemic. We show that copper from copper plumbing is leached into drinking water at high enough levels to be causal for AD, according to a rabbit AD model. We advise those who believe the hypothesis to be correct to curtail their ingestion of inorganic copper.

We also hypothesize that zinc deficiency is a risk factor for AD, and show that in patients age 70 and over, 6 months of zinc therapy significantly decreased cognition loss compared to age matched controls.

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Resveratrol and Alzheimer's disease: message in a bottle on red wine and cognition

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Cognitive impairment is the final outcome of a complex network of molecular mechanisms ultimately leading to dementia. Despite major efforts aimed at unraveling the molecular determinants of dementia of Alzheimer type (DAT), effective disease-modifying approaches are still missing. An interesting and still largely unexplored avenue is offered by nutraceutical intervention. For instance, robust epidemiological data have suggested that moderate intake of red wine may protect against several age-related pathological conditions (i.e., cardiovascular diseases, diabetes, and cancer) as well as DAT-related cognitive decline. Wine is highly enriched in many polyphenols, including resveratrol. Resveratrol is a well recognized antioxidant which may modulate metal ion deregulation outcomes as well as main features of the Alzheimer's disease (AD) brain. The review will discuss the potentiality of resveratrol as a neuroprotectant in dementia in relation to the oxidative stress produced by amyloid and metal dysmetabolism.

Keywords: resveratrol, Alzheimer's disease, aging, metal ions, aluminum, copper, iron, zinc

INTRODUCTION

The so-called *French paradox* arises from the epidemiological fact that French people, despite their indulgence to a high fat diet, show a relative low incidence of cardiovascular diseases (Renaud and De Lorgeril, 1992). Several epidemiological studies have shown that moderate wine consumption can be effective in slowing down age-related cognitive decline (Wang et al., 2006; Panza et al., 2012; Corona et al., 2013). A possible explanation of this phenomenon has been linked to the national high consumption of wine (20–30 g/day) (Renaud and De Lorgeril, 1992). Albeit moderate ethanol intake is, generally speaking, "beneficial", some more specific effects appear to be related to red wine. Red wine consumption seems in fact to promote far more protective effects than consumption of other ethanol containing beverages (Baur and Sinclair, 2006). Resveratrol, a natural polyphenol, is mainly present in red wine and has been suspected to be the major driving force behind the *French paradox* (Siemann and Creasy, 1992).

AD is one of the most common forms of dementia in the elderly. To date, no disease-modifying therapies are still available for AD.

The main four pathological features of the disease are: (1) extracellular deposition of misfolded β-amyloid (Aβ) in senile plaques (SPs); (2) intracellular accumulation of hyperphosphorylated tau in neurofibrillary tangles (NFTs); (3) severe brain atrophy; and (4) the presence of areas of chronic inflammation (Querfurth and Laferla, 2010; Medeiros et al., 2013).

In the last 20 years, deregulation of Aβ metabolism (amyloid oligomerization, aggregation, and plaques formation) has been considered the main trigger for AD-related synaptic dysfunction. Amyloid has been therefore the major target for therapeutic intervention (Hardy and Higgins, 1992; Mucke and Selkoe, 2012). Unfortunately, most of these attempts have dramatically failed or have produced only marginal effects (Reitz, 2012; Krstic and Knuesel, 2013; Doody et al., 2014).

Better therapeutic strategies are thus needed along with new acknowledgment that AD is a complex multifactorial syndrome.

Aging is the required paramount condition (Herrup, 2010) on which, in addition to Aβ together with tau deregulation, genes, chronic inflammation, mitochondrial, metabolic dysfunctions, impaired insulin signaling, oxidative stress, aberrant cell cycle reentry, cholesterol dysmetabolism as well as metal ion dyshomeostasis must synergistically work to promote AD pathological manifestation (Herrup, 2010; Querfurth and Laferla, 2010; Roberts et al., 2012). While a single-target therapeutic strategy seems to produce only suboptimal results a broader neuroprotective approach, at least theoretically, appears more appealing (Mudher and Lovestone, 2002).

In this review, we are providing some evidence for resveratrol as a broad-spectrum neuroprotective agent in aging and hopefully in AD.

RESVERATROL

Resveratrol has beneficial cardiovascular effects (Siemann and Creasy, 1992) throughout a great variety of molecular mechanisms (Howitz et al., 2003; Baur and Sinclair, 2006; Lagouge et al., 2006; Park et al., 2012).

A recent review on aging determinants has proposed nine hallmarks for the process (López-Otín et al., 2013). Not surprisingly, almost all of them are also involved in AD development and progression (**Figure 1;** Herrup, 2010; Querfurth and Laferla, 2010) and, notably, at least five, are well recognized target for resveratrol modulation.

In the following sections we have outlined potential effects of resveratrol on these aging and/or AD molecular targets.

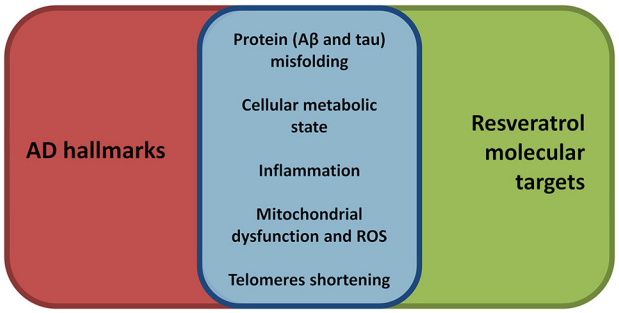

FIGURE 1 | Synoptic view of the overlap between aging, and thus AD, hallmarks (see López-Otín et al., 2013) and the molecular targets of resveratrol. Resveratrol, compared to many other disease modifying approaches, shows a broader range of beneficial effects targeting many molecular aspects of AD pathogenesis. Thus, resveratrol may represent a promising broad-spectrum neuroprotective agent in AD.

β-AMYLOID AND HYPERPHOSPHORYLATED TAU MISFOLDING

Blockade of Aβ deposition into SPs and inhibition of hyperphosphorylation of tau into NFTs has been considered mandatory to prevent or, at least, delay AD-related cognitive decline.

Resveratrol has been shown to inhibit Aβ fibrils formation (Porat et al., 2006; Rivière et al., 2007). Moreover, *in vitro* and *in vivo* studies have also indicated that resveratrol reduces amyloid toxicity by decreasing Aβ production through sirtuin-dependent activation of a disintegrin and metalloproteinase domain-containing protein 10 (Donmez et al., 2010). The compound also increases clearance and metabolism via an AMP-activated protein kinase-pathway and can induce autophagic and lysosomal Aβ degradation (Marambaud et al., 2005; Vingtdeux et al., 2010). Resveratrol can effectively interject in the amyloid cascade through its antioxidant and anti-inflammatory activity, thereby reducing Aβ-driven production of reactive oxygen species (ROS) as well as neuroinflammation (Liu and Bitan, 2012).

Effects on tau phosphorylation and deposition have been less investigated. However, resveratrol-mediated activation of sirtuin-1 (SIRT1) can lead to direct deacetylation of acetylated tau, thereby promoting its proteasomal degradation (Min et al., 2010). In addition, the compound can reduce phospho-tau toxicity (induced by cyclin-dependent kinase 5-p25 dependent tau phosphorylation) by favoring the deacetylation of peroxisome proliferator-activated receptor gamma, coactivator 1 alpha (PGC-1α) and p53 (Kim et al., 2007).

CELLULAR METABOLISM

Caloric restriction has been proposed to be effective in increasing lifespan in several animal models. Fasting has been observed to promote beneficial effects on preclinical models of AD and aging not only by extending lifespan but also by ameliorating cognitive performances (Halagappa et al., 2007). Caloric restriction can in fact promote release of brain-derived neurotrophic factor (BDNF), a neurotrophin critically involved in counteracting cognitive decline (Weinstein et al., 2014).

In this context, resveratrol efficiently mimics caloric restriction by inducing expression of SIRT1 (a nicotinamide adenine dinucleotide (NAD^+) dependent deacetylase) which in turn sets in motion a cascade of PGC-1α-dependent events that ultimately lead to improved mitochondrial functioning and biogenesis and boost cellular ROS scavenging (Gomes et al., 2013; López-Otín et al., 2013).

INFLAMMATION

Areas of localized inflammation and active microglia contribute to neurodegeneration and cognitive decline in AD brains (McGeer and McGeer, 2013). Pharmacological and genetic manipulations aimed at reducing brain inflammation appear to be effective in slowing/modifying the disease progression in AD animal models (Heneka et al., 2013; Giuliani et al., 2014).

Resveratrol is effective in reducing the inflammatory status (Rahman et al., 2006; Chen et al., 2013) in *in vitro* and *in vivo* settings of neuroinflammation (Capiralla et al., 2012; Frozza et al., 2013).

Mechanisms by which resveratrol attenuate neuroinflammation are still not completely clear. A major pathway seems to involve sirtuin-dependent arrest of nuclear factor kappa-light-chain-enhancer of activated B cells signaling cascades, a step that results in downstream blockade of microglia activation (Capiralla et al., 2012; Donmez, 2012; Ye et al., 2013).

MITOCHONDRIAL DYSFUNCTION AND ROS

Mitochondria play an essential role in the cell wellbeing. The organelles critically control cellular energy and metabolism as well as intracellular signaling (Rizzuto et al., 2012). On the dark side, mitochondria are also key players in modulating cellular death through release of apoptotic factors, blockade of energy supply and generation and release of ROS. Alterations of mitochondrial functioning are known in aging and early stages of AD (Wang et al., 2013).

Mitochondrial electron leakage, followed by ROS production occurs in neurodegenerative conditions paving the way to lipid peroxidation, nucleic acid damage, protein oxidation, and, eventually, neuronal death (Wang et al., 2013).

Resveratrol counteracts the production of mitochondrial ROS through two major mechanisms: (1) by efficiently scavenging hydroxyl, superoxide, and metal-induced radicals (Leonard et al., 2003); and (2) by increasing mitochondrial functioning and biogenesis through activation of the SIRT1–PGC-1α pathway, thereby boosting mitochondrial bioenergetic efficiency (Khan et al., 2012; Choi et al., 2013; Desquiret-Dumas et al., 2013).

TELOMERES SHORTENING

Telomeres shortening plays a key role in cellular aging and AD (Cai et al., 2013; Mathur et al., 2014). Short telomeres increase

DNA vulnerability to stressful insults (i.e., UV irradiation, ROS production) ultimately leading to aberrant cell functioning and cell death. Polyphenols has a positive impact upon maintenance of telomeres length (Jayasena et al., 2013). In that respect, resveratrol promotes the expression of Werner syndrome ATP-dependent helicase, a telomere maintenance factor (Uchiumi et al., 2011), increases the activity of telomerase via a SIRT1-dependent pathway (Palacios et al., 2010), and spares telomeres and DNA from ROS dependent damages thanks to its intrinsic scavenging properties (Jayasena et al., 2013).

METAL IMBALANCE IN THE AD BRAIN: A POTENT TRIGGER OF OXIDATIVE STRESS

In the brain, metal ions are involved in many essential processes such as intracellular signaling, modulation of cellular redox and metabolic states, enzymatic activities and channels functioning (Billard, 2006; Sensi et al., 2009; Rizzuto et al., 2012; Sekler and Silverman, 2012; Gaier et al., 2013). Metal homeostasis is strictly controlled by the interplay of transporters, channels, chaperones and metalloregulatory sensors (Finney and O'halloran, 2003). In neurodegenerative conditions and/or aging, this tightly controlled system is lost, thereby leading to disease-promoting metal imbalance (Bolognin et al., 2009; Breydo and Uversky, 2011; Jellinger, 2013).

Metal ion dyshomeostasis is in fact involved in several neurological disorders like Parkinson's disease (PD), Amyotrophic Lateral Sclerosis (ALS), Prion Protein disease, Huntington's disease (HD), and AD. All these neurodegenerative conditions share common pathological features that include deposition of misfolded proteins, metal ion deregulation and exposure to oxidative stress (Boillee et al., 2006; Duce and Bush, 2010; Roberts et al., 2012; Gonzalez-Dominguez et al., 2014).

In AD, metal ion dyshomeostasis represents a key, though too often overlooked, pathological step. A metal hypothesis for AD has been proposed by many authors (Bush, 2008). In that respect, copper, iron, zinc and aluminum are the metals found deregulated in AD. All of them are able to alter Aβ metabolism and deposition (Bolognin et al., 2011). SPs but also NFTs are highly enriched of these metals. Moreover, all these ions can promote ROS generation (Sayre et al., 2000; Granzotto and Zatta, 2011; Pithadia and Lim, 2012; Ayton et al., 2013).

Recent findings have shown that low levels of copper are sufficient to dramatically affect Aβ homeostasis by increasing Aβ accumulation and neuroinflammation related to Aβ-deposition (Singh et al., 2013). Compared to nondemented elderly controls, brains of AD patients show an increased presence of labile copper pools, which correlate with oxidative damage in these tissues (James et al., 2012). Resveratrol is a well known copper chelator (Tamboli et al., 2011) and, in theory, of some use in AD (Faux et al., 2010). Unfortunately, the copper-resveratrol complex seems to be more harmful than beneficial in the context of AD. Resveratrol promotes the reduction of copper (II) to copper (I) (de la Lastra and Villegas, 2007) and several studies have indicated a pro-oxidant activity of the compound when bound to copper (Zheng et al., 2006; de la Lastra and Villegas, 2007; Muqbil et al., 2012). Thus, resveratrol activity on copper homeostasis appears more harmful than neuroprotective if used as standalone therapeutic approach. A feasible and, in our opinion, clinically relevant approach might be represented by the administration of resveratrol in association with a higher affinity copper chelator. This would lead, at least in theory, to a dual beneficial effect: reduction of copper dyshomeostasis coupled with decreased ROS production.

Iron deregulation has been linked to AD (Weinreb et al., 2013; Crespo et al., 2014; Gonzalez-Dominguez et al., 2014). Role of iron in AD pathogenesis is substantiated by the effectiveness of metal homeostatic therapies aiming at reducing iron deregulation (Crouch et al., 2007), which results in (1) decreased free iron accumulation and ferroptosis (Dixon et al., 2012); (2) decreased iron-dependent ROS production; and (3) blockade of neurotoxic Aβ-iron conjugates formation (Liu et al., 2012). To date, *in vivo* evidence for iron chelation by resveratrol is missing, however the compound prevents iron-driven mitochondrial dysfunction by inhibiting glycogen synthase kinase-3 beta activity (a mechanism useful also to prevent tau hyperphosphorylation) (Shin et al., 2009), and by reducing peroxidation of lipoproteins and lipids through its activity as scavenger (Belguendouz et al., 1997; Tadolini et al., 2000).

Zinc dyshomeostasis has been proposed as a risk factor for AD. Accumulation of excessive zinc, or its deficiency, are both involved in the neuronal loss which leads to AD and aging related cognitive decline (Brewer, 2012). While zinc deficiency increases neuroinflammation and also affects BDNF maturation and ultimately cognition, aberrant intracellular zinc mobilization or accumulation leads to mitochondrial failure and ROS production. Extracellular zinc overload within SPs also inhibits the iron-export ferroxidase activity further increasing ROS production and ultimately neuronal death (Duce et al., 2010). Resveratrol does not directly affect zinc levels however it can be useful in preventing the full development of zinc-dependent injurious mechanisms. Actually, resveratrol inability to sequester zinc does not represent a limitation, as the compound can exert antioxidant activities without producing zinc deficiency.

Aluminum lacks modulatory functions in biological processes; however, its accumulation in the brain has been demonstrated to be linked to several neuropathological conditions (Zatta et al., 2003; Walton, 2013). To date three are the main mechanisms through which aluminum exerts its neurotoxic effects: (1) production of ROS; (2) induction of neuroinflammation; and (3) formation of toxic aggregates of misfolded proteins (Perl, 2006; Kumar et al., 2009; Wu et al., 2012; Bolognin et al., 2013). In AD, aluminum seems to act as an effective cross-linker between tau phospho-sites, to "freeze" Aβ in its toxic oligomeric state, and to induce exposure of Aβ hydrophobic clusters aggregates, thereby boosting toxic properties of these misfolded proteins (Zatta et al., 2009; Bolognin et al., 2011; Chen et al., 2011; Granzotto et al., 2011). Aluminum-related oxidative damage occurs through lipid peroxidation, alteration of the activity of antioxidant enzymes, alterations of mitochondrial functioning and biogenesis and promotion of DNA injury (Zatta et al., 2002; Sharma et al., 2013). Resveratrol shows a negligible ability to bind aluminum *in vitro*

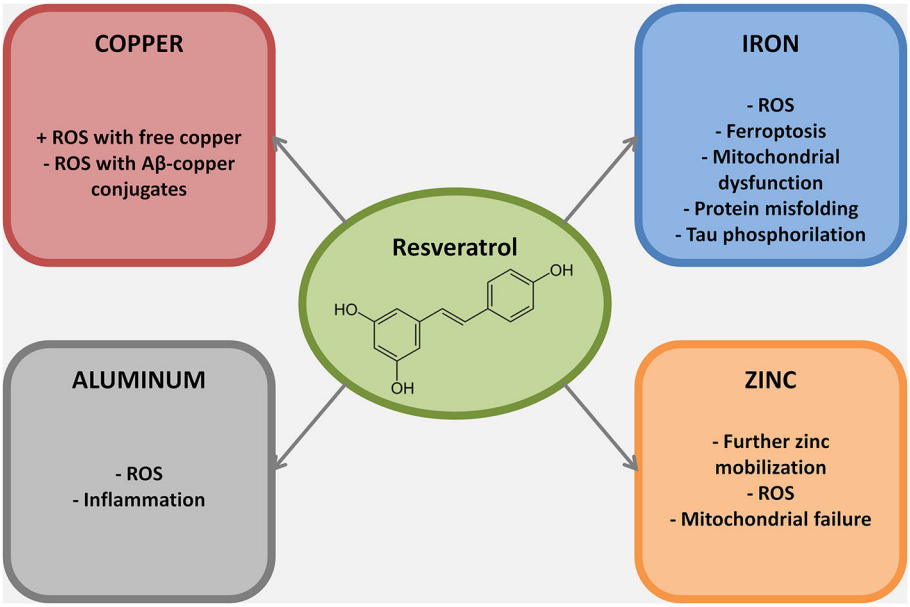

FIGURE 2 | Metal ions dyshomeostasis is closely related to different hallmarks of AD pathogenesis, mainly protein misfolding, ROS production, mitochondrial failure and inflammation. In this figure the mechanisms through which resveratrol exert its neuroprotective role against selected metal ions are reported. Of note, most of the resveratrol beneficial effects against metal ion dyshomeostasis belong to its scavenging properties.

(Granzotto and Zatta, 2011), nevertheless, it seems effective in reducing *in vivo* the downstream events of aluminum overload, namely the aluminum-related ROS production and neuroinflammatory response activation (Zaky et al., 2013).

CONCLUSIONS

Resveratrol is a multi target compound and may represent an effective therapeutic tool in aging-related neurodegenerative processes. Consistently, several clinical trials are ongoing to test its effectiveness as dietary supplement to slow dementia progression (ClinicalTrials.gov, 2014).

In summary, major effects are associated with its scavenging activity as well as in the activation of SIRT1 (see Bordone and Guarente, 2005; Herskovits and Guarente, 2014 for extensive reviews on the topic). The presence of non-SIRT1 neuronal targets of resveratrol is debated, suggesting that resveratrol *in vivo* may act on other uninvestigated biological targets (Herskovits and Guarente, 2014). The complementary role of modulator of metal dependent oxidative injury (**Figure 2**) represents a still largely unexplored field in resveratrol biochemistry.

Resveratrol is a multi-target, simple, safe, and cost-effective dietary supplement. Nevertheless, it should be reminded that its role as therapeutic agent is not devoid of potential problems. The pro-oxidant activity in presence of labile copper, the poor bioavailability and ease degradation all represent major issue that require new sophisticated efforts (Goldberg et al., 2003). Synthesis of novel resveratrol analogs is ongoing and improvement of drug delivery might represent in that regard the major targets to be considered in order to overcome current resveratrol limitations. In agreement, pterostilbene, a resveratrol derivative, has shown promise in preclinical models of neurodegeneration, resulting more efficient than resveratrol itself in modifying AD- and aging-related cognitive decline (Joseph et al., 2008; Chang et al., 2012). These results leave the door open for the use of newly synthesized resveratrol analogs in aging-related disorders (Bourzac, 2012; Ogas et al., 2013; Pezzuto et al., 2013).

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Aluminum and its potential contribution to Alzheimer's disease (AD)

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Alzheimer's disease (AD) is perhaps *the principal example* of cognitive failure in humans, and currently over 5.5 million Americans suffer from this incapacitating and progressive disorder of thought, reasoning and memory. Our laboratory has been evaluating the potential contribution of environmentally bioavailable neurotoxic metals to the onset, development and progression of AD for about 30 years (Lukiw et al., 1987). Largely because of its known multiple and potent neurotoxic effects, much of our research has focused on the potential contribution of aluminum to the AD process: (i) because of aluminum's remarkable abundance and bioavailability in the biosphere—in fact it is the most abundant naturally occurring neurotoxic element to which we are exposed; (ii) because of aluminum's remarkable cellular toxicity and genotoxicity at low nanomolar concentrations toward brain genetic processes, and (iii) because of aluminum's highly structured, specific and unique interactions with the phosphate-rich nucleic acids associated with the expression of genetic information in the human brain (Lukiw et al., 1989; Bryant et al., 2004; Alexandrov et al., 2005, 2013; Lukiw and Pogue, 2007; Pogue et al., 2009, 2012; Lukiw, 2010; Percy et al., 2011; Bhattacharjee et al., 2013; De Sole et al., 2013).

Aluminum's contribution to AD is based upon at least seven independently derived observations: (i) that at physiologically realistic concentrations, aluminum strongly promotes amyloid aggregation and accumulation, a key feature of AD neuropathology (Exley, 2005; Rodella et al., 2008; Walton and Wang, 2009; Yumoto et al., 2009); (ii) that both *in vitro* and *in vivo* aluminum promotes inflammatory signaling via the pro-inflammatory transcription factor NF-kB, another prominent feature characteristic of AD brain (Bondy, 2013; Walton, 2013); (iii) that out of the many thousands of brain gene messenger RNA (mRNAs) and micro RNAs (miRNAs), the family of mRNAs and miRNAs induced by aluminum are also strikingly similar to those found to be increased in AD; (iv) that in transgenic animal models of AD dietary aluminum enhances the development of pathological markers such as lipid peroxidation, oxidative stress, apoptosis, and gene expression deficits (Praticò et al., 2002; Bharathi et al., 2008; Zhang et al., 2012); (v) that many of the observed deficits in AD such as chromatin compaction, impaired energy utilization, impaired signaling involving chemical messengers such as adenine triphosphate (ATP) are recapitulated in aluminum-treated cellular or animal models of AD (Alexandrov et al., 2005; Lukiw and Pogue, 2007; Pogue et al., 2012; Bhattacharjee et al., 2013); (vi) that a very significant number of studies link the amount of aluminum in drinking water to the incidence of AD [worldwide, aluminum is added to drinking water as hydrated aluminum potassium sulfate $KAl(SO_4)_2{\cdot}12H_2O$, or alum, as a clarification and "finishing" agent (Flaten, 2001; Frisardi et al., 2010; Walton, 2013)], and (vii) perhaps most importantly, that of all pharmaceutical treatment approaches directed against AD to date, chelation using the anti-oxidant and trivalent iron/aluminum chelator desferrioxamine has been shown to be one of the most effective therapeutic strategies yet devised (Crapper McLachlan et al., 1991; Percy et al., 2011).

Abundant research indicates that aluminum is a particularly reactive metal toward multiple aspects of human neurobiology and the altered genetics that are associated with the development and propagation of sporadic AD (Lukiw et al., 1989; Lukiw, 2010; Bhattacharjee et al., 2013; Bondy, 2013; Shaw and Tomljenovic, 2013; Walton, 2013). Thirty years of research since the potent effects of aluminum on the genetic apparatus in AD were first described, the most recent evidence suggests a strong linkage between aluminum sulfates and induction of NF-kB-sensitive pro-inflammatory miRNAs (Lukiw et al., 1987; Alexandrov et al., 2013; Zhao et al., 2013). Aluminum has been previously shown to significantly induce the transcription factor NF-kB (Pogue et al., 2009; Bondy, 2013), and up-regulation of NF-kB drives synthesis of NF-kB-sensitive miRNAs which in turn down regulate the expression of many

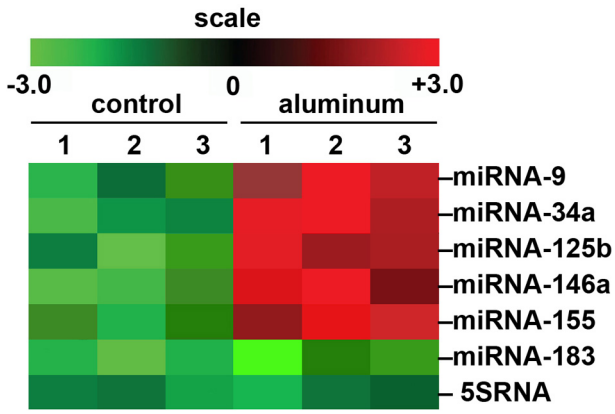

FIGURE 1 | Array-based cluster analysis of miRNA abundance in aluminum-fed Tg2576 mice vs. controls. In these experiments the brain (cortex) of 3 month-old Tg2576 mice fed aluminum-enriched diets were analyzed for miRNA speciation compared to age-matched controls (receiving standard diets); methodologies for aluminum-treatment of transgenic animals have been previously described in detail (Praticò et al., 2002; Zhang et al., 2012). Aluminum treatment of other transgenic murine AD models such as the amyloid over-expressing APP/PS1 show similar intensification of AD-type changes (Zhang et al., 2012). The up-regulation of the pro-inflammatory quintet of miRNA-9, miRNA-34a, miRNA-125b, miRNA-146a, and miRNA-155, depicted here, were amongst the most significantly increased miRNAs found to be 2- to 5-fold above normal diet, age-matched controls (compared to an unchanging internal control miRNA-183 and 5SRNA in the same samples). The results strongly suggest a potential contribution of aluminum to the AD processes associated with miRNA-mediated down-regulation of gene expression in the sporadic AD brain as is widely observed (Colangelo et al., 2002; Lukiw and Pogue, 2007; Ginsberg et al., 2012; Pogue et al., 2012; Alexandrov et al., 2013). Importantly, other common environmental neurotoxic divalent metals such as calcium, cadmium, copper, iron (2+), mercury, nickel, and lead, and neurotoxic trivalent metals such as boron, chromium, gadolinium, indium, iron (3+), and yttrium do not exhibit this potentially pathogenic effect (Walker et al., 1989; Alexandrov et al., 2005; Lukiw, 2010; unpublished observations); $N = 3$ control and $N = 3$ aluminum-treated mice; methodologies and data analysis have been extensively described elsewhere (Lukiw and Pogue, 2007; Alexandrov et al., 2013).

AD-relevant genes, including complement factor H (CFH) and neurotropic signaling in human brain cells (Pogue et al., 2009; Zhao et al., 2013).

We would like here to briefly include some recent genetic data on aluminum and its effects on miRNA abundance in a highly relevant transgenic animal model for AD that shows strong parallels to miRNA profiles which are found in AD brain (**Figure 1**). There are currently over 90 transgenic mouse models of AD (http://www.alzforum.org/research-models/). A commonly used Tg2576 mouse model overexpresses a mutant form of beta amyloid precursor protein (β APP), APPK670/671L, linked to early-onset familial AD, and develops amyloid plaques and progressive cognitive deficits as the mice age. Tg2576 mice exposed to dietary aluminum have been shown to develop oxidative stress and robust amyloidogenesis, key features of AD neuropathology (Praticò et al., 2002). Animals provided a 2 mg/kg aluminum-supplemented diet were analyzed for miRNA speciation and complexity in their brains using GeneChip and miRNA array technologies; intriguingly, the same quintet of up-regulated pro-inflammatory miRNAs (miRNA-9, miRNA-34a, miRNA-125b, miRNA-146a, and miRNA-155) as found in (i) AD and (ii) in aluminum-treated human brain cells in primary culture were also found to be amongst the most up-regulated in these aluminum-supplemented Tg2576 mice (Alexandrov et al., 2005; Lukiw and Pogue, 2007; Pogue et al., 2012; Bhattacharjee et al., 2013; Hill et al., 2014; **Figure 1**; unpublished observations). Up-regulated miRNAs are known to target susceptible mRNAs and down-regulate the expression of many AD-relevant brain genes as is widely observed in AD brain tissues (Colangelo et al., 2002; Guo et al., 2010; Ginsberg et al., 2012). Interestingly, these findings suggest some common miRNA-induced mechanism between two important *in vitro* and *in vivo* models for AD with AD itself. Indeed, the abundance of specific miRNAs are highly selective, and potential indicators and predictors of human health and disease, including progressive neurological disorders such as AD (Alexandrov et al., 2005, 2013; Lukiw and Pogue, 2007; Pogue et al., 2009; Maciotta et al., 2013; Zhao et al., 2013).

