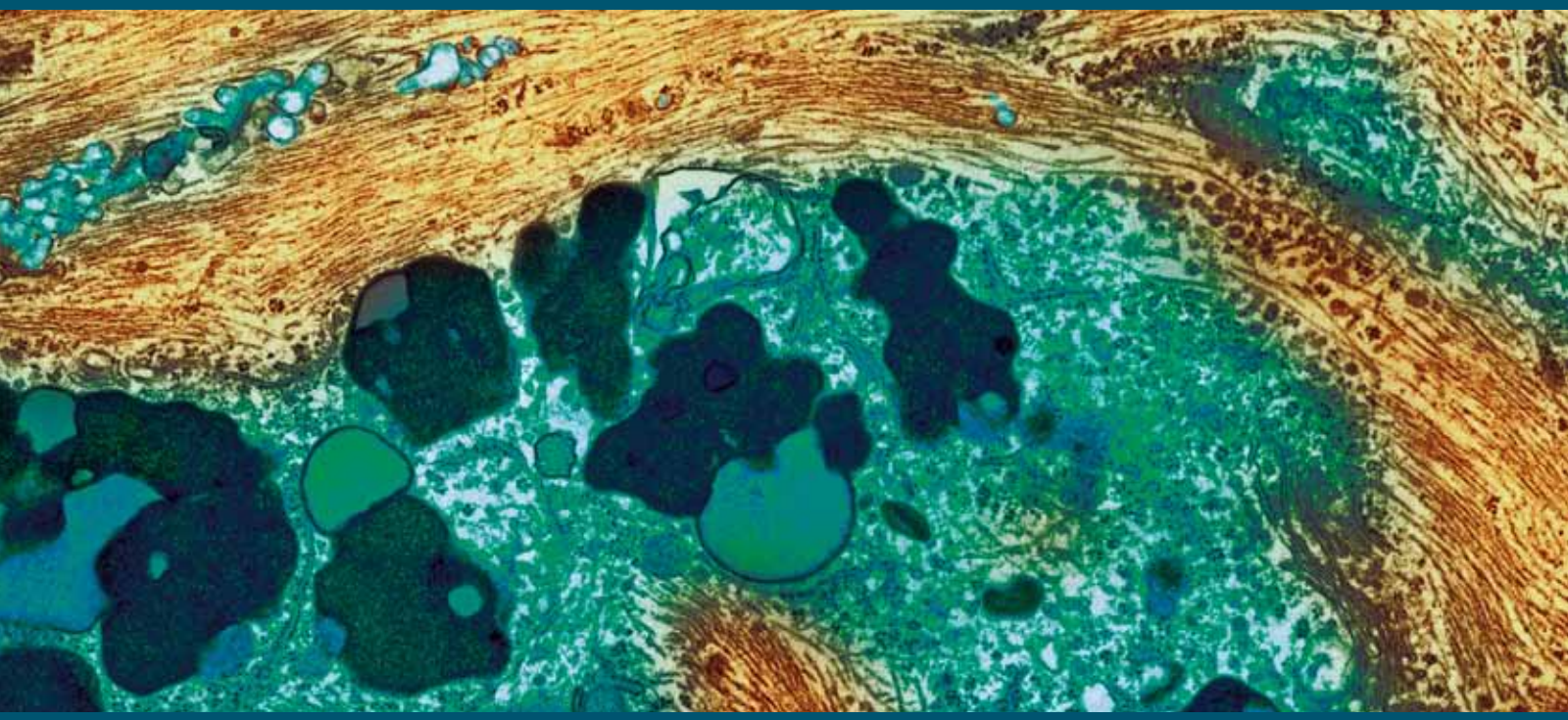
International Journal of Alzheimer's Disease

Metals and Alzheimer's Disease

Guest Editors: Anthony R. White, Peter Faller, Craig S. Atwood, and Paolo Zatta



Metals and Alzheimer's Disease

International Journal of Alzheimer's Disease

Metals and Alzheimer's Disease

Guest Editors: Anthony R. White, Peter Faller,
Craig S. Atwood, and Paolo Zatta

Copyright © 2011 SAGE-Hindawi Access to Research. All rights reserved.

This is a special issue published in volume 2011 of "International Journal of Alzheimer's Disease." All articles are open access articles distributed under the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original work is properly cited.

Editorial Board

D. Allsop, UK
Craig S. Atwood, USA
Brian Austen, UK
Jesus Avila, Spain
B. J. Bacskai, USA
Andrew Budson, USA
Roger A. Bullock, UK
A. I. Bush, USA
Gemma Casadesus, USA
Rudolph J. Castellani, USA
James R. Connor, USA
Suzanne M. de la Monte, USA
Justo G. de Yebenes, Spain
Sara M. Debanne, USA
Steven D. Edland, USA
Cheng-Xin Gong, USA
Paula Grammas, USA
George Grossberg, USA
Harald J. Hampel, Germany
K. Jellinger, Austria
Mark S. Kindy, USA
Amos D. Korczyn, Israel
Jeff Kuret, USA
Andrew J. Larner, UK
Hyoung-gon Lee, USA
Jerzy Leszek, Poland
Seth Love, UK
Michelangelo Mancuso, Italy
James G. McLarnon, Canada
P. Mecocci, Italy
Kenichi Meguro, Japan
Judith Miklossy, Canada
Paula I. Moreira, Portugal
Ricardo Nitrini, Brazil
Michal Novák, Slovakia
Leonardo Pantoni, Italy
Francesco Panza, Italy
Lucilla Parnetti, Italy
George Perry, USA
M. Cristina Polidori, Germany
John Powell, UK
Jeffrey R. Powell, USA
Marcella Reale, Italy
Vincenzo Solfrizzi, Italy
Akihiko Takashima, Japan
Matti Viitanen, Sweden
Gunhild Waldemar, Denmark
Bengt Winblad, Sweden
David Yew, Hong Kong
Henrik Zetterberg, Sweden

Contents

SAGE-Hindawi Access to Research
International Journal of Alzheimer's Disease
Volume 2011, Article ID 659424, 2 pages
doi:10.4061/2011/659424

Editorial

Metals and Alzheimer's Disease

Anthony R. White,[1] Peter Faller,[2,3] Craig S. Atwood,[4] and Paolo Zatta[5]

[1] *Department of Pathology, The University of Melbourne, Parkville, VIC 3010, Australia*
[2] *CNRS, (Laboratoire de Chimie de Coordination) LCC, 205, route de Narbonne, 31077 Toulouse, France*
[3] *LCC, INPT, UPS, Université de Toulouse, 31077 Toulouse, France*
[4] *Department of Medicine, Geriatric Research, Education and Clinical Center, Veterans Administration Hospital, University of Wisconsin, Madison, WI 53705, USA*
[5] *Metalloproteins Unit, Department of Biology, CNR-Institute for Biomedical Technologies, University of Padova, Avenue G. Colombo, 3, 35121 Padova, Italy*

Correspondence should be addressed to Anthony R. White, arwhite@unimelb.edu.au

Received 6 March 2011; Accepted 6 March 2011

Copyright © 2011 Anthony White et al. This is an open access article distributed under the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original work is properly cited.

Research into the role of metals in Alzheimer's disease (AD) has rapidly advanced over the past two decades. Early studies described controversial links between trace metals and dementia. More recently, this concept has developed further with the understanding that a range of biometals and environmental metal toxins are likely to have a central role in determining the onset, progression, and clinical outcomes of AD and other forms of neurodegeneration. Key studies were instigated in the early 1990s by Ashley Bush and colleagues, demonstrating that metals such as zinc and copper were critical in amyloid beta (Aβ) aggregation and (neuro)toxicity. Subsequent studies on metals in AD have opened up vast areas of research including the role for metals in amyloid precursor protein (AβPP) metabolism and processing, Aβ cleavage and degradation, synaptic Aβ-metal interactions, and neuroprotection associated with modulation of metal homeostasis.

In this issue we have provided a broad insight into some of these exciting areas of research, and the conclusion seems to be that a far greater level of understanding is still needed to allow development of treatments to halt the progression of AD. Tabner et al. and Kawahara and Kato-Negishi have described how interaction of Aβ with metals such as copper and iron is associated with (neuro)toxic action of small, soluble Aβ oligomers, likely mediated through generation of reactive oxygen species (ROS) and in particular, the highly reactive hydroxyl radical. In a related article, Yang et al. have provided valuable research into the importance of helical sheet formation in ROS generation associated with copper interactions. While metal interactions with Aβ are clearly critical for its (neuro)toxic processes, metals also have a key role in generation of Aβ. Ho et al. have described research demonstrating that calcium and magnesium are required for γ-secretase activity, which is the final cleavage step in Aβ generation. An important, but still undefined role for iron in AD is supported by the study described by Squitti et al. where they associated ceruloplasmin/transferrin ratios and transferrin saturation with cognitive decline and increased oxidative stress in AD. A broad review by Salvador et al. brings together an interesting overview of how neurons handle iron and how iron dysregulation may lead to neuronal degeneration in AD. Likewise, zinc has a clear and important role in AD. It is involved in many processes central to the disease, including AβPP and Aβ processing and degradation, and zinc is released into the synapse where it can modulate neurotoxic action of Aβ and glutamate neurotoxicity, two factors that are closely associated with AD (Watt et al. and Takeda).

B. Weiss takes us in a different direction, reviewing the importance of exposure to environmental metals during development and their possible association with AD later in life. Such interactions may have key modulating roles in neurodegeneration and have not been adequately investigated. Of course, the aim of understanding metals in AD, like any AD research, is primarily to form the basis of potential therapeutic treatments. In relation to this, El Ghazi et al. describe interesting interactions between metallothionein and proteins associated with transport, signaling and chaperoning, highlighting the complexity of metal interactions

with AD, both direct (Aβ-metal) and indirect, through metallothionein. Amadoruge and Barnham review the potential modulation of membrane receptor function by metals. In addition, Srinivasan et al. report on the potential use of melatonin to provide neuroprotective action early in the course of AD. Finally, the paper by Braymer et al. highlights the importance of developing novel tools to investigate the role of metals in AD and has described the generation of molecules that may target Aβ and metals simultaneously. It is hoped that the research and reviews described here will help to update the AD research community on the state of play in regards to metals in AD and offer insights into where future research should be directed.

Anthony R. White
Peter Faller
Craig S. Atwood
Paolo Zatta

SAGE-Hindawi Access to Research
International Journal of Alzheimer's Disease
Volume 2011, Article ID 546380, 6 pages
doi:10.4061/2011/546380

Review Article

Hypothesis: Soluble Aβ Oligomers in Association with Redox-Active Metal Ions Are the Optimal Generators of Reactive Oxygen Species in Alzheimer's Disease

Brian J. Tabner, Jennifer Mayes, and David Allsop

Division of Biomedical and Life Sciences, School of Health and Medicine, Lancaster University, Lancaster LA1 4YQ, UK

Correspondence should be addressed to David Allsop, d.allsop@lancaster.ac.uk

Received 12 August 2010; Accepted 8 October 2010

Academic Editor: Anthony R. White

Copyright © 2011 Brian J. Tabner et al. This is an open access article distributed under the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original work is properly cited.

Considerable evidence points to oxidative stress in the brain as an important event in the early stages of Alzheimer's disease (AD). The transition metal ions of Cu, Fe, and Zn are all enriched in the amyloid cores of senile plaques in AD. Those of Cu and Fe are redox active and bind to Aβ *in vitro*. When bound, they can facilitate the reduction of oxygen to hydrogen peroxide, and of the latter to the hydroxyl radical. This radical is very aggressive and can cause considerable oxidative damage. Recent research favours the involvement of small, soluble oligomers as the aggregating species responsible for Aβ neurotoxicity. We propose that the generation of reactive oxygen species (i.e., hydrogen peroxide and hydroxyl radicals) by these oligomers, in association with redox-active metal ions, is a key molecular mechanism underlying the pathogenesis of AD and some other neurodegenerative disorders.

1. Introduction

A large body of evidence supports an important role for oxidative stress in the pathogenesis of several different neurodegenerative diseases, including Alzheimer's disease (AD), Parkinson's disease (PD), the prion disorders, and motor neuron disease. Under normal physiological conditions, a balance would be anticipated between pro-oxidant and antioxidant levels in brain tissue. However, should there be an imbalance between the two, either as a result of excessive pro-oxidant levels or deterioration in antioxidant levels, a build up of reactive oxygen species (ROS) will occur, resulting in oxidative damage. Evidence of oxidative damage in AD and other neurodegenerative diseases has been found in a whole range of studies via a number of established markers (for recent reviews relevant to AD see, e.g., [1–4]).

Biometal ions, particularly those of Cu, Fe, and Zn, are also implicated in neurodegenerative disease, including AD (for some recent reviews see, e.g., [5–7], and references quoted therein). These ions were found to be enriched in the amyloid cores of senile plaques in AD sometime ago (see [8] for a summary) and are important for a number of reasons. Both Cu and Fe have two readily available oxidation states (i.e., Cu(II) and Cu(I) and Fe(III) and Fe(II)) which aid the transfer of electrons and are fundamental to redox chemistry. Consequently they are readily available for protein binding, can facilitate the Fenton reaction (illustrated in (2) with respect to Fe ions), and are able to transfer electrons to oxygen (to form H_2O_2). They are found as trace-level contaminants in laboratory buffers [8] and are present as impurities in synthetic Aβ [9]. It has been reported that even trace concentrations of Zn(II), Cu(II), and Fe(III) are all sufficient to enhance the Aβ(1–42) initiated seeding of Aβ(1–40) aggregation [8].

In this paper, we focus our attention on two ROS, that is, hydrogen peroxide (H_2O_2) and the hydroxyl radical ($^{\bullet}OH$). H_2O_2 is a cytotoxic oxidant which is freely permeable across cell membranes and consequently has effects that extend well beyond its site of generation. It is, therefore, likely to be responsible for global increases in oxidative damage. On the other hand, the hydroxyl radical is quite different. It is readily formed via Fenton chemistry from H_2O_2 (see (2)). It is very reactive, with a reaction rate largely controlled by its rate of diffusion and thus exerts its effects on a very local scale.

Largely because of its high reactivity, it is also very unselective in the site of attack and so will react with any biomolecule that is immediately adjacent to its site of formation. The most common reaction of $^{\bullet}$OH is hydrogen atom abstraction from the biomolecule, leaving the latter with a radical centre. The newly formed radical is then available to attack any other local biomolecule in a reaction typical of a classical chain reaction. These latter reactions will continue until such a time as termination occurs.

Until recently, Aβ amyloid fibrils themselves were believed to be an important toxic agent in AD. Support for this idea is now under question and presently there is growing interest in (a) the role of metal ions, and (b) the involvement of early stage Aβ assemblies (i.e., soluble oligomers) as the aggregating species potentially responsible for neurodegeneration in AD. A hypothesis that brings these two concepts together is the main focus of the remainder of this brief article.

2. Metal Ions: The Direct Generation of ROS and Aβ Oxidation

We now focus on the fascinating possibility that Aβ (and possibly several other amyloidogenic proteins) might have the ability to self-generate two important ROS, that is, H_2O_2 and $^{\bullet}$OH, during aggregation *in vitro*. In 1994, Behl et al. [10] reported results suggesting that Aβ could cause increased levels of H_2O_2 to accumulate in cells during incubation over an extended period although the source of the peroxide was not investigated. However, their results clearly indicate that, at least in the case of Aβ(25–35), H_2O_2 levels go through a maximum before subsequently declining. We believe this latter finding to be significant and this aspect is discussed further below.

Two key publications by Huang et al. followed in 1999 [11, 12]. These authors highlighted the direct generation of H_2O_2 and $^{\bullet}$OH during the incubation of solutions of both Aβ(1–40) and Aβ(1–42). They were able to establish that Aβ binds to Cu, Fe, and Zn ions and that the bound peptide can reduce Cu(II) to Cu(I) and Fe(III) to Fe(II). The binding of these metal ions is most significant since they are elevated in the amyloid deposits found in individuals with AD [13, 14]. Huang and colleagues were further able to show that the bound transition metal ions Cu(I) and Fe(II) were able to reduce molecular oxygen to H_2O_2 thus establishing the direct generation of the latter ROS during peptide incubation. Finally, and of prime importance, is that their results established that, when bound, both the Fe and Cu ions are redox active (note that Zn ions are not redox-active). This redox-active nature of the metal ion when bound to the peptide enables the ready formation of $^{\bullet}$OH via the well-known Fenton reaction. This latter observation has important implications, as discussed in more detail below. The essential reactions are

$$O_2 + 2\,Cu(I) + 2H^+ \longrightarrow H_2O_2 + 2\,Cu(II) \quad (1)$$

$$Fe(II) + H_2O_2 \longrightarrow Fe(III) + {}^{\bullet}OH + {}^{-}OH \quad (2)$$

These two reactions have important consequences. The reduction of oxygen to form H_2O_2 requires two electrons, whilst the formation of $^{\bullet}$OH from H_2O_2 requires one electron. It is clear that, in order for (1) and (2) to proceed, the bound metal ion needs to be in its lower oxidation state (i.e., either Cu(I) or Fe(II)). If the metal ions are in their higher oxidation state (i.e., Cu(II) or Fe(III)) the necessary conversion to their lower oxidation state requires an electron source. In the absence of any other electron donor it is possible that, (*in vitro*), the peptide itself could act as an electron donor resulting in the formation of the peptide radical cation:

$$A\beta/Cu(II) \longrightarrow (A\beta)^{\bullet+}/Cu(I) \quad (3)$$

The Aβ radical cation, thus formed, could then undergo a variety of rapid rearrangement reactions resulting in the formation of oxidation products consistent with those commonly observed [1–4]. This process could continue "long term" providing the bound metal ion: peptide complex remains redox active; that is, there is an interchange between the two oxidation states of the appropriate metal ion. However, these reactions could be limited by an inadequate supply of electrons and it has been questioned if the bound peptide has the ability to act as an electron donor to Cu(II) [15].

These results prompted us to carry out our own investigations, in which only impurity levels of Cu and Fe were present in samples of Aβ [9, 16]. Purified synthetic Aβ has significant metal ion content (estimated in our samples as 5.7×10^{-4} and 1.2×10^{-3} moles of Fe and Cu, resp., per mole of Aβ(1–40)) and these levels would be augmented by additional metal ions present in the buffers used [8, 9]. The experimental technique for H_2O_2 detection was quite different to that employed by Huang et al. and was based on electron spin resonance (ESR) spectroscopy. Here, H_2O_2 is converted to $^{\bullet}$OH via Fenton chemistry and the latter is detected by employing a well-established spin-trapping agent [9, 16]. Thus we were able to show, with many measurements undertaken over an extended incubation period, that Aβ(1–40), Aβ(1–42), and Aβ(25–35) can all self-generate H_2O_2 during incubation in solution. On the other hand, related nontoxic peptides, such as the reverse peptide Aβ(40–1) were inactive [9, 16]. Some typical ESR spectra obtained during the incubation of different Aβ peptides are shown in Figure 1.

At about the same time, a further publication from Opazo et al. [17] indicated that Huang's earlier data were flawed in the sense that a reagent required in their detection technique, tris(2-carboxyethyl)phosphine hydrochloride (TCEP), (which was present during incubation) was found to act, in its capacity as nonbiological reducing agent, to significantly enhance the concentration of H_2O_2 generated. Also, when several biological reducing agents such as vitamin C, dopamine (DA), and L-3,4-dihydroxyphenylalanine (L-DOPA) were added (separately) they were found to also considerably enhance H_2O_2 levels, although, except in the case of DA, measurements were taken after only a single time point. These latter observations are consistent with other research, covering several different

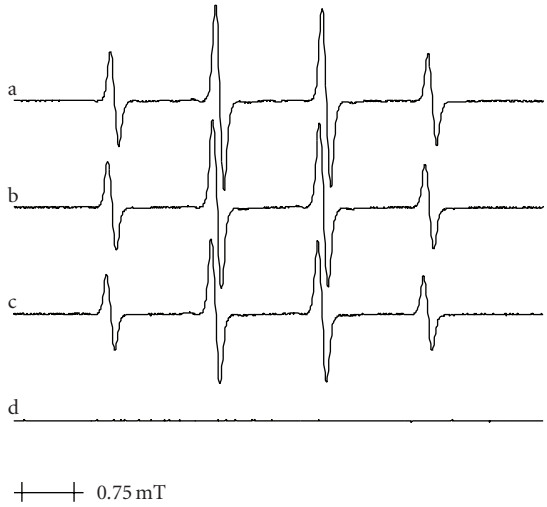


Figure 1: ESR spectra of the •OH adduct of 5,5-dimethyl-1-pyrroline *N*-oxide (DMPO) recorded after 48 h incubation of (a) Aβ(1–40), (b) Aβ(1–42), (c) Aβ(25–35) (intensity ×1.5) and (d) Aβ(40–1) at 37°C and all at 100 μM. The intensity of the spectra observed in (a), (b), and (c) varied with incubation time as described in the text whereas in (d) no DMPO adduct spectrum was observed after any period of incubation. Control samples gave straight line spectra, such as that illustrated in (d), at every time point examined. The experimental methodology, reaction conditions, and ESR spectrometer settings are fully detailed elsewhere [9, 16].

biological reducing agents [18–20]. The ability of these biological reducing agents to act as an electron source for the reduction in the oxidation state of the bound metal ions reduces the necessity of the peptide itself to do so (3) and, consequently, enhances the long-term formation of ROS, especially the •OH radical, so increasing the potential for oxidative damage.

It is apparent, however, that the presence of biological (and other) reducing agents is not the only factor influencing the amount of H_2O_2 generated as the metal ion : peptide ratio is also significant [21]. For example, H_2O_2 production is very much enhanced when the Cu(II) : peptide ratio in Aβ(1–40) is increased from 0 : 1 to 1 : 1, and it is now apparent that great care must be taken in assigning enhanced generation of $H_2O_2$when both the reducing agent : Aβ and metal ion : Aβ ratios are not examined separately. Also measurements over an extended time period covering many time points are required as these give information on H_2O_2 levels as aggregation progresses.

We mentioned above that particular importance should be attached to H_2O_2 generated via the bound peptide. Not only is there a possibility of a direct reaction between H_2O_2 and the bound peptide (and any other accessible biomolecules) but also the conversion of H_2O_2 to •OH via electron transfer from the bound metal ion is significant as this would occur in the immediate vicinity of the peptide. As outlined above •OH is extremely aggressive and will react with adjacent molecules, including Aβ itself, with a reaction rate virtually controlled by diffusion. A common reaction is hydrogen atom abstraction, with the resulting peptidyl radical undergoing subsequent internal transformation reactions, resulting in oxidation products. If these reactions do not terminate the radical centre then these peptidyl radicals could themselves attack other peptide sites (again via hydrogen atom abstraction) leading to further oxidative damage until the radical centre is eventually terminated.

As noted above, there is now evidence that, *in vivo*, ROS could be involved in the pathogenesis of AD (and other neurodegenerative diseases) particularly under conditions where antioxidant defences have deteriorated to the point where they are unable to cope with the general ROS burden, possibly as part of the normal ageing process. Under these conditions not only could H_2O_2 undergo direct reaction with any biomolecules encountered but, when arriving at any appropriate site, such as a redox-active metal bound Aβ, conversion to •OH in the immediate vicinity of the peptide would lead to the aggressive reactions described immediately above.

3. Hypothesis: Soluble Oligomers Are the Optimal Generators of ROS

A key finding is that oxidative damage has been established to occur as an early event in AD [22, 23] indicating that ROS activity would be expected to be present during an early stage of the Aβ aggregation process. Soluble oligomers are likely to be the major type of Aβ aggregate present during this phase [24] and so these species become the prime candidate for involvement in early ROS generation.