Lastly, more research into the potential contribution of aluminum to the AD process is clearly warranted. There are currently no treatments for AD that effectively prevent or cure AD's insidious onset or propagation. We think it important to emphasize that the most effective clinical treatment yet devised for moderate- to late-stage AD patients was the implementation of the first generation anti-oxidant and trivalent iron/aluminum chelator desferrioxamine to attempt to remove aluminum from the brains of AD patients (Crapper McLachlan et al., 1991; Percy et al., 2011). Second generation aluminum chelators such as Feralex-G, either alone or in combination with other chelators, has shown higher specificity, increased selectivity and higher efficacy in aluminum sequestration and chelation in preliminary *in vitro* studies (Kruck et al., 2004, 2008; Percy et al., 2011; unpublished observations). Certainly, laboratory experimentation in cultured primary human brain cells, in transgenic AD animal models, and clinical studies employing next-generation aluminum chelators, perhaps in combination with other drug strategies, are one of the research areas needing more focused attention—to more effectively address the exact role and mechanism of aluminum neurotoxicity in this rapidly expanding healthcare concern.

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Metal and complementary molecular bioimaging in Alzheimer's disease

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Alzheimer's disease (AD) is the leading cause of dementia in the elderly, affecting over 27 million people worldwide. AD represents a complex neurological disorder which is best understood as the consequence of a number of interconnected genetic and lifestyle variables, which culminate in multiple changes to brain structure and function. These can be observed on a gross anatomical level in brain atrophy, microscopically in extracellular amyloid plaque and neurofibrillary tangle formation, and at a functional level as alterations of metabolic activity. At a molecular level, metal dyshomeostasis is frequently observed in AD due to anomalous binding of metals such as Iron (Fe), Copper (Cu), and Zinc (Zn), or impaired regulation of redox-active metals which can induce the formation of cytotoxic reactive oxygen species and neuronal damage. Metal chelators have been administered therapeutically in transgenic mice models for AD and in clinical human AD studies, with positive outcomes. As a result, neuroimaging of metals in a variety of intact brain cells and tissues is emerging as an important tool for increasing our understanding of the role of metal dysregulation in AD. Several imaging techniques have been used to study the cerebral metallo-architecture in biological specimens to obtain spatially resolved data on chemical elements present in a sample. Hyperspectral techniques, such as particle-induced X-ray emission (PIXE), energy dispersive X-ray spectroscopy (EDS), X-ray fluorescence microscopy (XFM), synchrotron X-ray fluorescence (SXRF), secondary ion mass spectrometry (SIMS), and laser ablation inductively coupled mass spectrometry (LA-ICPMS) can reveal relative intensities and even semi-quantitative concentrations of a large set of elements with differing spatial resolution and detection sensitivities. Other mass spectrometric and spectroscopy imaging techniques such as laser ablation electrospray ionization mass spectrometry (LA ESI-MS), MALDI imaging mass spectrometry (MALDI-IMS), and Fourier transform infrared spectroscopy (FTIR) can be used to correlate changes in elemental distribution with the underlying pathology in AD brain specimens. Taken together, these techniques provide new techniques to probe the pathobiology of AD and pave the way for identifying new therapeutic targets. The current review aims to discuss the advantages and challenges of using these emerging elemental and molecular imaging techniques, and highlight clinical achievements in AD research using bioimaging techniques.

Keywords: LA-ICPMS, metals, Alzheimer's disease, bioimaging, MALDI, FTIR

INTRODUCTION

Alzheimer's disease (AD) is the most common progressive age-related neurodegenerative disorder, affecting about 2% of the population in the developed world (Mattson, 2004). Clinically, AD is characterized by devastating effects such as memory loss and decline in other cognitive abilities resulting in loss of independent functioning (Teri et al., 1989; Baddeley et al., 1991; Terry et al., 1991). Pathologically, AD is characterized by two main pathological hallmarks. These include extracellular amyloid plaques composed of insoluble amyloid beta (Aβ) protein produced by irregular cleavage of the amyloid precursor protein (APP), and intra-neuronal neurofibrillary tangles (NFTs) containing hyperphosphorylated tau protein (Khachaturian, 1985; Joachim et al., 1987; Selkoe et al., 1987; Mirra et al., 1991; Brun and Englund, 2002). Although the exact function of Aβ and APP remains unclear, recent studies suggest that APP may

play a crucial role in modulating neuronal survival, neurite outgrowth, synaptic plasticity and cell adhesion (Mattson, 1997). NFTs are not restricted to AD, and are also present in other neurodegenerative diseases such as fronto-temporal dementia (Filley et al., 1994).

AD is a complex multifactorial disorder associated with irregular protein aggregation (Pimplikar et al., 2010). Interestingly, accumulation of Aβ protein has been observed in cognitively normal brain, and sometimes an absence of Aβ deposits has been noted in some postmortem in patients who had been clinically diagnosed with AD (Edison et al., 2007). Moreover, various pathobiological mechanisms that are un-related to amyloid accumulation have been associated with the development and progression of AD. For instance, familial mutations in APP and presenilin-1 have been shown to induce autophagic dysfunction and impaired lysosomal proteolysis, cerebral hypoperfusion, and AD (Lee et al., 2010; Pimplikar et al., 2010; Wong and Cuervo, 2010). Furthermore, excess or deficiency in several nutritional, environmental or genetic factors may also potentiate AD-like pathology, making the etiology of this debilitating disorder difficult to elucidate (Russ et al., 2012).

Metals have a diversity of roles in medical biology encompassing both health and disease states (Olanow and Arendash, 1994; Oteiza et al., 2004; Farina et al., 2013; Jellinger, 2013; Grubman et al., 2014). Metals such as lead and mercury cause well established neuropathologies. By contrast several types of metal ions, such as potassium, sodium and calcium are vital for normal nerve cell function. Several other metals (copper, zinc, iron, magnesium, manganese, cobalt) have functional roles in enzymes and proteins (Yokel, 2006; Molina-Holgado et al., 2007; Farina et al., 2013). For example, brain iron is used by lipid and cholesterol synthesizing enzymes (Bartzokis, 2004) and up to 70% of brain iron is found in association with myelin (de los Monteros et al., 2000; Bartzokis, 2004). However, the careful control of metal ion compartmentalization and usage in the brain is critical, so that metal associated toxicity is avoided. The etiology of several neuropathologies includes a dysfunctional association between otherwise important trace elements and particular proteins or peptides (**Table 1**). Consequently the pathophysiology of metal-protein interactions in neurodegenerative diseases generally and in AD specifically is an area of growing interest. Divalent metal cations accumulate in plaque deposits and the inflammatory and oxidative processes which are well documented in AD may be mediated through chemistries involving metals (**Table 2**). However, the biochemistry of metal-protein interactions, sources of accumulating metals and chelation mechanisms are yet to be fully explored in AD.

The toxicity of Aβ is linked to changes in its structure from the soluble α-helical form to the insoluble β-pleated sheet form with consequent plaque formation, in which metals such as copper, zinc and iron are sequestered (Lovell et al., 1998a). It is not clear what molecular events trigger plaque formation, a process which may begin much earlier in life than the clinical symptoms of AD (Almkvist and Winblad, 1999). However, dissolution of plaque with metal chelating agents such as clioquinol is a potential new treatment (Cherny et al., 2000, 2001), highlighting the significant role that metals play in the etiology of this disease (Richardson, 2004).

Metal ions, such as those sequestered in plaques, also participate in oxidation and free radical production (**Figure 1**) (Multhaup et al., 1996). These processes are well documented in AD as are inflammatory processes, mediated by the presence of activated microglia and astrocytes, which generate high levels of Aβ (Busciglio et al., 1993). Metals such as copper, zinc, iron and aluminum have been implicated as possible contributors to neurodegenerative processes. In a few cases, well established links between metals and the function of specific proteins have been demonstrated (**Table 1**). However, as a subset of all the proteins studied in neuropathology, the metalloproteins are under-represented (Dobson, 2001). Since metal containing active sites of proteins are often involved in oxidation reactions and/or free radical generation, alterations to their biochemistry may be of particular interest in neurodegenerative conditions. Links between protein dysfunction and the role of metals in AD are emerging; (i) divalent metal cations are sequestered in Aβ plaques, (ii) oxidative processes are well documented in AD and metal cations, particularly iron, are a potential source of reactive species. Though metals are likely to play a significant role in AD and other inflammatory diseases, relatively little is known about their sources, mechanisms of transport and chelation, biochemistry and interactions with proteins.

Apart from redox active metals associated with the pathological hallmarks of AD, the presence of other trace metals may also be related to impaired cognitive function in AD. Several toxic heavy metals, including arsenic, lead, mercury, and cadmium are present in the environment due to their high industrial demand (Park et al., 2014). These metals serve no biological function, and their accumulation in the brain is attributed to contact between humans and the environment (Chowdhury and Chandra, 1987). Exposure to arsenic induces neuropathological and behavioral abnormalities similar to clinical features reported in AD and other related neurodegenerative disorders (Gong and O'Bryant, 2010). Lead, which is a well-established neurotoxic pollutant, can induce tau hyperphosphorylation, white matter degeneration, cellular apoptosis, and changes in cellular morphology, and impaired neuronal function (Yun and Hoyer, 2000; Rahman et al., 2012). While cadmium can induce hepatic and renal toxicity, cadmium and lead can also disrupt cholinergic transmission by reducing the turnover of the essential neurotransmitter, acetylcholine (Webster and Valois, 1981; Costa and Fox, 1983; Patra et al., 1999; Singh et al., 2012b). Inorganic mercury can mimic all the pathological hallmarks of AD in animal models (Saxe et al., 1999; Rusina et al., 2006; Mutter et al., 2010). Under normal physiological conditions, sequestration of arsenic, lead, cadmium, and mercury by the lateral choroid plexus represents a protective mechanism to prevent the influx of heavy metals from the blood and into the brain. However, elevated levels of cadmium and mercury can directly damage the choroid plexus, thus limiting the function of this endogenous defense mechanism (Gerhardsson et al., 2011). The toxicity of these metals in human neurodegenerative disorders is dependent on the concentration of the environmental contaminant, and chronic exposure to heavy metals can induce toxicity at relatively low levels (Llobett et al., 2003).

Table 1 | Metal Protein Interactions in Neurodegenerative Diseases.

Neurodegenerative disease	Metal/s	Metal binding protein with a link to neurodegeneration	References
AD	Zn^{2+}, Cu^{2+}, Fe^{2+}, Al^{3+}	Zn^{2+}, Cu^{2+}, Fe^{2+} are sequestered by Aβ fibrils and oligomers leading to oxidative stress. Al^{3+} is potentially involved in the formation of NFTs	Rodella et al., 2008; Thinnes, 2010; Savelieff et al., 2013; Watt et al., 2013
Down's syndrome	Zn^{2+}, Cu^{2+}, Fe^{2+}	Aβ fragment of the amyloid precursor protein associates with a number of divalent metals resulting in amyloid plaque formation	Kedziora et al., 1978; Prasher et al., 1998; Savelieff et al., 2013
Amyotrophic lateral sclerosis (Motor Neuron Disease)	Cu^{2+}, Zn^{2+}	Mutations in the metalloprotein superoxide dismutase (SOD) are associated with MND	Ince et al., 1994; Divers et al., 2006
Spongiform encephalopathies	Cu^{2+}	Prion Protein (Sc)	Basu et al., 2007; Singh et al., 2009, 2012a; Singh and Singh, 2010
Wilson's disease	Cu^{2+}	Mutations in ATP7B, a putative Cu^{2+} transporting gene product, leads to decrease in ceruloplasmin and consequent Cu^{2+} accumulation	Peng et al., 2012; Walshe, 2012; Liggi et al., 2013; Ni et al., 2013
Friedreich's ataxia	Fe^{2+}	Deficiency of mitochondrial protein frataxin is linked to altered Fe^{2+} homeostasis	Michael et al., 2006; Koeppen et al., 2007; Popescu et al., 2007; Lim et al., 2008
NBIA1 (Hallerverden-Spatz Syndrome)	Fe^{2+}	Brain Fe^{2+} deposition possibly in association with the protein synuclein	Valentin et al., 2006
Parkinson's disease	$Fe^{2+}Zn^{2+}$	Aggregates of α-synuclein form and release H_2O_2 in the presence of Fe^{2+} Increased localized brain Ferritin levels	Dashdorj et al., 2012; Lucas, 2012; Binolfi and Fernandez, 2013; Björkblom et al., 2013
Aceruloplasminemia	Cu^{2+}, Fe^{2+}	Mutations in the Cu^{2+} binding metalloprotein ceruloplasmin gene result in accumulation of Fe^{2+} in neurons	Dunaief et al., 2005; Kono et al., 2006; Oide et al., 2006; Gonzalez-Cuyar et al., 2008
Effects of Mn^{2+} in other neurodegenerative diseases	Mn^{2+}	Manganism can lead to Huntington's disease and Parkinsonian-like symptoms. The precise mechanism how manganese can damage the CNS is unclear	Bowman et al., 2011

The presence of sequestered biometals such as copper, zinc, and iron in β-amyloid plaques of AD-affected brain tissue, and the presence of toxicological metals as potential pathological cofactors in AD, has led to a focus on metal imaging (Hutchinson et al., 2005; Lelie et al., 2011; Pithadia and Lim, 2012; Stavitski et al., 2013). We should note that not only metals, but a wide range of elements may be imaged, down to ultratrace levels, and at length scales from micron to tens of nanometers. In certain cases isotopes, and even oxidation state and the coordination environment around specific elements can be imaged, potentially increasing the scope of trace element research in neurological disease beyond what has been studied to date. Visualizing changes in element concentration and matching them to anatomical and pathological features enhances our traditional approach to exploring the role of metal ions in neurological disease. Reviews on metal imaging in neurobiology have been presented recently (Bourassa and Miller, 2012) and a comprehensive range of instrumental techniques is available from McRae et al. (2009). However, the field continues to expand rapidly as spatially resolved elemental analysis is now a well-recognized method to investigate chemical changes associated with pathology in biological tissues. The experimental techniques used to obtain elemental information from tissues are quite diverse, with a range of different capabilities in spatial resolution, sensitivity and quantification. This review provides an overview of common instrumental techniques and examples of biological imaging with an emphasis on Alzheimer's studies. Elemental imaging is the main topic of this review; although a selection of molecular imaging examples are presented to demonstrate how these techniques can supplement the elemental bioimaging. Selective colorimetric and fluorescent

Table 2 | Processes in the Alzheimer's Disease Brain linked with metals.

Process	Chemistry	Mechanism	Products or outcome	References
Oxidative stress	Fenton reaction	$H_2O_2 + Fe^{2+} \rightarrow OH^{\bullet} + Fe^{3+} + OH^-$	Oxidized amino acids (e.g., *o/m*-tyrosine, DOPA, methionine sulfoxide), side-chain and peptide bond cleavage, carbonyls	Novellino et al., 1999; Tangkosakul et al., 2009; Thomas et al., 2009
	Haber-Weiss reaction	$O_2^{\bullet-} + Fe^{3+} \rightarrow Fe^{2+} + O_2 \rightarrow$ Fenton reaction	Oxidized amino acids, side-chain and peptide bond cleavage, carbonyls	Winterbourn, 1987; Burkitt and Gilbert, 1990; Egan et al., 1992; Khan and Kasha, 1994
	Metal catalyzed oxidation (MCO)	$2e^-$ (metal e^- donor) $+ O_2 + 2H^+ \rightarrow H_2O_2$ And H_2O_2 + protein(lys) ligated $Fe^{2+} \rightarrow$ protein alkyl radical/s	Protein carbonyls	Chan and Newby, 1980; Litwin, 1982; Ahmed et al., 1993; Wang et al., 2004
Protein aggregation	Protein unfolding and metal binding	α-helical ⇨ β-pleated sheet Aβ β-pleated sheet Aβ + redox-active metals ⇨ Aβ oligomers Aβ oligomers + metals (Cu^{2+}, Zn^{2+}, Fe^{2+}) ⇨ plaque Tau may also have adverse interactions with metal ions in AD	Aβ oligomers Aβ plaque	Giese et al., 2004; Fu et al., 2005; Ricchelli et al., 2006; Long et al., 2008; Salgado et al., 2008; Torosantucci et al., 2013
Neuronal toxicity and/or Cell death	Direct toxicity to brain cells Dysregulation of Calcium ion homeostasis	Altered permeability of cation channels (ionotropic glutamate receptors)	Cell Death by apoptosis or necrosis	Yoshida et al., 1987; Salanki et al., 1996; Xing et al., 2009; Gu and Lin, 2010
Effects on myelination	Iron is needed for myelination	Features of AD include white matter hyperintensity, axonal, neurite and synaptic changes	Impaired neurotransmission	Ortiz et al., 2004; Bartzokis et al., 2007; Baeten et al., 2010; Romero et al., 2010; Paling et al., 2012

staining is not covered here, but has been recently reviewed with a focus on neurobiology (Que et al., 2008).

GENERAL OVERVIEW OF ELEMENTAL IMAGING TECHNIQUES FOR BIOLOGICAL TISSUES

A significant challenge in this field is measuring a specific area on a sample that is small enough to remain biologically relevant, but large enough to enable the elements to be detected. When visual imaging is required to match anatomical features with the elemental distribution, the measurement needs to be carefully planned to leave any destructive analysis as the last step. Most of these techniques will damage the sample to an extent, for example radiation damage in the case of synchrotron techniques, or ablation of the sample surface into gas or ions in the case of laser and ion beam sampling.

It can be instructive to present the most common methods for elemental imaging by their sampling modes, as this will influence the achievable spatial resolution and detection limit. The most common techniques can be classified as (a) ablation of material off the surface that is then directed into an elemental analyzer, (b) ion generation within the sample, and (c) ion generation and ablation from the sample surface. Most of these techniques create spatially resolved elemental data by moving a flat specimen on a stage in precise intervals under the incident beam, and recording the change in analyte flux (ion, electron, photon) that is associated with a specific element.

ABLATION TECHNIQUES

A pulsed laser can be used to ablate material from a selected area of the sample surface and the gaseous plume swept into another instrument for elemental analysis. A laser ablation (LA) sampling accessory can be integrated with a more traditional atomic spectroscopy system for sensitive multi-elemental analysis. These are usually inductively coupled plasma (ICP) systems using mass spectrometry (LA-ICPMS), or optical emission spectroscopy (LA-ICPOES) for detection of the elements (Qin et al., 2011). Mass spectrometry has the advantage of higher detection limits than optical emission spectroscopy, however there are drawbacks with mass interferences, and the time taken to sweep through the selected ion set, resulting in fewer available ions

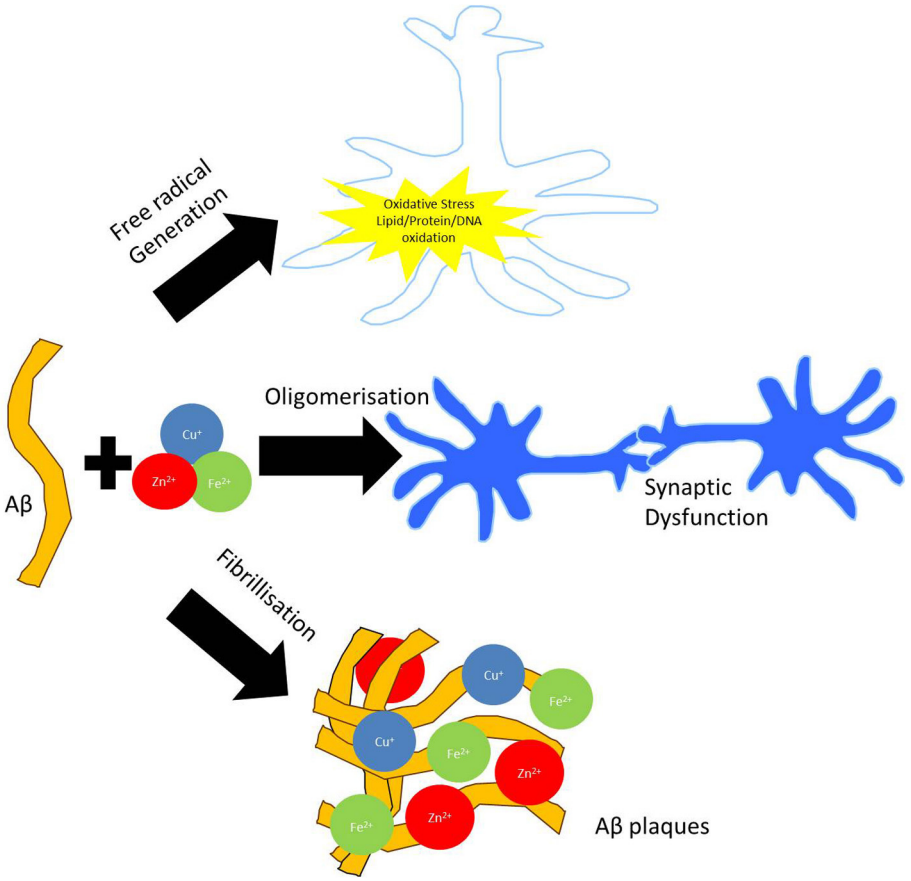

FIGURE 1 | Involvement of metal dyshomeostasis in AD pathology. Aggregation of Aβ can bind redox active metals such as copper, iron, and zinc in amyloid plaques. Sequestration of these biometals on Aβ fibrils and oligomers can potentiate synaptic dysfunction. Redox cycling of Cu^{2+}/Cu^{+} and Fe^{3+}/Fe^{2+} in the amyloid plaques are capable of producing hydrogen peroxide (H_2O_2), which can enter the cell. Through Fenton chemistry this can lead to the production of hydroxyl radical ($OH^{\bullet}$) capable of inducing oxidative modifications to both extracellular (i.e., proteins and lipids) as well as intracellular (DNA) macromolecules.

in order to create an image within a practical length of time. For example, imaging 6 metals across a 4 × 4 mm tissue section with a step size of 30 micron might take 12–24 h (Ketola and Mauriala, 2012). Nevertheless, LA-ICPMS is by far the more common technique for elemental imaging of biometals and toxicological metals in tissues than LA-ICPOES. Another variation on laser sampling is to detect the atomic excitation spectrum directly from the ablated plume, a technique known as laser induced breakdown spectroscopy (LIBS) (Pareja et al., 2013). LA techniques provide excellent analytical sensitivity in atmospheric or relatively low vacuum conditions. However, it is a destructive technique, and delivering sufficient energy to the sample to allow detection tends to limit the spatial resolution. As a result, LA techniques are well suited for analysis of whole tissue sections, but individual cells or pathological features such as amyloid plaques ∼20 micron are represented in an image as a single measured point (Hare et al., 2010, 2011; Lear et al., 2012; Chou et al., 2014). Metal imaging of an individual cell or plaque requires the higher resolution available from some of the techniques described below.

SAMPLE IONIZATION TECHNIQUES

Highly focused X-rays, electrons, or proton ion beams in a high-vacuum chamber can be used to eject an electron from the core shell of an atom in the sample (Fahrni, 2007). The energy of the ejected electron can be measured using X-ray photoelectron spectroscopy (XPS) to determine the element from which it originated. In certain cases, XPS is able to provide information on oxidation states and the chemical environment around an element, although spatial resolution is limited to 5–50 micron, and detection limits are relatively poor (around 0.1 atomic%), virtually ruling out the technique for trace metal studies (Paunesku et al., 2006). The majority of sample ionization techniques utilize the secondary process where outer shell electrons fill the core shell hole in the ion fluorescing X-rays with a characteristic wavelength for each element in the sample. When atoms are ionized using an electron beam, usually in an electron microscope, the technique is known as energy dispersive X-ray spectroscopy (EDX or EDS), sometimes referred to as electron photon micro analysis (EPMA). If ionization is achieved using an X-ray beam, the technique is X-ray fluorescence microscopy (XFM) or synchrotron

radiation micro-X-ray fluorescence (SR-μXRF) (Paunesku et al., 2006; Ralle and Lutsenko, 2009). Ionization can also be performed using a focused beam of protons in a technique called particle induced X-ray emission (PIXE). All of these techniques are performed in high-vacuum environments, so steps such as cryopreservation or careful drying must be taken to protect biological samples or specimen degassing that can reduce the performance of the instrument (de Silva et al., 2006; George et al., 2011; Ramsay et al., 2011; Weekley et al., 2013).

SECONDARY IONIZATION TECHNIQUES

Ablation-ionization directs a highly focused beam of ions, such as oxygen or cesium in the case of secondary ionization mass spectrometry (SIMS), onto a tissue surface under vacuum (Altelaar and Piersma, 2010). This is a destructive process that results in ions being ejected from the surface. The ions are usually detected with a magnetic sector (NanoSIMS) or time-of-flight (TOF SIMS) mass spectrometer (Pacholski and Winograd, 1999; Eller et al., 2013; Fernandez-Lima et al., 2013). A recent review is available detailing the general capabilities of mass spectrometry-ablation techniques such as SIMS (Amstalden van Hove et al., 2010). The ability to focus ion beams down to very small spot sizes enables excellent spatial resolution, with features of 50 nanometers having been reported in the case of the NanoSIMS. However, micron to submicron imaging is more common since, in order to generate sufficient secondary ions for detection with a very small spot size, the ablation depth needs to increase. Submicron imaging at hundreds of nanometers is more common, and is sufficient for cellular differentiation or observing small pathological features (Quintana et al., 2007; Musat et al., 2012). It is notable that the mass spectrometry techniques also enable more specialized imaging of isotopes across a surface, as well as providing more general elemental imaging.

OTHER TECHNIQUES

Electron energy loss spectroscopy (EELS) measures the energy loss due to scattering processes when a low energy, monoenergetic electron beam interacts with a sample. When used in a transmission electron microscope, EELS can provide atomic-scale resolution with excellent detection limits although biological applications are limited (Quintana et al., 2000; Terada et al., 2002). There are a variety of X-ray techniques that have evolved as a result of the high-intensity X-ray sources available at numerous synchrotron facilities around the world. X-ray Absorption Near Edge Structure (XANES), also known as Near edge X-ray absorption fine structure (NEXAFS), is a technique where the element composition change the absorption spectrum of the X-ray beam, providing information on elemental oxidation state and coordination geometry around metal ions (Bourassa and Miller, 2012). Although potentially powerful, imaging of biological materials using this technique is still in development.

Magnetic resonance imaging (MRI) remains the most widely used metal imaging technique in the clinical setting (Helpern et al., 2004). Although recent advances in MRI have made it possible to detect the levels of iron at physiological concentrations, copper and manganese are still not widely detectable, since they are present in low concentrations in the brain (Schenck and Zimmerman, 2004). Current MRI techniques exhibit lower spatial resolution compared to elemental imaging techniques mentioned above, but demonstrate the advantage of imaging live patients rather than cryo-cut postmortem tissue sections (Schenck and Zimmerman, 2004).

Positron emission tomography (PET) is another technique which facilitates *in vivo* medical imaging, usually of small molecules including glucose and more recently Aβ plaques using Pittsburg Compound B (PiB PET). More recently a novel metal imaging PET approach has been developed, using radioactive coordination bis(thiosemicarbazonato)copper complex of ^{64}Cu. This targets copper homeostasis and has been designed to bind selectively to amyloid plaques (Hickey et al., 2013). Copper radiolabels are essential for increasing our understanding on of the mechanisms of copper dyshomeostasis in AD.

COMBINED BIOIMAGING TECHNIQUES IN TISSUE SECTIONS

Complementary information regarding the role, uptake, transport, and storage of redox active metals associated with irregular protein abnormalities can be obtained using a combination of elemental imaging techniques, such as LA-ICPMS, and other biomolecular mass spectrometry imaging techniques such as laser ablation coupled with electrospray ionization mass spectrometry (LA-ESI-MS) or MALDI-IMS. While LA-ICPMS can be employed to identify the specific protein-bound metals, ESI-MS/MALDI enables the identification of the structure, dynamics and biological function of metal-protein complexes (Becker et al., 2008; Dobrowolska et al., 2008; Jakubowski et al., 2008; Wu et al., 2009).

ESI-MS is an ionization technique that is employed to detect polar compounds within a biological specimen (Fenn et al., 1989). This method is used to identify molecules that do not contain an intrinsic ionizable site through formation of adduct ions. Molecules which exhibit sufficient dipole potential to interact with a small anion or cation can be readily ionized and detected using ESI-MS. It is useful for the detection of triacylglycerols (TAGs) which contain long chain fatty acids. These molecules can be ionized and quantified with sensitivity in the low picomole range due to the formation of lithiated adducts which are formed when chelated lithium ions non-covalently bond with the carbonyl structures that are present in the infused solution (Han et al., 2000; Han and Gross, 2001). The benefits of using ESI-MS include more accurate quantification of lipid classes and subclasses, a greater signal-to-noise ratio in comparison to other mass spectrometry techniques, and an almost linear relationship between the relative intensities of molecular ions and the mass of individual lipids (Han and Gross, 1994).