Our own results, obtained during the early aggregation of Aβ(1–40), agree with the above inference [25]. They show that, *in vitro*, H_2O_2 is generated, during Aβ aggregation, as a "pulse" during the same time period that atomic force and electron microscopy images confirm the presence of small oligomers. Our results reveal a time delay before any H_2O_2 is observed, followed by a reasonably rapid increase in levels which reach a maximum before slowly declining back towards zero. The latter stage indicates that the rate of H_2O_2 removal (either through reacting directly with the peptide or via Fenton chemistry to form •OH) exceeds the rate of formation.

These observations raise a number of questions. In particular, what form of Aβ is optimal for the generation of ROS and why does the ability to generate H_2O_2 (and the associated toxicity) appear to decrease as the size of the Aβ aggregate increases beyond the oligomer stage?

Small soluble oligomers (such as dimers and trimers) will have a greater mobility than larger aggregates and so will be able to diffuse from their site of origin and insert into surrounding cell membranes, causing local damage, including reported changes in membrane permeability [27, 28]. The ability to generate ROS could also be a particular property of these small oligomers. A monomer bound to a single metal ion may not be particularly active in the formation of H_2O_2 if this reaction requires the synchronized transfer of two electrons [29]. Rapid diffusion would prevent the close

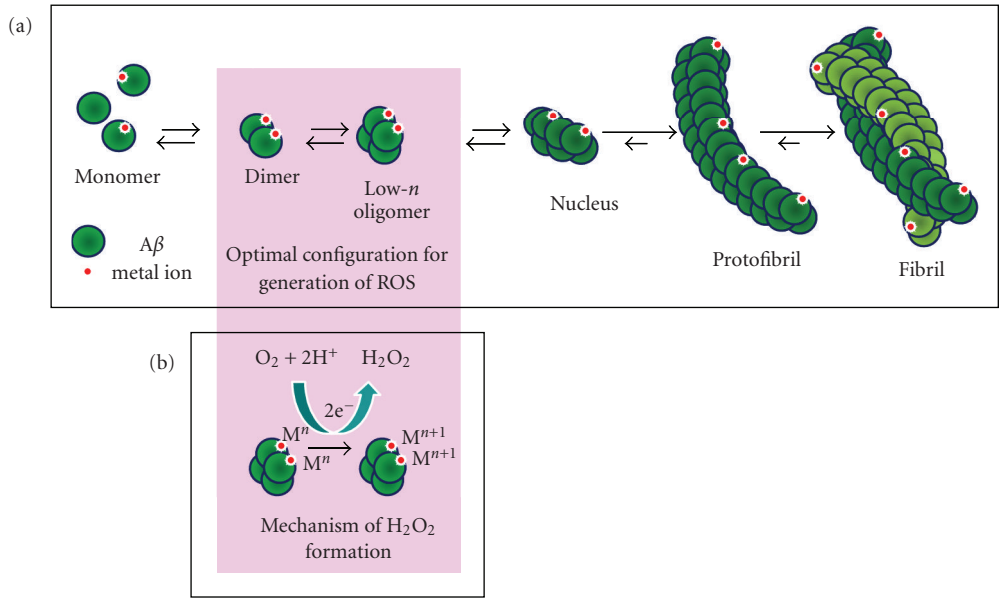


FIGURE 2: Soluble oligomers, together with their associated redox-active metal ions, are potentially responsible for the generation of H_2O_2. (a) Stages of Aβ aggregation, assuming that soluble oligomers are "on pathway" to fibril formation. The optimal configuration for generation of ROS may be at the small (low-n) oligomer stage. (b) Proposed mechanism for the generation of hydrogen peroxide. Here, M^n and M^{n+1} represent Cu(I) and Cu(II), or Fe(II) and Fe(III). The generation of H_2O_2 requires the synchronized transfer of two electrons from the bound metal ions and so reaction with the monomer is not favoured. Recent experimental evidence suggests that Cu ions remain bound to Aβ even in mature amyloid fibrils [26] and so the lack of activity of higher-order aggregates [25] is possibly due to some metal ions becoming sterically removed from any available O_2 molecules.

proximity of two such monomers to oxygen, thus impeding this reaction. Because the generation of H_2O_2 requires two electrons, the formation of early-stage oligomers would be expected to create a more favourable steric arrangement for this process to occur. One possible reason why higher-order aggregates, including mature amyloid fibrils, appear to lose the ability to generate H_2O_2 *in vitro* [25] could be that the supply of electrons becomes exhausted, although it is likely that available reducing agents would be plentiful *in vivo*. Another possible reason is that some of the metal ions associated with Aβ fibrils could lose their redox activity. The latter could be the result of a steric problem as the aggregate reaches its solubility limit (and beyond). Here, some metal ions could become sterically removed (i.e., hidden) from the O_2 molecule, with H_2O_2 generation becoming consequently more difficult. Under these circumstances, it is quite reasonable to suggest that the most suitable configuration for generating ROS lies within a range of optimum oligomer sizes. The latter could also explain the "time delay" observed in our aggregation experiments before levels rise, reach a maximum, and then decay [25]. These arguments lead us to hypothesize that ROS generation is greatest during the period when small (low-n) oligomers are the major species present. The many similarities between the properties of the amyloidogenic proteins/peptides implicated in other neurodegenerative diseases suggests that this type of mechanism could also apply to other disorders such as PD, the prion diseases and familial British dementia [9, 16, 25, 30–32].

Finally, it is becoming increasingly recognised that H_2O_2 acts as a diffusible signaling molecule in the CNS and is involved in the molecular mechanisms underlying synaptic plasticity and memory formation [33, 34]. Thus, the generation of ROS by soluble oligomers of Aβ could also, potentially, explain their potent effects on long-term potentiation (LTP), memory, and learning [35, 36].

Acknowledgment

The authors thank the Alzheimer's Society, UK, for a studentship to J. Mayes.

References

[1] R. Sultana, M. Perluigi, and D. A. Butterfield, "Oxidatively modified proteins in Alzheimer's disease (AD), mild cognitive impairment and animal models of AD: role of Abeta in pathogenesis," *Acta Neuropathologica*, vol. 118, no. 1, pp. 131–150, 2009.

[2] V. P. Reddy, X. Zhu, G. Perry, and M. A. Smith, "Oxidative stress in diabetes and Alzheimer's disease," *Journal of Alzheimer's Disease*, vol. 16, no. 4, pp. 763–774, 2009.

[3] D. Galasko and T. J. Montine, "Biomarkers of oxidative damage and inflammation in Alzheimers disease," *Biomarkers in Medicine*, vol. 4, no. 1, pp. 27–36, 2010.

[4] K. Gustaw-Rothenberg, A. Lerner, D. J. Bonda et al., "Biomarkers in Alzheimers disease: past, present and future," *Biomarkers in Medicine*, vol. 4, no. 1, pp. 15–26, 2010.

[5] Y. H. Hung, A. I. Bush, and R. A. Cherny, "Copper in the brain and Alzheimer's disease," *Journal of Biological Inorganic Chemistry*, vol. 15, no. 1, pp. 61–76, 2010.

[6] A. Rauk, "Why is the amyloid beta peptide of Alzheimer's disease neurotoxic?" *Dalton Transactions*, no. 10, pp. 1273–1282, 2008.

[7] A. Rauk, "The chemistry of Alzheimer's disease," *Chemical Society Reviews*, vol. 38, no. 9, pp. 2698–2715, 2009.

[8] X. Huang, C. S. Atwood, R. D. Moir, M. A. Hartshorn, R. E. Tanzi, and A. I. Bush, "Trace metal contamination initiates the apparent auto-aggregation, amyloidosis, and oligomerization of Alzheimer's Aβ peptides," *Journal of Biological Inorganic Chemistry*, vol. 9, no. 8, pp. 954–960, 2004.

[9] S. Turnbull, B. J. Tabner, O. M. A. El-Agnaf, S. Moore, Y. Davies, and D. Allsop, "α-synuclein implicated in Parkinson's disease catalyses the formation of hydrogen peroxide *in vitro*," *Free Radical Biology and Medicine*, vol. 30, no. 10, pp. 1163–1170, 2001.

[10] C. Behl, J. B. Davis, R. Lesley, and D. Schubert, "Hydrogen peroxide mediates amyloid β protein toxicity," *Cell*, vol. 77, no. 6, pp. 817–827, 1994.

[11] X. Huang, C. S. Atwood, M. A. Hartshorn et al., "The Aβ peptide of Alzheimer's disease directly produces hydrogen peroxide through metal ion reduction," *Biochemistry*, vol. 38, no. 24, pp. 7609–7616, 1999.

[12] X. Huang, M. P. Cuajungco, C. S. Atwood et al., "Cu(II) potentiation of Alzheimer aβ neurotoxicity. Correlation with cell-free hydrogen peroxide production and metal reduction," *Journal of Biological Chemistry*, vol. 274, no. 52, pp. 37111–37116, 1999.

[13] M. A. Lovell, J. D. Robertson, W. J. Teesdale, J. L. Campbell, and W. R. Markesbery, "Copper, iron and zinc in Alzheimer's disease senile plaques," *Journal of the Neurological Sciences*, vol. 158, no. 1, pp. 47–52, 1998.

[14] M. A. Smith, P. L. R. Harris, L. M. Sayre, and G. Perry, "Iron accumulation in Alzheimer disease is a source of redox-generated free radicals," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 94, no. 18, pp. 9866–9868, 1997.

[15] D. Jiang, L. Men, J. Wang et al., "Redox reactions of copper complexes formed with different β-amyloid peptides and their neuropathalogical relevance," *Biochemistry*, vol. 46, no. 32, pp. 9270–9282, 2007.

[16] B. J. Tabner, S. Turnbull, O. M. A. El-Agnaf, and D. Allsop, "Formation of hydrogen peroxide and hydroxyl radicals from Aβ and α-synuclein as a possible mechanism of cell death in Alzheimer's disease and Parkinson's disease," *Free Radical Biology and Medicine*, vol. 32, no. 11, pp. 1076–1083, 2002.

[17] C. Opazo, X. Huang, R. A. Cherny et al., "Metalloenzyme-like activity of Alzheimer's disease β-amyloid: Cu-dependent catalytic conversion of dopamine, cholesterol, and biological reducing agents to neurotoxic H_2O_2," *Journal of Biological Chemistry*, vol. 277, no. 43, pp. 40302–40308, 2002.

[18] K. J. Barnham, F. Haeffner, G. D. Ciccotosto et al., "Tyrosine gated electron transfer is key to the toxic mechanism of Alzheimer's disease β-amyloid," *FASEB Journal*, vol. 18, no. 12, pp. 1427–1429, 2004.

[19] T. J. Nelson and D. L. Alkon, "Oxidation of cholesterol by amyloid precursor protein and β-amyloid peptide," *Journal of Biological Chemistry*, vol. 280, no. 8, pp. 7377–7387, 2005.

[20] R. C. Nadal, S. E. J. Rigby, and J. H. Viles, "Amyloid β-Cu2+ complexes in both monomeric and fibrillar forms do not generate H_2O_2 catalytically but quench hydroxyl radicals," *Biochemistry*, vol. 47, no. 44, pp. 11653–11664, 2008.

[21] X. Dai, Y. Sun, Z. Gao, and Z. Jiang, "Copper enhances amyloid-β peptide neurotoxicity and non β-aggregation: a series of experiments conducted upon copper-bound and copper-free amyloid-β peptide," *Journal of Molecular Neuroscience*, vol. 41, no. 1, pp. 66–73, 2010.

[22] A. Nunomura, G. Perry, G. Aliev et al., "Oxidative damage is the earliest event in Alzheimer disease," *Journal of Neuropathology and Experimental Neurology*, vol. 60, no. 8, pp. 759–767, 2001.

[23] W. R. Markesbery, R. J. Kryscio, M. A. Lovell, and J. D. Morrow, "Lipid peroxidation is an early event in the brain in amnestic mild cognitive impairment," *Annals of Neurology*, vol. 58, no. 5, pp. 730–735, 2005.

[24] H. Fukumoto, T. Tokuda, T. Kasai et al., "High-molecular-weight β-amyloid oligomers are elevated in cerebrospinal fluid of Alzheimer patients," *FASEB Journal*, vol. 24, no. 8, pp. 2716–2726, 2010.

[25] B. J. Tabner, O. M. A. El-Agnaf, S. Turnbull et al., "Hydrogen peroxide is generated during the very early stages of aggregation of the amyloid peptides implicated in Alzheimer disease and familial British dementia," *Journal of Biological Chemistry*, vol. 280, no. 43, pp. 35789–35792, 2005.

[26] J. W. Karr and V. A. Szalai, "Cu(II) binding to monomeric, oligomeric, and fibrillar forms of the Alzheimer's disease amyloid-β peptide," *Biochemistry*, vol. 47, no. 17, pp. 5006–5016, 2008.

[27] R. Kayed, Y. Sokolov, B. Edmonds et al., "Permeabilization of lipid bilayers is a common conformation-dependent activity of soluble amyloid oligomers in protein misfolding diseases," *Journal of Biological Chemistry*, vol. 279, no. 45, pp. 46363–46366, 2004.

[28] A. Demuro, E. Mina, R. Kayed, S. C. Milton, I. Parker, and C. G. Glabe, "Calcium dysregulation and membrane disruption as a ubiquitous neurotoxic mechanism of soluble amyloid oligomers," *Journal of Biological Chemistry*, vol. 280, no. 17, pp. 17294–17300, 2005.

[29] A. I. Bush and R. E. Tanzi, "Therapeutics for Alzheimer's disease based on the metal hypothesis," *Neurotherapeutics*, vol. 5, no. 3, pp. 421–432, 2008.

[30] S. Turnbull, B. J. Tabner, D. R. Brown, and D. Allsop, "Copper-dependent generation of hydrogen peroxide from the toxic prion protein fragment PrP106–126," *Neuroscience Letters*, vol. 336, no. 3, pp. 159–162, 2003.

[31] S. Turnbull, B. J. Tabner, D. R. Brown, and D. Allsop, "Generation of hydrogen peroxide from mutant forms of the prion protein fragment PrP121–231," *Biochemistry*, vol. 42, no. 25, pp. 7675–7681, 2003.

[32] D. Allsop, J. Mayes, S. Moore, A. Masad, and B. J. Tabner, "Metal-dependent generation of reactive oxygen species from amyloid proteins implicated in neurodegenerative disease," *Biochemical Society Transactions*, vol. 36, no. 6, pp. 1293–1298, 2008.

[33] L. T. Knapp and E. Klann, "Role of reactive oxygen species in hippocampal long-term potentiation: contributory or inhibitory?" *Journal of Neuroscience Research*, vol. 70, no. 1, pp. 1–7, 2002.

[34] K. T. Kishida and E. Klann, "Sources and targets of reactive oxygen species in synaptic plasticity and memory," *Antioxidants and Redox Signaling*, vol. 9, no. 2, pp. 233–244, 2007.

[35] J. P. Cleary, D. M. Walsh, J. J. Hofmeister et al., "Natural oligomers of the amyloid-β protein specifically disrupt cognitive function," *Nature Neuroscience*, vol. 8, no. 1, pp. 79–84, 2005.

[36] G. M. Shankar, S. Li, T. H. Mehta et al., "Amyloid-β protein dimers isolated directly from Alzheimer's brains impair synaptic plasticity and memory," *Nature Medicine*, vol. 14, no. 8, pp. 837–842, 2008.

SAGE-Hindawi Access to Research
International Journal of Alzheimer's Disease
Volume 2011, Article ID 276393, 17 pages
doi:10.4061/2011/276393

Review Article

Link between Aluminum and the Pathogenesis of Alzheimer's Disease: The Integration of the Aluminum and Amyloid Cascade Hypotheses

Masahiro Kawahara[1] and Midori Kato-Negishi[2]

[1] *Department of Analytical Chemistry, School of Pharmaceutical Sciences, Kyushu University of Health and Welfare, 1714-1 Yoshino-cho, Nobeoka-shi, Miyazaki 882-8508, Japan*
[2] *Institute of Industrial Science (IIS), The University of Tokyo, 4-6-1 Komaba, Meguro-ku, Tokyo 153-8904, Japan*

Correspondence should be addressed to Masahiro Kawahara, kawamasa@phoenix.ac.jp

Received 15 September 2010; Revised 29 November 2010; Accepted 5 January 2011

Academic Editor: Paolo Zatta

Copyright © 2011 M. Kawahara and M. Kato-Negishi. This is an open access article distributed under the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original work is properly cited.

Whilst being environmentally abundant, aluminum is not essential for life. On the contrary, aluminum is a widely recognized neurotoxin that inhibits more than 200 biologically important functions and causes various adverse effects in plants, animals, and humans. The relationship between aluminum exposure and neurodegenerative diseases, including dialysis encephalopathy, amyotrophic lateral sclerosis and Parkinsonism dementia in the Kii Peninsula and Guam, and Alzheimer's disease (AD) has been suggested. In particular, the link between aluminum and Alzheimer's disease has been the subject of scientific debate for several decades. However, the complex characteristics of aluminum bioavailability make it difficult to evaluate its toxicity and therefore, the relationship remains to be established. Mounting evidence has suggested that significance of oligomerization of β-amyloid protein and neurotoxicity in the molecular mechanism of AD pathogenesis. Aluminum may play crucial roles as a cross-linker in β-amyloid oligomerization. Here, we review the detailed characteristics of aluminum neurotoxicity based on our own studies and the recent literatures. Our aim is to revisit the link between aluminum and AD and to integrate aluminum and amyloid cascade hypotheses in the context of β-amyloid oligomerization and the interactions with other metals.

1. Introduction

Aluminum (Al) is abundantly distributed in our environment, and compounds containing Al have been used in manufacturing (e.g., clays, glasses, and alum) for centuries. Despite its abundance, Al was first isolated as an element in 1827, and its use as being a silvery metal began only after 1886. Al is a new metal in this context. Because of its beneficial characteristics such as a lightweight, nonmagnetic, malleable, and ductile element, Al has a widespread and important use in industrial applications and consumer products. Al is also used in cooking utensils and in pharmacological agents including antacids and antiperspirants from which the element enters the human body.

Al is not essential for life. On the contrary, Al is a well established neurotoxin and is suspected to be linked with various neurodegenerative diseases including Alzheimer's disease (AD), amyotrophic lateral sclerosis (ALS), and Parkinsonism dementia in the Kii Peninsula and Guam [1], and the Gulf War syndrome [2].

In particular, a possible relationship between Al and the pathogenesis of AD has been discussed for several decades [3–7]. AD is a severe senile type of dementia first reported in 1906. The pathological hallmarks of AD are the deposition of extracellular senile plaques, intracellular neurofibrillary tangles (NFTs), and the selective loss of synapses and neurons in the hippocampal and cerebral cortical regions. The major component of NFTs is the phosphorylated tau protein. Senile plaques are largely comprised of β-amyloid protein (AβP) [8]. The hypothesis that Al is an environmental contributor to the pathogenesis of AD, termed the "aluminum hypothesis", was proposed in the 1960s based on

various neurotoxicological, analytical, and epidemiological findings [9–11]. In spite of these findings, the aluminum hypothesis has been the subject of much debate and criticism for several decades. During this period, great progress was made in AD research. Particularly, numerous studies have supported the idea termed "amyloid cascade hypothesis", namely that the conformational changes of AβP and its neurotoxicity play a central role in AD pathogenesis [12, 13]. Al^{3+} and other metals including Zn^{2+}, Cu^{2+}, and Fe^{3+} influence the oligomerization and conformational changes of AβP as cross-linkers, and, therefore, their implications are important in this context. Furthermore, increasing evidence suggests the implication of these metals in the pathogenesis of AD [14–16]. Al binds to various metal-binding proteins and influences homeostasis of other metals.

We review here the detailed characteristics of aluminum neurotoxicity based on our own studies and the recent literature. Our aim is to update the various adverse effects of Al and revisit the link between Al and AD based on new findings on Al-induced conformational changes and metal-metal interactions.

2. Neurotoxicity of Aluminum Update

2.1. Effects of Al on the Memory Disorder of Human: Historical Overview. An association between Al poisoning and memory disorder in humans was first reported in 1921 [17]. Later, it was shown that the intracerebral administration of Al induced epilepsy in experimental animals [18]. As a component of dialysis solutions or Al-containing pharmacological compounds, Al is known to cause various dialysis-related disorders, including osteomalacia (aluminum bone disease), microcytic anemia, β_2-microglobulin-associated amyloidosis [19], and dialysis encephalopathy in hemodialysis patients [20].

The accidental contamination of Al into drinking water occurred and more than 20,000 persons were exposed to high level of Al at 1988 in Camelford (Cornwall, UK). Residents exposed to contaminated Al exhibited various symptoms related to cerebral impairments such as loss of concentration and short term memory in a 10-year follow-up study [21].

Martyn et al. reported a high incidence of AD in areas with a high level of Al in the drinking water in England and Wales [11]. A considerable number of studies have provided evidence to support an association between AD and Al in drinking water after this initial report [22]. Frecker reported on a Norwegian area where high Al concentrations in drinking water were linked with high dementia mortality [23]. Neri and Hewitt found a positive relationship between Al in drinking water and AD risk in Canada [24]. Forbes and McLachlan demonstrated a greater risk of AD in Canadian areas where concentrations of Al are high and those of fluoride are low [25]. Rondeau et al. demonstrated that high daily intake of Al was correlated with increased risk of dementia or cognitive decline in a 15-year follow-up French cohort study [26–28]. These studies suggest that Al has adverse effects on human memories and causes dementia when it enters the brain.

2.2. Effects of Al on the Central Nervous System In Vitro or In Vivo. Despite it's environmental abundance, Al is not an essential element for living organisms, and no enzymatic reaction requires Al. Al is reported to influence more than 200 biologically important reactions and to cause various adverse effects on the mammalian central nervous system (CNS) (Table 1). These include crucial reactions for brain development such as the axonal transport, neurotransmitter synthesis, synaptic transmission, phosphorylation or dephosphorylation of proteins, protein degradation, gene expression, and inflammatory responses.

Al exhibits only one oxidation state, Al^{3+}. Al^{3+} has affinity for negatively charged, oxygen-donor ligands. Inorganic and organic phosphates, carboxylate, and deprotonated hydroxyl groups form strong bonds with Al^{3+}. Owing to these chemical characteristics, Al^{3+} binds to the phosphate groups of DNA and RNA, affecting DNA topology and influencing the expression of various genes essential for brain functions. Lukiw et al. reported that nanomolar levels of Al^{3+} were sufficient to influence neuronal gene expression [33, 35].

Al^{3+} also binds to the phosphate groups of nucleoside di- and triphosphates, such as ATP and can thus influence energy metabolism. Furthermore, Al inhibits the functions of various protein kinases and phosphatases.

Al^{3+} has very low ligand-exchange rate in comparison to other metals. For example, the ligand-exchange rate of Mg^{2+} is 10^5 times faster than that of Al^{3+}, and therefore, Al^{3+} inhibits enzymes with Mg^{2+} cofactors. Al^{3+} also inhibits biological processes involving rapid Ca^{2+} exchange: the exchange rate for Al^{3+} is 10^8 times slower than that of Ca^{2+}. These properties make Al useless in enzymatic reactions and increase its half-life in the human body. We show the typical effects of Al in Figure 1.

Al^{3+} has strong positive charges and a relatively small ionic radius in comparison to other metal ions such as Ca^{2+}, Zn^{2+}, and Na^{+} (Figure 2). Thus, Al^{3+} firmly binds to metal-binding amino acids (histidine (His), tyrosine (Tyr), arginine (Arg) etc.) or phosphorylated amino acids and acts as a cross-linker; this property has made it useful as a leather tanning agent. By binding to various proteins, Al can cause the oligomerization of proteins, inducing conformational changes that can inhibit their degradation by proteases. Strong binding of Al^{3+} to phosphorylated amino acids promotes the self-aggregation and accumulation of highly phosphorylated cytoskeleton proteins, including neurofilament and microtubule-associated proteins (MAPs), and so forth [58].

Consequently, Al causes apoptotic death of neurons and glial cells. Chronic administration of Al impairs long-term potentiation (LTP), which is a form of synaptic information storage well-known as a paradigm of memory mechanisms. Al also impairs various enzymes including those related to neurotransmitter synthesis and thus affects the neurotransmitter content. Al^{3+} also inhibits voltage-gated Ca^{2+} channels and neurotransmitter receptors, and impairs synaptic transmission. Finally, Al causes spatial memory deficit, influences emotional reactivity, and impairs various brain functions related to learning and memory.

Table 1: Effects of aluminum on the central nervous system.

	References
(1) Nucleus and gene expression	
Binding to DNA	
Binds to histone-DNA complex and induces conformational changes of chromatin.	[29]
Induces topological changes of DNA.	[30, 31]
Altered gene expression	
Induces decreased expression of neurofilament and tubulin.	[32]
Induces altered expression of genes of neurofilament, APP, and neuron specific enolase.	[33]
Induces decreased expression of transferrin receptor.	[34]
Induces altered expression of RNA polymerase I.	[35]
Induces downregulation of mitochondrial cytochrome c oxidase.	[36]
Induces altered expression of calbindin-D28k.	[37]
Induces decrease in the expression of nerve growth factor (NGF) and brain derived neurotrophic factor (BDNF).	[38]
Induces expression of pro-inflammatory genes and pro-apoptotic genes.	[39]
Induces elevated expression of APP.	[40, 41]
Induces altered expression of oxidative stress marker genes (SOD1, glutathione reductase, etc.).	[42]
Induces decreased expression of neprilysin.	[43]
Induces altered expression of β-APP secretase (BACE1 and BACE2).	[40, 44]
(2) Cellular functions	
Energy metabolism	
Inhibits the activity of hexokinase	[45]
Inhibits the activity of phosphofructokinase	[46]
Inhibits the activity of glucose-6-phosphate dehydrogenase	[47]
Causes mitochondrial dysfunction and depletion of ATP	[48, 49]
Decreases in activity and expression of TCA-cycle related enzymes (succinate dehydrogenase (SDH), alpha-ketoglutarate dehydrogenase (KGDH), isocitrate dehydrogenase-NAD^+ (IDH), fumarase (FUM), aconitase (ACN), and cytochrome c oxidase (Cyt C Ox)).	[50]
Phosphorylation and dephosphorylation	
Inhibits the activity of protein phosphatase.	[51]
Increases the activity of protein kinase C and cytoskeleton proteins.	[52]
Accelerates phosphorylation and accumulation of neurofilament.	[53]
Enhances Ca^{2+}/Calmodulin dependent protein kinase activity.	[54]
Accelerates phosphorylation of MAP 2 and neurofilament.	[55]
Inhibits dephosphorylation of tau.	[56]
Induces nonenzymatic phosphorylation of tau.	[57]
Abnormal accumulation of proteins	
Causes the conformational change and the accumulation of neurofilament and MAP1A, MAP1B.	[58]
Accelerates the phosphorylation of tau and its accumulation.	[59]
Causes the accumulation of tau protein in neuroblastoma cells or in primary cultured neurons.	[60, 61]
Causes the accumulation of tau protein in experimental animals.	[33, 62, 63]
Causes neurofibrillary degeneration *in vivo*.	[9]
Causes the accumulation of AβP in cultured neurons or in neuroblastoma cells.	[64, 65]
Causes the accumulation of AβP *in vivo*.	[44, 66, 67]
Neurotransmitter release	
Inhibits glutamate release.	[68]
Impairs synaptic transmission.	[69, 70]
Inactivates glutamate dehydrogenase.	[71]
Inhibits NMDA-type glutamate receptor.	[72]
Inhibits choline acetyl transferase and tyrosine hydroxylase, glutamate decarboxylase.	[73, 74]

Table 1: Continued.

	References
Influences acetyl-CoA and inhibits acetylcholine release.	[75]
Activates monoamine oxidase.	[76, 77]
Inhibits dopamine beta-hydroxylase.	[78]
Inhibits uptake of serotonin and noradrenalin in synaptosomes.	[79]
Channel inhibition	
Influences the activities of Na^+ channels and K^+ channels.	[80]
Enhances the voltage-activated Na^+ channels.	[81]
Inhibits the voltage-gated calcium channel.	[70, 82]
Inhibits the IP^3-mediated Ca^{2+} release.	[83]
Others	
Influences GTP binding proteins as aluminum fluoride.	[84]
Inhibits GAP junction.	[85]
Inhibits axonal transports.	[86]
Binds to calmodulin and inhibition of calmodulin-binding enzymes.	[87]
Induces inflammatory responses.	[88]
(3) Membrane lipids	
Peroxidation	
Accelerates iron-induced membrane lipid peroxidation.	[89]
Enhances lipid peroxidation in liposomes.	[90]
Induces peroxidation of myelin lipids *in vivo*.	[91]
Increases peroxidation products (malondialdehyde).	[59]
Membrane properties	
Causes the change the lipid/phospholipids profiles of myelin *in vivo*.	[92]
Induces the change in membrane physical properties (surface potential, lipid fluidity, and lipid arrangement).	[91]
Induces the change of membrane fluidity.	[93]
(4) Higher functions	
Cell death	
Causes the apoptotic neuronal death.	[94, 95]
Causes the apoptosis of astrocytes.	[96]
Causes the death of motor neuron.	[97, 98]
Behavior, learning, and memory, others	
Inhibits long term potentiation (LTP).	[99, 100]
Causes learning disorder or memory deficit in experimental animals.	[101–103]
Influences electrical activity in hippocampus and inhibits spatial learning memory deficit in aging rats.	[104]
Causes memory deficit in AD model mice.	[105, 106]
Causes encephalopathy in dialysis patients.	[20]
Causes encephalopathy in patients with renal failure.	[107]

These adverse effects may be involved in the mechanisms that underlie Al-induced memory disorder.

3. Link between Al and AD

3.1. Historical Overview of Aluminum Hypothesis and Arguments. A link between Al and AD is supported on many fronts, beginning in 1965 with the finding of Klatzo et al. that the intracerebral administration of Al to experimental animals induced neurofibrillary degeneration and the appearance of tangle-like structures that were similar to the NFTs found in the brains of AD patients [9]. Crapper et al. reported an increased level of Al in the brains of AD patients [10]. In the 1970s, Al in dialysis solutions or pharmacological compounds was found to cause dementia in dialysis patients (dialysis encephalopathy) [20]. As noted previously, several epidemiological studies reported a high percentage of AD cases in areas with high Al level in drinking water [11, 19].

Despite supporting evidence, the aluminum hypothesis of AD remains controversial and has been the subject of much debate in the past few decades. There were at least three arguments against the aluminum hypothesis. *First*,

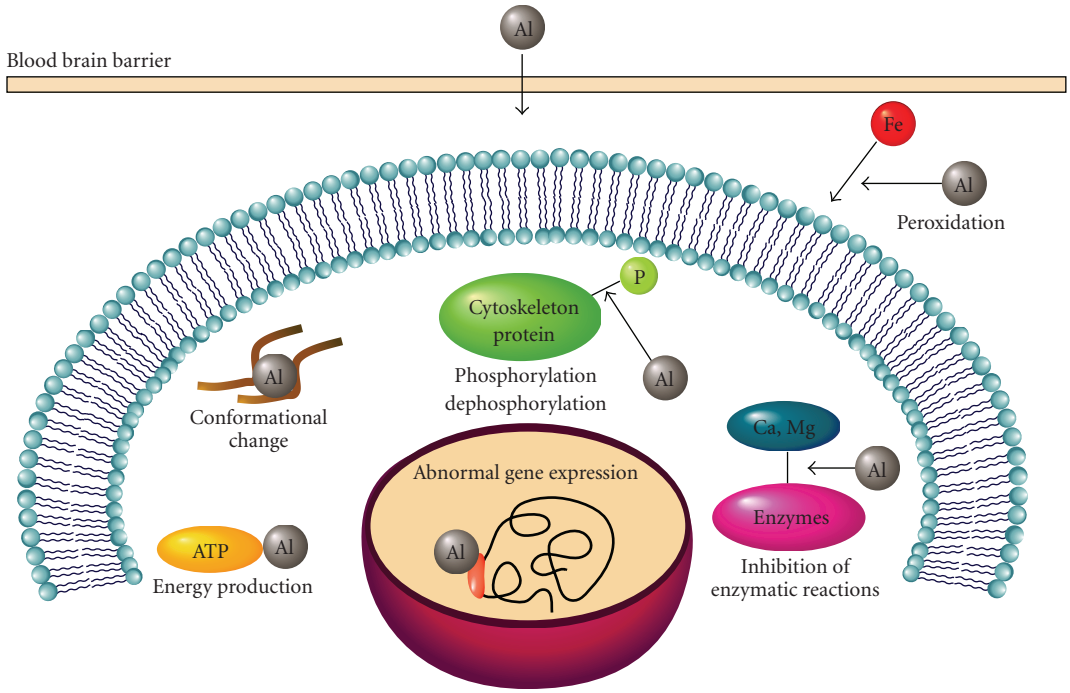


Figure 1: Effects of aluminum on the central nervous system. Major biological effects of Al on the central nervous system are depicted.

it has been argued that neurofibrillary changes in Al-intoxicated animals (Al-NFTs) are different from those in AD patients (AD-NFTs) [108]. Arguments cite morphological and biochemical differences such as the lack of paired helical filamental (PHF) structures, their different distributions in nerve terminals, and the absence of immunoreactivity for tau protein, which is the main component of NFTs in AD patients. *Second*, there is no significant difference in Al levels of AD patients and age-matched controls [109]. *Third*, the epidemiological studies on Al in drinking water are immature and inconclusive [110]. However, most of these criticisms were made in the 1990s. We would like to reinvestigate these early arguments in the context of new findings in the study of AD.

Regarding the first argument, more recent immunohistochemical studies have indicated that depositions in the brains of Al-intoxicated animals are stained with the anti-tau antibody [62, 63]. The accumulation of tau protein was reported in patients with dialysis encephalopathy [111], and in Al-intoxicated cultured neuronal cells [60, 61]. Al inhibits the dephosphorylation of tau [56] and enhances its aggregation *in vitro* [112]. Furthermore, NFTs in some AD patients have been shown to be composed of straight-type filaments rather than PHF-type filaments as is observed in Al-NFT [113]. These data indicate that attempts to discredit the aluminum hypothesis on the basis of differences between Al-NFTs and AD-NFTs are no longer tenable.

3.2. Accumulation of Al in AD Brain. Another argument cites a lack of significant difference between Al levels in AD patients and age-matched controls. One reason for the controversy may be Al contamination of the solutions used in the process of tissue fixation and staining. Therefore, prior studies in fixed tissues cannot be relied upon for precise measures of Al; quantitative analysis of nonfixed and freshly frozen tissues is necessary. One such study showed that the amount of Al in whole brains of AD patients was not significantly different in comparison to controls [114]. Landsberg et al. claimed that they could not detect Al in senile plaques or NFTs using nuclear microscopy [115]. However, this failure could simply be due to low detection limits of their analytical method. Bouras et al. used highly sensitive laser microprobe mass analysis (LAMMA) with nonfixed brain samples and reported an accumulation of Al in NFT-bearing neurons of AD brains [116]. An accumulation of Al in both senile plaques and NFTs has been reported in renal failure patients [117]. Recently, Yumoto et al. analyzed Al using energy-dispersive X-ray spectroscopy combined with transmission electron microscopy (TEM-EDX), a method which yields a high-resolution and low detection limit. Their detailed analysis demonstrated that Al was present in cores of senile plaques at a concentration of 35–50 ppm [118].

3.3. Epidemiological Studies of AD and Al in Drinking Water. Some epidemiological studies have failed to demonstrate the relationship between Al and AD [119, 120]. However, there are a number of possible explanations for this inconsistency, particularly when considering the difficulty in making side-by-side comparisons of epidemiological studies of Al (e.g., intake estimations, effect of move, changes in water-treatment processes, etc.). Using strict neuropathological criteria to discriminate between AD patients and controls (including histopathological verification), McLachlan et al.

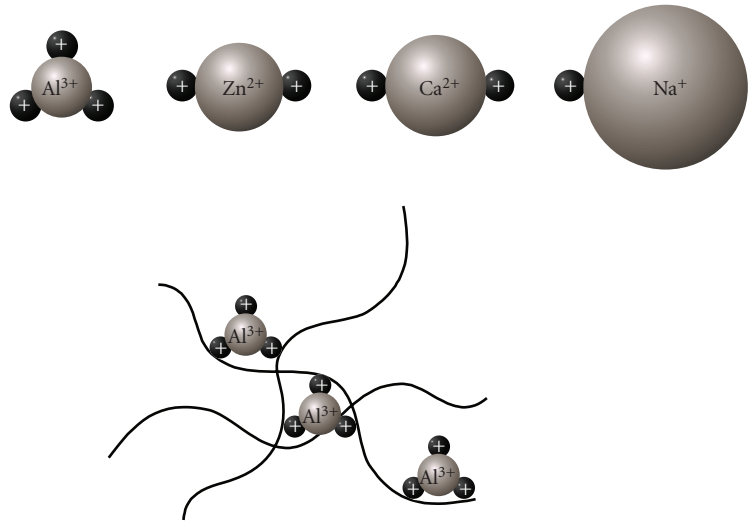


Figure 2: Cross-linking of protein by Al^{3+}. Al^{3+} has a relatively small ionic radius (50 pm) with 3 positive charges; here it is compared to other metal ions such as Zn^{2+} (74 pm), Ca^{2+} (99 pm), and Na^{+} (95 pm). These characteristics enable Al to be an effective cross-linker of proteins.

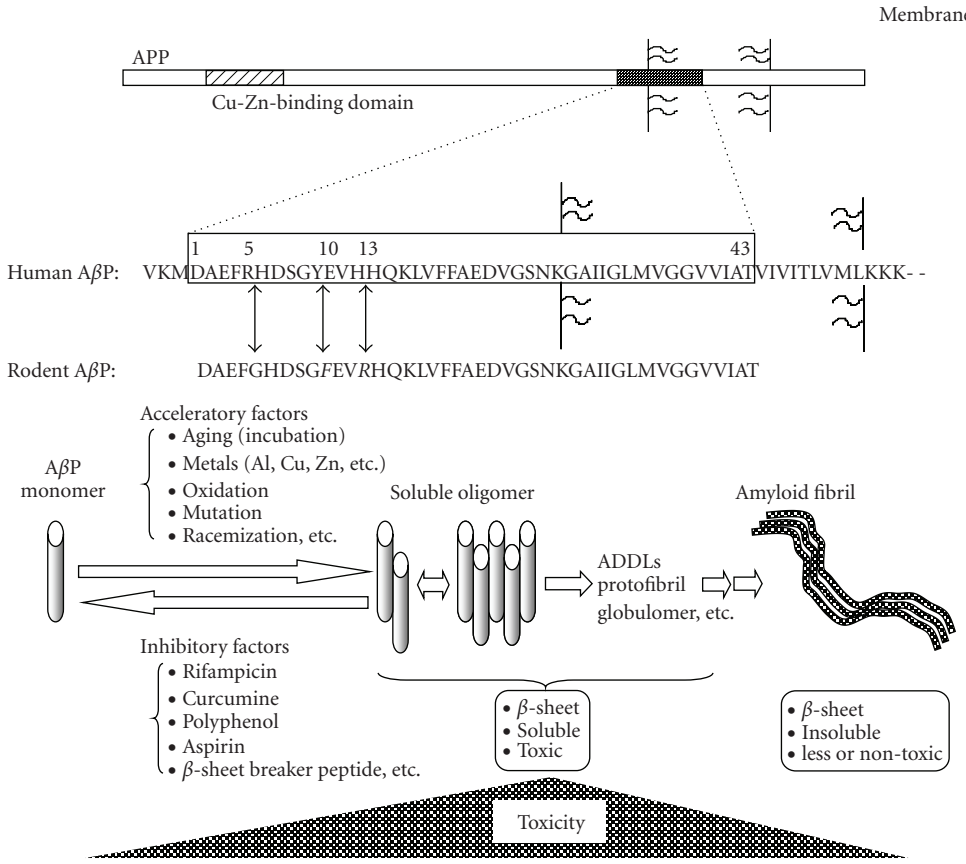


Figure 3: Secretion of AβP from APP and its oligomerization. AβP is secreted by the cleavage of the APP N-terminus by β-secretase (BACE), followed by the intramembrane cleavage of the C-terminus by γ-secretase. APP also binds to Cu or Zn. Human AβP and rodent AβP differ by 3 amino acids (Arg^5, Tyr^{10}, and His^{13}). AβP monomers form random-coil structures. However, under aging conditions or the existence of trace metals such as Al, Zn, and Cu, AβP self-aggregates and oligomerizes (dimmer to protofibrils), and then forms insoluble amyloid fibrils. Although monomeric AβPs are not toxic, oligomeric AβPs induce marked neuronal death.

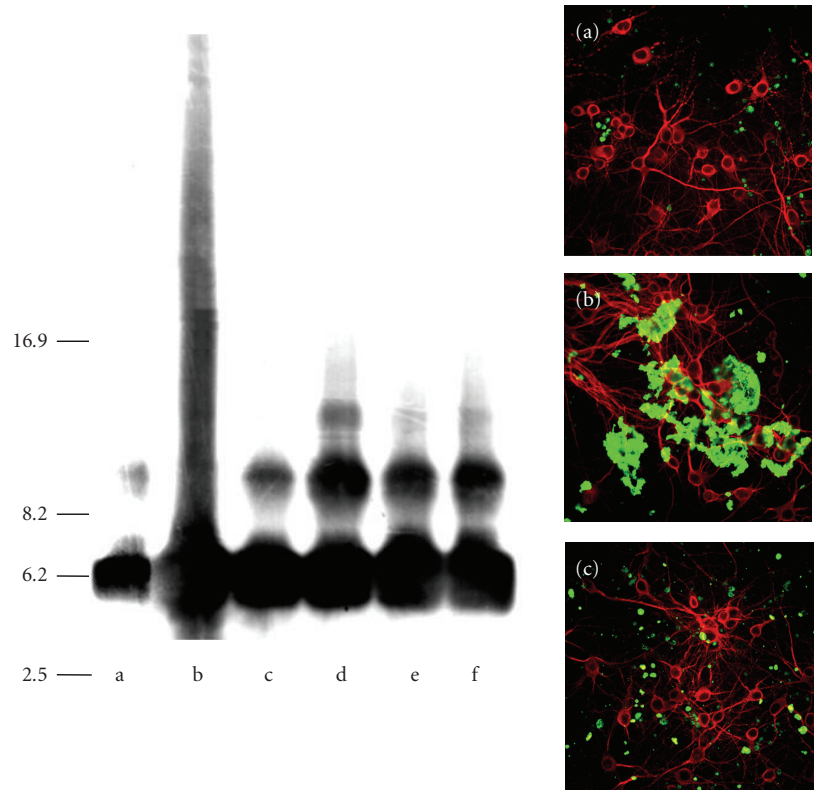


Figure 4: Aggregation of AβP by Al and other metals. (A) Immunoblotting of AβP preincubated with Al and other metals. The solutions of AβP(1–40) were incubated at 37°C for 24 h with or without 1 mM of various metals, and were analyzed by SDS-PAGE and immunoblotting using an antibody to AβP. Each lane contained 4 μg AβP(1–40). Lane a: control, b: $AlCl_3$, c: $ZnCl_2$, d: $CuCl_2$, e: $FeCl_3$, f: $CdCl_2$, Modified from [134]. (B) Deposition of AβP on surfaces of cultured neurons. Solutions of AβP(1–40) preincubated at 37°C for 24 h (a), with 1 mM $AlCl_3$ (b), or 1 mM $ZnCl_2$ (c) were applied to cultured rat cortical neurons. After 2 days of exposure, cells were washed and double immunostained with a polyclonal antibody to AβP (green) and a monoclonal antibody to MAP2 (red), and observed by laser confocal microscope. Scale bar: 50 μm, modified from [64].

found an elevated risk of histopathologically verified AD to be associated with the consumption of higher concentrations of Al in drinking water [121]. More detailed analysis revealed an association between exposure to organic monomeric Al and AD, even after adjustment for education level, family history and presence of the apoE4 allele [122].

The amount of Al consumed in drinking water is approximately 5% of the total daily intake. Thus, it is possible that some factors that prevent or accelerate Al absorption may exist in drinking water. Silicate in the water was reported to interact with Al and prevent Al toxicity to fish [123, 124]. Therefore, the level of silicate in drinking water may also be important. In a French cohort study, the relationship between Al and cognitive impairment is suggested to be influenced by the silica concentration [29]. Cognitive impairment among women was correlated with low concentrations of silica in drinking water [125].

In considering the above new lines of evidence about the neurotoxicity and epidemiology of Al, it is difficult to agree with the early criticisms of the aluminum hypothesis.

3.4. Effects of Al on the Oligomerization of AβP. In the 1990s when the early arguments were claimed, Al-induced Alzheimer-like pathological changes were first attributed to tau proteins (NFT). However, numerous biochemical, toxicological, cell biological, and genetic studies have supported the "amyloid cascade hypothesis", namely, that the accumulation of AβP and its neurotoxicity play a central role in the pathogenesis of AD [12, 13].