MALDI-IMS allows the analysis of a diversity of biopolymers with a variety of mass ranges. This approach has a lower spatial resolution but much higher mass range than TOF-SIMS, which is limited to identification of analytes with a molecular mass of less than 1 kDa (McArthur et al., 2004). A variety of analytes can be examined using MALDI-IMS, including metabolites, lipids, proteins, peptides, carbohydrates, and drugs. However, this method is limited by signal suppression effects. For instance, some analytes are more efficiently ionized during

MALDI-IMS. These artifacts are not only due to their unique chemical structure, but also to relative amounts present in the biological tissue (Knochenmuss et al., 1998). Alternatively, proteins can be extracted from the tissue section using hydrophobic materials, while preserving their specific location (Chaurand et al., 2004). Adaptation of MALDI-TOF to 2D and even 3D tissue imaging applications has necessitated use of rapid fire long lived lasers, such as the 2 kHz Nd-YAG, to accommodate the need to acquire 1000s of spectra across a tissue section. High end MALDI imaging mass spectrometers currently combine high mass resolution of 40,000 (1 ppm mass accuracy), wider mass range (50–300,000 Da), spatial resolution down to 10 μm, and TOFTOF capabilities for peptide sequencing. This combination of features allows detailed characterization of a diversity of tissue constituents, top-down sequencing of proteins as well as the more commonly used bottom-up techniques involving enzymatic/tryptic digestion and peptide sequencing, analysis of posttranslational modifications such as glycosylation. A growing body of literature recognizes the power of this approach (Cornett et al., 2007; Schuerenberg et al., 2007). A combination of mass spectrometry imaging techniques using LA-ICPMS and detailed proteomics analysis can be performed using thin cryo-cut sections of brain. MALDI-IMS is a relatively non-destructive technique so the tissue remaining after initial proteomic, metabolomic or lipidomic analysis can then be analyzed for elemental composition using LA-ICPMS.

Fourier transform infrared spectroscopy (FTIR) is another molecular imaging tool that can be combined with LA-ICPMS. These tools have been used to image the secondary structure of metal-protein complexes (Haris and Severcan, 1999). FTIR is a non-destructive technique, allowing further analyses to provide complementary information and to show spatial relationships between diverse analytes and/or functional groups, which may provide insight into biological/functional relatedness. The protein's FTIR consists of two main features: the Amide I band ($\sim$1650 cm^{-1}) which arises from the C=O stretching vibration, and the Amide II band ($\sim$1540 cm^{-1}) which is due to the N-H bending and C-N stretching vibrations of the peptide backbone. The vibrational frequency of an aggregated protein is about 1620–1625 cm^{-1}, owing to its hydrophobic environment (Goormaghtigh et al., 2006; Miller et al., 2006). Apart from examining the protein structure *in vitro*, FTIR can also be used to directly investigate irregular protein misfolding and aggregation both *in vitro* and *in vivo*. Protein aggregates are generally small, ranging from nanometers, to 20–30 μm for larger aggregates. As well, the spectral differences related to changes to protein conformation are subtle, requiring spectra with high signal to noise ratio (Choo et al., 1996; Miller et al., 2006). These difficulties have been resolved using the greater brightness of a synchrotron infrared source to directly assess protein aggregation and misfolding in AD tissue.

RECENT APPLICATIONS OF BIOIMAGING IN ALZHEIMER'S RESEARCH

Metals have been shown to be associated with the pathogenesis of AD for over 50 years since the discovery of significant iron deposition in postmortem AD brain tissue using Prussian blue stain (Goodman, 1953). Since then, other redox active metals have been implicated in AD, including copper, zinc, and aluminum. Several metal bioimaging strategies have been utilized to examine the distribution of metals in human clinical AD brain tissue and AD mouse models to better understand the relationship between metal dyshomeostasis and the etiology and progression of AD.

METALS AND Aβ PLAQUES

It has been well established that Aβ plaques are rich in metal ions (Opazo et al., 2002). These relatively high concentrations of metals within the plaques compared to adjacent tissue have been reaffirmed using a variety of bioimaging techniques. PIXE and XFM has been used to show that both the outer and central regions of the Aβ plaques contain elevated levels of iron, copper and zinc in human AD brain specimens (Lovell et al., 1998a,b). Although copper and zinc binding sites are present on the Aβ peptide, iron does not appear to directly interact with Aβ (Atwood et al., 2000; Bush, 2003; Roberts et al., 2012). Recently, synchrotron X-ray absorption, diffraction, and tomography techniques have been used to identify the presence of biogenic magnetite and/or maghematite in the plaque cores, implicating the likely role of a novel biomineralization process to account for the accumulation of iron in Aβ plaques (Collingwood et al., 2005, 2008).

Transgenic mouse models have provided additional advantages over postmortem human clinical AD specimens in the control of both genetics and onset of AD-like symptoms. Using XFM, no abnormal increase in copper or iron were reported in with disease progression in the PSAPP double transgenic mouse which expresses a chimeric mouse/human amyloid precursor protein (Mo/HuAPP695swe) and a mutant human presenilin 1 (PS1-dE9) both directed to CNS neurons. This mouse model develops amyloid pathology as well as learning and memory deficits by 6 months of age, independent of signs of neurodegeneration (Leskovjan et al., 2011). Moreover, only a slight upregulation in zinc concentrations was reported at the late stages of the disease. By contrast, the CRND mouse which expresses two familial mutations in the human Swedish (K595N/M596L) and Indiana (V717F) APP gene exhibited a 2–3-fold increase in the concentration of iron, copper, and zinc in the plaques after 6 months of age using PIXE. This unique mouse model develops diffuse and compact plaques by 10 weeks of age and Aβ deposition continues with advanced age (Rajendran et al., 2009). Similar findings have been reported using LA-ICPMS analysis of plaques present in the brains of TASTPM mice, which carry both the APP K670N/M671L and PS1M146V mutation and develop plaques by 4 months of age (Hutchinson et al., 2005).

METAL DYSHOMEOSTASIS IN AGING AND AD

Since ageing is a major risk factor for the development of AD, examining the age-related changes in metal distribution is critical for understanding the role that metals play during pathological and physiological conditions. Using LA-ICPMS, one study showed that iron levels were increased in the "physiologically" aged brain of a non-transgenic mice (14 months) compared to a young (2 month) mice (Becker et al., 2010). These increases were observed in the substantia nigra, thalamus, and the CA1 region of the hippocampus which are associated with development of

neuropathologies. Remarkably, zinc levels remained unchanged and zinc-enrichment in the CA3 of the hippocampus was already detected in young mice. This may be associated with the important role of zinc as an essential neuro-co-transmitter that is released from synaptic vesicles (Becker et al., 2010).

Evidence of metal dyshomeostasis has also been reported in AD. Studies using PIXE have shown increased levels of zinc in the amygdala, hippocampus and neuropils of human AD brains (Danscher et al., 1997; Lovell et al., 1998a,b). This is likely to be associated with the increased distribution of zinc enriched neurons (ZEN) which are located in these regions. ZENs maintain intracellular pools of zinc which is necessary as a neuromodulator and neuro-co-transmitter. One hypothesis suggests that zinc released from these neurons can interact with Aβ and promote aggregation (Bush et al., 1994; Frederickson et al., 2005). Zinc deficiency can also lead to excitotoxicity and neurodegeneration (Sensi et al., 2009). Moreover, zinc reuptake is an energy dependent process, and mitochondrial dysfunction can lead to increased free zinc which can interact with Aβ and lead to further neurotoxicity (Mony et al., 2009).

Altered iron levels have also been suggested to play a prominent role in ageing and AD. Iron levels have been shown to increase in the substantia nigra, motor rotex, hippocampus, basal ganglia, putamen, cerebellum and cortex of human normal subjects during ageing (Connor et al., 1992; Deibel et al., 1996; Bartzokis et al., 2000). A similar increase was also reported iron, copper and zinc content was also reported in the PSAPP mouse model in the cortex and hippocampus, and coincided with increased plaque formation using XFM (Leskovjan et al., 2011). Ferritin, the main protein responsible for iron storage, has been shown to increase in the coronal region of human AD plaques using TEM and NanoSIMS (Quintana et al., 2006). It is likely that ferritin, which stores inactive iron (III) under normal physiological conditions may bind redox active iron (II) in the AD brain leading to cell death via oxidative stress.

METALS AND NFTs

Metal dyshomeostasis may also play a role in the formation of NFTs. A 10-fold increase in iron and a 6-fold increase in copper, with a smaller increase in zinc, have been previously reported in NFTs (Morawski et al., 2005). Furthermore, hyperphosphorylated tau, which forms paired helical filaments (PHFs) that lead to NFTs, contains several binding domains which demonstrate some affinity to copper, and the presence of copper can enhance the formation of NFTs (Ma et al., 2006). Iron (III) can also induce NFT formation similar to copper (Yamamoto et al., 2003). Apart from copper, iron and zinc, aluminum has also been associated with the development of AD since it was first identified in neurons with NFTs (Perl and Brody, 1980). However, increased aluminum is also present in non-diseased brain tissue fixed with osmium tetroxide, which contains aluminum (Tokutake et al., 1995; Makjanic et al., 1997). Further work is warranted to validate the involvement of aluminum in AD.

LIPIDOMIC STUDIES USING ESI/MS

ESI-MS techniques have been used to investigate the lipidome in patients with dementia. These studies have demonstrated specific changes to the lipidome in the postmortem gray and white matter in the frontal, temporal and parietal cortex at the earliest clinically-recognizable stage of AD compared to cognitively normal control (Han et al., 2001, 2002). Specifically, plasmenylethanolamine (PlsEtn) mass was reduced by up to 40 mol% of total plasmalogens, in white matter in early AD subjects compared to age-matched controls. PlsEtn mass levels were depleted by 10% in the gray matter in patients with severe AD. Sulfatides, which form specialized components in the myelin sheath which encapsulate neurons, were depleted by 93 and 58 mol% in gray and white matter, respectively, in AD patients in all brain regions that were investigated (Han et al., 2001, 2002). Additionally, a significant increase (>3 fold) in ceramide content was observed in the white matter of all investigated brain regions during early AD. No significant changes have been observed in the levels of other lipid classes, including phosphatidylglycerols, phosphatidylinositols, phosphatidylserines, and phosphatidic acids in early stages of AD although significant reduction (∼15 mol%) of these lipids occurred in severe AD cases (Han et al., 2001, 2002). Taken together, these results suggest that changes to the lipidome may play a vital role in the pathogenesis of AD and may be associated with early molecular and cellular events which occur in the development of AD, such as neurodegeneration and synaptic dysfunction.

MALDI-MS IMAGING IN AD

Recently, MALDI-MS has been used to examine the spatial distribution and molecular contents of Aβ plaques. One study showed that Aβ-(1–40) and Aβ-(1–42) are the most abundant amyloid peptides in APP23 transgenic mice encoding the hAPP751 with Swedish mutation (Rohner et al., 2005). In support of this work, other studies have shown that vascular amyloid is primarily composed of Aβ-(1–40) and Aβ-(1–42) (Miller et al., 1993). Additionally, Aβ-(1–40) is the major peptide that is found in aqueous cerebral cortical extracts from AD brains. By contrast, the insoluble amyloid Aβ-(1–42) peptide is primarily localized in the senile plaque cores. Therefore, MALDI-MS can not only be used to identify known targets, but also facilitates mapping of the different peptide targets with high precision and accuracy, that is otherwise not possible when examining whole-brain extracts (Rohner et al., 2005).

FTIR SPECTROSCOPIC IMAGING IN AD

In AD, Aβ plaques are formed by the transformation of Aβ from a soluble form through to an oligomeric intermediate, culminating in the formation of an aggregated, fibrillary structure (Ii, 1995). The molecular mechanism which mediates the structural changes and cytotoxicity of Aβ during the aggregative process has been previously investigated in several *in vitro* studies using dichroism (CD) and nuclear magnetic resonance (NMR) to show the structural conversion of Aβ from a soluble α-helical protein to a fibrillar β-sheet protein (Barrow et al., 1992; Zhang and Rich, 1997). FTIR spectroscopy has been essential to examine the specific alignment of β-sheet strands within Aβ fibrils. A study by Petty and Decatur (2005) showed that β-sheets are antiparallel and in alignment across all strands (Petty and Decatur, 2005). Recently, it has been suggested that oligomeric Aβ is more

neurotoxic than Aβ fibrils and can form pore-like structures in lipid membranes that can disrupt ion homeostasis, culminating in cell death. FTIR spectroscopy has shown that Aβ oligomers exhibit an antiparallel β-sheet structure, which is closely related to that of bacterial outer membrane porins (Komatsu et al., 2007).

Apart from the Aβ protein, FTIR spectroscopy has also been used to gain a greater understanding of the structural conformation of the tau protein, which is hyperphosphorylated in AD, leading to the formation of NFTs. *In vitro* FTIR analysis provided confirmatory evidence that soluble tau protein is natively unfolded and composed of random coil structures, whilst PHFs which are present in the AD brain have a greater level of β-structure (Berriman et al., 2003). These results provide evidence to support the hypothesis that the repeat domain of tau (which is located within the core of PHFs) displays an enhanced level of β-structure during aggregation, while the N- and C-terminal domains which venture away from the central PHF core are largely random coils (Barghorn et al., 2004).

CONCLUSION

Metal imaging techniques are currently primed to facilitate an understanding of the pathobiology of AD, as well as identifying novel diagnostics and therapeutics. Bioimaging techniques are important for elucidating the role of metals in neurodegenerative diseases generally and AD in particular. Advancements in methodology and improved spatial resolution and detection sensitivities are essential for greater insight into the localization and distribution of metal ions at the cellular and tissue level, and their role in disease development and progression. A combination of other imaging techniques such as ESI- and MALDI-IMS, FTIR spectroscopy, and clinical techniques allowing *in vivo* analysis, such as MRI and PET, are invaluable in obtaining further understanding on the molecular mechanisms involved in the pathogenesis of AD and to confirm the diagnosis of AD through the identification of unique biomarkers present in the metabolome, lipidome and/or proteome. Additionally, the techniques described in this review have the potential to follow disease progression in AD patients from early to severe stages and assess the effect of novel therapeutic interventions which may retard, stop or reverse progressive neurodegeneration, the ultimate goal being a cure for this debilitating neurodegenerative disorder.

AUTHOR CONTRIBUTIONS

Nady Braidy, Christopher Marjo, Anne Poljak, Tharusha Jayasena, Nibaldo C. Inestrosa, and Perminder Sachdev wrote the draft, reviewed and interpreted the bioimages. Helen Rutlidge and Anne Rich processed the images. Nibaldo C. Inestrosa and Perminder Sachdev provided the conceptual foundation of the review, writing of drafts and interpretation of data.

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Aging- and injury-related differential apoptotic response in the dentate gyrus of the hippocampus in rats following brain trauma

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The elderly are among the most vulnerable to traumatic brain injury (TBI) with poor functional outcomes and impaired cognitive recovery. Of the pathological changes that occur following TBI, apoptosis is an important contributor to the secondary insults and subsequent morbidity associated with TBI. The current study investigated age-related differences in the apoptotic response to injury, which may represent a mechanistic underpinning of the heightened vulnerability of the aged brain to TBI. This study compared the degree of TBI-induced apoptotic response and changes of several apoptosis-related proteins in the hippocampal dentate gyrus (DG) of juvenile and aged animals following injury. Juvenile (p28) and aged rats (24 months) were subjected to a moderate fluid percussive injury or sham injury and sacrificed at 2 days post-injury. One group of rats in both ages was sacrificed and brain sections were processed for TUNEL and immunofluorescent labeling to assess the level of apoptosis and to identify cell types which undergo apoptosis. Another group of animals was subjected to proteomic analysis, whereby proteins from the ipsilateral DG were extracted and subjected to 2D-gel electrophoresis and mass spectrometry analysis. Histological studies revealed age- and injury-related differences in the number of TUNEL-labeled cells in the DG. In sham animals, juveniles displayed a higher number of $TUNEL^+$ apoptotic cells located primarily in the subgranular zone of the DG as compared to the aged brain. These apoptotic cells expressed the early neuronal marker PSA-NCAM, suggestive of newly generated immature neurons. In contrast, aged rats had a significantly higher number of $TUNEL^+$ cells following TBI than injured juveniles, which were NeuN-positive mature neurons located predominantly in the granule cell layer. Fluorescent triple labeling revealed that microglial cells were closely associated to the apoptotic cells. In concert with these cellular changes, proteomic studies revealed both age-associated and injury-induced changes in the expression levels of three apoptotic-related proteins: hippocalcin, leucine-rich acidic nuclear protein and heat shock protein 27. Taken together, this study revealed distinct apoptotic responses following TBI in the juvenile and aged brain which may contribute to the differential cognitive recovery observed.

Keywords: aging, apoptosis, dentate gyrus, neurogenesis, proteomics, traumatic brain injury

INTRODUCTION

Traumatic brain injury (TBI) is a major cause of death and disability world-wide. With the increase in numbers of the aging population in the United States, the epidemiology of TBI has shifted toward this demographic, with falls representing the leading cause of brain injuries involving the elderly. Although no overt neuronal cell loss is observed in the brain with aging, several subtle structural, chemical, and metabolic changes occur that render the aged brain more vulnerable to TBI as compared to the young brain. These changes include a reduction in the complexity of dendritic arborization, dendritic length, and spine numbers (Dickstein et al., 2007), increased oxidative stress and altered metabolic functions (Mattson and Magnus, 2006), and increased glial cell reaction and neuroinflammation (Frank et al., 2006). Collectively, these changes result in less plasticity and repair potential for the aged following TBI and lead to more enduring functional deficits.

The hippocampus, a region responsible for learning and memory functions, is particularly vulnerable to TBI. The learning and memory deficits observed following TBI are likely a reflection of differential susceptibility of neurons in different hippocampal subregions to injury (Small et al., 2011). Under normal conditions, new cells are constantly generated in the dentate gyrus (DG) in the hippocampus. Of these newly generated granule cells, approximately half of them die via apoptosis within the first month following their generation (Dayer et al., 2003); those that survive ultimately mature into functional granule neurons (van Pragg et al., 2002; Ramirez-Amaya et al., 2006) involved in hippocampal-dependent learning and memory functions (Clelland et al., 2009;

Deng et al., 2009). With normal aging, the neurogenic capacity of the DG is significantly decreased, with a concomitant increased vulnerability of neurons in this region and the decline in cognitive function (Pavlopoulos et al., 2013). Following TBI, in young adult animals, the injured brain displays a significantly enhanced neurogenic response in the DG (Chirumamilla et al., 2002; Sun et al., 2005). However, heightened levels of hippocampal neuronal degeneration and cell death, particularly among the newly generated neurons in the DG, are also observed following TBI (Gao et al., 2008).

As neuronal generation and degeneration concomitantly exist following TBI, the observed age-related differences in recovery may be due to not only the level of neurogenesis but also differences in the degree of cell death occurring after brain injury. Neural cell loss in the hippocampus has been linked to multiple neurochemical pathways and cell death cascades leading to necrosis and apoptosis (Raghupathi, 2004). Apart from necrotic cell death due to focal tissue damage following TBI, cell death consistent with apoptosis has been observed in the cortex, hippocampus, and thalamus both in clinical and experimental brain injury (Clark et al., 1997; Conti et al., 1998; Fox et al., 1998; Newcomb et al., 1999). Underscoring the incredible scope of the cellular response after injury is the finding that apoptotic neurons have been observed in the human hippocampus up to 12 months after injury (Williams et al., 2001).

In order to ascertain the potential link between apoptotic cell death and the observed age-related differences in functional recovery following TBI, the current study was undertaken to investigate the levels of aging and injury associated apoptotic cell death and the proteomic profiles of apoptosis-related proteins in the DG of the juvenile and aged rats.

MATERIALS AND METHODS

ANIMALS

Juvenile (postnatal day 28; weighing approximately 70 g) and aged (24 months; weighing approximately 575 g) male Sprague–Dawley rats (Harlan Inc., IN, USA) were used. Animals were housed in the animal facility, with a 12-h light/dark cycle, water and food provided *ad libitum*. All procedures were approved by our Institutional Animal Care and Use Committee.

FLUID PERCUSSION INJURY

Animals were subjected to moderate TBI ($n = 7$ for each age group) or sham injury ($n = 7$ for each age group) using the lateral fluid percussion injury (FPI) model as previously described (Chirumamilla et al., 2002; Sun et al., 2005, 2007). Briefly, rats were anesthetized in a plexiglass chamber with 3% isofluorane in 30% O_2/70% N_2, intubated and ventilated with 2% isofluorane in 30% O_2/70% N_2 and secured in a stereotaxic frame. Since intubation was not feasible in juvenile rats, these animals received continuous anesthesia via nose cone with the gas mixture described above. A midline incision was made to expose the skull and a 4.9 mm craniotomy was made on the left parietal bone halfway between the sutural landmarks lambda and bregma. A modified Luer lock fitting was then secured to the skull using cyanoacrylate adhesive and dental acrylic. A moderate fluid pressure pulse (2.00 ± 0.05 Atm) was administered through the craniotomy onto the intact dura, using a pre-calibrated FPI device. After injury, the Luer lock fitting was removed, the wound sutured and after a 3-h observation, the rats were returned to the vivarium. Sham animals underwent the same surgical procedure, but did not receive the injury pulse. Animals were allowed to survive for 48 h following injury, at which point they were anesthetized and brain tissue processed for either histochemical or proteomic analysis.

TISSUE PREPARATION FOR HISTOCHEMICAL PROCEDURES

Forty-eight hours following injury, animals ($n = 4$/group) were anesthetized with isofluorane, euthanized with euthasol (pentobarbital sodium, 780 mg/kg; phenytoin sodium 100 mg/kg), and transcardially perfused with phosphate buffered saline (PBS) immediately followed by 1% paraformaldehyde in PBS. The brains were rapidly dissected and immediately frozen in dry ice-chilled isopentane at −30°C. Ten μm-thick coronal sections spanning the rostro-caudal extent of the hippocampal DG (corresponding to the Paxinos and Watson stereotaxic rat atlas coordinates of −2.5 to −5.2 relative to bregma (Paxinos and Watson, 1986) were cut by cryostat, collected onto Superfrost®/Plus Slides (Fisher Scientific) and stored at −80°C until histochemical procedures were conducted.

TUNEL HISTOCHEMISTRY

In order to assess apoptosis levels in the DG, TUNEL histochemistry was performed according to the manufacturer's protocol using the ApopTag® Plus Fluorescein *In Situ* Apoptosis Detection Kit (Millipore, Billerica, MA, USA). Briefly, sections were post-fixed in pre-cooled ethanol:acetic acid (2:1) for 5 mins at −20°C, followed by two 5 mins washes in PBS. An equilibration buffer was applied for 10 s followed by 1 h incubation in a labeling solution of working strength TdT enzyme (composed of reaction buffer and TdT enzyme) in a humidified 37°C chamber. Following a 15 s agitation, sections were incubated for 10 mins in the stop/wash buffer to terminate the reaction and subsequently washed three times (one minute each) in PBS. In order to visualize the DNA fragments, sections were incubated in working strength anti-digoxigenin conjugate (composed of blocking solution and anti-digoxigenin antibody conjugated to fluorescein) for 30 min in a dark, humidified chamber at room temperature. Following four PBS washes, a mounting medium containing 0.5 μg/mL of a DAPI nuclear counter stain (Vector Lab, Burlingame, CA, USA) was applied to sections and slides were coverslipped.

QUANTIFICATION OF APOPTOTIC CELLS

TUNEL-stained sections were examined at 40× with an Olympus BX51 microscope with a BX-RFA fluorescence illuminator (Olympus, Tokyo, Japan). To quantify the degree of apoptotic cell death in the DG, every tenth coronal section throughout the rostro-caudal extent of the hippocampus was examined (for a total of 25 sections at 10 μm thickness per brain) and TUNEL-positive cells were systematically counted in a defined sampling region. The sampling region of interest extended from the hippocampal fissure down to the border of the lateral ventricle, including the suprapyramidal and infrapyramidal blades, granule cell layer (GCL), subgranular zone, and hilus of the DG (**Figure 1A**). A cell was considered apoptotic only if its nucleus was labeled with both

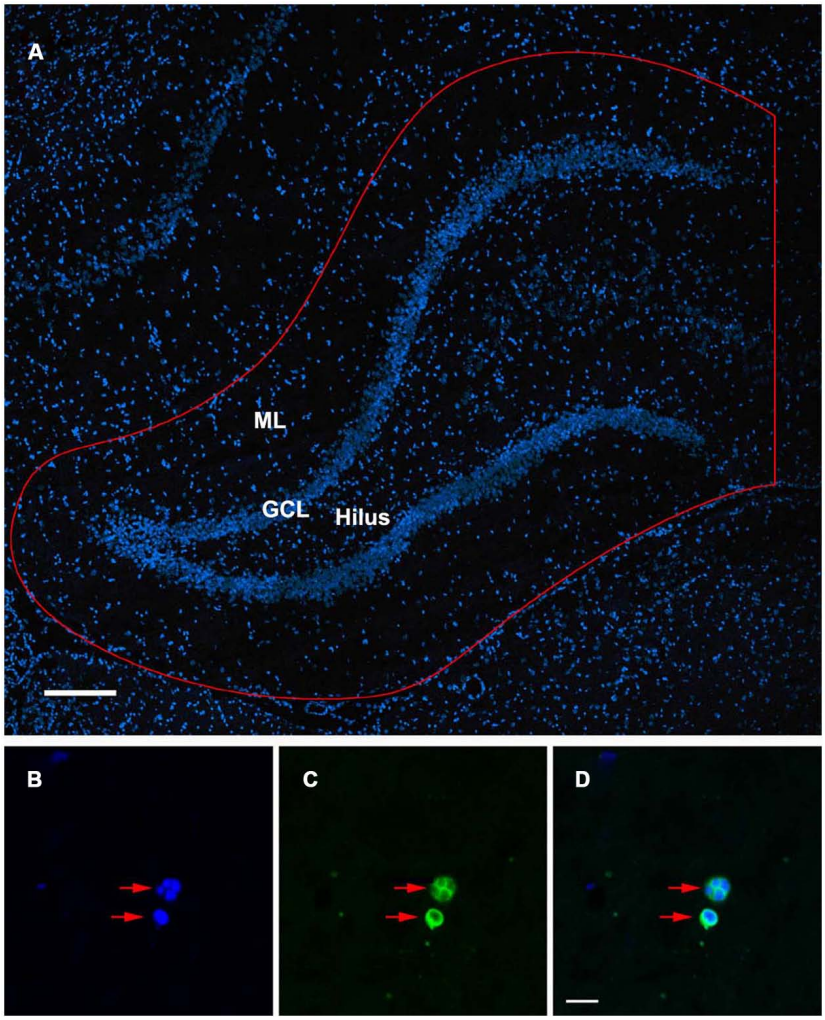

FIGURE 1 | TUNEL-labeled and DAPI-stained nuclei of the rat dentate gyrus. (A) Confocal image showing DAPI-stained nuclei in the dentate gyrus of the ipsilateral hippocampus. Sampling region of interest for quantification is outlined in red including the molecular layers (ML), suprapyramidal and infrapyramidal blades, granule cell layer (GCL), subgranular zone, and hilus of the dentate gyrus. Scale bar = 200 μm. **(B–D)** Condensed, fragmented nuclei exhibiting the morphological characteristics of apoptosis (red arrows) are stained with DAPI **(B)** and TUNEL **(C)**. The co-localization of DAPI/TUNEL **(D)**, in combination with the morphological features of the labeled nuclei would indicate an apoptotic phenotype. Scale bar = 10 μm.

fluorescein and a DAPI nuclear counter stain. The apoptotic state of these TUNEL-positive cells was further confirmed by the presence of condensed or fragmented nuclei when visualized by DAPI (**Figures 1B–D**). The estimated number of TUNEL-positive cells reported in the results represents the sum of apoptotic cell counts from all quantified sections throughout the DG of an individual rat multiplied by 10 to account for the intervals between quantified sections. The number of TUNEL-positive cells reported was the average of four animals in each group. In addition to evaluating TUNEL-positive cells in the DG of injured juvenile and aged animals, the extent of apoptotic cell death was also examined in age-matched shams.

IMMUNOFLUORESCENT LABELING

To determine the phenotype of cells undergoing apoptosis in the DG following brain injury, triple immunofluorescent labeling was performed with mature neuronal marker NeuN, immature neuronal marker PSA-NCAM, astrocytic marker GFAP, and microglia cell marker Iba1. Briefly, sections were incubated in a blocking solution (5% normal horse serum and 1% bovine serum albumin in PBS) with 1% Triton for 1 h at room temperature. Sections were then incubated overnight at 4°C with mouse monoclonal NeuN (1:500, Millipore), PSA-NCAM (1:500, Millipore), or rabbit polyclonal GFAP (1:1000, Dako) combined with goat polyclonal Iba1 (1:1000, Wako) diluted in the serum blocking solution. Following over night primary antibody incubation at 4°C, sections were washed in PBS and then incubated for 1 h at room temperature with Alex Fluor 568 anti-mouse IgG (for NeuN), IgA (for PSA-NCAM), or anti-rabbit IgG (for GFAP) combined with Alex Fluor 488 anti-goat IgG (1:200, Molecular Probes) in the serum blocking solution. Following incubation, sections were then washed three times in PBS and incubated with DAPI (1:1000) for

10 min. After a PBS wash, sections were coverslipped with Vectorshied. Sections were examined by confocal microscopy (Leica TCS SP2).