AβP is a small peptide of 39–43 amino acid residues, secreted by cleavage of the amyloid precursor protein (APP) N-terminus by β-APP cleaving enzyme (BACE) and intramembrane cleavage of its C-terminus by γ-secretase. Genetic studies of early-onset cases of familial AD indicated that APP mutations and AβP metabolism are associated with AD [126]. Yankner et al. reported that the first 40 amino acid residues of AβP (AβP(1–40)) caused the death of cultured rat hippocampal neurons or neurodegeneration in the brains of experimental animals [127]. AβP is a hydrophobic peptide with an intrinsic tendency to self-assemble and form SDS-stable oligomers in aqueous solution. The monomeric form

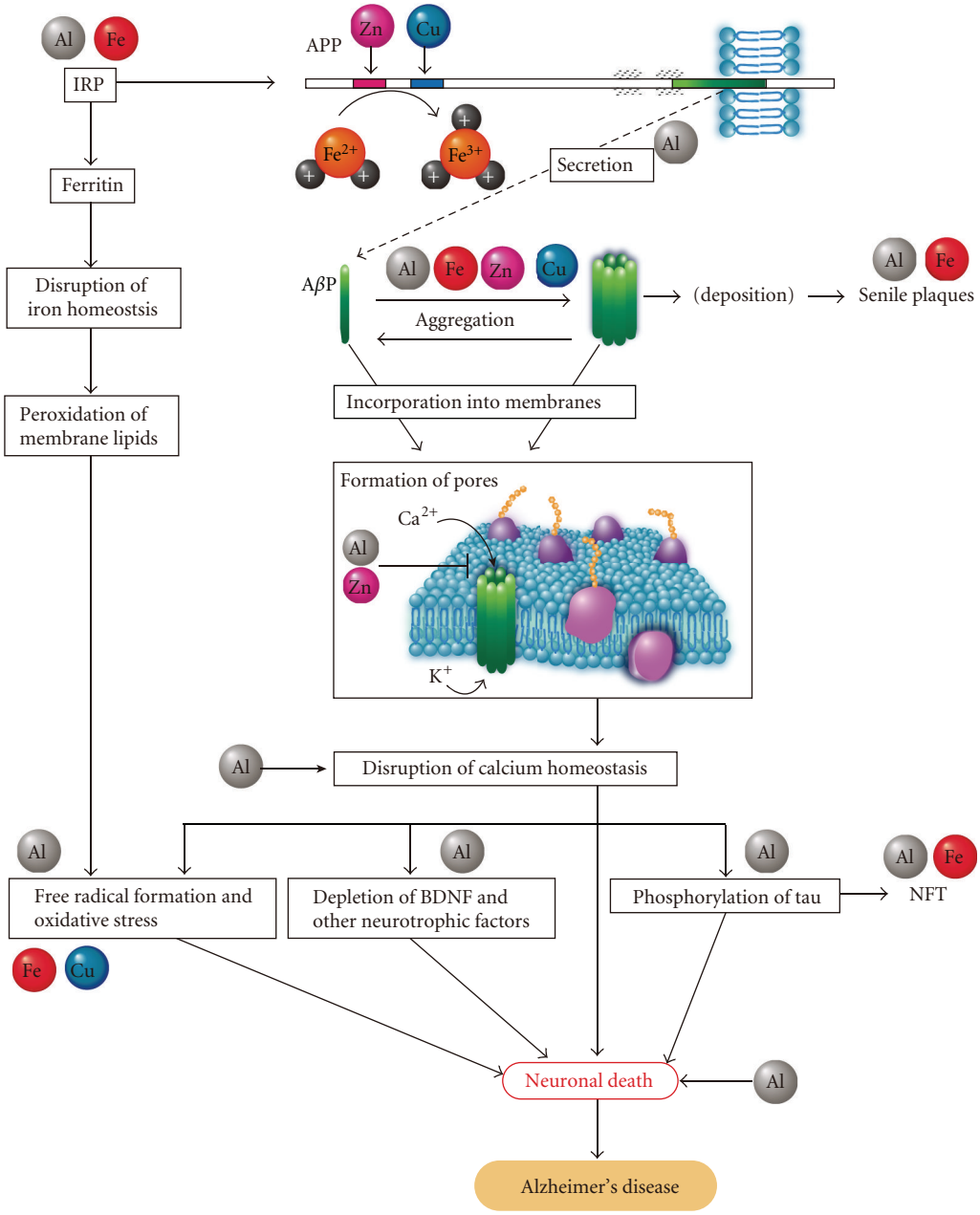


Figure 5: Modified aluminum hypothesis addressing the implications of Al and other trace metals in the pathogenesis of Alzheimer's disease. This model describes the implication of Al and other trace metals including Fe, Cu, and Zn in APP processing, generation and oligomerization of AβP, and the neurotoxic effects caused by AβP. Details are descried in the text.

of AβP has a random coiled structure. Oligomeric AβPs have β-pleated sheet structures and form insoluble aggregates, termed amyloid fibrils. Neurotoxicity of AβP(1–40) peptides was enhanced by the process of "aging" (aggregated under incubation at 37°C for several days) compared to freshly prepared AβP(1–40) in cultured neurons [128], and were correlated with its β-sheet contents [129]. Recent approaches using size-exclusion chromatography, gel electrophoresis, and atomic force microscopy have demonstrated that the soluble oligomers are synaptotoxic and neurotoxic [130]. Figure 3 exhibits the oligomerization of AβP and its neurotoxicity.

Considering that AβP is secreted in the cerebrospinal fluid (CSF) of young individuals as well as in aged or dementia patients [146], factors that accelerate or inhibit oligomerization may play essential roles in the pathogenesis

Table 2: Al-induced conformational changes of various proteins.

Proteins	References
Disease-related proteins	
Alzheimer's disease	
AβP (1-40)	[64, 131–134]
DAEFRHDSGYEVHHQKLVFFAEDVGSNKGAIIGLMVGGVV	
AβP (1-42):	[135, 136]
DAEFRHDSGYEVHHQKLVFFAEDVGSNKGAIIGLMVGGVVIA	
AβP (25-35):	[137]
GSNKGAIIGLMV	
APP	[138]
Tau or hyperphosphorylated tau (PHF-tau)	[32, 57, 139]
Perkinson's disease and other diseases with Lewy body	
α-synuclein (NACP)	[140, 141]
Type 2 diabetes mellitus	
Amylin:	[142]
KCNTATCATQRLANFLVHSSNNFGAILSSTNVGSNTY	
Familial British dementia	
ABri:	[143]
ASNCPAIRHPGNKPAVGTLICSRTVKKNIIGGN	
Spinocerebellar ataxia	
Ataxin 3	[144]
Dialysis-related arthropathy	
β_2-microglobulin	[145]

of AD. Several factors such as peptide concentration, pH or composition of solvents, and temperature can influence the oligomerization processes [147].

Interestingly, rodent AβP exhibits less tendency to oligomerization than human AβP *in vitro* [148] and the accumulation of AβP is rarely observed in the brains of rodents (rats or mice) as compared to primates (humans or monkeys). As shown in Figure 3, the amino acid sequences of human and rodent AβP are similar, but rodent AβP differs from primate only 3 amino acids (Arg^5, Tyr^{10}, and His^{13}) from primate AβP. All 3 amino acids have the ability to bind metals. Therefore, trace elements including Al^{3+} are of particular interest as potential acceleratory factors and may play important roles in the accumulation of AβP in the human brain.

Table 2 summarizes the effects of Al^{3+} on conformational changes of AβP and other various disease-related proteins. Exley et al. first demonstrated by CD spectroscopy that Al induces a conformational change in AβP(1–40) [131]. Al has also been shown to promote the aggregation of ^{125}I-labelled AβP(1–40), with similar findings for Fe and Zn [132]. Bush et al. demonstrated that Zn^{2+} and Cu^{2+} caused the oligomerization of AβP [149, 150]. However, role of Zn^{2+} in AD is complex and enigmatic. Lovell et al. reported that zinc has the protective effects against AβP-induced neurotoxicity [151]. We have demonstrated that Zn^{2+} blocks AβP-channels formed on membranes and inhibits the neurotoxicity [152].

We have developed a system for investigating AβP oligomerization that involves immunoblotting and precipitation. Using this system, we have demonstrated that Al enhances the polymerization of AβP(1–40) and forms SDS-stable oligomers *in vitro* [64, 133, 134]. The aggregated AβP(1–40) is redissolved by adding deferoxamine (DFO), an Al chelator. The oligomerization induced by Al is more marked than that induced by other metals, including Zn^{2+}, Fe^{3+}, Cu^{2+}, and Cd^{2+} (Figure 4(A)). Furthermore, while Al-aggregated AβPs bind tightly to the surface of cultured neurons and form fibrillar deposits, Zn-aggregated AβPs are rarely observed on the surface of cultured neurons (Figure 4(B)). These results suggest that Al-aggregated AβPs have a strong affinity for membrane surfaces as a result of minimal degradation by proteases. Indeed, Al has been shown to inhibit the degradation of AβP as the result of conformational changes [43, 153]. Furthermore, AβP coupled with Al is more toxic than normal AβP causing membrane disruption or perturbation of neural Ca^{2+} homeostasis and mitochondrial respiration [154–156].

The chronic application of Al caused the accumulation of AβP in cultured neurons of rat cerebral cortex [64] and in neuroblastoma cells [65]. Praticó et al. (2002) found that orally administered Al caused a marked increase in the amount of AβP both in its secreted and accumulated forms, and increased deposition of senile plaques in AD-model mice transfected with the human APP gene (Tg 2576) [66]. These results are consistent with other studies demonstrating that oral Al exposure causes the accumulation of AβP and impairs spatial learning memory in AD-model mice [67].

Exposure to Al causes the accumulation of AβP and induces adverse effects in humans as seen in the aftermath of the accidental Al exposure in 1988 at Camelford [157]. The

neuropathological case study of a 58 year-old woman who was exposed to Al and died 15 years later with unspecified neurological symptoms demonstrated the rare form of sporadic cerebral amyloid angiopathy, which is characterized by the deposition of AβP in blood vessels and has a causative link with AD [158]. The deposition of high amounts of Al in the patient's brain was also observed.

Al has also been reported to bind and cause conformational changes in other AD-related proteins, including APP [138], tau protein [32, 57], and PHF-tau protein [139] and in proteins related to other neurodegenerative diseases such as α-synuclein (Parkinson's disease (PD) and dementia with Lewy bodies; DLB) [140, 141], amylin (diabetes mellitus) [142], ABri (familial British dementia) [143], and ataxin 3 (spinocerebellar ataxia type 3) [144], β_2-microglobulin (dialysis-related arthropathy) [145] (Table 2).

3.5. Metal-Metal Interactions in the Pathogenesis of AD. The evidence now suggests that the significance of Al in the pathogenesis of AD should be concerned. Other metals usually share the binding site of one metal ion, although their binding constants differ. Al binds to various metal-binding proteins and influences metal homeostasis. The interactions between Al and other metals should be considered owing to the implications of various trace elements in the pathogenesis of AD. Figure 5 illustrates the modified aluminum hypothesis that accounts for the implications of Al and other trace metals in AD pathology from the secretion of AβP to its neurotoxicity as mentioned below.

3.5.1. Al^{3+} Affects Iron-Homeostasis and Generates Free Radicals. Al has similar characteristics to iron (Fe) and binds to Fe-binding proteins such as ferritin, transferrin, iron regulatory protein (IRP) or to iron chelators such as DFO. The iron responsive element/iron regulatory protein (IRE/IRP) network regulates the production of iron binding proteins which prevent the formation of free Fe^{2+}, which causes toxic free radicals [159]. In iron-deficient conditions, IRP binds to IRE and regulates the expression of genes that contain IREs in their mRNA, such as ferritin or transferrin. As the concentration of free Fe^{2+} increases, the binding of iron to IRP, expression of transferrin is downregulated and that of ferritin is upregulated, and the amount of free Fe^{2+} is thereby decreased. Al^{3+} also binds to IRP [34, 160], and thus influences the expression of Fe-binding proteins with IREs in their mRNA causing an elevated Fe concentration [161]. Al also influences the uptake of iron into cultured neurons or glial cells [34, 162]. Thus, Al^{3+} affects iron homeostasis and the expression of various iron-regulated proteins with IREs. Important findings are that APP mRNA contains an IRE as well as ferritin, and its expression is regulated by iron [163]. Indeed, Al caused elevated expression of APP in experimental animals [40, 41]. Recently, Duce et al. demonstrated that APP has ferroxidase activity, which converts Fe^{2+} to Fe^{3+} and regulates free pro-oxidant Fe^{2+} concentrations [164]. They also found that Zn^{2+} inhibits the ferroxidase activity of APP. APP also possesses copper/zinc binding sites in its amino-terminal domain and in the AβP domain and may be involved in homeostasis of these metals [165]. Al^{3+} stimulates Fe-induced membrane lipid peroxidation and causes oxidative damage *in vitro* and *in vivo*, although Al^{3+} does not directly affect peroxidation [89, 90]. There are other important findings implicating iron homeostasis in AD pathogenesis. Iron related genes such as transferrin C2 or hemochromatosis were revealed to be risk factors for AD [166, 167]. Imagawa et al. (1992) reported that iron supplementation was effective for the recovery of cognitive functions in AD patients [168].

3.5.2. Al^{3+} and Other Metals Enhance the Oligomerization of AβP. An abnormal expression of APP could lead to an increased secretion of AβP, and then enhance its accumulation. Secreted AβP is usually degraded by various proteases such as neprilysin within a short period. The downregulation of neprilysin induced by Al can cause the accumulation AβP [43]. Furthermore, AβP becomes oligomerized in the presence of trace metals such as Al^{3+}, Zn^{2+}, Fe^{3+}, and Cu^{2+}, could be resistant to proteases, and thus accumulates in the brain.

3.5.3. Al^{3+} Impairs Calcium Homeostasis. AβP oligomers could be readily incorporated into cell membranes, resulting in the formation of ion channels [147]. A subsequent influx of Ca^{2+} through these amyloid channels would lead to the phosphorylation of tau, depletion of neurotrophic factors, and the formation of free radicals, and so forth, with the outcome of these effects being neuronal death. Al^{3+} blocks various Ca^{2+} channels and influences Ca^{2+} homeostasis. We found that Al also inhibits the increase in Ca^{2+} levels induced by brain-derived neurotrophic factor (BDNF) [94]. As described previously, Al is implicated in most of these neurodegenerative pathways such as dephosphorylation of tau [56], depletion of neurotrophic factor [38], formation of free radicals [89], and induction of neuronal death.

This working hypothesis may be useful in developing an understanding of the link between AD and trace elements including Al, Zn, Cu, and Fe. Considering the implications of metals in AD pathogenesis, chelation therapy for AD treatment is of great interest [169]. Clioquinol (quinoform), a chelator of Cu^{2+} or Zn^{2+}, inhibits oligomerization of AβP and attenuates the accumulation of amyloid in the brains of experimental animals. Clinical trials using its analogue PBT2 are under investigation [170]. DFO, a chelator of Al and Fe, attenuates the decline of daily living skills in AD patients [171]. Silicates, which couple with Al and reduce its toxicity, are also candidates for chelation therapy in AD [172].

4. Conclusion: Al and Human Health

In this review, we have summarized the properties associated with various aspects of Al neurotoxicity. There is growing evidence for a link between Al and AD, and between other metals and AD. Nevertheless, because the precise mechanism of AD pathogenesis remains unknown, this issue is controversial. However, it is widely accepted that Al is a recognized neurotoxin, and that it could cause cognitive deficiency

and dementia when it enters the brain and may have various adverse effects on CNS. In general, the absorption of metals by the gastrointestinal tract is widely variable and is influenced by various factors including an individual difference, age, pH, stomach contents [173]. Recent studies using mass spectrometry of ^{26}Al have demonstrated that small, but a considerable amount of Al crosses the blood brain barrier, enters into the brain, and accumulates in a semipermanent manner [174, 175]. Therefore, Al can cause severe health problems in particular populations, including infants, elderly people, and patients with impaired renal functions, and unnecessary exposure to Al should be avoided for such patients [176].

In 1989, a joint FAO/WHO Expert Committee on Food Additives (JECFA) recommended a provisional tolerable weekly intake (PTWI) of 7.0 mg/kg body weight Al; however, this was changed in 2007 to 1.0 mg/kg body weight because of potential effects on the reproductive system and the developing nervous system. The characteristics of Al neurotoxity are complex, and further research is needed especially in relation to bioavailability, cellular effects, metabolism, and metal-metal interactions.

Abbreviations

AD: Alzheimer's disease
AβP: β-amyloid protein
Al: Aluminum
ALS: Amyotrophic lateral sclerosis
APP: Amyloid precursor protein
BACE: β-APP cleaving enzyme
BDNF: Brain derived neurotrophic factor
CSF: Cerebrospinal fluid
CNS: Central nervous system
DFO: Deferoxamine
DLB: Dementia with Lewy bodies
JECFA: FAO/WHO Expert Committee on Food Additives
IRE: Iron responsive element
IRP: Iron regulatory protein
LAMMA: Laser microprobe mass analysis
LTP: Long-term potentiation
MAP: Microtubule-associated protein
NFT: Neurofibrillary tangle
NGF: Nerve growth factor
PD: Parkinson's disease
PHF: Paired helical filament

PTWI: Provisional tolerable weekly intake
TEM-EDX: Energy-dispersive X-ray spectroscopy combined with transmission electron microscopy.

Acknowledgments

This work was partially supported by a Grant-in-Aid for Scientific Research from the Ministry of Education, Culture, Sports, Science and Technology of Japan and by a Grant from Cooperation for Innovative Technology and Advanced Research in Evolutional Area (CITY AREA) from the Miyazaki Prefectural Industrial Support Foundation.

References

[1] H. Shiraki and Y. Yase, "Amyotrophic lateral sclerosis and Parkinsonism-dementia in the Kii peninsula: comparison with the same disorders in Guam and with Alzheimer's disease," *Handbook of Clinical Neurology*, vol. 15, pp. 273–300, 1991.

[2] M. S. Petrik, M. C. Wong, R. C. Tabata, R. F. Garry, and C. A. Shaw, "Aluminum adjuvant linked to Gulf War illness induces motor neuron death in mice," *NeuroMolecular Medicine*, vol. 9, no. 1, pp. 83–100, 2007.

[3] A. Campbell, "The potential role of aluminium in Alzheimer's disease," *Nephrology Dialysis Transplantation*, vol. 17, no. 2, pp. 17–20, 2002.

[4] D. R. McLachlan, "Aluminium and the risk for Alzheimer's disease," *Environmetrics*, vol. 6, no. 3, pp. 233–275, 1995.

[5] P. Zatta, Ed., "Recent topics in aluminium chemistry," *Coordination Chemistry Reviews*, vol. 228, no. 2, 2002.

[6] M. Kawahara, "Effects of aluminum on the nervous system and its possible link with neurodegenerative diseases," *Journal of Alzheimer's Disease*, vol. 8, no. 2, pp. 171–182, 2005.

[7] P. Zatta, R. Lucchini, S. J. van Rensburg, and A. Taylor, "The role of metals in neurodegenerative processes: aluminum, manganese, and zinc," *Brain Research Bulletin*, vol. 62, no. 1, pp. 15–28, 2003.

[8] D. J. Selkoe, "The molecular pathology of Alzheimer's disease," *Neuron*, vol. 6, no. 4, pp. 487–498, 1991.

[9] I. Klatzo, H. Wisniewski, and E. Streicher, "Experimental production of neurofibrillary degeneration I. Light microscopic observation," *Journal of Neuropathology & Experimental Neurology*, vol. 24, pp. 187–199, 1965.

[10] D. R. Crapper, S. S. Krishnan, and A. J. Dalton, "Brain aluminum distribution in Alzheimer's disease and experimental neurofibrillary degeneration," *Science*, vol. 180, no. 4085, pp. 511–513, 1973.

[11] C. N. Martyn, C. Osmond, J. A. Edwardson, D. J. P. Barker, E. C. Harris, and R. F. Lacey, "Geographical relation between Alzheimer's disease and aluminium in drinking water," *The Lancet*, vol. 1, no. 8629, pp. 59–62, 1989.

[12] J. Hardy and D. J. Selkoe, "The amyloid hypothesis of Alzheimer's disease: progress and problems on the road to therapeutics," *Science*, vol. 297, no. 5580, pp. 353–356, 2002.

[13] O. Wirths, G. Multhaup, and T. A. Bayer, "A modified β-amyloid hypothesis: intraneuronal accumulation of the β-amyloid peptide—the first step of a fatal cascade," *Journal of Neurochemistry*, vol. 91, no. 3, pp. 513–520, 2004.

[14] S. L. Sensi, P. Paoletti, A. I. Bush, and I. Sekler, "Zinc in the physiology and pathology of the CNS," *Nature Reviews Neuroscience*, vol. 10, no. 11, pp. 780–791, 2009.

[15] P. Zatta, D. Drago, S. Bolognin, and S. L. Sensi, "Alzheimer's disease, metal ions and metal homeostatic therapy," *Trends in Pharmacological Sciences*, vol. 30, no. 7, pp. 346–355, 2009.

[16] Y. H. Hung, A. I. Bush, and R. A. Cherny, "Copper in the brain and Alzheimer's disease," *Journal of Biological Inorganic Chemistry*, vol. 15, no. 1, pp. 61–76, 2010.

[17] J. Spofforth, l. R. C. Edin, and M. R. C. Eng, "Case of aluminium poisoning," *The Lancet*, vol. 197, no. 5103, p. 1301, 1921.

[18] J. G. Chusid, B. L. Pacella, L. M. Kopeloff, and N. Kopeloff, "Chronic epilepsy in the monkey following multiple intracerebral injection of alumina cream," *Proceedings of the Society for Experimental Biology and Medicine*, vol. 78, pp. 53–54, 1951.

[19] M. R. Wills and J. Savory, "Aluminum and chronic renal failure: sources, absorption, transport, and toxicity," *Critical Reviews in Clinical Laboratory Sciences*, vol. 27, no. 1, pp. 59–107, 1989.

[20] A. C. Alfrey, G. R. LeGendre, and D. Kaehny, "The dialysis encephalopathy syndrome. Possible aluminium intoxication," *The New England Journal of Medicine*, vol. 294, no. 4, pp. 184–188, 1976.

[21] P. Altmann, J. Cunningham, U. Dhanesha, M. Ballard, J. Thompson, and F. Marsh, "Disturbance of cerebral function in people exposed to drinking water contaminated with aluminium sulphate: retrospective study of the Camelford water incident," *British Medical Journal*, vol. 319, no. 7213, pp. 807–811, 1999.

[22] T. P. Flaten, "Aluminium as a risk factor in Alzheimer's disease, with emphasis on drinking water," *Brain Research Bulletin*, vol. 55, no. 2, pp. 187–196, 2001.

[23] M. F. Frecker, "Dementia in Newfoundland: identification of a geographical isolate?" *Journal of Epidemiology and Community Health*, vol. 45, no. 4, pp. 307–311, 1991.

[24] L. C. Neri and D. Hewitt, "Aluminium, Alzheimer's disease, and drinking water," *The Lancet*, vol. 338, no. 8763, p. 390, 1991.

[25] W. F. Forbes and D. R. C. McLachlan, "Further thoughts on the aluminum—Alzheimer's disease link," *Journal of Epidemiology and Community Health*, vol. 50, no. 4, pp. 401–403, 1996.

[26] H. Jacqmin, D. Commenges, L. Letenneur, P. Barberger-Gateau, and J. F. Dartigues, "Components of drinking water and risk of cognitive impairment in the elderly," *American Journal of Epidemiology*, vol. 139, no. 1, pp. 48–57, 1994.

[27] V. Rondeau, D. Commenges, H. Jacqmin-Gadda, and J. F. Dartigues, "Relation between aluminum concentrations in drinking water and Alzheimer's disease: an 8-year follow-up study," *American Journal of Epidemiology*, vol. 152, no. 1, pp. 59–66, 2000.

[28] V. Rondeau, H. Jacqmin-Gadda, D. Commenges, C. Helmer, and J. F. Dartigues, "Aluminum and silica in drinking water and the risk of Alzheimer's disease or cognitive decline: findings from 15-year follow-up of the PAQUID cohort," *American Journal of Epidemiology*, vol. 169, no. 4, pp. 489–496, 2009.

[29] W. J. Lukiw, T. P. A. Kruck, and D. R. Crapper McLachlan, "Alterations in human linker histone-DNA binding in the presence of aluminum salts in vitro and in Alzheimer's disease," *NeuroToxicology*, vol. 8, no. 2, pp. 291–301, 1987.

[30] K. S. Bharathi, R. Jagannatha, and R. Stein, "First evidence on induced topological changes in supercoiled DNA by an aluminium D-aspartate complex," *Journal of Biological Inorganic Chemistry*, vol. 8, no. 8, pp. 823–830, 2003.

[31] K. S. Latha, S. Anitha, K. S. J. Rao, and M. A. Viswamitra, "Molecular understanding of aluminum-induced topological changes in $(CCG)_{12}$ triplet repeats: relevance to neurological disorders," *Biochimica et Biophysica Acta*, vol. 1588, no. 1, pp. 56–64, 2002.

[32] N. A. Muma and S. M. Singer, "Aluminum-induced neuropathology: transient changes in microtubule-associated proteins," *Neurotoxicology and Teratology*, vol. 18, no. 6, pp. 679–690, 1996.