TISSUE PREPARATION FOR PROTEOMIC ANALYSIS

Forty-eight hours following injury, animals ($n = 3$ for TBI and sham animals in each age group) were anesthetized with isofluorane, euthanized with euthasol (pentobarbital sodium, 780 mg/kg; phenytoin sodium 100 mg/kg) and transcardially perfused with ice-cold PBS. Brains were then rapidly dissected on ice and placed in a rat brain mold with coronal divisions, so that three 1 mm coronal sections encompassing the rostro-caudal extent of the DG were cut using a single edge razor blade. The hippocampus ipsilateral to the site of injury in each slice was visualized using an Olympus SZX9 dissecting microscope and the dentate gyri (corresponding to the sampling region of interest described above for TUNEL analysis) from the three slices were dissected, pooled, and snap frozen on dry ice. This was repeated on three samples for each of the four groups (young sham, aged sham, young TBI, and aged TBI). Tissues samples were then thawed on ice and homogenized in an osmotic lysis buffer containing protease inhibitors and nucleases. Samples were quantified with a bicinchoninic acid (BCA) protein assay to determine the protein concentration of each sample and to ensure that equal quantities of proteins (50 μg) were loaded onto each gel for two dimensional polyacrylamide gel electrophoresis (2D-PAGE).

PROTEOMIC ANALYSIS

In order to assess group differences in protein expression profiles, a comparative proteomic analysis was performed on tissue extracted from the DG of juvenile and aged rats subjected to either a sham or FPI. To identify candidate proteins in the DG that exhibited differential expression patterns with aging and injury, tissue was processed for 2D-PAGE (by Kendrick Laboratories, Madison, WI, USA) according to the method of O'Farrell (1975) and stained with a mass spectrometry (MS)-compatible special silver stain according to the Vorum method as previously described (Colello et al., 2002). 2D-PAGE, which separates proteins based first according to charge and then according to molecular weight (MW), was performed on 10% acrylamide slab gels capable of resolving proteins in the 15–200 kDa MW range with an isoelectric point (pI) between 3.5 and 10. 50 ng of an internal standard (purified tropomyosin – MW 33,000; pI 5.2) was added to the samples before gel running to serve as a reference marker. Duplicate 2D gel proteomic map sets were generated from DG tissue derived from both sham and fluid percussion injured animals of both age groups ($n = 3$/group) resulting in a total of six gels per experimental group.

The objective of this proteomic analysis was twofold: (1) to identify apoptosis-related proteins that are differentially expressed in the DG during the normal aging process and (2) to identify apoptosis-associated proteins that exhibit age-dependent alterations in expression following TBI. To this end, a manual and computer-automated subtractive comparative analysis was performed using Discovery Series PDQuest 2D-Gel Analysis software (Version 7.3.1, Bio-Rad, Hercules, CA, USA). Briefly, gels were digitized using a Bio-Rad GS-800 scanner (BioRad) and digital gels were cropped, prepped for spot detection, filtered, and smoothed to clarify spots using PDQuest software. Spot volume and density (optical density) parameters were used to quantitatively compare corresponding protein spots between experimental groups. To account for any inconsistencies in silver staining, the total staining intensity in a gel image was used to normalize spot density. Optical density measurements were further normalized via background subtraction and according to the internal tropomyosin standard. A conservative twofold selection threshold was applied to control the number of 2D gel spots processed for tandem mass spectrometric identification. Thus, only the more prominent protein changes were selected in this initial study and were subsequently excised from the 2D gels, tryptic digested, and processed for mass spectrometric analysis.

Liquid chromatography-electrospray ionization-tandem MS (LC-ESI-MS/MS; performed by the Stanford Mass Spectrometry Laboratory, Stanford University) was then used to determine the amino acid sequence of each protein, which was then compared to theoretical MS spectra of known proteins using a MASCOT database search in order to determine the identity of the protein.

DATA ANALYSIS

The TUNEL data was analyzed using SPSS software with analysis of variance (ANOVA) with *post hoc* Fisher LSD test or the Student *t*-test with an applied Bonferroni correction for multiple groups was utilized, with p value less than 0.05 considered statistically significant. Densitometric data for identified gel spots for log(2) transformed to a normal distribution and tested using a two-way ANOVA method for factors of injury and age and the interaction between the two using a Holm–Sidak method for multiple comparisons based on an initial alpha of 0.05. All values are reported as mean ± SEM in all figures.

RESULTS

INCREASED NUMBERS OF APOPTOTIC CELLS IN THE AGED BRAIN FOLLOWING TBI

Age-associated differences in the apoptotic response of the brain to traumatic insult may represent a mechanistic underpinning of the heightened vulnerability of the aged brain to TBI, as well as contributing to the poor cognitive recovery observed in elderly patients following TBI. In this study, TUNEL labeling was employed to assess the level of apoptosis in the DG of juvenile and aged rats following a moderate lateral fluid percussion or sham injury. Varying levels of TUNEL-positive cells were observed in the DG in all groups assessed (juvenile-sham; aged-sham; juvenile-TBI; aged-TBI), localized predominately in the GCL and subgranular zone **(Figures 1** and **2)**. Quantitative analysis to compare the number of TUNEL labeled cells between juvenile and aged animals revealed an age-related difference, both in the uninjured (sham) condition as well as following TBI (**Figure 3**). Specifically, sham juvenile animals exhibited significantly higher levels of apoptosis as compared to aged sham counterparts (**Figure 3**, $^{*}p < 0.05$). Furthermore, while significantly increased TUNEL labeling was observed in the DG of both age groups following TBI, the magnitude of the injury-induced apoptotic response was significantly more pronounced in aged animals as compared to juveniles

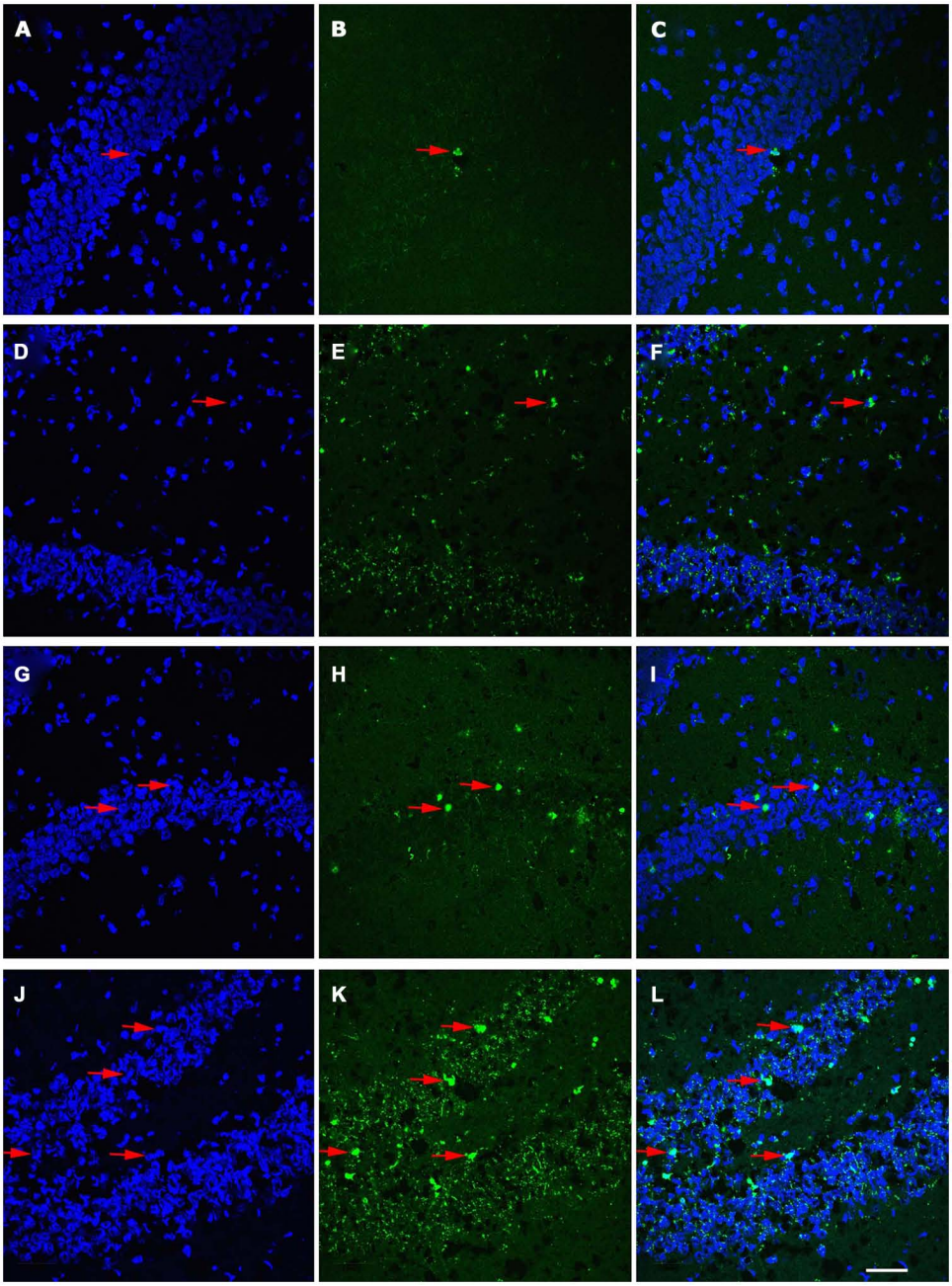

FIGURE 2 | Distribution of TUNEL-labeled apoptotic cells in the dentate gyrus (DG) of juvenile and aged rats following TBI. Confocal images showing TUNEL-labeled (green) apoptotic cells counterstained with DAPI (blue) in the hippocampus of a juvenile sham **(A–C)**, aged sham **(D–F)**, juvenile injured **(G–I)**, and an aged injured rat **(J–L)** at 48 h following injury. DAPI-stained sections **(A,D,G,J)** label the nuclei within the DG, while TUNEL-labeling **(B,E,H,K)** reveals apoptotic cells in this region. Co-localization of TUNEL/ DAPI **(C,F,I,L)** in conjunction with morphological analysis provides verification of their apoptotic phenotype. It should be noted that sections from aged brain in both sham and injured aged animals **(E,F,K,L)** have high levels of background staining as a result of the autoflourescence of lipofuscin, pigment granule product found in neurons that is associated with aging. Arrows denote apoptotic cells. Scale bar = 50 μm.

(**Figure 3**, $p < 0.05$). By comparing the number of apoptotic cells in the injured brain to the sham base level between age-matched counterparts, the injured-aged brain exhibited a 38.9-fold increase while the injured-juvenile showed a more modest 4.8-fold increase.

CELLS UNDERGOING APOPTOSIS WERE PREDOMINANTLY NEURONS

To determine the cellular constituents of apoptotic cells in the DG, we used neuronal markers NeuN (for mature neurons) and PSA-NCAM (for immature neurons) and the astrocytic marker GFAP combined with microglial cell marker Iba1. Apoptotic cells

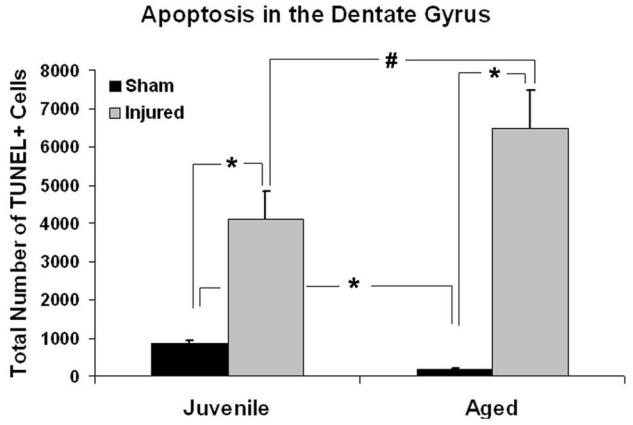

FIGURE 3 | Quantitative analysis of the apoptotic response in the dentate gyrus (DG) following aging and TBI. Graph showing estimated numbers of TUNEL-labeled cells in the DG of juvenile and aged rats subjected to a FPI or sham injury at 48 h post-injury. In sham animals, juvenile animals displayed approximately five times more TUNEL-positive cells than their aged sham counterparts ($^{*}p < 0.01$). Following injury, the number of TUNEL-positive apoptotic cells was significantly increased in both juvenile ($^{*}p < 0.05$) and aged animals ($^{*}p < 0.01$) as compare to their age-matched shams. While aged animals exhibited a 38.9-fold increase in the number of TUNEL-labeled cells following TBI, juvenile rats displayed a more modest 4.8-fold increase in apoptotic cell numbers over sham levels in response to injury. The injured aged brain also had significantly higher number of TUNEL-labeled cells than the injured juvenile brain ($^{\#}p < 0.05$).

were identified by the presence of condensed, fragmented nuclei with DAPI staining (**Figure 1**). The majority of apoptotic cells in the DG of the juvenile rats as characterized by dense DAPI labeling were NeuN-negative, PSA-NCAM-positive immature neurons located in the subgranule layer of the DG (**Figure 4**, arrows). In contrast, there were very few PSA-NCAM-positive cells in the DG in the aged brain. Rather, most of the apoptotic cells with dense DAPI labeling observed in the aged DG were NeuN-positive, PSA-NCAM-negative localized to the GCL (**Figure 5**, arrows). In both age groups, the apoptotic cells were enveloped by Iba+ microglial cells suggesting that the apoptotic cells were taken up by microglial cells (**Figures 4** and **5**, arrows). No GFAP-labeled astrocytes displayed apoptotic nuclei morphology (data not shown).

THE EXPRESSION LEVEL OF APOPTOSIS-ASSOCIATED PROTEINS IN THE DG CHANGED DURING AGING AND AFTER INJURY

To gain insight into the underlying mechanisms of the observed aging/injury-associated differences in the DG apoptotic response, we examined protein expression profiles in the DG in sham and injured juvenile and aged rats. Specifically, each individual protein sample from the DG was subjected to a 2D electrophoresis. Proteins spots from each 2D gel were then semi-quantitatively analyzed to identify apoptosis-associated proteins that are differentially expressed in the DG during aging process as well as following TBI. A comparison of 2D gel protein expression profiles between the experimental groups revealed several age- and injury-induced alterations in the DG proteome (**Figure 6**). Sixty distinct protein spots were detected recurrently across 2D gels with 10 exhibiting a twofold or greater change in abundance with injury or aging as a factor. Using tandem MS, 3 of the 10 gel spots were identified as proteins implicated in apoptotic processes: hippocalcin (P23k), acidic nuclear phosphoprotein pp32 (LANP), and heat shock protein (Hsp27).

Hippocalcin, a calcium binding protein that has been shown to protect neurons against apoptosis, exhibited a significantly

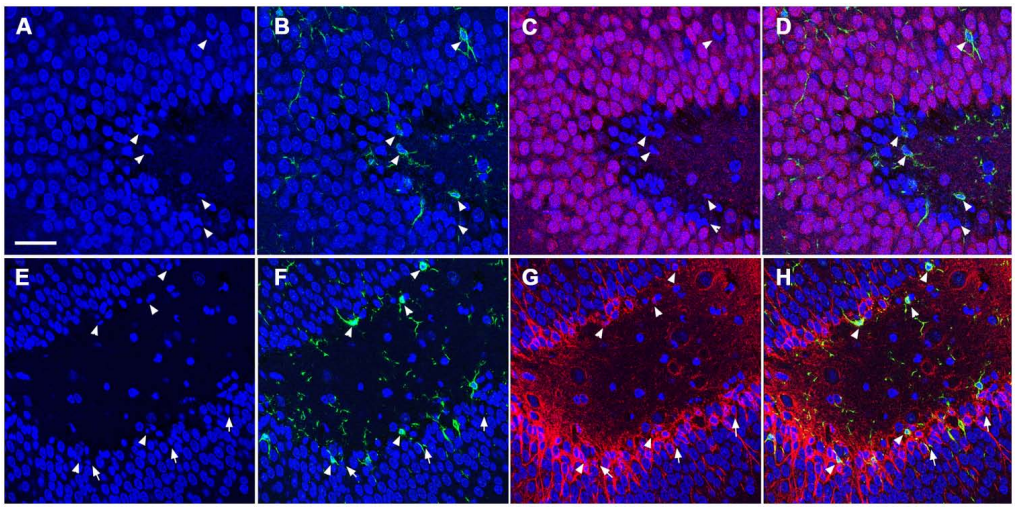

FIGURE 4 | TUNEL-positive apoptotic cells in the DG of the juvenile rat following TBI are newly generated immature neurons. Confocal microscopic images show triple labeling of the neuronal marker NeuN (red, **C,D**) or PSA-NCAM (red, **G,H**), the microglia marker Iba1 (green), and the nuclei marker DAPI (blue). **(A–H)** DAPI-labeling revealed apoptotic cell nuclei with condensed, fragmented morphology located mostly in the subgranular zone of the DG (arrows and arrowheads). Many of these nuclei are labeled with Iba1 **(B,D,F,H,** arrowheads**)**, and are NeuN-negative **(C,D)**, but PSA-NCAM-positive **(G,H,** arrows and arrowheads**)** suggesting that the majority of apoptotic cells in the juvenile brain are immature neurons in the subgranular zone of the DG. Scale bar = 30 μm.

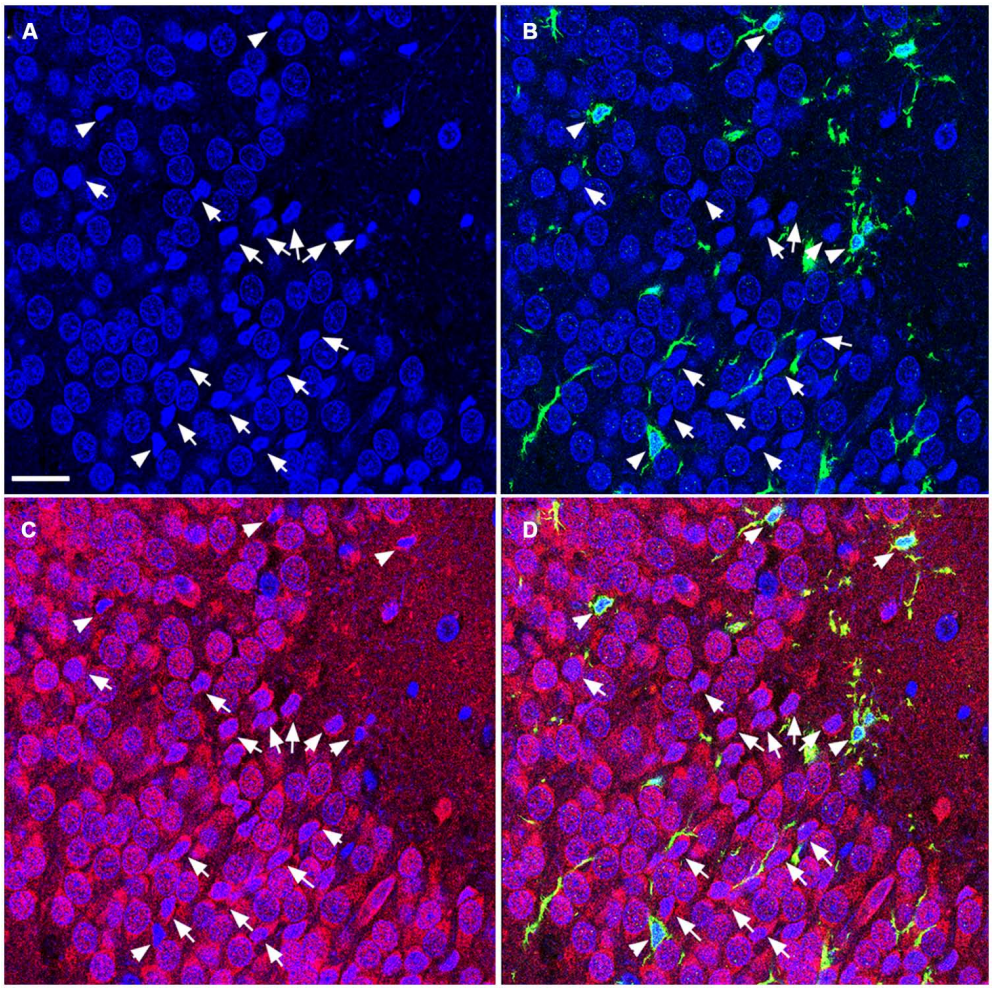

FIGURE 5 | TUNEL-positive apoptotic cells in the DG of the aged rat following TBI are predominantly mature neurons. Confocal microscopic images show triple labeling of the neuronal marker NeuN (red), the microglia marker Iba1 (green), and the nuclei marker DAPI (blue). **(A–D)** DAPI-labeling showing cells undergoing apoptosis with condensed, fragmented morphology located in the granule cell layer of the DG (arrows and arrowheads). These cells are NeuN-positive **(C,D**, arrows), some of them are co-labeled with Iba1 **(B,D**, arrowheads) suggesting that the majority of the apoptotic cells in the injured aged brain are mature neurons in close association with microglia, in the granule cell layer of the DG. Scale bar = 60 μm.

higher level (2.4-fold; $F = 6.2$; $p = 0.025$) in the DG of sham aged animals as compared to sham juveniles (**Figures 6A,B**). The elevated expression of this anti-apoptotic protein in the aging brain appears related to the declining levels of apoptosis that are observed during the aging process within the DG (as assessed by TUNEL staining). In addition to the differential expression of hippocalcin between sham animals of different ages, alterations in the expression levels of this anti-apoptotic protein were also observed in response to injury. A comparison between sham and injured aged animals revealed a significant decrease (−3.3-fold; $F = 13.4$; $p = 0.002$) in hippocalcin expression following TBI (**Figures 6A,B**) which corresponded to the marked injury-induced increase in TUNEL-positive cells observed in this aged population (**Figure 3**). In contrast to that seen in aged animals, no significant change in hippocalcin expression level was observed in juvenile rats as a consequence of injury. This hippocalcin expression pattern observed by proteomic analysis was further confirmed by immunohistochemistry. As shown in **Figure 7**, strong hippocalcin immunoreactivity was observed in the stratum oriens, stratum lucidum, and stratum radiatum of the CA1 region in all groups but particularly in the sham aged brain (**Figure 7C**). In the DG, hippocalcin was expressed in the molecular layers (ML) and hilus region, with robust expression observed in the aged sham animals (**Figure 7C**) as compared to that of juvenile sham animal (**Figure 7A**). Furthermore, while hippocalcin immunoreactivity remained relatively unchanged in the DG of juvenile animals following injury (**Figure 7B**), there was a substantial decline in hippocalcin immunoreactivity in the DG of aged rats in response to TBI (**Figure 7D**).

LANP, a protein that has been shown to exhibit pro-apoptotic properties, revealed a significantly greater level (2.7-fold; $F = 14.6$; $p = 0.002$) in the DG of juvenile sham animals as compared to aged shams (**Figures 6A,C**). The significantly decreased expression

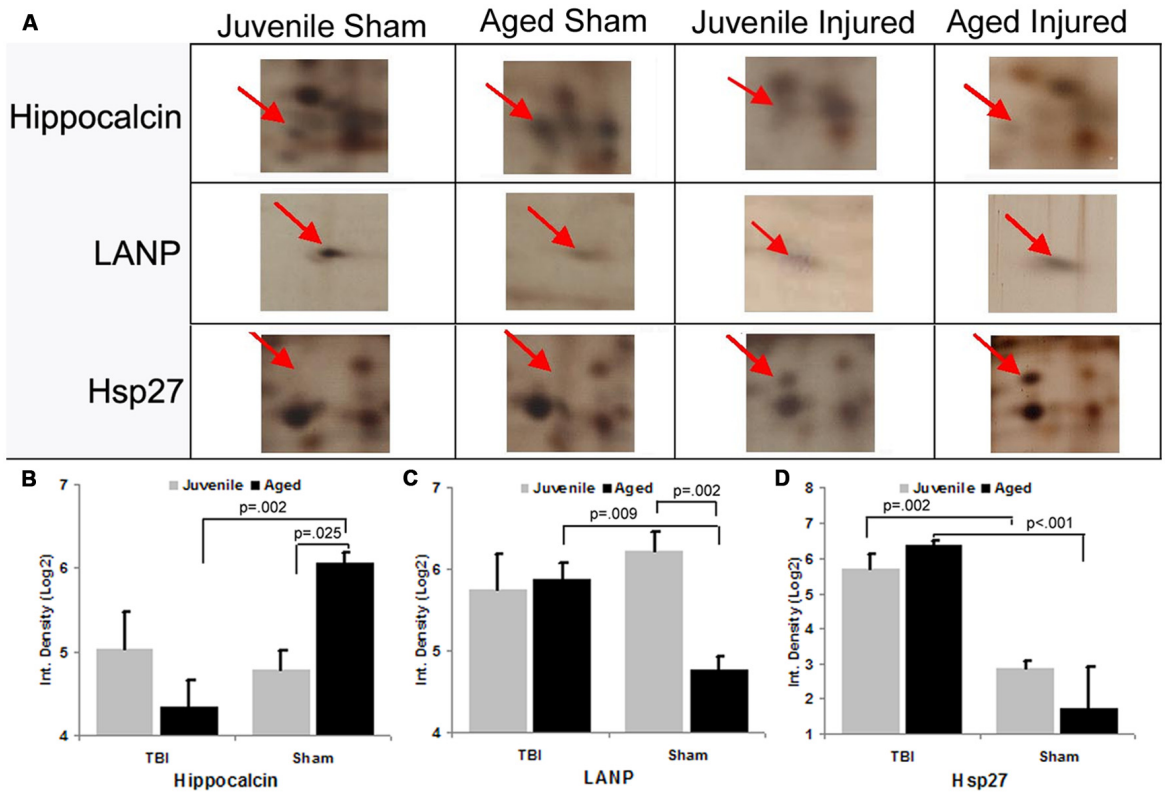

FIGURE 6 | Differential expression of apoptosis-associated proteins in the dentate gyrus (DG) following aging and injury. (A) Standard format, silver-stained 2D gels showing apoptosis-associated proteins expressed in the DG of juvenile and aged rats in both sham and injured groups at 48 h post-injury. Arrows point to distinct apoptosis-related proteins which exhibit changes in expression as a consequence of aging and/or injury, the identity of which were determined using LC-ESI-MS/MS. **(B–D)** Graph shows the expression levels of apoptosis-related proteins (hippocalcin, LANP and Hsp27) in the DG in relation to normal aging and injury. In sham animals, the expression levels of hippocalcin was increased with aging, while LANP was decreased with aging. HSP27 was low in both age groups. Following TBI, hippocalcin was sharply decreased in the injured aged brain but slightly increased in the juvenile brain. Whereas the level of LANP was increased in the injured aged brain, but slightly decreased in the injured juvenile brain. The expressed level of Hsp27 is drastically increased in both juvenile and aged brain following TBI. Results are reported as the log(2) transformed value of the optical density measure (each unit = doubling in density). Mean ± SE; $n = 3$; $^*p < 0.05$.

of this apoptosis-promoting protein in the DG during the normal aging process parallels the declining levels of apoptosis observed with aging (as assessed by TUNEL staining). Furthermore, while no change in LANP abundance was observed in the DG of injured juveniles, a significant increase (2.2-fold; $F = 9.5$; $p = 0.009$) in LANP expression over sham levels was observed in aged animals following TBI (**Figure 6**). This injury-induced increase in LANP expression in the aged DG following TBI corresponds to the markedly enhanced levels of apoptosis in this population.

Unlike the aforementioned proteins, no significant alterations ($F = 0.2$; $p = 0.638$) in Hsp27 expression were observed in the DG as a factor of normal aging (**Figures 6A,D**). However, the expression of this anti-apoptotic/anti-necrotic protein was significantly enhanced (13-fold; $F = 47$; $p < 0.001$) as a factor of injury, irrespective of age.

DISCUSSION

The aging population is the most vulnerable group to TBI and display heightened levels of cognitive deficits as a result, in part, from the progressive neuronal cell death in the hippocampus. Among the pathological responses that occur following TBI, apoptosis plays an important contributing role to the secondary insults that lead to neuronal loss. In this study, we have observed aging- and injury-related differences in apototosis levels and in the cell types that undergo apoptosis in the DG of the hippocampus. In sham animals, juveniles exhibited higher baseline levels of apoptosis as compared to their aged counterparts. Following injury, the number of apoptotic cells was significantly increased in both age groups with aged animals exhibiting a more marked increase. Furthermore, the cell types that undergo apoptosis, as well as their localization within the DG, were different for the two age groups. In the juvenile brain, the majority of apoptotic cells were newly generated PSA-NCAM+ immature neurons located in the SGZ, whereas in the aged brain, the majority of apoptotic cells were mature neurons residing in the GCL. Using proteomic approaches, we have also identified age-and injury-associated alterations in the expression levels of three apoptotic-related proteins in the DG. Specifically, we observed changes in the expression levels of heat

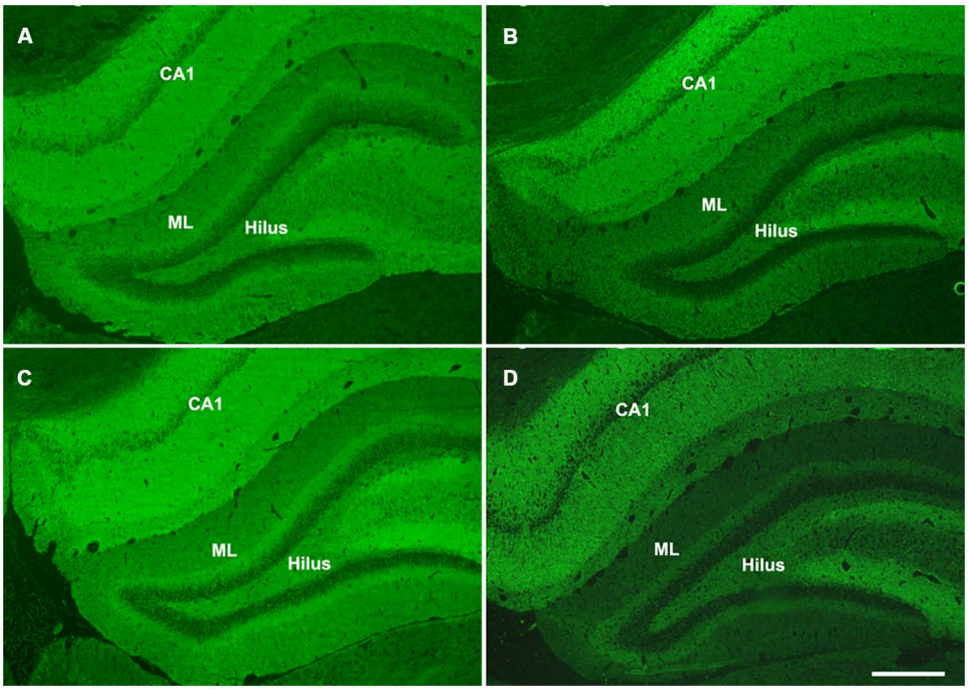

FIGURE 7 | Hippocalcin expression patterns in the hippocampus of juvenile and aged rats following TBI. Representative images of hippocalcin immunofluorescent staining of coronal sections of the DG of a juvenile sham **(A)**, aged sham **(C)**, juvenile injured **(B)**, and an aged injured **(D)** rat at 48 h post-injury. Hippocampal CA1 region, molecular layers (ML) and hilus region of the DG show strong hippocalcin staining. The staining was done at the same time for all sections and pictures were taken at the same time with same exposure, minimal adjustment of brightness and contrast was made to maintain the true staining patterns. Note that the intensity of hippocalcin immunoreactivity mirrors the protein expression pattern observed via 2-D gel proteomic analysis. Scale bar = 200 μm.

shock protein 27, hippocalcin, and LANP (acidic nuclear phosphoprotein), which suggested the presence of differential regulating pathways of apoptosis in the normal aging process and following TBI.

Apoptosis is an important mechanism during brain development for regulating neuronal cell numbers and to ensure the appropriate formation of neuronal circuitries. Apoptosis also occurs in the neurogenic regions of the adult brain, with a significant portion of newly generated cells in the adult brain eliminated within the first months following generation (Dayer et al., 2003). Cells undergoing apoptosis are removed by resident microglia cells (Sierra et al., 2010). These apoptotic processes that occur in the neurogenic regions of the normal adult brain are thought to be mediated through Bax, Blc pathways. Studies have revealed that transgenic mice with Bax-deficiency or over-expression of bcl-2 show increased numbers of new neurons in the DG, a phenomenon that results from decreased apoptosis and not increased cell proliferation (Sun et al., 2004; Kuhn et al., 2005). The continuous increase of DG cell numbers in Bax-knock out mice resulted in a readjustment of afferent and efferent synaptic connections, with reductions in dendritic arborization, synaptic transmission, and reduced performance in hippocampal-dependent learning and memory functions (Kim et al., 2009). In this context, elimination of excess newly generated neurons via apoptosis in the adult brain is essential for the normal organization and function of the hippocampus. Following aging, the degree of neurogenesis in the DG is sharply decreased as a result of the normal aging process (Rao et al., 2006; Olariu et al., 2007; Walter et al., 2011). Studies have shown that decreased neurogenesis in the DG with aging is accompanied by declines in apoptotic cells in this region (Heine et al., 2004; Sierra et al., 2010). Our current findings in sham animals, which show that TUNEL$^+$ cells are predominantly localized to the SGZ and that the number of TUNEL$^+$ cells decline with aging, are in agreement with these published studies.

Following TBI, the injured brain undergoes a cascade of secondary events which include apoptosis and neurogenesis. We have previously shown that TBI enhances hippocampal neurogenesis and that this enhancement is more prominent in the juvenile brain as compared to adults (Sun et al., 2005). In the current study, we observed increased TUNEL$^+$ cells in the injured juvenile brain as compared to sham counterparts, with apoptotic cells localizing predominantly to the SGZ and displaying an immature neuronal phenotype (PSA-NCAM$^+$). This observation is in agreement with previously published findings that adult-generated immature neurons in the SGZ are vulnerable to TBI, with many cells undergoing cell death due to the injury impact (Gao et al., 2008). In the current study, one of the striking findings is the marked increase in TUNEL$^+$ cells in the DG of aged animals following TBI. The majority of the dying cells in the injured aged DG area are NeuN$^+$ mature neurons localized to the GCL as opposed to the PSA-NCAM$^+$ cells in the SGZ, as found in the juvenile DG. These age-related differences reflect changes in the degree

of neurogenesis as very few PSA-NCAM$^+$ cells are found in the aged brain. The observed cell death of mature neurons in the aged brain is likely due to the vulnerability of the aged brain to TBI. It is known that heightened levels of oxidative stress, inflammation, excitotoxicity, etc. lead to increased neuronal death in the aged brain, particularly in the hippocampus, following TBI (Shao et al., 2006; Sandhir et al., 2008; Timaru-Kast et al., 2012).

Neuronal death following TBI occurs through several processes and via many mechanisms involving both necrosis and apoptosis. Calcium dysregulation, excitotoxicity, activation of cysteine proteases, mitochondrial permeability transition, and mechanical pertubation of neuronal membranes are all mechanisms that contribute to apoptotic and/or necrotic neuronal cell death after TBI (Shapira et al., 1989; Fineman et al., 1993; Zipfel et al., 2000). The precise mechanism that determines the fate of a particular cell type has yet to be precisely defined. The cell type and regional differences in the apoptotic response that we have observed in the aged and juvenile brain following injury suggest that different mechanisms are at play.

Developmental apoptosis is thought to selectively remove unviable cells in order to promote overall growth whereas cell death after TBI may play a much more detrimental role in recovery after injury. One mechanism for modulating apoptosis is a shift between pro and anti-apoptotic factors that promote the expression of proteins responsible for cell death (Raghupathi, 2004). In the current study, using proteomic approaches, we identified aging and injury associated changes of three apoptosis related proteins (Heat shock protein beta-1, Hippocalcin, LANP) which may be responsible for the differences observed in the aging and injury-induced apoptotic response.

Heat shock protein beta-1 (Hsp27) was present on 2D-electrophoresis gels run for this study at a MW of 23 kDa and with an isoelectric point (pI) of 6.12. In our study, Hsp27 expression level was low in sham animals regardless of age. Its expression level was drastically increased after injury in both age groups indicating that Hsp27 is an injury-induced protein, which is in agreement with previous findings that report enhanced synthesis of Hsp27 after stress. To date, no published literature has reported HsP27 expression following TBI. Hsp27 is a member of the small heat shock or stress protein (shsp) families that are known to display enhanced synthesis after heat or oxidative stress (Landry et al., 1989; Mehlen et al., 1995). The increased production of hsp27 following TBI in both juvenile and aged brain may represent a protective response of the CNS following injury-induced excitotoxicity, oxidative stress, and inflammation. Hsp27 functions as molecular chaperones (Horwitz, 1992) or actin capping/decapping enzymes (Guay et al., 1997) involved in several fundamental cellular processes including protein intracellular transport, cytoskeleton architecture, translation regulation, intracellular redox homeostasis, and most relevant to the current study, protection against spontaneous or stimulated programmed cell death (reviewed by Vidyasagar et al., 2012). Hsp27 has been shown to have significant anti-apoptotic properties via different pathways including Fas-FasL, Bax, and cytochrome *c*, as well as caspase-dependent apoptosis (Bruey et al., 2000; Charette and Landry, 2000; Havasi et al., 2008).

Hsp27 has also been studied in relation to necrosis, a cell death process that has been shown to dominate after moderate to severe experimental TBI (Conti et al., 1998). Necrosis has been shown to occur more often in the CA1, CA3, and hilus regions of the hippocampus after TBI, whereas apoptosis has been shown to occur more often in the DG (Clark et al., 1997). Over expression of hsp27 protects against both programmed cell death and necrosis (Wagstaff et al., 1999). Hsp27 expression has been shown to decrease intracellular reactive oxygen species levels, a condition that often triggers necrosis. Hsp27 has also been shown to block cell death induced by TNFα by increasing cellular content of the antioxidant glutathione (Mehlen et al., 1995, 1996). In addition, shsp expression has been shown to protect against cellular necrosis induced by oxidative stress (Mehlen et al., 1993), and inflammatory cytokines (Mehlen et al., 1996). The increased production of Hsp27 following TBI in both juvenile and aged brain observed in our study suggests that the brain exerts a protective effect against excessive cell death induced by excitotoxicity, oxidative stress, and inflammation following injury. Consequently, an increased exogenous expression of hsp27 may be a potential therapeutic target for the prevention of massive cell death after neurotrauma specifically via protection against apoptosis, necrosis, and neurodegeneration.

Hippocalcin, also known as p23k, was present on 2D-electrophoresis gels at a MW of 23 kDa and with a pI of 4.87. This protein displayed an increase in expression with aging in sham animals, and a decrease in expression after injury in the aged brain only. The differential expression correlating with aging is in conflict with a previously published study reporting decreased hippocalcin immunostaining in the hippocampus of aged rats during normal aging (Furuta et al., 1999). This discrepancy may due to the differences of sub-regional expression of hippocalcin and the method used. To date no other published study has reported the changes of hippocalcin expression level following injury in aged animals.

Hippocalcin is a member of the small neuronal calcium-sensor family (NCS) (Kobayashi et al., 2005; Palmer et al., 2005). In the hippocampus, hippocalcin is strongly expressed in the pyramidal cell layer and is modest in the DG (Furuta et al., 1999). Although the physiological role of hippocalcin is not completely understood, it is implicated in regulating neuronal viability and plasticity. For example, studies have found that hippocalcin can protect hippocampal neurons against excitotoxicity induced damage by enhancing Ca^{++} extrusion and maintaining ideal intracellular Ca^{++} levels (Masuo et al., 2007). It is also reported that hippocalcin acts to abate apoptosis by interfering with the programmed cell death cascades. For example, hippocalcin interacts with neuronal apoptosis inhibitory protein to protect neurons against Ca^{++}-induced cells death by decreasing caspase 3 and caspase 7 activities (Mercer et al., 2000). In addition, hippocalcin has been shown to protect against caspase 12-induced and age-dependent neuronal degeneration (Korkohonene et al., 2005). It also plays a critical Ca^{++}-sensing role in NMDA receptor-mediated hippocampal LTD (Palmer et al., 2005) suggesting that hippocalcin may be involved in the downstream $Ca2^{++}$-signaling cascade leading to synaptic plasticity and learning and memory function.

In the current study, in the injured brain, hippocalcin level was sharply decreased in the aged DG while remaining constant in the

juvenile animals. This decrease of hippocalcin expression in the injured aged DG corresponds to the sharp increase in the number of TUNEL$^+$ cells observed. As hippocalcin plays roles in neuronal viability and plasticity, the decreased expression of hippocalcin in the DG following injury may contribute to the vulnerability of the aged brain to TBI leading to increased neuronal cell death and cognitive dysfunction. Taken together, our findings suggest that hippocalcin may play a role in endogenous repair or homeostasis after TBI.

LANP, also known as ANP21-A, PHAPI (putative HLA-associated protein 1), and pp32 (phosphoprotein with a molecular mass of 32 kDa), was present on 2D-electrophoresis at a MW of 29 kDa and with a pI of 4.00. In sham animals, this protein was expressed at high level in the DG of juvenile brain and was low in the aged brain. Following TBI, an increase in expression was observed only in the aged animal while remaining constant in the juvenile animal. This expression pattern suggests that the aged brain was stimulated to produce high level of LANP to recapitulate the level in the juvenile brain.

LANP is a nucleocytoplasmic shuttling protein with a diverse functions including signaling, protein degradation, cytoskeletal dynamics, and morphogenesis, due to the leucine rich repeat domains which serve as versatile protein binding sites (Kobe and Deisenhofer, 1994). Functionally, the most defined biological function of LANP is its role as a tumor suppressor owning to its apoptotic enhancer function by stimulating apoptosome-mediated caspase activation (Pan et al., 2009). Other known functions of LANP include inhibition of protein phosphatase 2A (PP2A) and histone acetyltransferase (HAT) (Habrukowich et al., 2010). In the brain, LANP is abundantly expressed during early weeks of postnatal life and the level is decreased in adult (Matsuoka et al., 1994). To date there is no published study reporting the changes of LANP expression in the DG related to aging and following brain injury. Consequently, its role in the injured brain is unknown. In the current study, in sham animals, we found a high level of expression of LANP in the DG of juvenile brain and low in the aged. This expression pattern is in agreement with published report about its expression in the cerebella (Matsuoka et al., 1994). In the injured brain, we found a sharply increased expression of LANP in the aged brain following injury. The high expression level of LANP in the sham juvenile and injured aged DG is in parallel with the higher level of TUNEL$^+$ cells observed in these groups, which support the role of LANP as an apoptosis promoter. Nevertheless, other roles of LANP such as its function in regulating neurite extension (Kular et al., 2009), COX-2 (cyclooxygenase-2) by interaction with sphingoshine (Habrukowich et al., 2010) may be also at play.

In summary, this study revealed the degree of apoptosis and the differential changes of three apoptosis-related proteins in the DG during normal aging and following TBI. The present design focused on proteins with a sizeable twofold or greater change in level as a response to injury or aging, precluding the detection of more subtle changes in the proteome. Also, the relative lower animal number (three per group), we may miss out on some other proteins that may have significant association to age and injury related changes. Nevertheless, the differential expression of the three apoptosis proteins that we have identified in the current study may suggest any one of these proteins may play an important role in contributing to the decreased capacity of recovery of the aged population following injury. Our results suggest that therapeutic strategies for treating TBI sufferers need to consider age-related differences in pathological changes and cellular pathways in order to be effective for the particular age group.

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Role of metal ions in the cognitive decline of Down syndrome

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Down syndrome (DS), caused by trisomy of whole or part of chromosome 21 is the most common mental impairment. All people with DS suffer from cognitive decline and develop Alzheimer's disease (AD) by the age of 40. The appearance of enlarged early endosomes, followed by Amyloid βpeptide deposition, the appearance of tau-containing neurofibrillary tangles and basal forebrain cholinergic neuron (BFCN) degeneration are the neuropathological characteristics of this disease. In this review we will examine the role of metal ion dyshomeostasis and the genes which may be involved in these processes, and relate these back to the manifestation of age-dependent cognitive decline in DS.

Keywords: Down syndrome, Alzheimer disease, iron, copper, zinc, calcium

INTRODUCTION

This review will discuss the role of intrinsic metals in cognitive decline in people with Down syndrome (DS). DS, caused by trisomy of whole or part of human chromosome 21 is the most common mental impairment with an incidence of about 1 in 700 live births (Epstein, 1995). Characteristics of DS individuals have been described such as stunted growth, mental impairment, congenital disorders of multi-organ systems such as the heart, haematological, musculoskeletal, thyroid, gastrointestinal, dental, nervous and immune systems, and having a higher incidence of diabetes and age-associated diabetes (Burch and Milunsky, 1969; Peiris et al., 2012). Life expectancy of people with DS has increased from 12 years in the 1940s to 60 years at the present time, largely due to enhanced medical and social care (Contestabile et al., 2010). Because of the increased longevity, it has become evident that by the age of 40 (Mann et al., 1990), all individuals with DS have the cognitive decline and the neuropathology seen in Alzheimer's disease (AD).

AD is the most common form of dementia. It was first described by a German psychiatrist and neuropathologist named Alois Alzheimer in 1906 (Alzheimer et al., 1995). One of the earlier symptoms is short term memory loss. As the disease progresses long-term memory loss, confusion and mood swing occur. Sporadic AD and DS share similar neuropathological features (Mann, 1988), these include the enlargement of early endosomes in certain neurons, deposition of Aβ plaques, the presence of tau-containing neurofibrillary tangles and the degeneration of basal forebrain cholinergic neuron (BFCN). The pattern of pathology is similar but the symptoms appear earlier in individuals with DS (Ikeda et al., 1989). In DS, enlarged endosomes in neurons are seen as early as 28 weeks of gestation (Cataldo et al., 2000). The presence of enlarged endosomes precedes Aβ peptide deposition which appears at around 12 years of age in the form of diffuse plaques, followed by mature Aβ plaques when the individuals are in their 30's (Lemere et al., 1996). In a review of the literature, it was reported that most studies have found that tau-containing neurofibrillary tangles occur in the BFCN along with neuronal degeneration, gliosis and dementia at ages of between 35 to 45 years (Wilcock and Griffin, 2013). An immunohistochemical study of the brain tissues from post-mortem DS individuals showed that in earlier years of life (37–38) brain neuron loss was present and this progressed with age (over 50s) with abundant, mature amyloid deposits. Taken together, these observations suggest that in DS there is an accelerated progression occurring through the different stages of AD-like neuropathology.

There are 127 known genes, 98 predicted genes and 59 pseudo genes located on chromosome 21 (Hattori et al., 2000). It is putatively thought that having an extra copy of one or more of these genes could be responsible for the manifestation of AD-like neuropathology and cognitive decline in DS. Some genes of interest are: Amyloid precursor protein (*APP*), Down syndrome candidate region 1 (*DSCR1*), Intersectin 1(*ITSN1*), Superoxide dismutase 1(*SOD1*), Beta-site APP-cleaving enzyme 2 (βsecretase or *BACE 2*), S100 calcium binding protein β(*S100*β).

In the next section, how these genes may be involved in cognitive decline of DS will be discussed.

GENES POTENTIALLY INVOLVED IN COGNITIVE DECLINE OF DS

AMYLOID PRECURSOR PROTEIN (APP)

Studies have suggested that over-expression of the APP is the key element for the manifestation of AD in DS (Schupf and Sergievsky, 2002). A case study where an individual had partial trisomy 21 that did not include an extra copy of the *APP* gene confirmed this concept (Prasher et al., 1998). This individual showed some characteristics of DS but did not suffer from AD and the associated cognitive decline.

Amyloid peptides (Aβ 1–40/42) are cleavage products of the APP. These form β-sheet rich aggregates and soluble oligomers. The cleavage products are generated by the proteolytic enzymes βsecretase and γsecretase. There is also an alternative pathway where α-secretase cleaves APP. The products of this pathway do not include Aβ peptides and aggregates do not form (Vassar et al., 1999). The gene encoding βsecretase 2 is located on chromosome 21, therefore, an extra copy would potentially contribute to the production of higher levels of toxic Aβ, hence formation of amyloid plaques early in DS.

However, this extra dosage of APP and BACE may not be required to produce the AD-like phenotype in DS. This idea can be derived from observations from an individual who had an over-expression of APP, but exhibited normal levels of BACE (Cheon et al., 2008). In a recent study, polymorphisms in BACE2 were shown to be a major factor for the age of onset of AD in the DS population (Mok et al., 2014) suggesting a significant role for BACE2 in the development of AD in DS.

In addition, it was shown that the amyloid truncated Aβ peptides (Aβ 9–42 and Aβ 17–42) form ion channels by formation of a β-barrel secondary structure (βstrand-turn-βstrand) that alters calcium (Ca^{2+}) homeostasis providing additional avenues for the manifestation of AD (Jang et al., 2010). An alternative mechanism may be that N-terminal truncated amyloids are neurotoxic because they form fibrils that aggregate more readily (Pike et al., 1995). These studies suggest that it is not only Aβ but smaller truncated amyloid peptides which are also involved in the development of AD.

AD research has focused heavily on APP. However studies have suggested that APP may not be the only factor involved in the manifestation of AD. This was demonstrated in mice where the APP protein was over-expressed in a mouse model of DS (Ts65Dn). No change in the endosomal phenotype was observed indicating other factors are necessary for this alteration (Cataldo et al., 2003). As mentioned above, there are other genes that could contribute to the development of AD-like neuropathology that will be discussed later in this review. Although APP is necessary to develop the disease, it appears that it alone is not sufficient to cause it. This is why it is interesting to study DS as it narrows the search done to trisomy genes.

Importantly, amyloid plaques are formed as the result of inappropriate interaction between biometals (iron, copper and zinc) and beta-amyloid. Indeed, increased levels of zinc promote Aβ precipitation (Bush et al., 1994). There is also increased levels of iron in AD patients (Gerlach et al., 1994). These observed increases in the levels of metals in AD give rise to the questions of why, how and when these increases occur? This so-called metal theory of AD and DS will be discussed later in this review.

DOWN SYNDROME CANDIDATE REGION 1

Studies showed that DSCR1 mRNA expression was increased in post-mortem brains of AD patients compared with aged-matched non-AD controls (Ermak et al., 2001). DSCR1 is a regulator of calcineurin and in some cases has been shown to inhibit calcineurin signaling pathways (Fuentes et al., 2000). Over-expression of DSCR1 contributes to the formation of neurofibrillary tangles and Aβ deposition (Ermak et al., 2011). As discussed earlier, formation of neurofibrillary tangles is the first step of memory loss. Hence, having an extra copy of DSCR1 in DS could contribute to earlier formation of neurofibrillary tangles and premature cognitive decline. The formation of tangles could be facilitated by the enhanced ability of over-activate calcineurin to dephosphorylate tau but the mechanism has not been fully elucidated (Ermak et al., 2001; Ma et al., 2004).

INTERSECTIN 1

Another potential gene of interest on chromosome 21 is *ITSN1*. This gene appears to be involved in metal homeostasis since it is involved in receptor mediated endocytosis and one of its cargos is the transferrin/transferrin receptor complex required for iron transport into the cell. Transferrin (and its iron cargo) is transferred from the blood into cells by the formation of a vesicle (clathrin-coated pit) that internalizes the complex. First the recruitment of adaptor protein complex 2 (AP-2) to the plasma membrane occurs, and then clathrin is recruited to the cytoplasm where AP-2 has been recruited. The plasma membrane forms a vesicle by budding inward. The receptors and bound ligand (transferrin) are thus taken up by the cell. ITSN1 is involved in the recruitment of AP-2 and the formation of clathrin-coated pits. Having an extra copy of the *ITSN1* gene in DS could alter receptor trafficking and consequently iron transport.

METALS

THE METAL THEORY

As discussed above, excess APP and/or its cleavage products is necessary for the development of AD but, it is not sufficient to cause it. In addition, clinical trials targeting Aβ (by anti- Aβ antibodies) have failed to treat AD (Greenberg et al., 2003). Bush et al. (on the basis of the studies done by them and others) have proposed that a number of proteins involved in neurodegeneration (Aβ, AβPP, tau, presenilin, and β-secretase1) fail in their ability to regulate metals in the AD brains (Finefrock et al., 2003; Bonda et al., 2011; Bush, 2013), and consequently these proteins are overwhelmed by the increased levels of these metals. This leads to accumulation of extracellular zinc and copper in amyloid, and an accumulation of intracellular iron in neurons (Bush, 2013).

Iron

Iron is essential for normal neurological function. It is required for the synthesis of myelin and neurotransmitters (Piñero and Connor, 2000; Bush, 2013). It is known that free iron accumulates in the brains of AD patients, and that iron transport and storage are disrupted (Sayre et al., 2000). Another study

showed that iron stimulates the production of hydroxyl radicals from hydrogen peroxide, and hence exacerbates oxidative stress. Iron was increased in all areas of the AD brains compared with controls and transferrin was increased in the frontal cortex of AD brains. The transferrin/iron ratio which is indicative of iron mobilization was reduced in AD brains, indicating a disturbance of iron metabolism in the AD population (Loeffler et al., 1995).

Iron transport into the brain is accomplished by Transferrin (Tf). Tf is a glycoprotein that carries iron in the blood and regulates iron homeostasis. As mentioned above, ITSN1 is required for iron internalization. It has been reported that there is an increase of Tf in brains of DS individuals compared with controls (Leveugle et al., 1994). An extra copy of *ITSN1* could be the reason for this elevated level of Tf in DS brains, and this level increases with age in DS. The increased level of Tf is also evident in the amniotic fluid obtained from women carrying DS foetuses (Perluigi et al., 2011), suggesting iron-mediated damage starts during pregnancy.

Ferritin, the iron storage protein is available in the cytosol to bind the iron that has been endocytosed into the cell. Excess iron can be transported out of the cell by ferroportin (Fpn). For this to occur, a ferroxidase enzyme is required. In the glia, ceruloplasmin is the known ferroxidase (Klomp et al., 1996) but ceruloplasmin is not produced by neurons. Instead, APP has been implicated in the process of iron export in neuronal cells. This occurs through the stabilization of Fpn (Duce et al., 2010). Interestingly, excess dietry iron fed to APP-knock-out mice induced iron accumulation and damaged the neocortex (Duce et al., 2010).

Iron regulatory protein (IRP) is a cytoplasmic protein that regulates iron uptake, storage and usage in response to the concentration of cellular iron. The level of iron regulatory protein (IRP-2) in the whole brain was the same in both AD patients and controls. However IRP-2 is co-localized to the specific areas with AD neuropathology (Farrar et al., 1990; Smith et al., 1998), suggesting the regulation of iron uptake, storage and usage changes in specific parts of AD brains which leads to the development of the AD neuropathology. The role of IRP-2 and indeed iron in the patho-biology of AD has been well established, whereas in DS this needs to be investigated.

Tau protein stabilizes microtubules which are involved in cellular transport. In AD, excess tau phosphorylation causes formation of tau clumps (neurofibrillary tangles). This leads to a break down of nutrient transport and death of the neurons which is believed to be the first step of memory loss. The appearance of tau-containing neurofibrillary tangles is one of the later neuropathological hallmarks in AD brains. Tau can bind to iron and this could contribute to tau aggregation (Adlard and Bush, 2006). Tau protein is needed for iron-export as it was shown that iron accumulated in tau-knock-out mice (Lei et al., 2012). Therefore, excess iron levels in the brain could contribute to memory loss as the consequence of an increased level of tau aggregation.

Copper

The brain is enriched with copper. Copper has been localized in mitochondria, synaptosomes and myelin (Nalbandyan, 1983). In a normal nervous system, copper plays a role in regulating neuronal excitability (Kardos et al., 1989), myelination, iron metabolism and the function of copper containing enzymes such as superoxide dismutase (SOD), dopamine-β monooxygenase and tyrosinase (Nalbandyan, 1983). As mentioned above, the *SOD1* gene is located on chromosome 21. An extra copy of *SOD1* in DS might contribute to a dysfunctional SOD1 enzyme. SOD is a copper and zinc binding enzyme which is responsible for destroying free superoxide radicals in the body.

Studies have also shown that APP knockout mice have higher levels of copper in brain and liver (White et al., 1999) while the APP-overexpressing transgenic mice have reduced levels (Maynard et al., 2002) indicating that APP acts as a major regulator of neuronal copper homeostasis. On the other hand, a recent study showed that copper promotes APP trafficking through the secretory pathway (Acevedo et al., 2014). Collectively, these studies suggest there is a two way relationship between copper and APP.

It is known that copper binding to APP reduces the production of Aβ *in vitro* (Barnham et al., 2003). As DS individuals have an extra copy of the *APP* gene, it may follow that they would have reduced levels of copper in their brains and hence increased production of Aβ leading to the formation of amyloid plaques. This has yet to be investigated.

It has also been shown that in AD brains, there is an overall deficiency of copper (Cater et al., 2008) and an increased level of extracellular copper in amyloid (Bush, 2013) suggesting a change in the homeostasis of copper in AD. Excess cellular copper can compromise cell viability by acting as a pro-oxidant and generating toxic reactive oxygen species via Fenton-type reactions, involving copper ions and hydrogen peroxide. Therefore, cellular copper homeostasis must be tightly regulated (Huang et al., 1999) to maintain healthy brain function.

Zinc

Zinc is essential for the function of numerous enzymes and transcription factors. In the normal brain, zinc is bound to membrane-bound metalloproteins, or loosely bound within the cytoplasm to proteins and enzymes as well as being in synaptic vesicles that are enriched with zinc (exceeding 1 mmol/L in concentration) (Frederickson et al., 2000). During synaptic transmission, high concentrations of zinc are released into the synapse from mossy fibers (Sensi et al., 2011). Zinc regulates ion channels and transmitter receptors such as α-Amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) and N-methyl-D-aspartate (NMDA) receptors which are implicated in synaptic plasticity and memory consolidation (Sindreu and Storm, 2011). Therefore, any change in homeostasis of zinc can have an impact on learning and memory.

Within the ectodomain 2 (E2) of APP, a metal binding site has been identified which binds competitively to zinc or copper (Dahms et al., 2012). Both zinc and copper can interact with Aβ to form aggregates (Adlard and Bush, 2006). An increase in level of extracellular zinc in amyloid of AD has been observed (Bush, 2013). Earlier studies demonstrated that a small increase in brain zinc concentration (>3 micromolar) increased the adhesiveness of Aβ (Bush et al., 1994) and changed Aβ metabolism. Intriguingly, the area of the brain with the highest level of zinc, the

cerebral cortex (Frederickson et al., 1983), exhibits the most severe pathological lesions of AD (Hyman et al., 1986).

Zinc transporters (ZnT) are also crucial to maintain memory and cognitive function. There are 10 ZnT known to date. Zinc uptake into synaptic vesicles needs zinc-transporter-3 (Cole et al., 1999). It has been demonstrated that zinc-transporter-3 knock-out mice develop amyloid pathology characteristic of AD (Adlard et al., 2010) supporting a role for the zinc transporter in the manifestation of AD. Another study showed that zinc-transporter-6, which is located in the Golgi apparatus, is involved in the accumulation of zinc (Lyubartseva et al., 2010). The role of ZnT is yet to be investigated in DS.