[33] I. M. Parhad, C. A. Krekoski, A. Mathew, and P. M. Tran, "Neuronal gene expression in aluminum myelopathy," *Cellular and Molecular Neurobiology*, vol. 9, no. 1, pp. 123–138, 1989.

[34] S. Oshiro, M. Kawahara, S. Mika et al., "Aluminum taken up by transferrin-independent iron uptake affects the iron metabolism in rat cortical cells," *Journal of Biochemistry*, vol. 123, no. 1, pp. 42–46, 1998.

[35] W. J. Lukiw, H. J. LeBlanc, L. A. Carver, D. R. C. McLachlan, and N. G. Bazan, "Run-on gene transcription in human neocortical nuclei: inhibition by nanomolar aluminum and implications for neurodegenerative disease," *Journal of Molecular Neuroscience*, vol. 11, no. 1, pp. 67–78, 1998.

[36] F. Bosetti, G. Solaini, E. A. Tendi, E. G. Chikhale, K. Chandrasekaran, and S. I. Rapoport, "Mitochondrial cytochrome c oxidase subunit III is selectively down-regulated by aluminum exposure in PC12S cells," *NeuroReport*, vol. 12, no. 4, pp. 721–724, 2001.

[37] K. A. Cox and M. A. Dunn, "Aluminum toxicity alters the regulation of calbindin-D28k protein and mRNA expression in chick intestine," *Journal of Nutrition*, vol. 131, no. 7, pp. 2007–2013, 2001.

[38] V. J. Johnson and R. P. Sharma, "Aluminum disrupts the pro-inflammatory cytokine/neurotrophin balance in primary brain rotation-mediated aggregate cultures: possible role in neurodegeneration," *NeuroToxicology*, vol. 24, no. 2, pp. 261–268, 2003.

[39] W. J. Lukiw, M. E. Percy, and T. P. Kruck, "Nanomolar aluminum induces pro-inflammatory and pro-apoptotic gene expression in human brain cells in primary culture," *Journal of Inorganic Biochemistry*, vol. 99, no. 9, pp. 1895–1898, 2005.

[40] R. Lin, X. Chen, W. Li, Y. Han, P. Liu, and R. Pi, "Exposure to metal ions regulates mRNA levels of APP and BACE1 in PC12 cells: blockage by curcumin," *Neuroscience Letters*, vol. 440, no. 3, pp. 344–347, 2008.

[41] J. R. Walton and M. X. Wang, "APP expression, distribution and accumulation are altered by aluminum in a rodent model for Alzheimer's disease," *Journal of Inorganic Biochemistry*, vol. 103, no. 11, pp. 1548–1554, 2009.

[42] T. García, J. L. Esparza, M. Giralt, M. Romeu, J. L. Domingo, and M. Gómez, "Protective role of melatonin on oxidative stress status and RNA expression in cerebral cortex and cerebellum of aβpp transgenic mice after chronic exposure to aluminum," *Biological Trace Element Research*, vol. 135, no. 1–3, pp. 220–232, 2010.

[43] Y. Luo, F. Niu, Z. Sun et al., "Altered expression of Aβ metabolism-associated molecules from d-galactose/$AlCl_3$ induced mouse brain," *Mechanisms of Ageing and Development*, vol. 130, no. 4, pp. 248–252, 2009.

[44] A. Castorina, A. Tiralongo, S. Giunta, M. L. Carnazza, G. Scapagnini, and V. D'Agata, "Early effects of aluminum chloride on beta-secretase mRNA expression in a neuronal model of β-amyloid toxicity," *Cell Biology and Toxicology*, vol. 26, no. 4, pp. 367–377, 2010.

[45] J. M. Socorro, R. Olmo, C. Teijon, M. D. Blanco, and J. M. Teijon, "Analysis of aluminum-yeast hexokinase interaction: modifications on protein structure and functionality," *Journal of Protein Chemistry*, vol. 19, no. 3, pp. 199–208, 2000.

[46] J. C. K. Lai and J. P. Blass, "Inhibition of brain glycolysis by aluminum," *Journal of Neurochemistry*, vol. 42, no. 2, pp. 438–446, 1984.

[47] S. W. Cho and J. G. Joshi, "Inactivation of bakers' yeast glucose-6-phosphate dehydrogenase by aluminum," *Biochemistry*, vol. 28, no. 8, pp. 3613–3618, 1989.

[48] V. Kumar, A. Bal, and K. D. Gill, "Impairment of mitochondrial energy metabolism in different regions of rat brain following chronic exposure to aluminium," *Brain Research*, vol. 1232, pp. 94–103, 2008.

[49] J. Lemire, R. Mailloux, S. Puiseux-Dao, and V. D. Appanna, "Aluminum-induced defective mitochondrial metabolism perturbs cytoskeletal dynamics in human astrocytoma cells," *Journal of Neuroscience Research*, vol. 87, no. 6, pp. 1474–1483, 2009.

[50] R. J. Mailloux, R. Hamel, and V. D. Appanna, "Aluminum toxicity elicits a dysfunctional TCA cycle and succinate accumulation in hepatocytes," *Journal of Biochemical and Molecular Toxicology*, vol. 20, no. 4, pp. 198–208, 2006.

[51] K. Shetty, T. Veeranna, and S. C. Guru, "Phosphatase activity against neurofilament proteins from bovine spinal cord: effect of aluminium and neuropsychoactive drugs," *Neuroscience Letters*, vol. 137, no. 1, pp. 83–86, 1992.

[52] G. V. W. Johnson, K. W. Cogdill, and R. S. Jope, "Oral alumimum alters in vitro protein phosphorylation and kinase activities in rat brain," *Neurobiology of Aging*, vol. 11, no. 3, pp. 209–216, 1990.

[53] D. Julka and K. D. Gill, "Involvement of altered cytoskeletal protein phosphorylation in aluminum-induced CNS dysfunction," *Journal of Biochemical Toxicology*, vol. 11, no. 5, pp. 227–233, 1996.

[54] A. Kaur, K. Joshi, R. W. Minz, and K. D. Gill, "Neurofilament phosphorylation and disruption: a possible mechanism of chronic aluminium toxicity in Wistar rats," *Toxicology*, vol. 219, no. 1–3, pp. 1–10, 2006.

[55] J. C. Troncoso, J. L. March, M. Haner, and U. Aebi, "Effect of aluminum and other multivalent cations on neurofilaments in vitro: an electron microscopic study," *Journal of Structural Biology*, vol. 103, no. 1, pp. 2–12, 1990.

[56] H. Yamamoto, Y. Saitoh, S. Yasugawa, and E. Miyamoto, "Dephosphorylation of τ factor by protein phosphatase 2A in synaptosomal cytosol fractions, and inhibition by aluminum," *Journal of Neurochemistry*, vol. 55, no. 2, pp. 683–690, 1990.

[57] A. H. El-Sebae, M. E. Abdel-Ghany, D. Shalloway, M. M. A. Zeid, J. Blancato, and M. A. Saleh, "Aluminum interaction with human brain tau protein phosphorylation by various kinases," *Journal of Environmental Science and Health Part B*, vol. 28, no. 6, pp. 763–777, 1993.

[58] J. Diaz-Nido and J. Avila, "Aluminum induces the in vitro aggregation of bovine brain cytoskeletal proteins," *Neuroscience Letters*, vol. 110, no. 1-2, pp. 221–226, 1990.

[59] S. K. Abd-Elghaffar, G. H. El-Sokkary, and A. A. Sharkawy, "Aluminum-induced neurotoxicity and oxidative damage in rabbits: protective effect of melatonin," *Neuroendocrinology Letters*, vol. 26, no. 5, pp. 609–616, 2005.

[60] S. P. Guy, D. Jones, D. M. A. Mann, and R. F. Itzhaki, "Human neuroblastoma cells treated with aluminium express an epitope associated with Alzheimer's disease neurofibrillary tangles," *Neuroscience Letters*, vol. 121, no. 1-2, pp. 166–168, 1991.

[61] M. Kawahara, K. Muramoto, K. Kobayashi, and Y. Kuroda, "Functional and morphological changes in cultured neurons of rat cerebral cortex induced by long-term application of aluminum," *Biochemical and Biophysical Research Communications*, vol. 189, no. 3, pp. 1317–1322, 1992.

[62] J. Savory, Y. Huang, M. M. Herman, M. R. Reyes, and M. R. Wills, "Tau immunoreactivity associated with aluminum maltolate-induced neurofibrillary degeneration in rabbits," *Brain Research*, vol. 669, no. 2, pp. 325–329, 1995.

[63] T. Kihira, S. Yoshida, Y. Yase, S. Ono, and T. Kondo, "Chronic low-Ca/Mg high-Al diet induces neuronal loss," *Neuropathology*, vol. 22, no. 3, pp. 171–179, 2002.

[64] M. Kawahara, M. Kato, and Y. Kuroda, "Effects of aluminum on the neurotoxicity of primary cultured neurons and on the aggregation of β-amyloid protein," *Brain Research Bulletin*, vol. 55, no. 2, pp. 211–217, 2001.

[65] A. Campbell, A. Kumar, F. G. La Rosa, K. N. Prasad, and S. C. Bondy, "Aluminum increases levels of β-amyloid and ubiquitin in neuroblastoma but not in glioma cells," *Proceedings of the Society for Experimental Biology and Medicine*, vol. 223, no. 4, pp. 397–402, 2000.

[66] D. Praticò, K. Uryu, S. Sung, S. Tang, J. Q. Trojanowski, and V. M. Lee, "Aluminum modulates brain amyloidosis through oxidative stress in APP transgenic mice," *The FASEB Journal*, vol. 16, no. 9, pp. 1138–1140, 2002.

[67] L. F. Rodella, F. Ricci, E. Borsani et al., "Aluminium exposure induces Alzheimer's disease-like histopathological alterations in mouse brain," *Histology and Histopathology*, vol. 23, no. 4-6, pp. 433–439, 2008.

[68] S. D. Provan and R. A. Yokel, "Aluminum inhibits glutamate release from transverse rat hippocampal slices: role of G proteins, Ca channels and protein kinase C," *NeuroToxicology*, vol. 13, no. 2, pp. 413–420, 1992.

[69] J. J. Canales, R. Corbalán, C. Montoliu et al., "Aluminium impairs the glutamate-nitric oxide-cGMP pathway in cultured neurons and in rat brain in vivo: molecular mechanisms and implications for neuropathology," *Journal of Inorganic Biochemistry*, vol. 87, no. 1, pp. 63–69, 2001.

[70] H. Meiri, E. Banin, M. Roll, and A. Rousseau, "Toxic effects of aluminium on nerve cells and synaptic transmission," *Progress in Neurobiology*, vol. 40, no. 1, pp. 89–121, 1993.

[71] S. J. Yang, J. W. Huh, J. E. Lee, S. Y. Choi, T. U. Kim, and S. W. Cho, "Inactivation of human glutamate dehydrogenase by aluminum," *Cellular and Molecular Life Sciences*, vol. 60, no. 11, pp. 2538–2546, 2003.

[72] C. M. Nday, B. D. Drever, T. Salifoglou, and B. Platt, "Aluminium interferes with hippocampal calcium signaling in a species-specific manner," *Journal of Inorganic Biochemistry*, vol. 104, pp. 919–927, 2010.

[73] J. R. Hofstetter, I. Vincent, O. Bugiani, B. Ghetti, and J. A. Richter, "Aluminum-induced decreases in choline acetyltransferase, tyrosine hydroxylase, and glutamate decarboxylase in selected regions of rabbit brain," *Neurochemical Pathology*, vol. 6, no. 3, pp. 177–193, 1987.

[74] P. Zatta, M. Ibn-Lkhayat-Idrissi, P. Zambenedetti, M. Kilyen, and T. Kiss, "In vivo and in vitro effects of aluminum on the activity of mouse brain acetylcholinesterase," *Brain Research Bulletin*, vol. 59, no. 1, pp. 41–45, 2002.

[75] H. Bielarczyk, M. Tomaszewicz, and A. Szutowicz, "Effect of aluminum on acetyl-CoA and acetylcholine metabolism in nerve terminals," *Journal of Neurochemistry*, vol. 70, no. 3, pp. 1175–1181, 1998.

[76] K. S. J. Rao and G. V. Rao, "Effect of aluminium (Al) on brain mitochondrial monoamine oxidase-A (MAO-A) activity—an in vitro kinetic study," *Molecular and Cellular Biochemistry*, vol. 137, no. 1, pp. 57–60, 1994.

[77] P. Zatta, P. Zambenedetti, and M. Milanese, "Activation of monoamine oxidase type-B by aluminum in rat brain

homogenate," *NeuroReport*, vol. 10, no. 17, pp. 3645–3648, 1999.

[78] M. Milanese, M. I. Lkhayat, and P. Zatta, "Inhibitory effect of aluminum on dopamine β-hydroxylase from bovine adrenal gland," *Journal of Trace Elements in Medicine and Biology*, vol. 15, no. 2-3, pp. 139–141, 2001.

[79] J. C. K. Lai, L. Lim, and A. N. Davison, "Effects of Cd^{2+}, Mn^{2+}, and Al^{2+} on rat brain synaptosomal uptake of noradrenaline and serotonin," *Journal of Inorganic Biochemistry*, vol. 17, no. 3, pp. 215–225, 1982.

[80] M. Kanazirska, P. P. Vassilev, S. Y. Birzon, and P. M. Vassilev, "Voltage-dependent effect of Al^{3+} on channel activities in hippocampal neurons," *Biochemical and Biophysical Research Communications*, vol. 232, no. 1, pp. 84–87, 1997.

[81] T. Csóti, J. Gyri, J. Salánki, and L. Erdélyi, "pH-dependent actions of aluminum on voltage-activated sodium currents in snail neurons," *NeuroToxicology*, vol. 22, no. 1, pp. 109–116, 2001.

[82] B. Platt and D. Busselberg, "Combined actions of Pb^{2+}, Zn^{2+}, and Al^{2+} on voltage-activated calcium channel currents," *Cellular and Molecular Neurobiology*, vol. 14, no. 6, pp. 831–840, 1994.

[83] S. Pentyala, J. Ruggeri, A. Veerraju et al., "Microsomal Ca^{2+} flux modulation as an indicator of heavy metal toxicity," *Indian Journal of Experimental Biology*, vol. 48, no. 7, pp. 737–743, 2010.

[84] L. Li, "The biochemistry and physiology of metallic fluoride: action, mechanism, and implications," *Critical Reviews in Oral Biology and Medicine*, vol. 14, no. 2, pp. 100–114, 2003.

[85] C. Theiss and K. Meller, "Aluminum impairs gap junctional intercellular communication between astroglial cells in vitro," *Cell and Tissue Research*, vol. 310, no. 2, pp. 143–154, 2002.

[86] A. Bizzi, R. C. Crane, L. Autilio Gambetti, and P. Gambetti, "Aluminum effect on slow axonal transport: a novel impairment of neurofilament transport," *Journal of Neuroscience*, vol. 4, no. 3, pp. 722–731, 1984.

[87] N. Siegel, C. Suhayda, and A. Haug, "Aluminum changes the conformation of calmodulin," *Physiological Chemistry and Physics*, vol. 14, no. 2, pp. 165–167, 1982.

[88] A. Campbell, A. Becaria, D. K. Lahiri, K. Sharman, and S. C. Bondy, "Chronic exposure to aluminum in drinking water increases inflammatory parameters selectively in the brain," *Journal of Neuroscience Research*, vol. 75, no. 4, pp. 565–572, 2004.

[89] P. I. Oteiza, "A mechanism for the stimulatory effect of aluminum on iron-induced lipid peroxidation," *Archives of Biochemistry and Biophysics*, vol. 308, no. 2, pp. 374–379, 1994.

[90] N. Kaneko, T. Sugioka, and H. Sakurai, "Aluminum compounds enhance lipid peroxidation in liposomes: insight into cellular damage caused by oxidative stress," *Journal of Inorganic Biochemistry*, vol. 101, no. 6, pp. 967–975, 2007.

[91] S. V. Verstraeten and P. I. Oteiza, "Al-mediated changes in membrane physical properties participate in the inhibition of polyphosphoinositide hydrolysis," *Archives of Biochemistry and Biophysics*, vol. 408, no. 2, pp. 263–271, 2002.

[92] J. D. Pandya, K. R. Dave, and S. S. Katyare, "Effect of long-term aluminum feeding on lipid/phospholipid profiles of rat brain myelin," *Lipids in Health and Disease*, vol. 3, article no. 13, 2004.

[93] V. S. Silva, J. M. Cordeiro, M. J. Matos, C. R. Oliveira, and P. P. Gonçalves, "Aluminum accumulation and membrane fluidity alteration in synaptosomes isolated from rat brain cortex following aluminum ingestion: effect of cholesterol," *Neuroscience Research*, vol. 44, no. 2, pp. 181–193, 2002.

[94] O. Ghribi, M. M. Herman, M. S. Forbes, D. A. DeWitt, and J. Savory, "GDNF protects against aluminum-induced apoptosis in rabbits by upregulating Bcl-2 and Bcl-X and inhibiting mitochondrial Bax translocation," *Neurobiology of Disease*, vol. 8, no. 5, pp. 764–773, 2001.

[95] M. Kawahara, M. Kato-Negishi, R. Hosoda, L. Imamura, M. Tsuda, and Y. Kuroda, "Brain-derived neurotrophic factor protects cultured rat hippocampal neurons from aluminum maltolate neurotoxicity," *Journal of Inorganic Biochemistry*, vol. 97, no. 1, pp. 124–131, 2003.

[96] G. W. Guo and Y. X. Liang, "Aluminum-induced apoptosis in cultured astrocytes and its effect on calcium homeostasis," *Brain Research*, vol. 888, no. 2, pp. 221–226, 2001.

[97] M. J. Strong and R. M. Garruto, "Neuron-specific thresholds of aluminum toxicity in vitro: a comparative analysis of dissociated fetal rabbit hippocampal and motor neuron-enriched cultures," *Laboratory Investigation*, vol. 65, no. 2, pp. 243–249, 1991.

[98] C. A. Shaw and M. S. Petrik, "Aluminum hydroxide injections lead to motor deficits and motor neuron degeneration," *Journal of Inorganic Biochemistry*, vol. 103, no. 11, pp. 1555–1562, 2009.

[99] B. Platt, D. O. Carpenter, D. Busselberg, K. G. Reymann, and G. Riedel, "Aluminum impairs hippocampal long-term potentiation in rats in vitro and in vivo," *Experimental Neurology*, vol. 134, no. 1, pp. 73–86, 1995.

[100] M. Wang, J. T. Chen, D. Y. Ruan, and Y. Z. Xu, "The influence of developmental period of aluminum exposure on synaptic plasticity in the adult rat dentate gyrus in vivo," *Neuroscience*, vol. 113, no. 2, pp. 411–419, 2002.

[101] R. A. Yokel, D. D. Allen, and J. J. Meyer, "Studies of aluminum neurobehavioral toxicity in the intact mammal," *Cellular and Molecular Neurobiology*, vol. 14, no. 6, pp. 791–808, 1994.

[102] N. Kaneko, J. Takada, H. Yasui, and H. Sakurai, "Memory deficit in mice administered aluminum-maltolate complex," *BioMetals*, vol. 19, no. 1, pp. 83–89, 2006.

[103] J. R. Walton, "A longitudinal study of rats chronically exposed to aluminum at human dietary levels," *Neuroscience Letters*, vol. 412, no. 1, pp. 29–33, 2007.

[104] P. Sethi, A. Jyoti, R. Singh, E. Hussain, and D. Sharma, "Aluminium-induced electrophysiological, biochemical and cognitive modifications in the hippocampus of aging rats," *NeuroToxicology*, vol. 29, no. 6, pp. 1069–1079, 2008.

[105] D. Ribes, M. T. Colomina, P. Vicens, and J. L. Domingo, "Effects of oral aluminum exposure on behavior and neurogenesis in a transgenic mouse model of Alzheimer's disease," *Experimental Neurology*, vol. 214, no. 2, pp. 293–300, 2008.

[106] D. Ribes, M. T. Colomina, P. Vicens, and J. L. Domingo, "Impaired spatial learning and unaltered neurogenesis in a transgenic model of alzheimer's disease after oral aluminum exposure," *Current Alzheimer Research*, vol. 7, no. 5, pp. 401–408, 2010.

[107] P. Zatta, P. Zambenedetti, E. Reusche et al., "A fatal case of aluminium encephalopathy in a patient with severe chronic renal failure not on dialysis," *Nephrology Dialysis Transplantation*, vol. 19, no. 11, pp. 2929–2931, 2004.

[108] H. M. Wisniewski and G. Y. Wen, "Aluminium and Alzheimer's disease," *Ciba Foundation Symposium*, vol. 169, pp. 142–154, 1992.

[109] D. Shore and R. J. Wyatt, "Aluminum and Alzheimer's disease," *Journal of Nervous and Mental Disease*, vol. 171, no. 9, pp. 553–558, 1983.

[110] R. Doll, "Review: Alzheimer's disease and environmental aluminium," *Age and Ageing*, vol. 22, no. 2, pp. 138–153, 1993.

[111] C. R. Harrington, C. M. Wischik, F. K. McArthur, G. A. Taylor, J. A. Edwardson, and J. M. Candy, "Alzheimer's-disease-like changes in tau protein processing: association with aluminium accumulation in brains of renal dialysis patients," *The Lancet*, vol. 343, no. 8904, pp. 993–997, 1994.

[112] C. W. Scott, A. Fieles, L. A. Sygowski, and C. B. Caputo, "Aggregation of tau protein by aluminum," *Brain Research*, vol. 628, no. 1-2, pp. 77–84, 1993.

[113] R. A. Crowther, "Straight and paired helical filaments in Alzheimer disease have a common structural unit," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 88, no. 6, pp. 2288–2292, 1991.

[114] E. Bjertness, J. M. Candy, A. Torvik et al., "Content of brain aluminum is not elevated in Alzheimer disease," *Alzheimer Disease and Associated Disorders*, vol. 10, no. 3, pp. 171–174, 1996.

[115] J. P. Landsberg, B. McDonald, and F. Watt, "Absence of aluminium in neuritic plaque cores in Alzheimer's disease," *Nature*, vol. 360, no. 6399, pp. 65–68, 1992.

[116] C. Bouras, P. Giannakopoulos, P. F. Good, A. Hsu, P. R. Hof, and D. P. Perl, "A laser microprobe mass analysis of brain aluminum and iron in dementia pugilistica: comparison with Alzheimer's disease," *European Neurology*, vol. 38, no. 1, pp. 53–58, 1997.

[117] J. M. Candy, F. K. McArthur, A. E. Oakley et al., "Aluminium accumulation in relation to senile plaque and neurofibrillary tangle formation in the brains of patients with renal failure," *Journal of the Neurological Sciences*, vol. 107, no. 2, pp. 210–218, 1992.

[118] S. Yumoto, S. Kakimi, A. Ohsaki, and A. Ishikawa, "Demonstration of aluminum in amyloid fibers in the cores of senile plaques in the brains of patients with Alzheimer's disease," *Journal of Inorganic Biochemistry*, vol. 103, no. 11, pp. 1579–1584, 2009.

[119] A. Wettstein, J. Aeppli, K. Gautschi, and M. Peters, "Failure to find a relationship between mnestic skills of octagenarians and aluminum in drinking water," *International Archives of Occupational and Environmental Health*, vol. 63, no. 2, pp. 97–103, 1991.

[120] C. N. Martyn, D. N. Coggon, H. Inskip, R. F. Lacey, and W. F. Young, "Aluminum concentrations in drinking water and risk of Alzheimer's disease," *Epidemiology*, vol. 8, no. 3, pp. 281–286, 1997.

[121] D. R. C. McLachlan, C. Bergeron, J. E. Smith, D. Boomer, and S. L. Rifat, "Risk for neuropathologically confirmed Alzheimer's disease and residual aluminum in municipal drinking water employing weighted residential histories," *Neurology*, vol. 46, no. 2, pp. 401–405, 1996.

[122] E. Gauthier, I. Fortier, F. Courchesne, P. Pepin, J. Mortimer, and D. Gauvreau, "Aluminum forms in drinking water and risk of Alzheimer's disease," *Environmental Research*, vol. 84, no. 3, pp. 234–246, 2000.

[123] J. D. Birchall and J. S. Chappell, "The chemistry of aluminum and silicon in relation to Alzheimer's disease," *Clinical Chemistry*, vol. 34, no. 2, pp. 265–267, 1988.

[124] J. A. Edwardson, P. B. Moore, I. N. Ferrier et al., "Effect of silicon on gastrointestinal absorption of aluminium," *The Lancet*, vol. 342, no. 8865, pp. 211–212, 1993.

[125] S. Gillete-Guyonnet, S. Andrieu, F. Nourhashemi, V. de la Guéronnière, H. Grandjean, and B. Vellas, "Cognitive impairment and composition of drinking water in women: findings of the EPIDOS study," *American Journal of Clinical Nutrition*, vol. 81, no. 4, pp. 897–902, 2005.

[126] A. Goate, M. C. Chartier-Harlin, M. Mullan et al., "Segregation of a missense mutation in the amyloid precursor protein gene with familial Alzheimer's disease," *Nature*, vol. 349, no. 6311, pp. 704–706, 1991.

[127] B. A. Yankner, L. K. Duffy, and D. A. Kirschner, "Neurotrophic and neurotoxic effects of amyloid β protein: reversal by tachykinin neuropeptides," *Science*, vol. 250, no. 4978, pp. 279–282, 1990.

[128] C. J. Pike, D. Burdick, A. J. Walencewicz, C. G. Glabe, and C. W. Cotman, "Neurodegeneration induced by β-amyloid peptides in vitro: the role of peptide assembly state," *Journal of Neuroscience*, vol. 13, no. 4, pp. 1676–1687, 1993.

[129] L. K. Simmons, P. C. May, K. J. Tomaselli et al., "Secondary structure of amyloid β peptide correlates with neurotoxic activity in vitro," *Molecular Pharmacology*, vol. 45, no. 3, pp. 373–379, 1994.

[130] D. J. Selkoe, "Soluble oligomers of the amyloid β-protein impair synaptic plasticity and behavior," *Behavioural Brain Research*, vol. 192, no. 1, pp. 106–113, 2008.

[131] C. Exley, N. C. Price, S. M. Kelly, and J. D. Birchall, "An interaction of β-amyloid with aluminium in vitro," *FEBS Letters*, vol. 324, no. 3, pp. 293–295, 1993.

[132] P. W. Mantyh, J. R. Ghilardi, S. Rogers et al., "Aluminum, iron, and zinc ions promote aggregation of physiological concentrations of β-amyloid peptide," *Journal of Neurochemistry*, vol. 61, no. 3, pp. 1171–1174, 1993.

[133] M. Kawahara, K. Muramoto, K. Bobayashi, H. Mori, and Y. Kuroda, "Aluminum promotes the aggregation of Alzheimer's amyloid β-protein in vitro," *Biochemical and Biophysical Research Communications*, vol. 198, no. 2, pp. 531–535, 1994.

[134] Y. Kuroda and M. Kawahara, "Aggregation of amyloid beta-protein and its neurotoxicity: enhancement by aluminum and other metals," *Tohoku Journal of Experimental Medicine*, vol. 174, no. 3, pp. 263–268, 1994.

[135] G. D. Fasman, A. Perczel, and C. D. Moore, "Solubilization of β-amyloid-(1-42)-peptide: reversing the β-sheet conformation induced by aluminum with silicates," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 92, no. 2, pp. 369–371, 1995.

[136] E. House, J. Collingwood, A. Khan, O. Korchazkina, G. Berthon, and C. Exley, "Aluminium, iron, zinc and copper Influence the *in vitro* formation of amyloid fibrils of Aβ42 in a manner which may have consequences for metal chelation therapy in Alzheimer's disease," *Journal of Alzheimer's Disease*, vol. 6, no. 3, pp. 291–301, 2004.

[137] S. C. Bondy and A. Truong, "Potentiation of beta-folding of β-amyloid peptide 25–35 by aluminum salts," *Neuroscience Letters*, vol. 267, no. 1, pp. 25–28, 1999.

[138] Y. H. Chong and Y. H. Suh, "Aggregation of amyloid precursor proteins by aluminum in vitro," *Brain Research*, vol. 670, no. 1, pp. 137–141, 1995.

[139] H. Murayama, R.-W. Shin, J. Higuchi, S. Shibuya, T. Muramoto, and T. Kitamoto, "Interaction of aluminum with PHFtau in Alzheimer's disease neurofibrillary degeneration evidenced by desferrioxamine-assisted chelating autoclave method," *American Journal of Pathology*, vol. 155, no. 3, pp. 877–885, 1999.

[140] S. R. Paik, J. H. Lee, D. H. Kim, C. S. Chang, and J. Kim, "Aluminum-induced structural alterations of the precursor of the non-Aβ component of Alzheimer's disease amyloid,"

Archives of Biochemistry and Biophysics, vol. 344, no. 2, pp. 325–334, 1997.

[141] V. N. Uversky, J. Li, and A. L. Fink, "Metal-triggered structural transformations, aggregation, and fibrillation of human α-synuclein: a possible molecular link between parkinson's disease and heavy metal exposure," *Journal of Biological Chemistry*, vol. 276, no. 47, pp. 44284–44296, 2001.

[142] B. Ward, K. Walker, and C. Exley, "Copper(II) inhibits the formation of amylin amyloid in vitro," *Journal of Inorganic Biochemistry*, vol. 102, no. 2, pp. 371–375, 2008.

[143] A. Khan, A. E. Ashcroft, O. V. Korchazhkina, and C. Exley, "Metal-mediated formation of fibrillar ABri amyloid," *Journal of Inorganic Biochemistry*, vol. 98, no. 12, pp. 2006–2010, 2004.

[144] F. Ricchelli, P. Fusi, P. Tortora et al., "Destabilization of non-pathological variants of ataxin-3 by metal ions results in aggregation/fibrillogenesis," *International Journal of Biochemistry and Cell Biology*, vol. 39, no. 5, pp. 966–977, 2007.

[145] M. Chaussidon, P. Netter, M. Kessler et al., "Dialysis-associated arthropathy: secondary ion mass spectrometry evidence of aluminum silicate in β-microglobulin amyloid synovial tissue and articular cartilage," *Nephron*, vol. 65, no. 4, pp. 559–563, 1993.

[146] R. Fukuyama, T. Mizuno, T. Mizuno et al., "Age-dependent change in the levels of Aβ40 and Aβ42 in cerebrospinal fluid from control subjects, and a decrease in the ratio of Aβ42 to Aβ40 level in cerebrospinal fluid from Alzheimer's disease patients," *European Neurology*, vol. 43, no. 3, pp. 155–160, 2000.

[147] M. Kawahara, "Role of calcium dyshomeostasis via amyloid channels in the pathogenesis of Alzheimer's disease," *Current Pharmaceutical Design*, vol. 16, pp. 2779–2789, 2010.

[148] T. Dyrks, E. Dyrks, C. L. Masters, and K. Beyreuther, "Amyloidogenicity of rodent and human βA4 sequences," *FEBS Letters*, vol. 324, no. 2, pp. 231–236, 1993.

[149] A. I. Bush, W. H. Pettingell, G. Multhaup et al., "Rapid induction of Alzheimer Aβ amyloid formation by zinc," *Science*, vol. 265, no. 5177, pp. 1464–1467, 1994.

[150] C. S. Atwood, R. D. Moir, X. Huang et al., "Dramatic aggregation of alzheimer Aβ by Cu(II) is induced by conditions representing physiological acidosis," *Journal of Biological Chemistry*, vol. 273, no. 21, pp. 12817–12826, 1998.

[151] M. A. Lovell, C. Xie, and W. R. Markesbery, "Protection against amyloid beta peptide toxicity by zinc," *Brain Research*, vol. 823, no. 1-2, pp. 88–95, 1999.

[152] M. Kawahara, N. Arispe, Y. Kuroda, and E. Rojas, "Alzheimer's disease amyloid β-protein forms Zn^{2+}-sensitive, cation-selective channels across excised membrane patches from hypothalamic neurons," *Biophysical Journal*, vol. 73, no. 1, pp. 67–75, 1997.

[153] T. Sakamoto, H. Saito, K. Ishii, H. Takahashi, S. Tanabe, and Y. Ogasawara, "Aluminum inhibits proteolytic degradation of amyloid β peptide by cathepsin D: a potential link between aluminum accumulation and neuritic plaque deposition," *FEBS Letters*, vol. 580, no. 28-29, pp. 6543–6549, 2006.

[154] F. Ricchelli, D. Drago, B. Filippi, G. Tognon, and P. Zatta, "Aluminum-triggered structural modifications and aggregation of β-amyloids," *Cellular and Molecular Life Sciences*, vol. 62, no. 15, pp. 1724–1733, 2005.

[155] D. Drago, A. Cavaliere, N. Mascetra et al., "Aluminum modulates effects of βamyloid1-42 on neuronal calcium homeostasis and mitochondria functioning and is altered in a triple transgenic mouse model of Alzheimer's disease," *Rejuvenation Research*, vol. 11, no. 5, pp. 861–871, 2008.

[156] D. Drago, S. Bolognin, and P. Zatta, "Role of metal ions in the Aβ oligomerization in Alzheimer's disease and in other neurological disorders," *Current Alzheimer Research*, vol. 5, no. 6, pp. 500–507, 2008.

[157] C. Exley and M. M. Esiri, "Severe cerebral congophilic angiopathy coincident with increased brain aluminium in a resident of Camelford, Cornwall, UK," *Journal of Neurology, Neurosurgery and Psychiatry*, vol. 77, no. 7, pp. 877–879, 2006.

[158] D. L. Dickstein, J. Walsh, H. Brautigam, S. D. Stockton Jr., S. Gandy, and P. R. Hof, "Role of vascular risk factors and vascular dysfunction in Alzheimer's disease," *Mount Sinai Journal of Medicine*, vol. 77, no. 1, pp. 82–102, 2010.

[159] M. U. Muckenthaler, B. Galy, and M. W. Hentze, "Systemic iron homeostasis and the iron-responsive element/iron-regulatory protein (IRE/IRP) regulatory network," *Annual Review of Nutrition*, vol. 28, pp. 197–213, 2008.

[160] K. Yamanaka, N. Minato, and K. Iwai, "Stabilization of iron regulatory protein 2, IRP2, by aluminum," *FEBS Letters*, vol. 462, no. 1-2, pp. 216–220, 1999.

[161] R. R. Crichton, S. Wilmet, R. Legssyer, and R. J. Ward, "Molecular and cellular mechanisms of iron homeostasis and toxicity in mammalian cells," *Journal of Inorganic Biochemistry*, vol. 91, no. 1, pp. 9–18, 2002.

[162] S. Oshiro, M. Kawahara, Y. Kuroda et al., "Glial cells contribute more to iron and aluminum accumulation but are more resistant to oxidative stress than neuronal cells," *Biochimica et Biophysica Acta*, vol. 1502, no. 3, pp. 405–414, 2000.

[163] H.-H. Cho, C. M. Cahill, C. R. Vanderburg et al., "Selective translational control of the Alzheimer amyloid precursor protein transcript by iron regulatory protein-1," *Journal of Biological Chemistry*, vol. 285, no. 41, pp. 31217–31232, 2010.

[164] J. A. Duce, A. Tsatsanis, M. A. Cater et al., "Iron-export ferroxidase activity of β-amyloid precursor protein is inhibited by zinc in Alzheimer's disease," *Cell*, vol. 142, no. 6, pp. 857–867, 2010.

[165] G. K. W. Kong, L. A. Miles, G. A. N. Crespi et al., "Copper binding to the Alzheimer's disease amyloid precursor protein," *European Biophysics Journal*, vol. 37, no. 3, pp. 269–279, 2008.

[166] K. Namekata, M. Imagawa, A. Terashi, S. Ohta, F. Oyama, and Y. Ihara, "Association of transferrin C2 allele with late-onset Alzheimer's disease," *Human Genetics*, vol. 101, no. 2, pp. 126–129, 1997.

[167] D. J. Lehmann, M. Schuur, D. R. Warden et al., "Transferrin and HFE genes interact in Alzheimer's disease risk: the Epistasis Project," *Neurobiology of Aging*. In press.

[168] M. Imagawa, S. Naruse, S. Tsuji, A. Fujioka, and H. Yamaguchi, "Coenzyme Q_{10}, iron, and vitamin B_6 in genetically-confirmed Alzheimer's disease," *The Lancet*, vol. 340, no. 8820, p. 671, 1992.

[169] M. L. Hegde, P. Bharathi, A. Suram et al., "Challenges associated with metal chelation therapy in alzheimer's disease," *Journal of Alzheimer's Disease*, vol. 17, no. 3, pp. 457–468, 2009.

[170] A. I. Bush and R. E. Tanzi, "Therapeutics for Alzheimer's disease based on the metal hypothesis," *Neurotherapeutics*, vol. 5, no. 3, pp. 421–432, 2008.

[171] R. A. Yokel, "Aluminum chelation: chemistry, clinical, and experimental studies and the search for alternatives to desferrioxamine," *Journal of Toxicology and Environmental Health*, vol. 41, no. 2, pp. 131–174, 1994.

[172] C. Exley, "Organosilicon therapy in Alzheimer's disease?" *Journal of Alzheimer's Disease*, vol. 11, no. 3, pp. 301–302, 2007.

[173] M. Kawahara, K. Konoha, T. Nagata, and Y. Sadakane, "Aluminum and human health: its intake, bioavailability and neurotoxicity," *Biomedical Research on Trace Elements*, vol. 18, pp. 111–120, 2007.

[174] S. Yumoto, H. Nagai, K. Kobayashi, A. Tamate, S. Kakimi, and H. Matsuzaki, "Al incorporation into the brain of suckling rats through maternal milk," *Journal of Inorganic Biochemistry*, vol. 97, no. 1, pp. 155–160, 2003.

[175] N. D. Priest, "The biological behaviour and bioavailability of aluminium in man, with special reference to studies employing aluminium-26 as a tracer: review and study update," *Journal of Environmental Monitoring*, vol. 6, no. 5, pp. 375–403, 2004.

[176] M. Kawahara, "Auminum and human health: possible link with neurodegenerative disorders," in *Metals and Neurodegeneration*, S. Huang, Ed., pp. 15–56, Research Signpost, 2010.

SAGE-Hindawi Access to Research
International Journal of Alzheimer's Disease
Volume 2011, Article ID 607861, 7 pages
doi:10.4061/2011/607861

Research Article

Correlation of Copper Interaction, Copper-Driven Aggregation, and Copper-Driven H_2O_2 Formation with Aβ40 Conformation

Chia-Anne Yang,[1] Yung-Han Chen,[2] Shyue-Chu Ke,[2] Yi-Ru Chen,[3] Hsien-Bin Huang,[4] Ta-Hsien Lin,[5,6] and Yi-Cheng Chen[7]

[1] *Institute of Life Science, Tzu Chi University, Hualien 970, Taiwan*
[2] *Department of Physics, National Dong Hwa University, Hualien 974, Taiwan*
[3] *Institute of Pharmacology, National Yang-Ming University, Shihpai, Taipei 112, Taiwan*
[4] *Institute of Molecular Biology, National Chung-Cheng University, Chiayi 621, Taiwan*
[5] *Department of Biochemistry and Molecular Biology, National Yang-Ming University, Shihpai, Taipei 112, Taiwan*
[6] *Department of Medical Research & Education, Taipei Veterans General Hospital, Shihpai, Taipei 112, Taiwan*
[7] *Department of Medicine, Mackay Medical College, Taipei County 252, Taiwan*

Correspondence should be addressed to Ta-Hsien Lin, thlin@vghtpe.gov.tw and Yi-Cheng Chen, chen15@mmc.edu.tw

Received 15 September 2010; Accepted 15 November 2010

Academic Editor: Craig Atwood

Copyright © 2011 Chia-Anne Yang et al. This is an open access article distributed under the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original work is properly cited.

The neurotoxicity of Aβ is associated with the formation of free radical by interacting with redox active metals such as Cu^{2+}. However, the relationship between ion-interaction, ion-driven free radical formation, and Aβ conformation remains to be further elucidated. In the present study, we investigated the correlation of Cu^{2+} interaction and Cu^{2+}-driven free radical formation with Aβ40 conformation. The Cu^{2+}-binding affinity for Aβ40 in random coiled form is 3-fold higher than that in stable helical form. Unexpectedly but interestingly, we demonstrate in the first time that the stable helical form of Aβ40 can induce the formation of H_2O_2 by interacting with Cu^{2+}. On the other hand, the H_2O_2 generation is repressed at Aβ/Cu^{2+} molar ratio ≥ 1 when Aβ40 adopts random coiled structure. Taken together, our result demonstrates that Aβ40 adopted a helical structure that may play a key factor for the formation of free radical with Cu^{2+} ions.

1. Introduction

β-Amyloid (Aβ) peptide is the main cause of Alzheimer's disease (AD) [1, 2]. Aβ has two major types, Aβ40 and Aβ42, which are derived from a ubiquitous type I transmembrane protein—amyloid precursor protein (APP) by a two-step secretase (β- and γ-secretase) pathway [3–5]. The amyloid cascade hypothesis predicts that the aggregated Aβ peptides in the brain have an early and essential role in the neuronal degeneration that leads to dementia [1, 2, 6]. The aggregation of Aβ occurs following a conformational conversion from either α-helix or random coil to β-sheet in a time-dependent manner, indicating that the formation of β-sheet structure is the key step for the peptide aggregation [7, 8].

The formation of Aβ aggregates is modulated by several factors, including metal ions [9, 10]. In amyloid plaques of AD-affected brain, remarkably high concentration of metals, such as Cu (400 μM), Zn (1 mM) and Fe (1 mM), has been found [11, 12]. *In vitro* studies, micromolar levels of Cu^{2+}, Zn^{2+} and Fe^{3+} have been shown to sufficiently induce protease-resistant aggregation and precipitation of Aβ [13]. Copper ions are bound to the three His residues of Aβ located at the N-terminus, which forms a 3N1O square-planar motif as verified by EPR spectroscopy [14]. Aβ/Cu^{2+} complexes have been demonstrated to generate neurotoxic H_2O_2 from O_2• through Cu^{2+} reduction [15]. Copper-selective chelators can dissolve Aβ deposits extracted from AD postmortem brain specimens and reduce the level of free radical [16].

Curtain and his colleagues previously reported that the addition of Cu^{2+} to oligomeric Aβ40 in a negatively charged lipid membrane can induce Aβ peptides reinserted into lipid membranes, convert Aβ conformation from β-strand into α-helix, and cause the lipid peroxidation [17]. Their result is the first study to show that instead of β-sheet, Aβ in α-helical

form can also generate free radicals in the presence of Cu ions. Recently, our group has characterized the conformation of Aβ in the presence of Cu ions into three structural states, stable helix, unstable helix, and random coil, in 40%, 25%, and 5% TFE, respectively, [18]. Therefore, this provides us a possibility to re-examine the relationship between Aβ conformation, and Cu-driven free radical generation.

In the present study, we examined the correlation between Cu^{2+} binding affinity, H_2O_2 formation, and Aβ40 conformation. It was found that both Cu^{2+} binding affinity and H_2O_2 formation are well correlated with the conformation of Aβ40. In general, Cu^{2+} ions can be bound to the different structural forms of Aβ40. The copper-binding affinity of Aβ40 in random coiled form is about 3-fold higher than that of Aβ40 in stable helical form. For H_2O_2 formation, our result shows in the first time that the generation of H_2O_2 can also be induced by Cu^{2+} and the stable helical form of Aβ40. In contrast, in random coiled form, the formation of H_2O_2 is inhibited by Aβ40.

2. Materials and Methods

2.1. Peptide Synthesis, Purification, and Sample Preparation. The synthesis of full-length Aβ40 peptide was performed in an ABI-433A solid-phase peptide synthesizer using the Fmoc protocol. Cleavage and deprotection of the synthesized peptide were performed by treatment with a mixture of trifluoroacetic acid/distilled water/phenol/thioanisole/ethanedithiol. Then the peptide was extracted with 1 : 1 (v : v) ether : H_2O containing 0.1% 2-mercaptoethanol. The synthesized Aβ peptides were purified on a reverse-phase C-18 HPLC with a linear gradient from 0% to 100% acetonitrile, and the molecular weight of Aβ40 peptides and purity was verified by an MALDI-TOF mass spectroscopy. One mg of purified monomeric Aβ40 peptide was dissolved in 1 mL 100% trifluoroethanol (TFE) as stock solution.

2.2. Circular Dichroism (CD) Spectroscopy. A final Aβ peptide concentration of 30 μM in either 5% or 40% TFE was prepared from fresh peptide stock solution diluted in phosphate buffer, pH 7.4, with or without the same concentration of Cu^{2+}. A thousand μM of Cu^{2+} stock solution was prepared by dissolved $CuCl_2$ salt in distilled H_2O purged by N_2 in a sealed flask and freshly diluted into the designed concentration before it was used. CD spectra were recorded using an Aviv spectropolarimeter equipped with a thermal circulator accessory. All measurements were performed in quartz cells with a path length of 0.1 cm. Data were collected at wavelengths from 190 to 260 nm in 0.2 nm increments. Every CD spectrum is reported as the average from at least three individual samples. The reported CD spectra were corrected using phosphate buffer, pH 7.4 for the baseline. All measurements were carried out at 25.0 ± 0.2°C.

2.3. Electron Paramagnetic Resonance (EPR) Spectroscopy. An equal molar 300 μM of Aβ and Cu^{2+} in 30% glycerol phosphate buffer, pH 7.4, and 5% and 40% of TFE was employed for spin counting purposes. EPR spectra were obtained at X-band using a Bruker EMX ER073 spectrometer equipped with a Bruker TE102 cavity and an advanced research system continuous-flow cryostat (4.2–300 K). During EPR experiments, the sample temperature was maintained at 10 K. The microwave frequency was measured with a Hewlett-Packard 5246L electronic counter.

2.4. Tyrosine Fluorescence Spectroscopy. Tyrosine fluorescence spectroscopy was used to investigate the binding affinity for copper bound to Aβ. Aβ peptide stock solution was diluted in phosphate buffer, pH 7.4 to a final peptide concentration of 10 μM with designed concentration of Cu^{2+}. All measurements were performed in a 96-well plate using a microplate reader (FlexStation 3, MD). The excitation and emission wavelengths were selected at 278 and 305 nm, respectively. Reported data were the average obtained from three individual samples and three repeated measurements of each sample.

The binding affinity was calculated using the following equation:

$$\frac{F_x}{F_0} = \frac{I_x - I_\infty}{I_0 - I_\infty} = \frac{1}{1 + K_a\left[\mathrm{Cu}^{2+}\right]^n}, \quad (1)$$

where I_0 and I_x are the fluorescence intensity for free and Aβ/Cu^{2+}complex, respectively, I_∞ is the fluorescence intensity at saturation state, $[Cu^{2+}]$ is the copper concentration, and n is the copper binding number. The related parameters were fitted using nonlinear curve fitting program MicroOrigin v6.0 (Microcal Software, Inc., Nothampton, MA). In the initial fitting stage, the simplex method, which was set to 100 cycle runs, was used to calculate the initial parameter for further nonlinear curve fitting. A 0.95 confidence level was set to constrain the quality of curve fitting. The final fitting parameters were obtained when the value of χ^2 was less than 0.05 and the parameters and errors for the parameters reached the convergent and steady state.