Calcium (Ca^{2+})

Calcium plays an important role in the central nervous system. It acts as a cofactor, second messenger and signaling molecule, and a coenzyme when part of a protein. It is also part of excitatory function of neuronal cells, being involved in voltage-gated calcium ion channels. It has been shown that voltage-gated calcium ion channel activity in a mouse model of DS (trisomy 16 mouse; Ts16) was significantly higher than the wild type (Galdzicki et al., 1998). This increased activity could potentially lead to altering calcium homeostasis in the brains of DS individuals.

Human chromosome 21 has two genes of interest that modulate calcium; the *S100*β gene stimulates calcium influx (Mattson et al., 1993a,b) and the *DSCR1* gene, which is a regulator of calcineurin and in some circumstances, can inhibit calcineurin signaling pathways. It has been shown that calcineurin regulates Ca^{2+} pumps and exchangers to maintain Ca^{2+} homeostasis (Stark, 1996). Having an extra copy of *DSCR1* could potentially change calcium homeostasis in the brains of people with DS by influencing the activity of calcineurin, leading to formation of neurofibrillary tangles and consequently memory loss.

In AD, Aβ binds to neurons in close proximity to the NMDA-R which triggers NMDA-R- mediated calcium influx and alters calcium homeostasis (De Felice et al., 2007). Another report suggesting altered calcium homeostasis contributed to AD came from a study on L-type voltage gated calcium channels (LTVGCC) (Anekonda et al., 2011). In this study MC65 neuroblastoma cells were exogenously transfected with APP under the control of a tetracycline-responsive promoter. Upon the withdrawal of tetracycline, production of APP and Aβ fragments was observed along with up-regulation of LTVGCC, leading to increased calcium influx (Anekonda et al., 2011). Interestingly the use of calcium channel blockers prevented the neurotoxicity of Aβ. It has been suggested that the neurotoxicity of Aβ is due to the presence of elevated calcium ions, resulting in increased responses to excitatory amino acids such as glutamate (Mattson et al., 1993a). Due to these increased responses, glutamate receptors are over activated which result in dendritic pruning, increased immunoreactivity of tau, and an accumulation of filaments. These are all characteristics of neurofibrillary tangles of AD in DS and non-DS individuals.

CONCLUSION

An extra dosage of the genes on chromosome 21 could contribute directly or indirectly (by changing metal homeostasis) to the manifestation of AD-like neuropathology in DS. There might be more genes involved in these processes other than the ones discussed in this review. Although some studies have been conducted on cognitive decline in DS individuals, the molecular mechanisms involved in this cognitive decline remain unknown. More studies are required to investigate these mechanisms and thus provide avenues to explore for the prevention of the cognitive decline characteristic of AD in DS and in the non-DS population.

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Caenorhabditis elegans: a model to investigate oxidative stress and metal dyshomeostasis in Parkinson's disease

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Parkinson's disease (PD) is characterized by progressive motor impairment attributed to progressive loss of dopaminergic (DAergic) neurons in the *substantia nigra pars compacta*. Additional clinical manifestations include non-motor symptoms such as insomnia, depression, psychosis, and cognitive impairment. PD patients with mild cognitive impairment have an increased risk of developing dementia. The affected brain regions also show perturbed metal ion levels, primarily iron. These observations have led to speculation that metal ion dyshomeostasis plays a key role in the neuronal death of this disease. However, the mechanisms underlying this metal-associated neurodegeneration have yet to be completely elucidated. Mammalian models have traditionally been used to investigate PD pathogenesis. However, alternate animal models are also being adopted, bringing to bear their respective experimental advantage. The nematode, *Caenorhabditis elegans*, is one such system that has well-developed genetics, is amenable to transgenesis and has relatively low associated experimental costs. *C. elegans* has a well characterized neuronal network that includes a simple DAergic system. In this review we will discuss mechanisms thought to underlie PD and the use of *C. elegans* to investigate these processes.

Keywords: *C. elegans*, oxidative stress, metals, Parkinson's disease, α-synuclein, tau, microtubules, axonal transport

INTRODUCTION

Parkinson's disease (PD) is the second most prevalent age-related neurodegenerative disorder of the central nervous system, after Alzheimer's disease (AD). Idiopathic or sporadic PD affects approximately 1% of people over 65 years old (Hirtz et al., 2007). PD is characterized by severe motor impairment, which is attributed to profound depletion of striatal dopamine (DA) due to progressive loss of dopaminergic (DAergic) neurons in the *substantia nigra pars compacta*, a region in the basal ganglia that is crucial in voluntary motor functions (Hornykiewicz and Kish, 1987; Wooten, 1997; Braak et al., 2003). PD is also characterized by proteinaceous neuronal inclusions known as Lewy bodies (Irizarry et al., 1998). Current PD therapies focus mainly on correcting this DA depletion. Although effective in alleviating symptoms, these treatments lose their efficacy over time and do not halt the underlying neurodegeneration (Smith et al., 2012). Determining the mechanisms contributing to PD neurodegeneration is critical to facilitate the design of effective therapies to halt further neuronal loss.

While some PD cases are monogenic, arising from single point mutation in a specific gene, more than 90% of the cases are idiopathic (**Table 1**). The mechanisms underlying idiopathic PD are not fully understood. However, increasing evidence suggests that oxidative stress may be a major contributing factor to neuronal loss. This is evidenced by increased levels of oxidized lipids, proteins and nucleic acids in PD brains (Dexter et al., 1989a, 1994; Jenner and Olanow, 1996; Yoritaka et al., 1996; Alam et al., 1997a,b). Oxidative stress is thought to arise from a variety of mechanisms including mitochondrial dysfunction, neuroinflammation, perturbed DA metabolism and environmental toxins (Thomas and Beal, 2007; Hwang, 2013). Metal ion dyshomeostasis has also been hypothesized to cause oxidative stress, following evidence that PD brains exhibit increased total iron concentration (Dexter et al., 1991; Gotz et al., 2004; Oakley et al., 2007). In addition, levels of zinc are increased and copper decreased in the *substantia nigra* (Dexter et al., 1991).

Investigating the molecular basis of neurodegeneration *in vivo* relies on animal models, with mammalian models typically being used. All animal models have inherent experimental limitations and none fully replicate all aspects of a disease such as PD. As greater understanding of PD is gained and new hypotheses proposed there is a parallel need for animal models to be updated and modified to further our understanding. Establishing new transgenic models can have a significant lead-time with some animal systems being less suited to genetic modification. These particular limitations can be alleviated by use of a less complex animal, such as *Caenorhabditis elegans.*

C. ELEGANS AS A NEURODEGENERATION MODEL

C. elegans is a free-living nematode, approximately 1 mm in length, which exists as either a self-fertilizing hermaphrodite or as a male (**Figure 1**). *C. elegans* can be cultured inexpensively on an *E. coli* lawn on agar media and has a short defined life cycle (Brenner, 1974). The rapid life cycle coupled with a high reproductive capacity makes *C. elegans* a suitable tool for mutagenesis and compound screening approaches. *C. elegans* also has

Table 1 | PD associated and susceptibility genes and corresponding *C. elegans* homologs.

***PARK* designation[a]**	**PD-associated genes**			
	Gene	**Type of mutation**	**Status**	***C. elegans* homolog**
PARK1	*SNCA*	Dominant	Confirmed	No known homolog
PARK2	*Parkin*	Recessive	Confirmed	*pdr-1*
PARK3	Unknown	Dominant	Not validated since first publication	Unknown
PARK5	*UCHL-1*	Dominant or risk factor	Unconfirmed; conflicting reports (Healy et al., 2006)	*ubh-1*
PARK6	*PINK1*	Recessive	Confirmed	*pink-1*
PARK7	*DJ-1*	Recessive	Confirmed	*djr-1.1 and drj-1.2*
PARK8	*LRRK2*	Dominant	Confirmed	*lrk-1*
PARK9	*ATP13A2*	Recessive	Confirmed	*catp-6*
PARK11	*GIGYF2*	Dominant	Unconfirmed; conflicting reports (Pankratz et al., 2002; Bras et al., 2009; Tan et al., 2009)	No known homolog
PARK12	Unknown	Risk factor	Confirmed	Unknown
PARK13	*HTRA2*	Dominant or risk factor	Unconfirmed; conflicting reports (Strauss et al., 2005; Simon-Sanchez and Singleton, 2008)	No known homolog
PARK14	*PLA2G6*	Recessive	Confirmed	Potential homologs: *C45B2.6, D1037.5, F47A4.5, H23L24.2, T04B2.5,* and *W07A8.2*
PARK15	*FBXO7*	Recessive	Confirmed	No known homolog
PARK16	Unknown	Risk factor	Confirmed	Unknown
PARK17	*VPS35*	Dominant	Confirmed	*vps-35*
PARK18	*EIF4G1*	Dominant	Not validated since first publication (Chartier-Harlin et al., 2011)	*ifg-1*
PARK19	*DNAJC6*	Recessive	Recently published (Edvardson et al., 2012; Koroglu et al., 2013)	*dnj-25*
PARK20	*SYNJ1*	Recessive	Recently published (Krebs et al., 2013; Quadri et al., 2013)	*unc-26*

	PD susceptibility genes[b]	
Gene	**Protein**	***C. elegans* homolog**
MAPT	Tau	*ptl-1*
GBA	Beta-glucosidase	*gba-1, gba-2, gba-3,* and *gba-4*
MC1R	Melanocyte-stimulating hormone receptor	No known homolog
ADH1C	Alcohol dehydrogenase 1C	*H24K24.3* and *Y50D4C.2*
HLA locus	Major histocompatibility complex	No known homolog

[a] *PARK designation represents genes that are putatively linked to PD in chronological order of their identification.*

[b] *Certain polymorphisms or mutations in these genes pose a risk factor for PD.*

an adult lifespan of approximately 3 weeks and is an established model of biological aging. Additionally, the *C. elegans* genome has been fully sequenced which has revealed that about 80% of *C. elegans* genes have human homologs and at least 42% of human disease-related genes have a *C. elegans* homolog (Consortium, 1998; Culetto and Sattelle, 2000; Lai et al., 2000). Functional studies of corresponding or related human genes can be done via mutation (where available) or RNA interference (RNAi) (Fire et al., 1998; Hamamichi et al., 2008; Ruan et al., 2010).

To complement these approaches or in the absence of endogenous homologs, *C. elegans* can be transgenically manipulated to express human disease associated genes in specific cell types, including neurons (Faber et al., 1999; Lakso et al., 2003; Brandt et al., 2009; McColl et al., 2009, 2012). Adult hermaphrodite *C. elegans* have 302 neurons, a neuronal network that is stereotypical between animals and which possesses most of the major neurotransmitter systems found in mammals, including DAergic neurons (White et al., 1986; Rand and Nonet, 1997; Bargmann, 1998). *C. elegans* are also optically transparent, which in conjunction with fluorescent protein reporters, allows for *in vivo* visualization of neurons, such as in **Figure 2** (Chalfie et al., 1994; Nass et al., 2002; Chew et al., 2013).

As with other animal models, use of *C. elegans* to model disease is always tempered by an awareness of the limitations of cellular and anatomical differences. For example, *C. elegans* lack a vascular system and the somatic tissues of adult *C. elegans* are post-mitotic. Despite these obvious differences, key discoveries in *C. elegans* have been readily translated to vertebrate research.

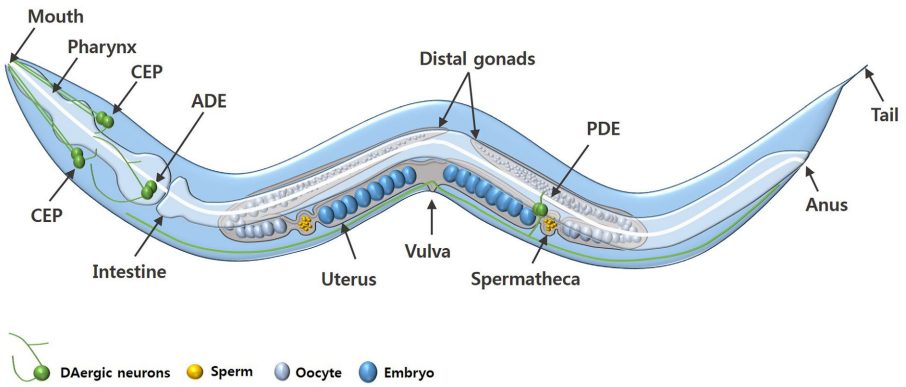

FIGURE 1 | An adult *C. elegans* hermaphrodite. The diagram shows the key anatomical features and the DAergic neurons (green) of *C. elegans*. The DAergic neurons include four cephalic (CEP) neurons, two anterior deirid (ADE) neurons, and two posterior deirid (PDE) neurons. Males have six additional DAergic neurons located in the tail (not shown).

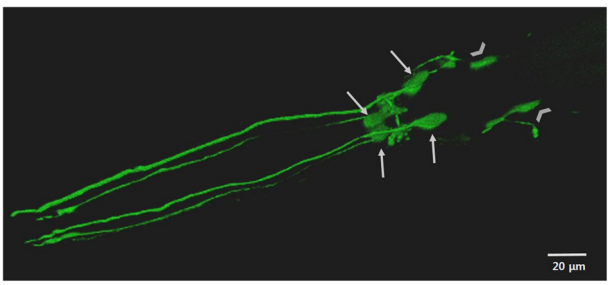

FIGURE 2 | The anterior DAergic neurons of an adult *C. elegans* hermaphrodite. The neurons are visualized by the translational expression of GFP driven by the promoter of the DA transporter (P_{dat-1}::GFP). The key features highlighted include the cell bodies and dendritic processes of the four CEP neurons (arrows) and the cell bodies of the two ADE (chevrons).

C. elegans was used to identify genes that are involved in regulating programmed cell death (Hedgecock et al., 1983; Ellis and Horvitz, 1986). dsRNA gene expression regulation was characterized in *C. elegans* and led to development of RNAi, a tool widely used in functional genomics (Fire et al., 1998). Additionally, the conserved effects of the insulin/insulin growth factor-1 signaling pathway on longevity were first noted in *C. elegans* mutants (Friedman and Johnson, 1988; Kenyon et al., 1993; Dorman et al., 1995; Murakami and Johnson, 1996; Kimura et al., 1997). *C. elegans* research has also linked iron metabolism to restless leg syndrome (Catoire et al., 2011). The findings in *C. elegans* were predictive of the role of ferritin in human tissue.

PARKINSON'S DISEASE

The hallmark PD symptoms are motor deficits, which include resting tremor, rigidity, slowness in movement (bradykinesia) and posture instability. In the majority of PD cases, these clinical manifestations only appear when approximately 50–70% of nigral neurons are lost and approximately 80% of striatal DA is depleted (Hornykiewicz and Kish, 1987; Kish et al., 1988; Orth and Schapira, 2002). This DA deficiency leads to the observed motor impairments because DA is an essential motor control neurotransmitter. In addition to DAergic degeneration, extensive neurodegeneration and atrophy occurs in other nerve cell types and brain regions as PD advances. The regions affected include the hippocampus, thalamus, and neocortex. This additional neurodegeneration leads to non-motor symptoms that include insomnia, depression, psychosis and cognitive impairment (Braak et al., 2003; Weintraub et al., 2011; Smith et al., 2012). These non-motor symptoms worsen over time, for example, an estimated 80% of PD patients with mild cognitive impairment develop dementia (Janvin et al., 2006; Buter et al., 2008; Hely et al., 2008). The etiology of the neurodegeneration leading to cognitive impairment remains unclear.

PD is also characterized by neuronal inclusions, Lewy bodies and Lewy neurites, which mainly contain aggregated α-synuclein (Forno, 1996; Spillantini et al., 1997; Irizarry et al., 1998). Alpha-synuclein is a 140-amino acid peptide encoded by the *SNCA* gene and is predominantly located at presynaptic terminals. It is highly expressed in the *substantia nigra*, hippocampus, neocortex, thalamus and cerebellum (Ueda et al., 1993; Nakajo et al., 1994; Iwai et al., 1995; Recchia et al., 2004). These brain regions are highly impacted by neurodegeneration in PD pathology. Several heritable point mutations, A30P, A53T, E46K, H50Q, and G51D, and a triplication of the *SNCA* gene are implicated in autosomal dominant forms of familial PD (Polymeropoulos et al., 1997; Kruger et al., 1998; Singleton et al., 2003; Zarranz et al., 2004; Appel-Cresswell et al., 2013; Proukakis et al., 2013). These findings have initiated numerous studies into the involvement of α-synuclein in idiopathic PD pathology.

Although several possibilities have been proposed, the function of α-synuclein remains unknown. Alpha-synuclein KO mice have impaired spatial learning and working memory suggesting some involvement in cognitive function (Kokhan et al., 2012). Sequestration of the protein in Lewy bodies may contribute to cognitive impairment seen in advanced PD. Alpha-synuclein over-expression in transgenic mice inhibits DA synaptic release while α-synuclein deficiency causes decreased vesicle-bound striatal DA (Abeliovich et al., 2000; Nemani et al., 2010). This

suggests that α-synuclein is involved in synaptic transmission by regulating DA release. Alpha-synuclein deficiency may lead to unregulated DA release, which when coupled with loss of DAergic neurons, leads to the striatal DA depletion observed in PD. Under normal physiological conditions, α-synuclein negatively modulates the dopamine transporter (DAT), which is required for re-uptake of synaptically released DA (Wersinger and Sidhu, 2003). This implies that α-synuclein deficiency caused by sequestration in Lewy bodies may lead to increased DA re-uptake, causing increased concentration of intracellular DA. High levels of unbound intracellular DA have been shown to be neurotoxic (Olanow and Arendash, 1994; Luo et al., 1998; Offen et al., 1999; Lee et al., 2001).

Other studies suggest that α-synuclein may be a microtubule-associated protein (MAP) as it interacts with tubulin (Alim et al., 2002, 2004), with α-synuclein deficiency postulated to lead to microtubule dysfunction. Microtubules provide structural scaffolding in neurons and so their dysfunction would compromise neuronal integrity leading to neuron death. Alpha-synuclein sequestration in Lewy bodies appears to have significant implications in PD pathology, potentially by inhibiting the normal functions of α-synuclein, which may include facilitating cognitive function, synaptic transmission and stabilizing neuronal morphology. However, the underlying mechanisms that trigger DAergic neuronal death and α-synuclein aggregation in idiopathic PD require further investigation.

C. ELEGANS AND DAergic NEURONS

C. elegans hermaphrodites have a comparatively simple DAergic system comprising eight neurons in total: six anterior DAergic neurons, which include four cephalic (CEP) neurons and two anterior deirid (ADE) neurons, and two posterior deirid (PDE) neurons (**Figures 1**, **2**). Males have six additional DAergic neurons located in the tail (Sulston et al., 1975). DA synthesis, storage and transport mechanisms are conserved in *C. elegans* and DAergic nerve endings and synaptic vesicles have DA levels similar to those in mammalian neurons (Fuxe and Jonsson, 1973; Bargmann, 1998).

The functions of DAergic neurons have been investigated using laser ablation, a technique which can target a specific neuron while leaving neighboring neurons intact. The loss of DAergic neurons revealed that they are important for food searching and the basal slowing response upon sensing food (Sawin et al., 2000; Hills et al., 2004). Exposure to exogenous DA resulted in decreased egg laying, slowed defecation and paralysis (Schafer and Kenyon, 1995; Weinshenker et al., 1995; Hills et al., 2004; McDonald et al., 2006). Studies of mutations in *cat-2*, the tyrosine hydroxylase which is the rate limiting enzyme in DA synthesis, showed loss of basal slowing response and decreased touch habituation suggesting that DA signaling is necessary for mechanosensation (Sawin et al., 2000; Sanyal et al., 2004). These findings suggest that DAergic neurons are important for locomotion, associative learning, food searching, food sensing, egg-laying and defecation.

Most models of DAergic neurodegeneration in *C. elegans* are induced through exposure to neurotoxins and some metals, which selectively ablate DAergic neurons. These toxins include 6-hydroxydopamine (6-OHDA), l-methyl-4-phenylpyridinium (MPP+), methylmercury (MeHg), and manganese (**Table 2**) (Nass et al., 2002; Braungart et al., 2004; Settivari et al., 2009; VanDuyn et al., 2010). When exposed to 6-OHDA, *C. elegans* show a progressive and selective DAergic neuron degeneration and loss as evidenced by formation of blebs in axonal and dendritic membranes. (Nass et al., 2002; VanDuyn et al., 2010).

C. ELEGANS AND α-SYNUCLEIN

Although *C. elegans* lacks a human α-synuclein homolog, α-synuclein expression has been investigated in transgenic *C. elegans*. The targeting of transgene expression in *C. elegans* body wall muscle cells has been used to explore the toxicity of several disease-associated proteins. Body wall muscles run longitudinally along the length of the nematode and are essential for locomotion. Functional disruption of these cells causes a clear and robust paralysis phenotype (McColl et al., 2009, 2012). In PD research, green or yellow fluorescent protein-tagged α-synuclein was expressed in the body-wall muscle of *C. elegans* to visualize α-synuclein aggregation *in vivo* (Hamamichi et al., 2008; van Ham et al., 2008). These lines have been used to screen RNAi libraries, revealing 20 neuroprotective genes whose knock down enhanced α-synuclein aggregation. One of these genes was the ortholog of human VSP41, a key lysosomal trafficking protein that protects against toxicity of DA-derived neurotoxins (Hamamichi et al., 2008; Ruan et al., 2010). Another genome-wide RNAi screen revealed 80 genes that when knocked down accelerated formation of α-synuclein inclusions. These genes, which appear to suppress inclusion formation, are predominantly involved in vesicular transport and lipid metabolism (van Ham et al., 2008). These findings suggest that defects in the endosomal-lysosomal and ER-Golgi vesicular trafficking system pathways may be implicated in α-synuclein toxicity.

Additionally, neurodegenerative processes can also be studied directly in *C. elegans* neurons. Over-expression of wild type and A53T mutant α-synuclein under the control of pan-neuronal promoter, *aex-3* and under the DAergic neuron specific promoter, *dat-1*, caused loss of DAergic neurons (Lakso et al., 2003; Cao et al., 2005). Two neuroprotective endoplasmic reticulum (ER) associated proteins, TorsinA and Rab1 A, were found to ameliorate α-synuclein toxicity and prevent neuron loss (Cao et al., 2005; Cooper et al., 2006), suggesting that α-synuclein toxicity affects the ER-Golgi vesicular trafficking system. Another model over-expressing wild type or mutant α-synuclein under the control of the pan-neuronal promoter, *unc-51*, was used to screen an RNAi library for genetic modifiers that either suppress or exacerbate α-synuclein toxicity. Knock down of four genes that are involved in synaptic endocytosis enhanced α-synuclein toxicity (Kuwahara et al., 2008), suggesting that impaired endocytosis may contribute to α-synuclein dysfunction seen in PD pathology.

Wild type and A53T mutant (human) α-synuclein have been transgenically over-expressed via the *C. elegans* DAergic neuron specific promoter, *dat-1* (Lakso et al., 2003; Cao et al., 2005). A screen of 115,000 compounds in cells and then *C. elegans* identified four 1,2,3,4-tetrahydroquinolinones antagonists of α-synuclein toxicity (Su et al., 2010). Another larger screen revealed that several 8-hydroxyquinolines could ameliorate α-synuclein

Table 2 | *C. elegans* Parkinson's disease models.

Gene	Construct/allele name[a]	Expression pattern	Phenotype	References
α-synuclein (human wild type)	$P_{dat\text{-}1}$ *::α-synuclein*	DAergic neurons	DAergic neurodegeneration, motor deficits, reduced DA and α-synuclein accumulation in DAergic neurons	Lakso et al., 2003; Kuwahara et al., 2006; Cao et al., 2005
	$P_{aex\text{-}3}$ *::α-synuclein*	Pan-neuronal	DAergic neurodegeneration	Lakso et al., 2003
	$P_{unc\text{-}51}$ *::α-synuclein*		Endocytosis, motor and developmental defects	Kuwahara et al., 2008
	$P_{snb\text{-}1}$ *::α-synuclein*		Mitochondrial stress	Ved et al., 2005
	$P_{unc\text{-}54}$ *::α-synuclein::GFP*	Body wall muscles	α-synuclein accumulation	Hamamichi et al., 2008
	$P_{unc\text{-}54}$ *::α-synuclein::YFP*		α-synuclein accumulation	van Ham et al., 2008
	$P_{acr\text{-}2}$ *::α-synuclein*	Motor neurons	Reduced motor movements	Lakso et al., 2003
	$P_{mec\text{-}7}$ *::α-synuclein*	Touch-receptor neurons	Impaired touch sensitivity	Kuwahara et al., 2008
α-synuclein (human mutant)	$P_{dat\text{-}1}$ *::α-synuclein (A30P), (A53T), (A56P),* and *(A76P)*	DAergic neurons	DAergic neurodegeneration	Karpinar et al., 2009
	$P_{dat\text{-}1}$ *::α-synuclein (A53T)*		DAergic neurodegeneration	Lakso et al., 2003
	$P_{dat\text{-}1}$ *::α-synuclein (A30P)* and *(A53T)*		Reduced DA and α-synuclein accumulation in DAergic neurons	Kuwahara et al., 2006
	$P_{unc\text{-}51}$ *::α-synuclein (A53T)* and *(A30P)*	Pan-neuronal	Endocytosis, motor and developmental defects	Kuwahara et al., 2008
	$P_{unc\text{-}119}$ *::α-synuclein (A53T)*		Mitochondrial stress	Ved et al., 2005
	$P_{aex\text{-}3}$ *::α-synuclein (A53T)*		DAergic neurodegeneration, motor deficits	Lakso et al., 2003
	$P_{acr\text{-}2}$ *::α-synuclein (A53T)*	Motor neurons	Reduced motor movements	Lakso et al., 2003
	$P_{mec\text{-}7}$ *::α-synuclein (A53T)*	Touch-receptor neurons	Impaired touch sensitivity	Kuwahara et al., 2008
GFP	$P_{dat\text{-}1}$ *::GFP*	DAergic neurons	Visualizes the DAergic neurons	Nass et al., 2002
MAPT (human tau)	$P_{aex\text{-}3}$ *::tau (WT)*	Pan-neuronal	Uncoordinated movement	Kraemer et al., 2003
	$P_{aex\text{-}3}$ *::tau (V337M)*		Insoluble tau accumulation	
	$P_{aex\text{-}3}$ *::tau (P301L)*		Nerve cord degeneration	
LRRK2	$P_{snb\text{-}1}$ *::LRRK2 (WT)*	Pan-neuronal	Mitochondrial stress	Saha et al., 2009
	$P_{snb\text{-}1}$ *::LRRK2 (R1441C)*		Mitochondrial stress	
	$P_{snb\text{-}1}$ *::LRRK2 (G2019S)*		Mitochondrial stress, DAergic neurodegeneration and reduced DA levels	
Protein with tau like repeats (ptl-1)	*ok621*	Null mutant	Early on-set neurodegeneration, egg hatching defects and reduced touch sensitivity	Gordon et al., 2008; Chew et al., 2013
	tm543	Partial deletion mutant	Early on-set neurodegeneration	Chew et al., 2013

Chemical treatment	Phenotype	References
6-hydroxydopamine (6-OHDA)	DAergic neurodegeneration	Nass et al., 2002; Cao et al., 2005
MPTP/MPP+	DAergic neurodegeneration	Braungart et al., 2004; Pu and Le, 2008
Methyl mercury (MeHg)	DAergic neurodegeneration	VanDuyn et al., 2010
Manganese	DAergic neurodegeneration and oxidative stress	Settivari et al., 2009
Aluminum	DAergic neurodegeneration	VanDuyn et al., 2013

[a] *Construct name includes the promoter used to drive the transgene (promoter::transgene).*

aggregation and toxicity in *C. elegans* (Tardiff et al., 2012). The underlying mechanism of protection is proposed to be via interplay between metal homeostasis and proteotoxicity of aggregation prone proteins. Interestingly another 8-hydroxyquinoline, PBT2, has been found to reduce (the Alzheimer's associated peptide) Aβ toxicity in transgenic *C. elegans* (McColl et al., 2012). This compound is currently under clinical trial as an AD therapeutic (Lannfelt et al., 2008; Crouch et al., 2011).

OXIDATIVE STRESS AND METAL HOMEOSTASIS

Oxidative stress occurs from an imbalance between toxic oxidant production and antioxidant activity, which leads to cellular damage followed by apoptosis (Sies, 1991; Jenner, 2003). The main reactive oxidants are the reactive oxygen species (ROS) and the reactive nitrogen species (RNS). RNS have been comprehensively reviewed elsewhere (Jomova et al., 2010). ROS, such as superoxide ($O_2^{\bullet -}$) and hydroxyl radical ($^{\bullet}OH$) are normal by-products of oxygen consumption during cellular metabolism, predominantly in the mitochondria (Kepp, 2012). ROS levels are tightly regulated by endogenous antioxidant enzymes, such as glutathione, superoxidase dismutase (SOD), and catalase (Bains and Shaw, 1997; Sohal and Orr, 2012). It is important to stress that ROS have essential functions in normal cell biology and are not always inherently detrimental. For example, ROS are a component of the innate immune system, particularly in phagocytes, which produce ROS to prevent colonization by microbes (Fang, 2004). ROS are also utilized in cellular signaling (Hekimi et al., 2011) to modulate the activity of kinases, phosphatases and transcription factors. However, ROS are detrimental when their production goes unchecked leading to damage of cellular lipids, proteins and nucleic acids, and ultimately cell death (Pattison et al., 2002; Niki, 2009).

A way to counter the detrimental effects of ROS overproduction could be to administer antioxidant supplements or drugs, such as, Vitamins A, C, and E and compounds that inhibit ROS production. However, antioxidant therapeutic interventions have not been successful in alleviating oxidative stress associated with neurodegenerative diseases. This is primarily due to the inability of these compounds to effectively cross the blood brain barrier (Halliwell, 2001). Additionally, these antioxidants when administered in high doses have negative side effects by affecting normal cellular processes that rely on ROS activity (Halliwell, 2001; Freeman and Keller, 2012). An understanding of the cause of oxidative stress is vital to design better therapies to prevent neurodegeneration.

Biological transition metals, such as iron, copper, zinc, magnesium, nickel, cobalt, and manganese, are essential co-factors for at least one-third to one-half of all proteins (Andreini et al., 2008; Waldron et al., 2009). Iron and copper are metabolically utilized due to their ability to redox cycle, with iron being the most abundant. However, in the event of metal ion misregulation, this redox ability has the potential to produce toxic radicals via Haber-Weiss and Fenton reactions leading to oxidative stress (Nunez et al., 2012). Levels of these metal ions are reported to be perturbed in brains affected by various neurodegenerative diseases. This has led to the metal ion dyshomeostasis hypothesis, which proposes that the metal ion imbalance triggers increased ROS production causing oxidative stress that eventually leads to neuronal death. It is plausible that the observed metal imbalance is just a symptom and not a cause of neurodegeneration. However, several heritable neurodegenerative diseases are directly caused by metal-ion misregulation. These progressive conditions include aceruloplasminaemia and neuroferritinopathy, which result from iron misregulation, and Menkes Disease and Wilson's Disease, which result from copper misregulation (Vulpe et al., 1993; Yoshida et al., 1995; Harris et al., 1998; Curtis et al., 2001). These diseases suggest that dyshomeostasis of brain metals is sufficient to initiate neurodegeneration.

Iron is an essential metal in organisms because of its redox ability (Cairo et al., 2002). For example, reactive iron is part of the cytochrome complex in the mitochondrial respiration chain, which is important for cellular energy production. It is a crucial co-factor for catalase, an antioxidant that regulates hydrogen peroxide levels and also for heme proteins, which are essential for vascular transport of oxygen and carbon-dioxide. In the *substantia nigra*, iron is essential for DA synthesis (Youdim et al., 1984). However, this reactivity also allows iron to catalyze production of toxic hydroxyl radicals via Fenton chemistry:

$$Fe^{3+} + H_2O_2 \rightarrow Fe^{2+} + HOO^{\bullet} + H^{+}$$

$$Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + OH^{-} + {}^{\bullet}OH$$

Therefore, the concentration of unbound intracellular iron must be kept low; a process regulated by iron storage and transport proteins, such as ferritin (iron storage), ferroportin (iron efflux), divalent metal transporter-1 (DMT-1, an iron transporter), and transferrin (iron shuttling/uptake) (Lee and Andersen, 2010; Gkouvatsos et al., 2012). Disruption in these homeostatic functions could result in iron accumulation leading to oxidative damage and loss of function of proteins that depend on iron as a co-factor. This could potentially disrupt cellular respiration, antioxidant activity, oxygen/carbon dioxide transport and DA synthesis.

Copper is an important co-factor in the activity of redox active proteins, such as ceruloplasmin (iron homeostasis), cytochrome c oxidase (mitochondrial cellular respiration), Cu/Zn-superoxide dismutase (antioxidant activity) and dopamine-b-hydroxylase and tyrosinase, which are key proteins in DA synthesis (Arredondo and Nunez, 2005; Kepp, 2012). Therefore, copper imbalance in neurons may affect the function of these proteins. Additionally, unbound copper concentration requires tight control due to its redox potential. Copper levels higher than 10^{-18} M can initiate oxidative damage by facilitating ROS production (Rae et al., 1999):

$$Cu^{+} + H_2O_2 \rightarrow Cu^{2+} + OH^{-} + {}^{\bullet}OH$$

Copper levels are predominantly regulated by ion importers, copper efflux pumps (ATP7A and ATP7B), metallochaperones, metalloregulators and other copper regulating proteins, such as, ceruloplasmin (Cp), (Camakaris et al., 1999; Waldron et al., 2009; Pang et al., 2013). Defects in these systems may result in increased levels of unbound copper causing oxidative damage. In addition, copper misregulation may cause loss of function of the

copper dependent proteins, with resultant negative implications on iron homeostasis, cellular energy metabolism, oxidative stress responses and DA synthesis.

PARKINSON'S DISEASE AND OXIDATIVE STRESS

PD brains show increased levels of oxidized macromolecules, which can be used as an indirect measure of ROS levels. Malondialdehyde, lipid hydroperoxides and 4-hydroxynonenal, which are lipid peroxidation products, are increased in PD brains (Dexter et al., 1989a, 1994; Yoritaka et al., 1996). PD brains also show increased levels of 8-hydroxydeoxyguanosine (8-OHdG) and protein carbonyls, which are products of DNA and protein oxidation, respectively, (Alam et al., 1997a,b). Another marker of elevated ROS levels in PD brains is increased SOD activity in the *substantia nigra* (Marttila et al., 1988; Saggu et al., 1989). SOD catalyzes the dismutation of superoxide ($O_2^{\bullet -}$) into oxygen and hydrogen peroxide, therefore its activity may increase as a neuroprotective measure to cope with increased ROS levels.

Increased ROS levels not only lead to cellular damage but also to production of oxidation by-products that are also potentially neurotoxic. For example, 4-hydroxynonenal irreversibly modifies α-synuclein aggregation *in vitro*, potentially leading to formation of protofibrils, which are neurotoxic to cultured DAergic neurons (Qin et al., 2007). Lipid hydroperoxides have been shown to lead to oxidation of DA to 6-OHDA, a known neurotoxin (Sauer and Oertel, 1994; Przedborski et al., 1995; Pezzella et al., 1997; Lotharius and O'Malley, 2000). Additionally, α-synuclein aggregation can be induced *in vitro* in the presence of hydrogen peroxide (Hashimoto et al., 1999). This suggests that increased ROS levels not only directly cause neuronal damage but also indirectly contribute to DA depletion and α-synuclein aggregation, which can further exacerbate PD progression.

Taken together, these findings suggest that PD brains are under oxidative stress, which leads to neurodegeneration. However, the mechanisms underlying the increase in ROS levels are not clearly understood. Mitochondrial dysfunction, neuroinflammation, DA autoxidation and environmental toxins have been implicated in the increase of ROS in PD brains (Thomas and Beal, 2007; Jomova et al., 2010; Hwang, 2013). Metal ion dyshomeostasis may also lead to increased ROS production in PD. Generally, the *substantia nigra* has the highest distribution of iron in the central nervous system. However, PD brains have more elevated levels of iron in this region (Dexter et al., 1989b; Riederer et al., 1989; Sofic et al., 1991; Good et al., 1992; Gerlach et al., 1994; Vymazal et al., 1999; Haacke et al., 2007). The infusion of iron into rat brains results in parkinsonism and behavioral changes (Ben-Shachar and Youdim, 1991; Sengstock et al., 1993). Additionally, in mice the 8-hydroxyquinoline metal ion chelator, clioquinol, and over-expression of ferritin, an iron storage protein, both prevent neurodegeneration in PD models (Kaur et al., 2003). These findings suggest that iron may play a significant role in PD neurodegeneration.

The elevated iron levels in the *substantia nigra* are proposed to directly and indirectly contribute to increased ROS production. Increased unbound iron levels can produce ROS, such as superoxide, via Fenton chemistry (Halliwell and Gutteridge, 1986). Additionally, ferric ions can precipitate oxidation of DA to 6-OHDA in the presence of hydrogen peroxide (Pezzella et al., 1997). Superoxide and 6-OHDA have the ability to release iron stored in ferritin and [4Fe-4S] cluster-containing enzymes (Liochev and Fridovich, 1994). This can potentially lead to a vicious cycle in which unbound iron increases levels of superoxide and 6-OHDA causing release of more unbound iron. This may contribute to the progressive neurodegeneration observed in PD.

Iron dyshomeostasis not only contributes to ROS production but also negatively impacts the function of proteins that use iron as a co-factor. For example, tyrosine hydroxylase, the rate-limiting enzyme in DA synthesis, depends on iron (Nagatsu, 1995; Ponting, 2001). Therefore, an increase in iron as seen in PD brains may increase DA synthesis, causing excess DA to be released into the cytoplasm, which may lead to increased ROS production. This iron-induced DA dysfunction not only inhibits the normal function of DA but may also lead to increased DA oxidation into the neurotoxin 6-OHDA (Pezzella et al., 1997; Jiang et al., 2013).

In addition to increased ROS production and iron dyshomeostasis, PD brains also exhibit a reduction in metal ion storage capacity and antioxidant activity. Ferritin is a key iron storage protein and disruption of its function perturbs iron homeostasis. PD brains have decreased ferritin levels (Dexter et al., 1991). This potentially leads to iron storage deficiency, which allows unbound reactive iron to accumulate in the *substantia nigra*, facilitating ROS production (White and Munro, 1988; Dexter et al., 1991; Connor et al., 1995). Neuroferritinopathy is a condition caused by a genetic mutation of the ferritin light chain which disrupts ferritin assembly, leading to iron accumulation and neurodegeneration in the basal ganglia, resulting in severe motor disorders (Curtis et al., 2001; Vidal et al., 2003). In addition to decreased ferritin levels, PD patients have decreased concentration and activity of Cp (Kristinsson et al., 2012). Cp is a multi-copper oxidase that oxidizes ferrous ions (Fe^{2+}) to less reactive ferric ions (Fe^{3+}). This oxidation is essential for cellular iron uptake and efflux by ferroportin and transferrin. Aceruloplasminaemia, a heritable condition resulting from Cp deficiency, leads to iron accumulation in the basal ganglia, neurodegeneration and motor problems including dystonia and tremors (Harris et al., 1998).

PD brains have approximately 40% lower reduced glutathione (GSH), an antioxidant enzyme that catalyzes the reduction of ROS (Sofic et al., 1992). GSH also forms complexes with other enzymes, such as glutathione peroxidase and glutathione S-tranferases, to facilitate ROS reduction (Smeyne and Smeyne, 2013). Decreased antioxidant capacity likely contributes to the oxidative stress seen in PD brains. These findings suggest that defective metal ion transport and storage, decreased antioxidant activity and increased reactive metal ion accumulation contribute to oxidative stress leading to neurodegeneration.

C. ELEGANS: OXIDATIVE STRESS AND METAL ION HYPOTHESIS

Another link between PD and oxidative stress is gleaned from studies of mutations in *DJ-1* and *PINK1*, which are associated with early onset PD (Bonifati et al., 2003; Valente et al., 2004). *DJ-1* and PINK1 have been shown to protect against oxidative stress (Junn et al., 2005; Pridgeon et al., 2007). This was confirmed in *C. elegans* by studying the nematode homologs, *djr1.1* and *pink-1*. The *djr-1.1* knock-down and *pink-1* mutant strains showed

increased sensitivity to toxin-induced oxidative stress (Ved et al., 2005; Samann et al., 2009). These *C. elegans* models complement the familial PD studies and strengthen the hypothesis that oxidative stress contributes to PD pathology.

Transgenic *C. elegans* expressing α-synuclein in neurons exhibited mitochondrial fragmentation attributed to α-synuclein interaction with mitochondrial membranes, affecting membrane fusion (Kamp et al., 2010). Alpha-synuclein is localized in mitochondria, suggesting that α-synuclein dysfunction potentially contributes to mitochondrial dysfunction in PD (Li et al., 2007). In turn, mitochondrial dysfunction leads to ROS overproduction in the *substantia nigra* of PD brains, which leads to cellular damage and cell death.

A recent study using electron paramagnetic resonance demonstrated that unbound reactive iron levels increased during oxidative stress in *C. elegans* (Rangel et al., 2012). Increased iron levels in *C. elegans* resulted in increased protein oxidation, suggesting that iron triggers increased ROS production. Iron chelation using deferoxamine and over-expression of ferritin (*ftn-1*) reduced protein oxidation (Valentini et al., 2012). Knock down or deletion of *C. elegans* SMF-1/2/3 (orthologs of human iron transporter, DMT-1) partially inhibits DAergic neuronal death (Settivari et al., 2009; VanDuyn et al., 2013). PD brains have increased iron levels, decreased ferritin levels and increased DMT-1 levels, consistent with defective iron transport and storage systems in PD brains (Dexter et al., 1991; Salazar et al., 2008).

Knock down of SKN-1 (a *C. elegans* ortholog of Nrf2), a transcription factor that regulates expression of glutathione S-transferase, increased susceptibility to metal-induced neurodegeneration in DAergic neurons (VanDuyn et al., 2010; Settivari et al., 2013). This finding and the observation that PD brains show decreased levels of glutathione suggests that decreased antioxidant activity contributes to PD pathology (Sofic et al., 1992).

Metal dyshomeostasis and oxidative stress may represent an important component underlying idiopathic PD. *C. elegans* possesses homologs of some of the iron homeostasis proteins (**Table 3**) therefore the nematode can be used to further our understanding of metal homeostasis in relation to PD. Even more broadly, any findings can potentially be extended to familial autosomal PD because *C. elegans* also has homologs for the majority of genes implicated in familial PD (**Table 1**). These features may be exploited to investigate these genes and their interactions with metal homeostasis.

Table 3 | *C. elegans* iron metal homeostasis proteins.

Human protein	*C. elegans* homolog
Ferritin	Ferritin 1 (FTN-1)
	Ferritin 2 (FTN-2)
Ceruloplasmin	F21D5.3
Ferroportin	Ferroportin 1.1 (FPN-1.1)
	Ferroportin 1.2 (FPN-1.2)
	Ferroportin 1.3 (FPN-1.3)
Divalent metal-ion transporter	SMF-1
	SMF-2
	SMF-3

THE MISSING LINK

Varied evidence supports the potential role of metal dyshomeostasis in PD neurodegeneration. However, the underlying mechanism that leads to metal imbalance still remains to be elucidated. The interplay between tau, α-synuclein and microtubules, may hold an answer to this question (**Figure 3**).

TAU, α-SYNUCLEIN, AND PARKINSON'S DISEASE

Tau is a MAP predominantly expressed in axons and is thought to regulate the assembly of microtubules (Weingarten et al., 1975; Kosik and Finch, 1987). Neurofibrillary tangles (NFTs) comprised of hyperphosphorylated tau aggregates are a pathological hallmark of AD (Kidd, 1963; Wischik et al., 1988). Although not often emphasized, tau has also been implicated in PD pathology. Some PD patients have NFTs and in older people with parkinsonian symptoms, the severity of gait impairment appears to correlate with the degree of NFT accumulation (Joachim et al., 1987; Bancher et al., 1993; Schneider et al., 2006). Certain single-nucleotide polymorphisms in the tau gene pose an increased risk factor for PD (Zabetian et al., 2007; Edwards et al., 2010). Tau KO mice have recently been reported to exhibit neuronal iron accumulation, *substantia nigra* neuronal loss, parkinsonism and cognitive deficits (Lei et al., 2012). Anti-psychotic DA D2 receptor antagonists, such as azaperone, suppress insoluble tau aggregation in *C. elegans* (McCormick et al., 2013), suggesting an interplay between tau and DA.

Increasing evidence highlights the importance of tau and α-synuclein in PD pathology and indicates that the two proteins significantly interact. Tau is co-localized with α-synuclein in Lewy bodies (Arima et al., 1999). Tau and α-synuclein can seed and promote each other's polymerization to form insoluble aggregates (Giasson et al., 2003; Geddes, 2005). Alpha-synuclein has been shown to directly facilitate tau phosphorylation and also to mediate glycogen synthase kinase 3 (GSK-3β, a serine/threonine protein kinase) catalyzed tau phosphorylation, which is increased in PD brains (Jensen et al., 1999; Muntane et al., 2008; Duka et al., 2009). This indicates that α-synuclein may contribute to the increased GSK-3β activity, which leads to tau hyperphosphorylation.

TAU, α-SYNUCLEIN, AND MICROTUBULE DYSFUNCTION

Based on the interaction between tau and α-synuclein, the dysfunction of the two proteins may disrupt two key functions of microtubules: axonal transport and maintaining neuronal morphology. Microtubule dysfunction precedes impaired axonal transport (Cartelli et al., 2013). This was deduced from altered mitochondria distribution and neurodegeneration in DAergic neurons of mice exposed to MPTP. MPTP is known to destabilize microtubules and impair axonal transport specifically in DAergic neurons (Cappelletti et al., 2005; Ren et al., 2005; Morfini et al., 2007). Administration of a microtubule stabilizer, Epothilone D, attenuated further nigrostriatal neurodegeneration (Cartelli et al., 2013), highlighting a potential link between axonal transport disruption, microtubule dysfunction and neurodegeneration.

As a MAP, tau not only stabilizes microtubules but also regulates transport by serving as a physical barrier and by interacting with transport motor proteins, dynein and kinesin, to regulate

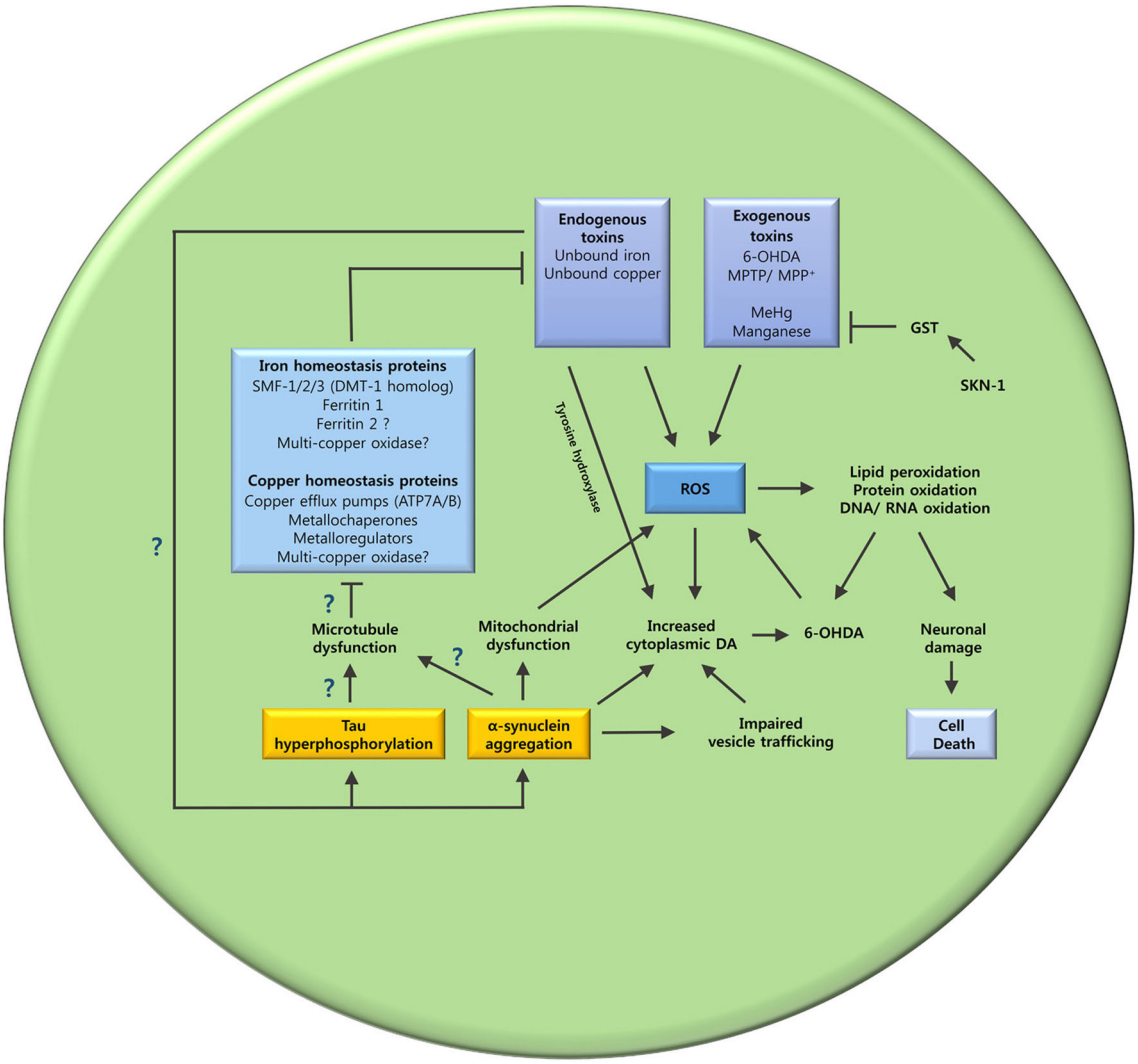

FIGURE 3 | The oxidative stress and metal ion dyshomeostasis cascade. This schematic summarizes the hypothesized mechanisms that may lead to oxidative stress and metal dyshomeostasis in DAergic neurons of *C. elegans* PD models. The unanswered questions are also highlighted. Does tau hyperphosphorylation and α-synuclein aggregation cause microtubule dysfunction? Are key metal ion homeostasis proteins dependent on microtubules? Does metal ion dyshomeostasis contribute to tau hyperphosphorylation and α-synuclein aggregation? GST (glutathione S-transferase); SKN-1 (Nrf2 ortholog).

microtubule attachment and detachment (Jancsik et al., 1996; Trinczek et al., 1999; Stamer et al., 2002; Mandelkow et al., 2003; Magnani et al., 2007; Dixit et al., 2008). Tau over-expression disrupts the transport of mitochondria and vesicles leading to accumulation of mitochondria in distal parts of the neuron (Ebneth et al., 1998; Stamer et al., 2002; Mandelkow et al., 2003). Hyperphosphorylated tau filaments have been shown to phosphorylate the kinesin light chain thereby triggering the dissociation of kinesin from its cargo (Lapointe et al., 2009). Phosphorylation of tau at the amino terminus can also impact its inhibitory effect on axonal transport (Kanaan et al., 2012). Mutant tau has been shown to cause "traffic jams" which inhibit axonal transport (Shemesh et al., 2008). The tau dysfunction observed in PD may negatively impact axonal transport, contributing to neurodegeneration.

Alpha-synuclein is co-localized with tubulin in Lewy bodies and co-purifies with microtubules. Additionally, when incubated with tubulin, α-synuclein polymerizes tubulin into microtubules (Alim et al., 2002, 2004). Immunofluorescence staining of α-synuclein transfected COS-1 cells with α-synuclein and tubulin antibodies, showed that α-synuclein co-localized predominantly with microtubules (Alim et al., 2004). Alpha-synuclein binds synaptic vesicles via its amino terminus and is involved in vesicle trafficking (Jensen et al., 1999; Cooper et al., 2006). These findings suggest that α-synuclein, like tau, is a MAP and is involved in axonal transport of vesicles. Alpha-synuclein dysfunction likely leads to impaired axonal transport.

Protein with tau-like repeats (PTL-1) is the only known tau/MAP2 ortholog in *C. elegans* (Goedert et al., 1996). PTL-1 is important for maintaining *C. elegans* neuronal morphology

(Chew et al., 2013). Null mutants for *ptl-1* show accelerated neurite branching and microtubule bundle disorganization in mechanosensory and GABAergic neurons (Chew et al., 2013). Microtubule changes in these neurons suggest a link between tau deficiency and compromised neuronal integrity. In addition, transfection of *ptl-1* into non-neuronal cells promotes microtubule assembly and bundling (Goedert et al., 1996).

Loss of function of tau and α-synuclein may result in significant microtubule disruption that leads to neurodegeneration seen in PD. Although the downstream effect of microtubule dysfunction in neurons remains to be elucidated, we can speculate that cellular functions which rely upon microtubules will be disrupted. The function of metal ion regulating proteins, such as ferroportin and copper transporter (ATP7A), are dependent on axonal transport (Cobbold et al., 2004; Moos and Rosengren Nielsen, 2006). Microtubule disruption would likely lead to disrupted trafficking of these metal ion homeostasis proteins. This in turn disrupts metal ion homeostasis leading to accumulation of unbound reactive metal ions and metal ion deficiency, resulting in oxidative stress followed by neuronal loss.

CONCLUSION

Despite evidence pointing to the involvement of metal ion imbalance and microtubule dysfunction in neurodegeneration, few studies have attempted to link these two elements. We propose that disrupted axonal transport and neuronal integrity greatly impacts metal ion balance by hindering the trafficking of metal ion homeostasis proteins and neuronal anti-oxidants (**Figure 3**). Disrupting metal ion homeostasis is likely to result in oxidative stress leading to neuronal loss. In addition, microtubule disruption may result in loss of synaptic connections due to altered neuronal morphology causing synaptic transmission impairment. The interplay between tau, α-synuclein and metal dyshomeostasis offers a new avenue of investigation. *C. elegans* has homologs for many of the genes involved in iron regulation (**Table 3**) and can be genetically manipulated to express transgenes in the absence of homologs (**Table 2**); this may represent an ideal system in which to investigate these questions.

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The molecular basis of memory. Part 3: tagging with "emotive" neurotransmitters

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Many neurons of all animals that exhibit memory (snails, worms, flies, vertebrae) present arborized shapes with many varicosities and boutons. These neurons, release neurotransmitters and contain ionotropic receptors that produce and sense electrical signals (ephaptic transmission). The extended shapes maximize neural contact with the surrounding neutrix [defined as: neural extracellular matrix (nECM) + diffusible (neurometals and neurotransmitters)] as well as with other neurons. We propose a *tripartite* mechanism of animal memory based on the dynamic interactions of splayed neurons with the "neutrix." Their interactions form cognitive units of information (*cuinfo*), metal-centered complexes within the nECM around the neuron. Emotive content is provided by NTs, which embody molecular links between physiologic (body) responses and psychic feelings. We propose that neurotransmitters form mixed complexes with *cuinfo* used for tagging emotive memory. Thus, NTs provide encoding option not available to a Turing, binary-based, device. The neurons employ combinatorially diverse options, with >10 NMs and >90 NTs for encoding ("flavoring") *cuinfo* with emotive tags. The neural network efficiently encodes, decodes and consolidates related (entangled) sets of *cuinfo* into a coherent pattern, the basis for emotionally imbued memory, critical for determining a behavioral choice aimed at survival. The *tripartite* mechanism with tagging of NTs permits of a causal connection between physiology and psychology.

Keywords: metal complex, *cuinfo*, neurotransmitters, emotion, mentation

INTRODUCTION

The neural circuitry of the brain has been likened to a biological computing device. But the process whereby a physiologic process (stimulus sensation) transforms into a psychical sensation (such as emotionally-tinged memory), which determines physical response to immediate stimuli, remains mysterious (**Figure 1**).

Each of the senses receives environmental stimuli (input) which are transformed into a synaptic cognitive information (cog-info) signals, which are somehow encoded and stored somewhere in the brain, later to be decoded (recalled), to determine a behavioral choice based on recalled experience. Much has been speculated in philosophical term (Romanes, 1883; James, 1884; Langer, 1967; Meshulam et al., 2011) and on the basis of biologic observations (Squire and Kandel, 2008; Kandel, 2009; Garcia-Lopez1 et al., 2010; DeFelipe, 2011; Murtya et al., 2011; Emmons, 2012; Jarrell et al., 2012; Hirano et al., 2013; Strausfeld and Hirth, 2013; Wright et al., 2013), but molecular details for the mentation of memory by neural animals are lacking.

Q: Does the brain operate like a Turing machine (Boole, 1853; Turing, 1950)?

A: Computer and machine circuits (**Figure 2A**) operate in dry condition. Wires in an electric circuit are insulated from one another by plastic, non-conducting, coatings and air gaps (or vacuum), to prevent short circuits.

By contrast, we previously pointed out that neurons are enclothed in a wet, electrically conducting hydrogel (nECM) with many component glycosaminoglycans (GAGs) and proteins (Marx and Gilon, 2012, 2013). The intimate contacts of the extended neural surface with the nECM permits iontophoretic/piezo-electric/visco-elastic actuators on the neural surface to metamorph cog-info into *cuinfo*. Images of "naked" neurons suspended in vacuous space are misleading, in that they ignore the nECM and the dopants (NTs and neurometals) distributed therein. Cajal did not perceive their roles in neural mentation (**Figure 2B**; see Garcia-Lopez1 et al., 2010). Rather, the intuitive painting of Pollock (1997) more closely represents the physical circumstances of neurons enclothed within a biogel lattice (**Figure 2C**).

ENCODING EMOTIONS

Neural memory recalls *emotive* as well as *objective* qualities. As conceived by the philosopher William James and other philosophers (Romanes, 1883; James, 1884; Langer, 1967; Meshulam et al., 2011), emotions have physical correlates.

Q: What kind of molecular structure or process endows memory with emotive quality?

A: Possibly, neurotransmitters (NTs) are involved. They eliciting a range of physiologic and psychic responses and most of them bind to metals.