2.5. Hydrogen Peroxide Assay. The production of H_2O_2 was analyzed using the dichlorofluorescein diacetate (DCFH-DA) assay [19]. Dichlorofluorescein diacetate was dissolved in 100% dimethyl sulfoxide, deacetylated with 50% v/v 0.05 M NaOH for 30 min, and then neutralized (pH 7.4) to a final concentration of 200 μM as stock solution. This stock solution was kept on ice and in dark until use. The reactions were carried out in Dulbecco's PBS, pH 7.4, in a 96-well plate (100 μL/well) containing different concentration of Aβ40, 30 μM $CuCl_2$, TFE (5% and 40%), deacylated DCF (20 μM), and horseradish peroxidase (5 μM) at 37°C. For negative control, an extra of 1.0 mM catalase (*Aspergillus niger*) was used to quench H_2O_2. Measurements were performed on the day of sample preparation. Fluorescence readings were recorded on a microplate reader (MD, FlexStation 3), with the excitation and emission wavelengths selected at 485 and 530 nm, respectively.

2.6. Turbidity Assay. UV/Vis turbidity assay was used to detect the aggregation process of Aβ. 200 μL of 30 μM Aβ40

in phosphate buffer, pH 7.4 containing either 5% or 40% TFE was freshly prepared from stock solution. Fresh prepared samples with or without 30 μM Cu^{2+} placed in a 96-well plate were incubated at 37°C. Turbidity was measured using a microplate reader (MD, FlexStation 3) at a wavelength of 450 nm.

3. Results

The aim of this study is to examine the relationship between Aβ40 conformation and other physical and chemical properties such as aggregation, Cu-binding affinity, and free radical formation. In the present study, we applied 5% and 40% TFE to mimic the conformation of Aβ40 in random coil and stable helix, respectively, [18]. Therefore, the effect of TFE on Cu^{2+} binding affinity and free radical generation has to be evaluated. As depicted in supplemental data (see Supplementary Material available online at doi:10.4061/2011/607861), the main effect of TFE on the system is only to quench the DCF fluorescence intensity. Neither was the solubility of Cu^{2+} ions nor the formation of H_2O_2 affected by TFE.

3.1. Secondary Structure of Aβ40 and Aβ40/Cu^{2+}. First of all, we characterized the structural state of Aβ40 with and without Cu^{2+} in either 5% or 40% TFE using circular dichroism (CD) spectroscopy. Figures 1(a) and 1(b) show the CD spectra for Aβ40 and Aβ40/Cu^{2+} in 5% and 40% TFE, respectively. It can be seen that, in 5% TFE, the pattern of CD spectrum for Aβ40 in the presence of Cu^{2+} showed a dramatic change compared to that for Aβ40 only, while the CD spectra of Aβ40 with or without Cu^{2+} in 40% TFE did not show any significant change. The secondary structure of Aβ40 in 5% TFE changes from 63% random coil, 34% β-sheet and 3% helix in Cu^{2+}-free state to 45% random coil, 50% β-sheet and 5% helix in Cu^{2+}-bound state, representing that there is a structural conversion from random coil into β-sheet. On the other hand, the conformation of Aβ40 with or without Cu^{2+} in 40% TFE remains stable in which the secondary structure is 74% helix, 14% β-sheet, and 12% random coil in Cu^{2+}-free state and 73% helix, 15% β-sheet, and 12% random coil in Cu^{2+}-bound state, indicating that there is no conformational conversion of Aβ40 in 40% TFE while adding Cu^{2+} ions.

3.2. Correlation of Aggregation and Aβ40 Structure. It has been shown that Cu^{2+} ions are able to accelerate the aggregation of Aβ40 [9, 15, 18, 20]. Hence, we characterized the aggregation process of Aβ40/Cu^{2+} and Aβ40 in 5% and 40% TFE. The aggregation process of Aβ40/Cu^{2+} and Aβ40 in 5% and 40% TFE was analyzed by turbidity assay as shown in Figure 2. In general, in both 5% and 40% TFE, Aβ40 in the presence of Cu^{2+} was aggregated much faster than that in the absence of Cu^{2+}. Furthermore, the aggregation rate in 5% TFE for both Aβ40/Cu^{2+} and Aβ40 was much faster than that in 40% TFE. The aggregation process in 5% TFE for Aβ40/Cu^{2+} and Aβ40 showed a typical sigmoidal profile. On the other hand, the aggregation process in 40% TFE for Aβ40 with and without Cu^{2+} mostly stayed in the nucleation or the early elongating stage within 24 hrs. The aggregation process for Aβ40 and Aβ40/Cu^{2+} in 5% and 40% TFE echoes with the result obtained in the analyses of secondary structure in which the aggregation ability is correlated with the ability of conformational formation of β-sheet [8].

3.3. EPR Spectroscopy of Aβ40/Cu^{2+} in 5% and 40%. In the present study, we showed that the conformational states for Aβ40/Cu^{2+} and Aβ40 in 5% are dramatically different, while no such conformational change can be observed between Aβ40/Cu^{2+} and Aβ40 in 40% TFE. Although Cu^{2+} ions have been shown to be bound to Aβ in aqueous solution [14], then, whether Cu^{2+} ions are bound to Aβ40 in 40% TFE is needed to be investigated since the structural state for Aβ40/Cu^{2+} and Aβ40 in 40% TFE shows no difference. To address this issue, EPR spectroscopy was applied to explore the interaction between Cu^{2+} and Aβ40 in 40% TFE. As shown in Figure 3, the EPR spectra of Aβ/Cu^{2+} both in 5% and 40% TFE showed two major groups of hyperfine peaks, while no hyperfine peak could be observed in the EPR spectrum for Cu^{2+} alone, indicating that Cu^{2+} ions were bound to Aβ40 in both 5% and 40% TFE. The pattern of hyperfine peaks for Aβ40/Cu^{2+} in 5% and 40% TFE is similar, but the position of hyperfine peaks in 40% TFE shifts to low magnetic field. The related EPR parameters of $g_{\|}$, A, and $g_{\perp}$ are 2.28, 156.8, and 2.06 for Aβ40/Cu^{2+} in 5% TFE, respectively. The $g_{\|}$ and $g_{\perp}$ values in 5% TFE are very close to those in the literature measured in aqueous condition [14], representing that the binding geometry of Aβ40/Cu^{2+} in 5% TFE should adopt a similar 3N1O coordination. On the other hand, the related EPR parameters of $g_{\|}$, A, and $g_{\perp}$ are 2.40, 119.6, and 2.09 for Aβ40/Cu^{2+} in 40% TFE, indicating that the copper-binding geometry is different from the 3N1O mode and may be locally distorted in 40% TFE. Meanwhile, the A value in 5% TFE is larger than that in 40% TFE. This further indicates that the binding affinity of Cu^{2+} to Aβ40 in random coiled form may be stronger than that to Aβ40 in stable helical form.

3.4. Cu^{2+}-Binding Affinity in 5% and 40% TFE. As shown in EPR studies, Cu^{2+} can be bound to different structural forms of Aβ40 with a different binding affinity. In order to further elucidate the binding property of Cu^{2+}, we applied the tyrosine fluorescence spectroscopy to determine the Cu^{2+}-binding constant, since tyrosine 10 is the only fluorophore in Aβ amino acid sequence and locates at the binding pocket, in which the binding of $Cu^{2+}$to Aβ40 may cause the change of tyrosine fluorescence.

The titration curves of Cu^{2+} concentration versus tyrosine fluorescence intensity in 5% and 40% TFE are shown in Figures 4(a) and 4(b), respectively. The binding constants, K_a, were estimated using the equation as described in the experimental section with molar ratio of Aβ : Cu^{2+} = 1 : 1, that is, $n = 1$. The calculated K_a values are 0.14 μM^{-1} ($R^2 > 0.97$) and 0.05 μM^{-1} ($R^2 > 0.96$) for Aβ/Cu^{2+} in 5% and 40% TFE, respectively. The K_a of Aβ40/Cu^{2+} in 5% TFE is around 3-fold higher than that in 40% TFE, suggesting that Cu^{2+} is bound to random coiled form of Aβ40 more strongly than to

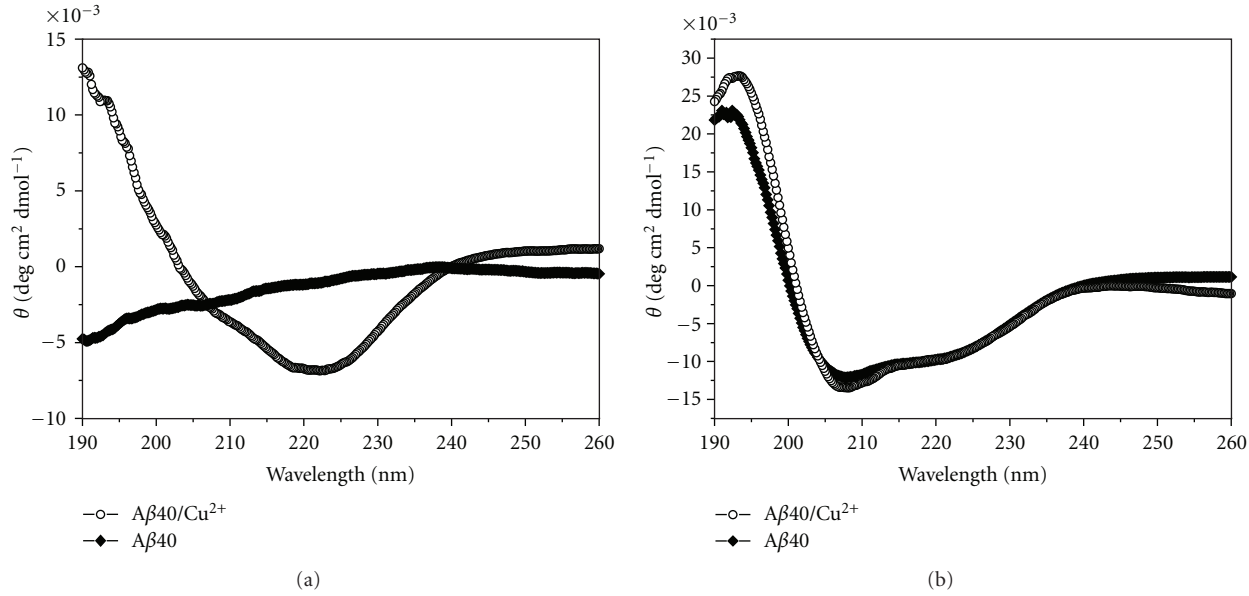


Figure 1: Circular dichroism spectra of Aβ40 peptide with or without Cu^{2+} in (a) 5% TFE and (b) 40% TFE. The concentration for both Aβ40 and Cu^{2+} used in measurements was 10 μM. It can be seen that the conformation of Aβ40 in 5% TFE shows a dramatic change in the presence of Cu^{2+}, while the conformation of Aβ40 in 40% TFE remains unchanged in the presence or absence of Cu^{2+}.

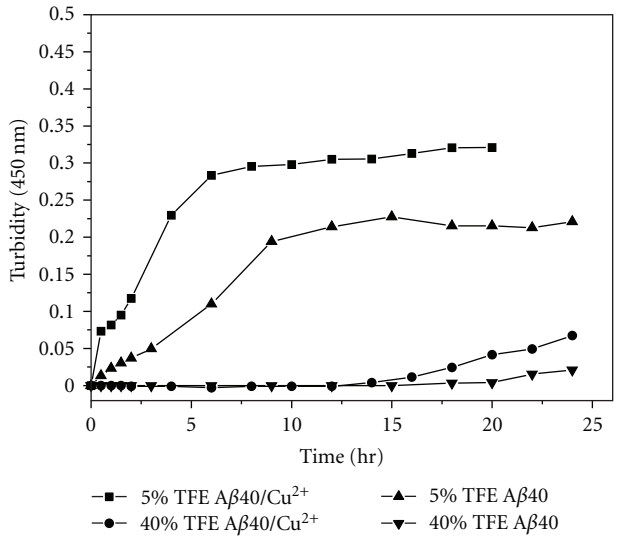


Figure 2: The aggregation process of Aβ40 and Aβ40/Cu^{2+} complex in 5% and 40% TFE measured by turbidity assay. (■) Aβ40/Cu^{2+} in 5% TFE, (▲) Aβ40 in 5% TFE, (•) Aβ40/Cu^{2+} in 40% TFE, and (▼) Aβ40 in 40% TFE.

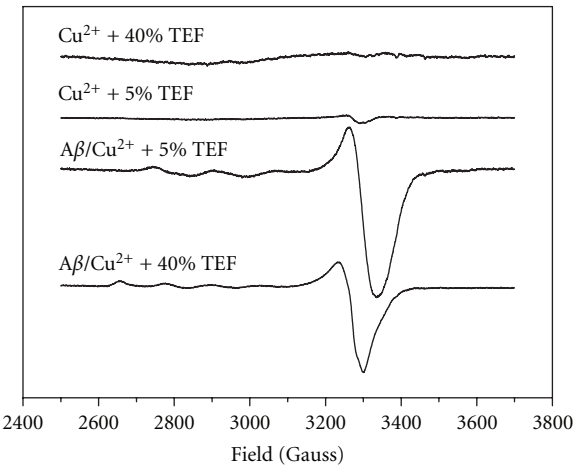


Figure 3: EPR spectra for Cu^{2+} only, Aβ40/Cu^{2+} in either 5% or 40% TFE. The characteristic $g_{\|}$ and $g_{\perp}$ hyperfine peaks appeared in 5% and 40% TFE represent that copper ion coordinates with Aβ40 in a 3N1O mode. The characteristic bands in 40% TFE are slightly shift downfield compared with the hyperfine peaks appeared in 5% TFE, suggesting that Aβ40 adopts different conformation in 5% and 40% TFE.

stable helical form of Aβ40 which is consistent with the EPR studies.

3.5. Hydrogen Peroxide Formation in 5% and 40% TFE. It has been demonstrated that Aβ40 coordinated with Cu^{2+} can cause the reduction of Cu^{2+} and then induce the formation of H_2O_2 from $O_2\cdot$ in a catalytic manner [15, 20]. To examine the relationship between free radical formation and Aβ40 conformation, DCF assay was used to detect the formation of H_2O_2. Figures 5(a) and 5(b) show the plots of DCF fluorescence intensity versus Aβ concentration in 5% and 40% TFE as incubated at 37°C for 1 hr.

In general, results clearly show that the formation of H_2O_2 is also correlated with Aβ40 conformation. In 5% TFE, the H_2O_2 level was decreased with an increase of Aβ40 concentration. The level of H_2O_2 was reduced to zero when

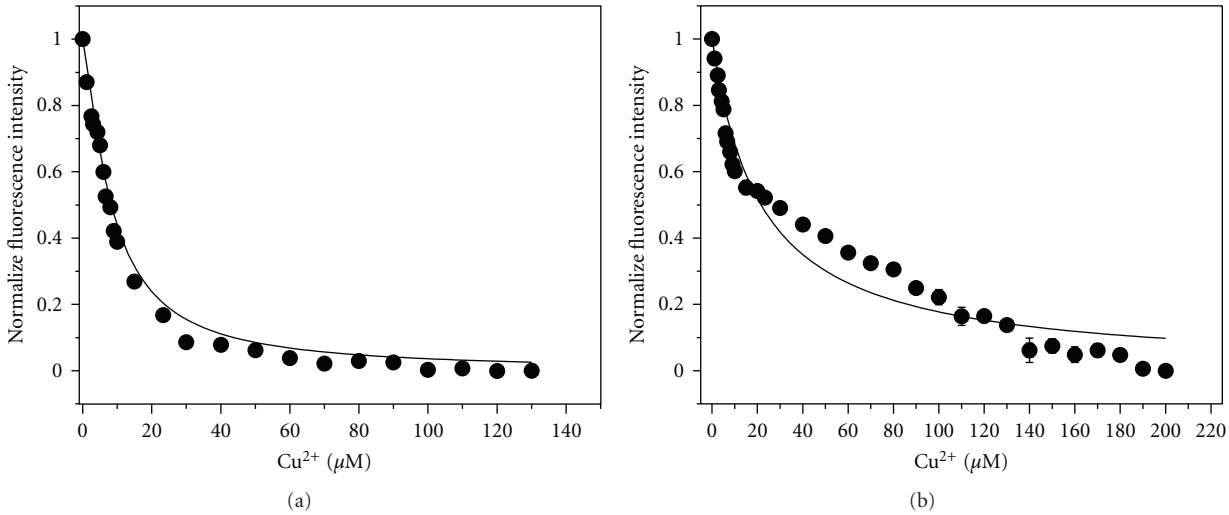


Figure 4: The binding affinity of Cu^{2+} to Aβ40 peptide in (a) 5% TFE and (b) 40% TFE measured by tyrosine fluorescence spectroscopy. Insets in (a) and (b) show the Jot plot. The concentration of Aβ40 was 10 μM. The solid lines represent the fitting curve using the equation as depicted in Section 2 with $n = 1$. The R^2 values in (a) and (b) are all >0.96, and the χ^2 values are <0.05.

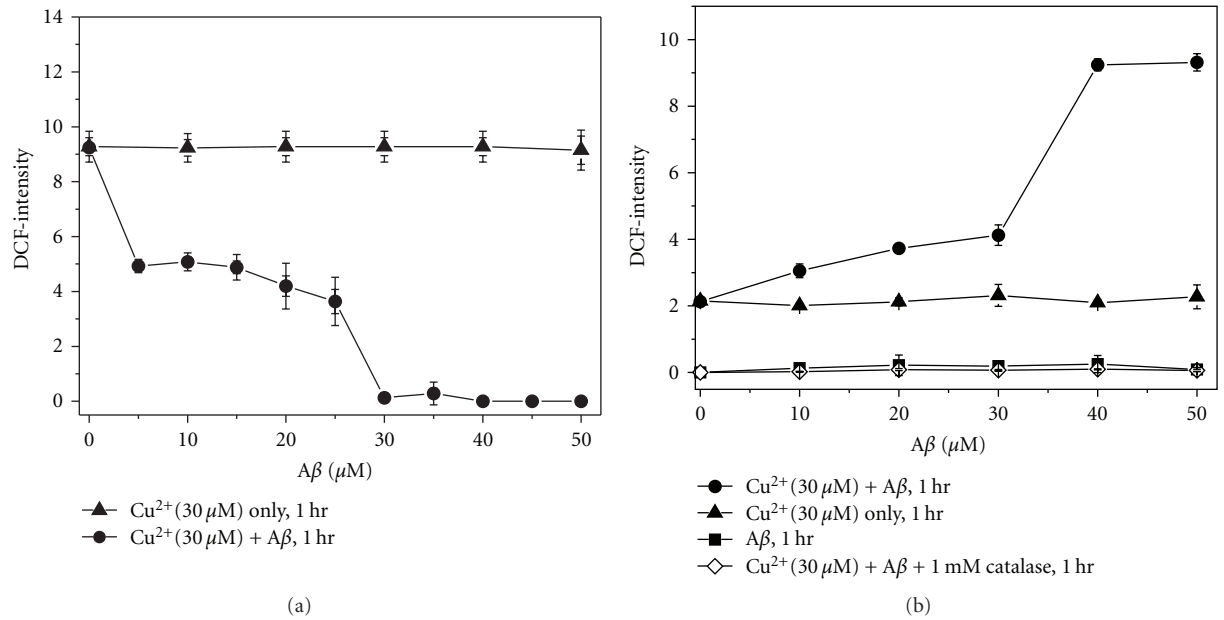


Figure 5: H_2O_2 generation assay for Aβ40 in (a) random coiled state (5% TFE) and (b) stable helical state (40% TFE). In (a), instead of generating free radicals, Aβ40 shows to inhibit the generation of free radical. (▲) 30 μM Cu^{2+} alone and (•) titration of Aβ40 with 30 μM Cu^{2+}. On the other hand, in stable helical structure (b), Aβ40 produces free radical with copper, in (▲) 30 μM Cu^{2+} alone, (■) Aβ40 alone, (•) titration of Aβ40 with 30 μM Cu^{2+}, and (◇) Aβ40 with 30 μM Cu^{2+} and 1 mM Catalase. All measurements were incubated at 37°C for 1 hour.

the Aβ40/Cu^{2+} molar ratio ≥1, indicating that the formation of H_2O_2 is inhibited when Aβ40 adopts the random coiled form. Unexpectedly but interestingly, unlike the formation of H_2O_2 which was inhibited in 5% TFE, the level of H_2O_2 in 40% TFE was increased with an increase of Aβ40 concentration and was much higher than those of either Cu^{2+} or Aβ40 alone, suggesting that the formation of H_2O_2 was induced when Aβ40 was in the stable helical form.

4. Discussion

According to amyloid cascade hypothesis, aggregated Aβ is the main toxic species to cause the Alzheimer's disease [6]. The various forms of Aβ aggregate have been shown to coordinate with redox active transition metals, such as Cu^{2+} and Fe^{3+}, and induce the generation of reactive oxygen species [15, 20]. However, the correlation of copper-binding

affinity, copper-driven aggregation, and free radical formation with Aβ40 conformation still needs to be elucidated.

For ion interaction, our results demonstrate for the first time that Cu^{2+} ions can interact with both random coiled and stable helical forms of Aβ40. As a larger A value and binding constant obtained for Aβ40 in 5% TFE, the Cu^{2+}-binding affinity for random coiled form of Aβ40 is much stronger than that for stable helical form of Aβ40. The possible explanation may be attributed to the different flexibility of Aβ40 conformation in 5% and 40% TFE. As shown in CD spectra, the conformation of Aβ40 in 5% TFE is relatively flexible and can easily convert structure from random coil into β-sheet while coordinating with Cu^{2+}. On the other hand, the overall conformation of Aβ40 with or without Cu^{2+} is relatively rigid and remains in stable helix in 40% TFE, and only the local geometry of Cu^{2+}-binding site is distorted while interacting with Cu^{2+} ions as indicated by the shift of $g_{\|}$ value. Therefore, the interaction between Cu^{2+} and Aβ40 is strong in 5% TFE and relatively weak in 40% TFE. The binding affinity for stable helical form of Aβ40 is almost 3-fold lower than that for random coiled form of Aβ40.

The Cu^{2+}-binding affinity can further be used to account for the differences for Cu^{2+}-driven aggregation between random coiled and stable helical forms of Aβ40. It is well known that the formation of β-sheet plays a key step for the aggregation of Aβ40 [8]. As shown in the present study, the conformation of Aβ40 in 5% TFE is much easier than that in 40% TFE to convert into β-strand structure by Cu^{2+} ions. Thus, in the presence of Cu^{2+}, the random coiled form of Aβ40 (in 5% TFE) aggregates faster and more severe than the stable helical form of Aβ40 (in 40% TFE).

Unlike the cascade hypothesis that only β-sheet form of aggregated Aβ can produce free radical, we demonstrate that helical form of Aβ40 can also induce the formation of H_2O_2. From the aggregation assay, this helical form of Aβ40 may possibly exist as a monomer during the early incubation period. Then, this further implies that monomeric Aβ40 in stable helical form by coordinating with Cu^{2+} can induce the formation of H_2O_2. In contrast, Aβ40 in random coiled form shows to inhibit the formation of H_2O_2 during the early incubation period. The inhibition of H_2O_2 formation becomes more significant with an increase of Aβ40 concentration, and the formation of H_2O_2 further is completely inhibited when the molar ratio of Aβ/Cu^{2+} molar ratio $\geq$1. Our observation is consistent with a recent study by Viles and his colleagues, in which they showed that, at Aβ/Cu^{2+} molar ratio =1, the formation of H_2O_2 is inhibited by both monomeric and fibrillar Aβ peptides [21]. Taken together, it may suggest that helical structure may play a key factor for the generation of H_2O_2 by Aβ40/Cu^{2+}.