We point out that the NTs are a class of molecules synthesized and secreted by neurons that elicit emotive reactions,

concomitant with physiologic responses. Thus, NTs can be considered as the molecular embodiments of emotions. In that they have strong affinity to metals, they can form ternary complexes with metals, as exemplified by the binding of bilirubin to albumin (Marx, 1984). We propose that the NTs (Hughes and Zubek, 1956; Colburn and Maas, 1965; Boggess and Martin, 1975; Chandra et al., 1980; Ludlam et al., 1980; Sigel and Martin, 1982; Jolles, 1983; Coffman and Dunn, 1988; Flood et al., 1990; Jefferys, 1995; Velez-Pardo et al., 1995; White and Rumbold, 1988; Buhot et al., 2000; Reith, 2002; Shaik, 2003; Álvarez and Ruarte, 2004; Siegel et al., 2005; Kroval et al., 2006; Marazziti et al., 2006; Neumann, 2007; Wyttenbach et al., 2008; Paoletti et al., 2009; van der Burgt et al., 2009; Burbach, 2010; Dere et al., 2010; Guastella et al., 2010; Brady et al., 2011; Lesburguères et al., 2011; Beets et al., 2012; García et al., 2012; Garrison et al., 2012; Ma et al., 2013; Pitt et al., 2013; Yanagita et al., 2013) provide the neural net with a new mode of processing (mentating) cognitive information (cog-info) not available to a binary Turing machine.

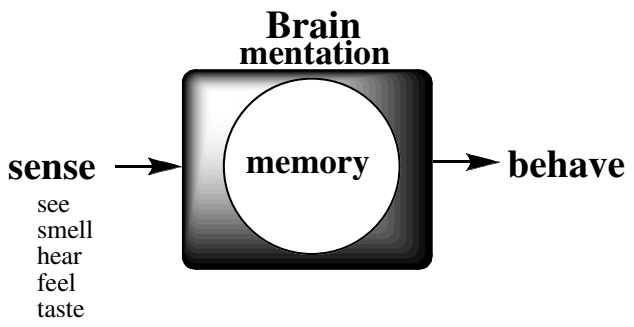


FIGURE 1 | Schematic of the process whereby an external stimulus is remembered to determine whole body response, critical for survival.

TRIPARTITE MECHANISM

To rationalize the phenomenon of biologic memory in physical-chemical terms, we have proposed (Marx and Gilon, 2012, 2013) a *tripartite* mechanism comprising 3 physiologic compartments:

- Neuron—elongated cell in synaptic and non-synaptic contact with others
- nECM—an anionic biogel lattice surrounding the neuron
- dopants—neurometals and neurotransmitters (NTs)—metals (e.g., Al^{+3}, Ca^{+2}, Co^{+2}, Cu^{+2}, $Fe^{+2/3}$, Mg^{+2}, $Mn^{+2/3}$, Zn^{+2}) and small molecular modulators, distributed in the nECM.

Though the term "space" is often used to refer to the neurons' environment, it is not quite correct. The neurons are not naked, floating in space. Rather, they are suspended (enmeshed) in a matrix composed of glycosamino-glycans (GAGs) and proteins (such as tenascins, and laminin), referred to as "nECM." Their shape (highly elongated with many dendrites, splayed, arborized) exposes the large surface to intimate contact with the nECM, through which chemical, as well as electrical, signaling occur.

Just like all other physiologic processes, mentation must be biochemically based. All three of the above compartments are involved in transforming (encoding) cognitive information (cog-info) incoming from the senses, into [nECM:metal] complexes, the molecular correlates of *cognitive units of information* (*cuinfo*), like computer bits. The incoming cog-info is transferred to the brain via into synaptic and non-synaptic networks. But what happens there?

We generalize a biochemical processes and notations, which feature sets of metal-centered complexes (*cuinfo*) which can undergo different types of redox, tagging and cross-linking reactions, thereby modulating the dielectric properties, viscoelasticity and stability of local, molecular ensembles. The neuron is atuned to such nECM ensembles and can thereby chemically affect/sense (encode/decode) cog-info.

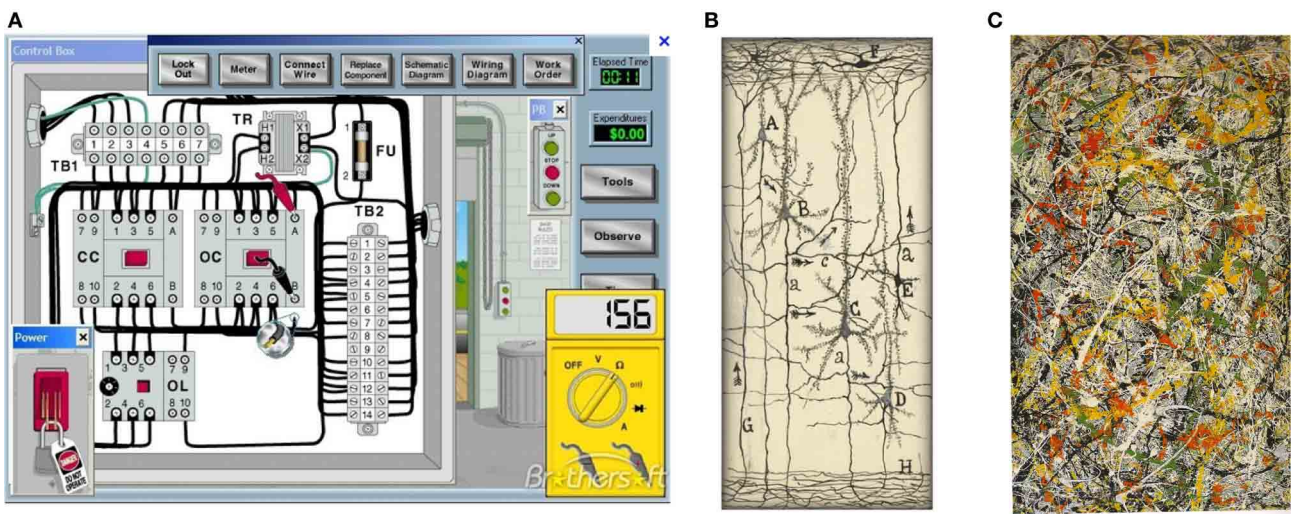
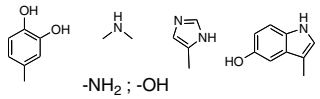
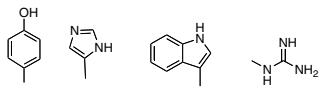


FIGURE 2 | (A) Circuit for electric motor. (Note the insulating spaces between wires and components). **(B)** Cajal drawing of a neural circuit (from Garcia-Lopez1 et al., 2010; DeFelipe, 2011). (Note the empty spaces around neurons) (with permission). **(C)** Painting by Jackson Pollok (Boole, 1853) that more closely represents neurons enmeshed within the nECM. (No empty spaces). (with permission).

Table 1 | Metal complexing neurotransmitters.

Type of Neurotransmitter	Number	MetalComplexingGroup
AminoAcids	5	$-NH_2$; -COOH; -OH
BiogenicAmines	5	 $-NH_2$; -OH
Neuropeptides	>75	$-NH_2$; -COOH; -OH; $-CONH_2$; -SH; $-S-CH_3$; -CO-NH-; -S-S- 
Others	>5	NO; -OH; -O-; -NH-CO- 

Types and structures (Hughes and Zubek, 1956; Colburn and Maas, 1965; Boggess and Martin, 1975; Chandra et al., 1980; Ludlam et al., 1980; Sigel and Martin, 1982; Jolles, 1983; Coffman and Dunn, 1988; Flood et al., 1990; Jefferys, 1995; Velez-Pardo et al., 1995; White and Rumbold, 1988; Buhot et al., 2000; Reith, 2002; Shaik, 2003; Álvarez and Ruarte, 2004; Siegel et al., 2005; Kroval et al., 2006; Marazziti et al., 2006; Neumann, 2007; Wyttenbach et al., 2008; Paoletti et al., 2009; van der Burgt et al., 2009; Burbach, 2010; Dere et al., 2010; Guastella et al., 2010; Brady et al., 2011; Lesburguères et al., 2011; Beets et al., 2012; García et al., 2012; Garrison et al., 2012; Ma et al., 2013; Pitt et al., 2013; Yanagita et al., 2013).

Focusing on the neurotransmitters (NTs) shown in **Table 1**, all present a variety of metal complexing moieties called ligands (e.g., glycine, glutamate catechol amines, neuropeptides, adenosine) that can form mixed complexes with *cuinfo*. Activated neurons release vesicles containing NTs along with neurometals (M^{+v}; such as $Ca^{+2,}$ $Cu^{+2,}$ $Fe^{+3,}$ Mn^{+2}, Zn^{+2}, etc.) into the nECM of the synaptic cleft and other extracellular locations, permitting the formation of ternary complexes [nECM: M^{+v}:NT]. Some larger one permit coordination with more than 1 metal centered *cuinfo* (bidendate, tridendate).

With the exception of acetylcholine and muscarine, which are true cationic entities due to their tetrasubstituted ammonium moiety, most NTs are electron donors, behaving as effective metal complexants (ligands) (**Table 1**). An individual NT can be considered to embody an "emotive" signal, if it elicits physiologic responses (pulse, breathing, dilation of blood vessels and pupil, erection, sweating, etc), as well as corresponding psychic reactions (attention, anxiety, anger, fear, hunger, pain, love, etc.), which are remembered. Chemically, an electron-rich NT molecule diffusing in the nECM can bind to a metal-centered *cuinfo*, confers an emotive tag to the ternary (mixed) complex, resulting in a tagged, *cuinfo:NT*. The stability of such complexes depends on the valency of the metal cation and binding affinity of the components (pK_D). Monovalent metal (Na^+, K^+, Li^+, Cs^+) complexes are relatively unstable; the resultant *cuinfo* tend to rapidly disintegrate. Small monodendate NTs bind to a single *cuinfo*; larger ones are polydendate and could bind to multiple *cuinfo*, thereby literally "entangling" them. **Table 2** below organizes the metal-complexing NT which have been shown to induce physiologically-linked psychic responses to stimuli (**Table 2**), are also imprinted in memory.

Table 2 | Bio-modulators (also called NTs) of physiologic responses to stimuli, which simultaneously elicit both physiologic responses and psychic (emotions) feelings, which also encode the stimuli, aiding the recall (memory).

Modulators (neurotransmitters)	Metal complexing ligands	Physiologic reactions	Psychic effects emotions
Acetylcholine (AcChol)	NO	Breathing	Anxiety
Epinephrine (EPI)	YES	Blinking	Aggression
Serotonin (SER)	YES	Blood pressure	Awareness
Histamine (HIS)	YES	Coughing	Depression
Nicotine	YES	Crying	Fear
Muscarine	NO	Dilation of pupil	Hate
Amino acids	YES	Drooling	Heat
>75 neuropeptides	YES	Erection	Hunger
		Evacuation	Joy
		Fever	Love
		Goose-bumps	Pain
		Heart beat	Sadness
		Itching	Sex drive
		Orgasm	
		Pulse	
		Salivation	
		Secretion	
		Spasms	
		Sweating	
		Tremors	
		Urination	
		Vasodilation	
		Vomiting	

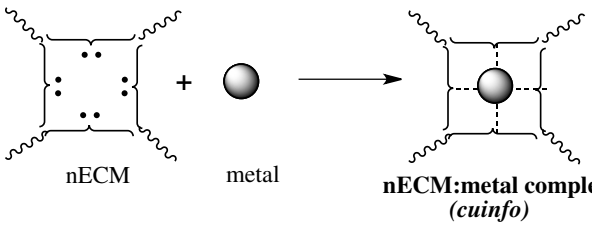


FIGURE 3 | Iconographic representation of formation of anECM:metal complex (*cuinfo*). The chelating node (address) within the nECM is presented as square electron-rich hole fixed within the nECM lattice, with 2 dots representing ligands available for capturing a metal. The metal-bonded to the complexing group electrons is indicated by a dotted line, within the *cuinfo*. It can serve as a binding focus for metabolites and neurotransmitters. The nECM array with metal complexes is called neutrix.

ICONOGRAPHY

We offer an iconography to visualize the formation of *cuinfo* (**Figure 3**) and their transformation by tagging with NT (**Figure 4**). To stay within the IUPAC guidelines for chemical notations, the graphic notation previously employed has been slightly modified (Boole, 1853; Marx and Gilon, 2012, 2013). The complexing moieties (ligands) in the nECM are presented as two dots (non-bonding pair of electrons). The metal bonded to the

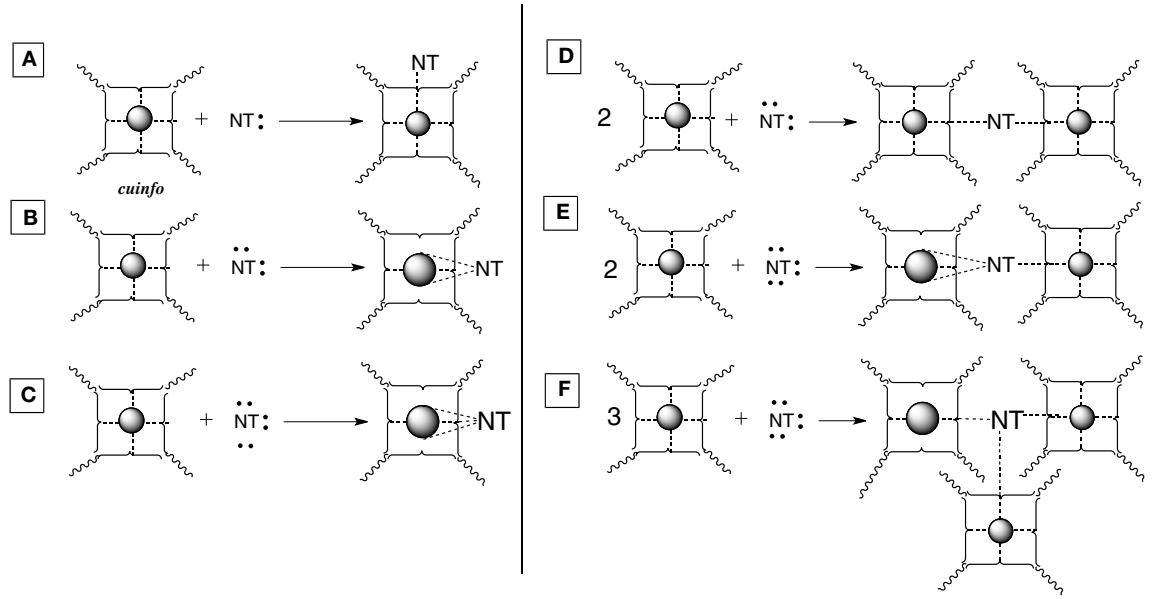


FIGURE 4 | Various types of [*cuinfo*:NT] complexes. (A) A monodentate NT replaces one neutrix metal bond. **(B)** A bidentate NT couples (entangles) 2 *cuinfo*. **(C)** A tridentate NT replaces three neutrix metal bonds. **(D)** A bidentate NT entangles two *cuinfo*. **(E)** A tridentate NT entangles two *cuinfo*, via double and single complexing bonds. **(F)** A tridentate NT entangles three *cuinfo*.

complexing groups in the nECM is indicated by a dotted line (e.g., **Figure 3**). We have defined an arbitrary unit of cognitive information as *cuinfo*. We call the nECM array with metal complexes: neutrix.

EXAMPLES OF METAL CHELATING NEUROTRANSMITTERS (NTs)

The NT can be considered to embody "emotive" signals, in that they elicit emotive physiologic reactions (attention, anxiety, anger, fear, love, pain, etc) that are remembered. Many NT are also effective metal chelators. Chemically, the presence of an NT can "flavor" a *cuinfo*. The resultant ternary complex is more stable, crowned with an emotive tag. Such chelate complexes are reversible depending on their binding strength to a particular *cuinfo* (pK_D). Some redox and crosslinking reaction can stabilize these.

Mono-, bi- and tridentate complexes of electron-rich NTs with metal-centered *cuinfo* can be conceived (**Figure 4**). In such mixed complexes, the NT replaces one or more neutrix metal bond also indicated by a dotted line

Of course, cross-linking (from either redox or enzymatic) reactions would render the entire ensemble much more stable, relevant to long term memory.

TYPES OF NTs

A: Catecholamines: epinephrine (EPI), norepinephrine (NE), dopamine (DA) (Colburn and Maas, 1965; Boggess and Martin, 1975; Chandra et al., 1980; Kroval et al., 2006; García et al., 2012).

Catecholamines are "emotive" neurotransmitters associated with fear, fright, anxiety, all emotions strongly recalled in memory. Physiologically, they elicit responses such an altered heart rate, blood flow, pupil dilation, muscle contraction, etc. Chemically, they comprise an ortho-dihydroxy benzene structure, a potent metal chelating moiety; stored within neuron's vesicles and released upon signaling. The NT of this class, the dopamine (DA), norepinephrine (NE) and epinephrine (EPI) can form ternary complexes with *cuinfo*, generaly described in **Figure 6**.

The catecholamines present 2 independent chelate centers (the ortho hydroxy benzene and the distal amino terminus) which can bridge two adjacent *cuinfo*, effectively entangling a pair of *cuinfo*, rendering them more stable as well as more identifiable (tagging) for linked (entangled) recall. They permit of emotive memory associated with physiologic reactions.

B: Amino acids (Hughes and Zubek, 1956; White and Rumbold, 1988; Flood et al., 1990; Velez-Pardo et al., 1995; Buhot et al., 2000; Álvarez and Ruarte, 2004; Siegel et al., 2005; Paoletti et al., 2009; Dere et al., 2010; Lesburguères et al., 2011) and other small molecules.

In the same manner, other NT such as glutamine, histidine, and seratonin, which affect numerous physiologic responses (water balance, immune reactions, blood clotting, fever, sweating), as well as emotion, can form mixed complexes with *cuinfo*. A lineup of some NT's capable of adorning the *cuinfo* by chelate complexation is iconographically presented in **Figure 7**.

C: Metal chelating neuropeptides (Jolles, 1983; Ludlam et al., 1980; Coffman and Dunn, 1988; Marazziti et al., 2006;

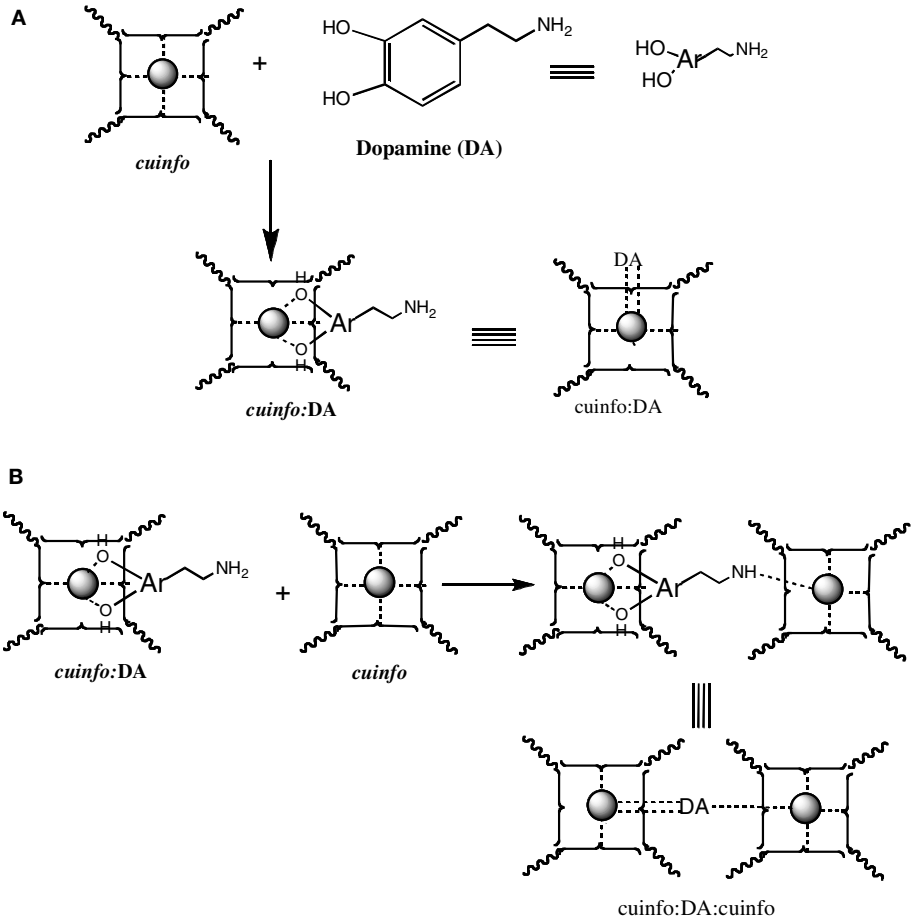


FIGURE 5 | Formation of mixed dopamine:*cuinfo* complexes. (A) Formation of ternary *cuinfo* complex with one catecholamine. **(B)** Formation of a entangled dopamine (DA) complex ($cuinfo_2$:DA).

Neumann, 2007; Wyttenbach et al., 2008; van der Burgt et al., 2009; Guastella et al., 2010; Beets et al., 2012; Garrison et al., 2012; Pitt et al., 2013).

Neuropeptides are an important class of molecular communicators in the central and peripheral nervous systems, acting as neurotransmitters, neuromodulators, and hormones. They also connect the nervous system to other physiological networks regulating breathing, pulse, etc. Many neuropeptides are abundantly expressed in brain regions involved in emotional processing and anxiety behaviors.

Neurotransmitters (NTs) and neuropeptides (NP), having various physiological effects have also been implicated in cognitive functions such as learning and memory. The peptides include corticotropin releasing factor, urocortin, neuropeptide Y, vasoactive intestinal polypeptide, neurotensin, galanin, opioid peptides, tachykinins, nociceptin, oxytocin, vasopressin, and angiotensin. In addition to their many physiological functions, NTs elicit psychic effects on mood (anxiety and depression) and memory.

For example oxytocin is cyclic nona-peptides (9 aa), capable of eliciting numerous physiological responses [lactation, blood coagulation (factor VIII)]. It also affects cognitive functions related to memory as well as to emotions love, mood, appetite, sexual behavior, social behavior. The 3-D structure of complexes of oxytocin with Cu^{+2} and Zn^{+2} and insulin have been described. For example, the groups in oxytocin, which participates in the

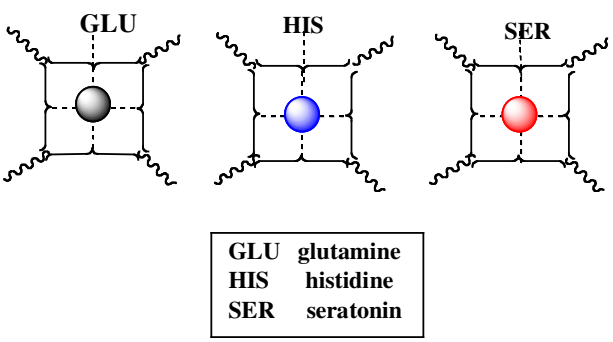


FIGURE 6 | Icons of *cuinfo* tagged with various neurotransmitters, as ternary complexes.

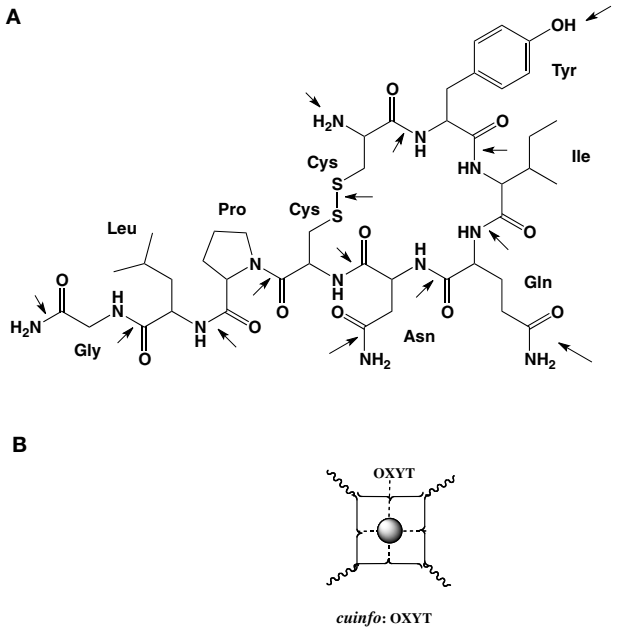


FIGURE 7 | (A) Molecular structure of oxytocin (OXYT), with 14 potential metal-complexing moieties marked with arrows. **(B)** Iconographic representation of a monodendate *cuinfo*:OXYT complex.

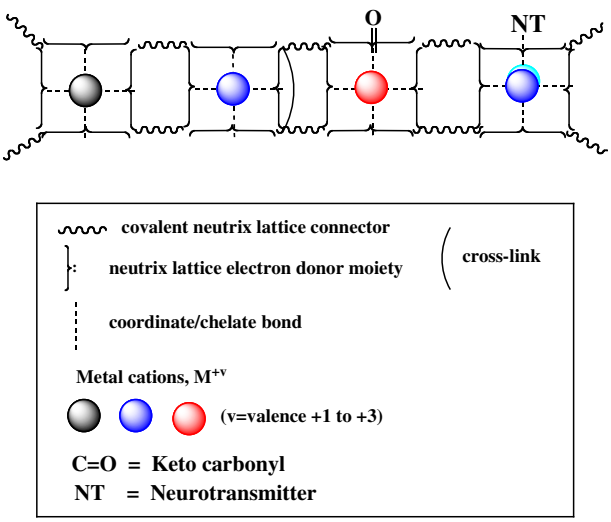


FIGURE 8 | A set of *cuinfo*, tagged with keto group and a generic neurotransmitter (NT).

formation of these complexes can also permit the formation of mixed metal complexes with *cuinfo*. The metal complexing moieties (indicated be arrows in **Figure 7**) include: the amino terminal Cys^1 group, the disulfide bond between Cys^1 and Cys^6, the phenol group of Tyr^2, the carboxamide groups of Gln^4 and Asn^5, and the terminal carboxamide group of Gly^9. In addition the peptide bonds constitute multiple metal bonding ligands.

A set of *cuinfo* might be represented as adjacent units adorned by redox or NT tags.

DISCUSSION

Memory is a mental function that permits recall of past events, to guide future behavior. One could say: "No cognition without memory." How are different memories assigned value or significance? What are the molecular-scale details? What are the molecular encoders of emotions or feeling (James, 1884; Langer, 1967)?

We point out that the NTs elicit not only physiologic effects but concommitantly elicit psychic effects described as emotions (see **Table 2**). For the purposes of discussing memory, the NTs can be considered to be the encoders of emotions. With the exception of acetylcholine and muscarine, which both express a tetra-substituted ammonium moiety and are true cations regardless of the pH, the other NTs are all electron donors, capable of forming ternary, metal-centered complexes, described as *cuinfo:NT.*

Consider the computer using binary code. Each bit is anonymous, (100111001110), exhibiting no flavor, color, value or priority, one over the other. The Turing machine computes (performs a series of discrete procedures) inexorably according to the laws of logic, mathematics and communication theory, with no emotional context (Boole, 1853; Turing, 1950) or survival import. The NTs provide the neural system with a novel encoding modality that is missing in binary codes, the emotive option for encoding cog-info, critical for providing value and significance to the memory consolidated from tagged *cuinfo:NT,* aiding survival.

The brain is first and foremost an emotive organ, mentating emotionally with combinatorially large sets of chemical "encoders" (Lehn, 2002, 2012) to ensure survival. Emotions such as fear, anger, love, etc., drive behavior, are the "coins of significance," which provide a priority value to cog-info, are strongly remembered.

We may not be able to penetrate the realm of subjective experience, but we can describe the molecular correlates and chemical dispositions of psychical processes (mentation) of which memory is an example. The molecular correlates of emotions could be considered to be encoded by NTs (**Tables 1**, **2**), relatively small molecules that are secreted into the nECM by activated neurons, as part of non-synaptic "chemical signaling" (volume transmission) (Wu et al., 2004; Delgado et al., 2006; Ortega et al., 2007; Syková and Nicholson, 2008; Adlard et al., 2010; Vizi et al., 2010; Kaler, 2011; Sadiq et al., 2012; Trueta and De-Miguel, 2012; Goyal and Chaudhury, 2013; Vizi, 2013). Vesicles containing psychoactive neurometals (Al, Ca, Co, Cu, Fe, Mg, Mn, Zn) are also released by the neuron into the nECM upon firing, combinatorially encoding cog-info as *cuinfo*, ternary (mixed) complexes capable of combing with NTs, described by the iconographic notations in **Figures 4–8**.

Emotion without memory to guide behavior would be very short-lived. . . the organism would not long survive. Emotions provide value/priority to (incoming) sensorial cog-info. Emotion and memory are functionally linked phenomena. . . providing motivational significance (value) to guide adaptive behavior. Emotions could be considered as responses that "flavor" cog-info with value, to aid recall and guide behavior.

All animals need to respond emotionally to a specific circumstance, and to remember the specific situation within the

limitations of its evolved capabilities to recall. The NTs are capable of eliciting both physiologic and psychic responses to a significant experience. Thus, they can affect behavior and also imprint *cuinfo* with emotive tags, to enable "prioritized recall," enabling survival. The above-described *tripartite* mechanism with NTs, brings emotion-laden mental sensibility into the compass of biochemical fact, applicable to all neural creatures exhibiting memory.

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