In summary, our present results demonstrate that both ion interaction and copper-driven free radical formation are well correlated with Aβ40 conformation. When Aβ40 adopts a stable helical structure, the Cu^{2+} binding affinity is weaker, and the free radical is produced. On the other hand, when Aβ40 adopts a random coil in 5% TFE, the Cu^{2+} binding affinity is stronger, and the formation of H_2O_2 is inhibited.

Acknowledgments

This work was supported by Grants from the National Science Council of Taiwan, Taiwan (NSC 962113M320-001 and NSC 972627M320 to Y. C. Chen) and Taipei Veterans General Hospital, Taiwan, Republic of China (V97C1-036, V97S4-007 to T. H. Lin). C. A. Yang and Y. H. Chen contributed equally to the paper.

References

[1] D. J. Selkoe, "The molecular pathology of Alzheimer's disease," *Neuron*, vol. 6, no. 4, pp. 487–498, 1991.

[2] D. M. Skovronsky, R. W. Doms, and V. M. Y. Lee, "Detection of a novel intraneuronal pool of insoluble amyloid β protein that accumulates with time in culture," *Journal of Cell Biology*, vol. 141, no. 4, pp. 1031–1039, 1998.

[3] R. E. Tanzi, E. D. Bird, S. A. Latt, and R. L. Neve, "The amyloid β protein gene is not duplicated in brains from patients with Alzheimer's disease," *Science*, vol. 238, no. 4827, pp. 666–669, 1987.

[4] J. Kang, H.-G. Lemaire, and A. Unterbeck, "The precursor of Alzheimer's disease amyloid A4 protein resembles a cell-surface receptor," *Nature*, vol. 325, no. 6106, pp. 733–736, 1987.

[5] C. Haass, A. Y. Hung, M. G. Schlossmacher, D. B. Teplow, and D. J. Selkoe, "β-Amyloid peptide and a 3-kDa fragment are derived by distinct cellular mechanisms," *Journal of Biological Chemistry*, vol. 268, no. 5, pp. 3021–3024, 1993.

[6] E. Levy, M. D. Carman, I. J. Fernandez-Madrid et al., "Mutation of the Alzheimer's disease amyloid gene in hereditary cerebral hemorrhage, Dutch type," *Science*, vol. 248, no. 4959, pp. 1124–1126, 1990.

[7] A. Lomakin, D. S. Chung, G. B. Benedek, D. A. Kirschner, and D. B. Teplow, "On the nucleation and growth of amyloid β-protein fibrils: detection of nuclei and quantitation of rate constants," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 93, no. 3, pp. 1125–1129, 1996.

[8] C. J. Barrow, A. Yasuda, P. T. M. Kenny, and M. G. Zagorski, "Solution conformations and aggregational properties of synthetic amyloid β-peptides of Alzheimer's disease: analysis of circular dichroism spectra," *Journal of Molecular Biology*, vol. 225, no. 4, pp. 1075–1093, 1992.

[9] C. S. Atwood, R. D. Moir, X. Huang et al., "Dramatic aggregation of alzheimer by Cu(II) is induced by conditions representing physiological acidosis," *Journal of Biological Chemistry*, vol. 273, no. 21, pp. 12817–12826, 1998.

[10] A. I. Bush, G. Multhaup, R. D. Moir et al., "A novel zinc(II) binding site modulates the function of the βA4 amyloid protein precursor of Alzheimer's disease," *Journal of Biological Chemistry*, vol. 268, no. 22, pp. 16109–16112, 1993.

[11] M. A. Lovell, J. D. Robertson, W. J. Teesdale, J. L. Campbell, and W. R. Markesbery, "Copper, iron and zinc in Alzheimer's disease senile plaques," *Journal of the Neurological Sciences*, vol. 158, no. 1, pp. 47–52, 1998.

[12] M. A. Smith, P. L. R. Harris, L. M. Sayre, and G. Perry, "Iron accumulation in Alzheimer disease is a source of redox-generated free radicals," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 94, no. 18, pp. 9866–9868, 1997.

[13] W. Garzon-Rodriguez, M. Sepulveda-Becerra, S. Milton, and C. G. Glabe, "Soluble amyloid Aβ-(1-40) exists as a stable

dimer at low concentrations," *Journal of Biological Chemistry*, vol. 272, no. 34, pp. 21037–21044, 1997.

[14] C. D. Syme, R. C. Nadal, S. E. J. Rigby, and J. H. Viles, "Copper binding to the amyloid-β (aβ) peptide associated with Alzheimer's disease: folding, coordination geometry, pH dependence, stoichiometry, and affinity of Aβ-(1–28): insights from a range of complementary spectroscopic techniques," *Journal of Biological Chemistry*, vol. 279, no. 18, pp. 18169–18177, 2004.

[15] X. Huang, C. S. Atwood, M. A. Hartshorn et al., "The Aβ peptide of Alzheimer's disease directly produces hydrogen peroxide through metal ion reduction," *Biochemistry*, vol. 38, no. 24, pp. 7609–7616, 1999.

[16] R. A. Cherny, C. S. Atwood, M. E. Xilinas et al., "Treatment with a copper-zinc chelator markedly and rapidly inhibits β-amyloid accumulation in Alzheimer's disease transgenic mice," *Neuron*, vol. 30, no. 3, pp. 665–676, 2001.

[17] C. C. Curtain, F. Ali, I. Volitakis et al., "Alzheimer's disease amyloid-β binds copper and zinc to generate an allosterically ordered membrane-penetrating structure containing superoxide dismutase-like subunits," *Journal of Biological Chemistry*, vol. 276, no. 23, pp. 20466–20473, 2001.

[18] Y. R. Chen, H. B. Huang, C. L. Chyan, M. S. Shiao, T. H. Lin, and Y. C. Chen, "The effect of Aβ conformation on the metal affinity and aggregation mechanism studied by circular dichroism spectroscopy," *Journal of Biochemistry*, vol. 139, no. 4, pp. 733–740, 2006.

[19] C. Opazo, X. Huang, R. A. Cherny et al., "Metalloenzyme-like activity of Alzheimer's disease β-amyloid: cu-dependent catalytic conversion of dopamine, cholesterol, and biological reducing agents to neurotoxic HO," *Journal of Biological Chemistry*, vol. 277, no. 43, pp. 40302–40308, 2002.

[20] K. J. Barnham, F. Haeffner, G. D. Ciccotosto et al., "Tyrosine gated electron transfer is key to the toxic mechanism of Alzheimer's disease β-amyloid," *FASEB Journal*, vol. 18, no. 12, pp. 1427–1429, 2004.

[21] R. C. Nadal, S. E. J. Rigby, and J. H. Viles, "Amyloid β-Cu complexes in both monomeric and fibrillar forms do not generate HO catalytically but quench hydroxyl radicals," *Biochemistry*, vol. 47, no. 44, pp. 11653–11664, 2008.

SAGE-Hindawi Access to Research
International Journal of Alzheimer's Disease
Volume 2011, Article ID 950932, 10 pages
doi:10.4061/2011/950932

Research Article

Effect of Metal Chelators on γ-Secretase Indicates That Calcium and Magnesium Ions Facilitate Cleavage of Alzheimer Amyloid Precursor Substrate

Michael Ho,[1] David E. Hoke,[1,2] Yee Jia Chua,[1] Qiao-Xin Li,[1,3] Janetta G. Culvenor,[1,4] Colin Masters,[3] Anthony R. White,[1,4] and Geneviève Evin[1,3]

[1] *Department of Pathology, The University of Melbourne, Parkville, VIC 3010, Australia*
[2] *Department of Biochemistry and Molecular Biology, Monash University, Clayton, VIC 3800, Australia*
[3] *Mental Health Research Institute, The University of Melbourne, Parkville, VIC 3052, Australia*
[4] *Centre for Neuroscience, The University of Melbourne, Parkville, VIC 3010, Australia*

Correspondence should be addressed to Geneviève Evin, gmevin@unimelb.edu.au

Received 15 September 2010; Revised 22 November 2010; Accepted 25 November 2010

Academic Editor: Peter Faller

Copyright © 2011 Michael Ho et al. This is an open access article distributed under the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original work is properly cited.

Gamma-secretase is involved in the production of Aβ amyloid peptides. It cleaves the transmembrane domain of the amyloid precursor protein (APP) at alternative sites to produce Aβ and the APP intracellular domain (AICD). Metal ions play an important role in Aβ aggregation and metabolism, thus metal chelators and ligands represent potential therapeutic agents for AD treatment. A direct effect of metal chelators on γ-secretase has not yet been investigated. The authors used an *in vitro* γ-secretase assay consisting of cleavage of APP C100-3XFLAG by endogenous γ-secretase from rodent brains and human neuroblastoma SH-SY5Y, and detected AICD production by western blotting. Adding metalloprotease inhibitors to the reaction showed that clioquinol, phosphoramidon, and zinc metalloprotease inhibitors had no significant effect on γ-secretase activity. In contrast, phenanthroline, EDTA, and EGTA markedly decreased γ-secretase activity that could be restored by adding back calcium and magnesium ions. Mg^{2+} stabilized a 1,000 kDa presenilin 1 complex through blue native gel electrophoresis and size-exclusion chromatography. Data suggest that Ca^{2+} and Mg^{2+} stabilize γ-secretase and enhance its activity.

1. Introduction

Gamma-secretase is a key protease activity involved in the production of Alzheimer's disease Aβ amyloid peptides and in regulated intramembrane processing of a subset of membrane receptors, including Notch (reviewed in [1]). Aβ is proteolytically derived from the type I integral amyloid precursor protein (APP) [2] by two sequential cleavages. Shedding of the large APP ectodomain by β-secretase (β-APP cleaving enzyme1, BACE1) [3] produces a 99 amino acid membrane-tethered stub (β-secretase generated APP C-terminal fragment, βCTF, or C99) that is further processed by γ-secretase to liberate Aβ peptides in the extracellular/luminal space. γ-Secretase cleaves the transmembrane domain of APP at multiple sites. Cleavage at the ε-site [4], at position 49-50 according to numbering from Aβ N-terminus mediates the cytosolic release of APP intracellular domain (AICD) together with its binding partners and regulates their nuclear translocation [5]. Further cleavages at the ζ/(46-47) and γ/(40-41) sites generate Aβ_{40} [6]. Pathogenic mutations in APP or in the presenilins result in shifting of ε-cleavage to the (48-49) site and production of Aβ_{42} [7]. APP also undergoes ectodomain shedding by cleavage within the Aβ domain by α-secretase, in a nonamyloidogenic cellular pathway, followed by γ-secretase processing of the corresponding membrane stub (C83) to release AICD and a p3 fragment (reviewed in [8]).

γ-Secretase activity is contained within high molecular complexes formed by assembly of four integral membrane proteins, presenilin, nicastrin, Aph-1, and Pen-2 [9]. Gene knockout experiments [10] and mutation of two conserved aspartates [11] have revealed that the presenilins,

either PS1 or PS2, are membrane proteases and constitute the catalytic subunits of γ-secretase complexes.

The mechanism of γ-secretase and its modulation are yet to be elucidated. We aimed to investigate the effect of metal chelators on γ-secretase activity *in vitro*. Indeed, biometals and metalloenzymes play an important role in the metabolism of APP and Aβ. APP itself comprises two zinc/copper binding sites, one of them located within the Aβ sequence [12–14]. Although the precise function of APP remains unclear, a wealth of experimental evidence indicates that it plays a role in copper homeostasis [15]. The reduction of Cu^{2+} to Cu^{+} by APP is accompanied by the production of hydrogen peroxide resulting in oxidative stress [16]. Also, metal ions, particularly copper, mediate Aβ oligomerization and toxicity [17], therefore metal chelators and ionophores are currently being evaluated as drug candidates for AD treatment (reviewed in [18]). To support the merit of this therapeutic approach, the copper chelator clioquinol (CQ) has been shown to reduce Aβ deposition in the brain of an AD transgenic mouse model [19].

Metals are also implicated in Aβ clearance as the enzymes that metabolize Aβ peptides are zinc-dependent (for a complete review, see [20]), in particular the insulin-degrading enzyme (IDE) [21–24], neprilysin (NEP) [25–27], and the matrix-metalloproteinases MMP2 and MMP9 [28–30]. Secretase processing of APP is also influenced by metal ions since the α-secretase enzymes belong to the family of ADAM proteases, which are zinc-metalloproteases [31], and the β-secretase enzyme; BACE1 comprises a copper-binding site within its cytoplasmic tail, which may regulate its enzymatic activity [32].

In this paper, we have evaluated the direct effects of metal chelators on γ-secretase in an *in vitro* assay using endogenous enzyme extracted from guinea pig and mouse brains, or from human neuroblastoma SH-SY5Y cells, together with C100-3XFLAG substrate, an analogue of APP β-CTF.

2. Materials and Methods

2.1. Materials and Reagents. Adenosine 5′-triphosphate disodium salt (ATP), ethylene glycol-bis[2-aminoethyl ether]-N,N,N′,N′-tetraacetic acid (EGTA), glycerol, 5-chloro7-iodo -8-hydroxyquinoline (clioquinol or CQ), D,L-thiorphan, P-2714 protease inhibitor cocktail, [3-[(3-cholamidopropyl)dimethylammonio-]-2-hydroxy-1-propanesulfonate] (CHAPSO), M2 monoclonal antibody, and anti-FLAG M2-agarose were purchased from Sigma-Aldrich (Sydney, Australia). Glycerol, phosphoramidon and 1,10-phenanthroline were from Merck Biosciences (Victoria, Australia). GM6001 (ilomastat) was obtained from Chemicon (Boronia, Victoria, Australia). L-685,458 inhibitor was obtained from Dr. Mark Shearman (Merck, Sharpe, and Dohme).

2.2. C100-3XFLAG Preparation and Purification. E. Coli transformed with the C100-3XFLAG vector were grown and induced for C100-3XFLAG expression as described before [33]. The cells were harvested, resuspended in 50 mM HEPES, 5 mM $MgCl_2$, 5 mM $CaCl_2$, 150 mM KCl, and pH 7.4, supplemented with 1% (w/v) P-2714 (Sigma) protease inhibitor cocktail, and disrupted by sonication. Membranes were isolated by centrifugation for 1 hour at 100,000 g, and were resuspended in homogenisation buffer containing 1% (v/v) CHAPSO, using a Dounce homogenizer, followed by repeated passages through syringe needles of decreasing bore size. The resultant fine suspension was incubated for 1 hour at 4°C, with end-over-end rocking, then centrifuged at 18,000 g for 1 hour at 4°C, and the supernatant containing the solubilized proteins was brought up to 10% glycerol (v/v). C100-3FLAG was affinity-purified on anti-FLAG M2-agarose column and the purified substrate was stored as aliquots at −80°C.

2.3. Preparation of γ-Secretase Activity. All steps were carried out on ice and the centrifugation steps done at 4°C. Guinea pigs and mouse (C57/Bl6) brains were obtained from the Animal Facility of the University of Melbourne Department of Pathology as secondary tissue usage of excess animals. Homogenates from whole brains, minus cerebellum, were prepared as described previously [33]. The crude tissue homogenate prepared in 20 mM HEPES, pH 7.4, containing 250 mM sucrose, and 1% protease inhibitor cocktail (with no pepstatin), was centrifuged at 3,000 g for 30 minutes to remove cellular debris. The supernatant was collected and centrifuged further at 100,000 g for 1 hour in a L8-80M ultracentrifuge equipped with a Ti70.1 rotor (Beckman-Coulter). The pellet was washed with 20 mM HEPES, 150 mM KCl, and pH 7.4, and resuspended in the same buffer plus 1% (v/v) CHAPSO, with mixing on a rotating platform for 1 hour, at 4°C. CHAPSO-extracted membranes were removed by centrifugation at 18,000 g for 20 minutes The supernatant, containing solubilized γ-secretase, was brought up to 10% glycerol (v/v), and stored as aliquots at −80°C.

Alternatively, SH-SY5Y cells were resuspended in 20 mM HEPES, 80 mM KCl, and pH 7.3, supplemented with 1% protease inhibitor cocktail, and homogenized by repeated passages through a 25-gauge needle. The cell homogenate was centrifuged at 1,000 g for 20 min to remove nuclei and cell debris. The resultant supernatant was collected and centrifuged for 1 hour at 100,000 g. The sediment pellet was extracted with 1% CHAPSO.

2.4. Gamma-Secretase Assays. Purified recombinant substrate (~1.5 μM) and CHAPSO-solubilized membranes (5 μg of guinea pig or mouse brain preparation, or 15 μg of SH-SY5Y membrane extract) were incubated for 4 to 18 hours at 37°C in presence of ATP (1.25 mM) and a mixture of phosphotidyl-ethanolamine (PE) and phosphotidylcholine (PC) (50 ng each; Sigma-Aldrich) in a final reaction volume of 20 μL of 20 mM HEPES (pH 7.4) and 0.5% CHAPSO, plus either 2 mM EDTA (buffer E) or 5 mM $CaCl_2$, 5 mM $MgCl_2$ and 150 mM KCl (buffer A). Inhibitors were prepared as stocks in DMSO and used at dilutions so that DMSO concentration in incubations was no more than 2.5%. Incubations were terminated by adding Laemmli SDS sample buffer and heating at 95°C for 5 minutes. The samples were electrophoresed for 5 hours on

Tris-tricine gels (two-layer, 10–15% acrylamide discontinuous gradient) using a miniPROTEAN 3 system (Bio-Rad) followed by electrotransfer to nitrocellulose (Trans-blot, Bio-Rad). The blots were boiled in phosphate buffer saline, pH 7.4 (PBS) for 3 min prior to blocking for 1 hour with 0.5% casein in PBS and probing with primary antibody for 2 hours. The blots were developed with the Super Signal West Dura kit (Pierce, Rockford, Il) and exposed to a GeneGnome digital imaging system (Syngene, Cambridge, UK). Data were analysed using GeneTools software (Syngene). γ-Secretase activity was expressed as the ratio of AICD signal to the sum of C100 plus AICD signals after subtracting blank values. Aβ was quantified by DELFIA as described before [34]. In brief, plates were coated with mouse monoclonal antibody G210 (specific for Aβ_{40}) and developed with biotinylated WO2 (anti-Aβ 1–16). Bound antibody was detected with streptavidin-labeled Europium (Perkin Elmer, Inc, Melbourne, Victoria). Results were calculated from a standard curve obtained with Aβ_{40} synthetic peptide.

2.5. BN-PAGE Analysis. SH-SY5Y membrane extract (0.5% CHAPSO), prepared as described above, was mixed with an equal volume of 20 mM HEPES (pH 7.4) containing 0.5% CHAPSO plus one of the following, 2 mM EDTA, 2 mM EGTA, 5 mM $MgCl_2$, or 5 mM $CaCl_2$, and incubated for 18 hours at 19°C. BN-PAGE sample buffer was added and the samples resolved on 3–8% NuPAGE Tris-acetate gels (Invitrogen) as described previously [35].

2.6. Size-Exclusion Chromatography. SH-SY5Y membrane extracts (diluted to 0.5% CHAPSO) prepared as for BN-PAGE were fractionated on a Superose-6 column (GE Healthcare) equilibrated in 20 mM HEPES, 150 mM KCl buffer, and pH 7.3 and eluted at a flow rate of 0.5 mL/min. 0.5 mL fractions were collected and analysed by western blotting with 98/1 antibody [36].

3. Results

3.1. In Vitro γ-Secretase Assay. C100 substrate, expressed in *E. coli,* is based on the human APP C-terminal sequence, which corresponds to the C-terminal fragment produced by β-secretase cleavage of APP (β-CTF, the direct precursor to Aβ) plus N-terminal Met_{596}. C100 is expressed with a 3XFLAG C-terminal tag to improve solubility, stability, and detection of AICD [33]. Incubations of C100-3XFLAG with γ-secretase preparations from guinea pig brain membranes produced a 10 kDa C-terminal fragment that was inhibited in a dose-response manner by two specific γ-secretase inhibitors, L-685,458 [37], and DAPT [38] (Figure 1(a)). AICD production was time dependent (Figure 1(b)). Detection of Aβ with WO2 shows an increased production between 2 and 4 hours but the results could not be quantified due to high background and merging of the bands between the lanes.

The effects of phospholipids and ATP were tested to further validate the AICD detection assay and optimize conditions. Adding phospholipids (PC and PE, 2.5 μg/mL each) enhanced the production of AICD (Figure 1(c)), a result consistent with reports by others [39, 40]. Adding ATP (1.25 mM) to the γ-secretase/APP substrate reaction was also found to cause an average 1.6-fold increase of AICD production (Figures 1(d) and 1(e)), corroborating the report by Fraering et al. [41] who used recombinant γ-secretase solubilized from membranes of cells overexpressing the γ-secretase components. The positive effect of ATP was also obtained using γ-secretase preparations from SH-SY5Y-membranes (data not shown).

3.2. Effect of Zinc and Copper Chelators on γ-Secretase Activity. The effect of metal ion chelators and metalloprotease inhibitors on γ-secretase was evaluated using various compounds (Figures 2(a) and 2(b)). The zinc chelators, thiorphan and phosphoramidon, and the hydroxamate inhibitor ilomastat, which is an inhibitor of α-secretase, were all assayed at 250 nM, a concentration sufficient to completely inhibit their target metalloprotease activities in cells and tissues. There was no significant effect of these compounds in the γ-secretase assay, although thiorphan showed a trend for an increase in AICD signal. Thiorphan is an inhibitor of neprilysin, which might be present in the assay as it is associated with brain membranes. The modest decrease in γ-secretase activity in the presence of the copper chelator clioquinol was not statistically significant. In contrast a 65% decrease in AICD production ($P = .024$; $N = 3$) was observed in the presence of phenanthroline (5 mM), suggesting that some metal ions facilitate γ-secretase activity.

3.3. Metal Chelators of Broad Specificity Decrease γ-Secretase Activity. The reduction of activity in the presence of phenanthroline led us to test the effect of EDTA, another chelator of broad specificity. Indeed, reports in the literature indicate the use of alternative buffers for *in vitro* γ-secretase assays. Notably, these involve the use of buffers that either contain EDTA [41, 42], or that are supplemented with Ca^{2+} and Mg^{2+} [39, 40, 43–45]. Therefore, we compared both conditions in our assay (Figures 3(a) and 3(b)). In the presence of EDTA, AICD production was 28% of that in the presence of buffer with Ca^{2+} and Mg^{2+} ($P = .0002$, $N = 5$). Measurement of Aβ40 production by a sandwich ELISA (Figure 3(c)) showed that 0.035 ± 0.01 ng/mL Aβ40 was produced in the presence of Ca^{2+} and Mg^{2+} whereas 0.011 ± 0.004 ng/mL was produced in the presence of EDTA, further supporting that Ca^{2+} and Mg^{2+} ions facilitate the processing of C100 by γ-secretase. Similar results were obtained when using solubilised SH-SY5Y membranes as the source of γ-secretase, with AICD production in the presence of EDTA being 30% to that in the presence of Ca^{2+} and Mg^{2+} (Figures 3(d) and 3(e)). Comparing incubations with and without Ca^{2+} and Mg^{2+} showed that the addition of these metal ions ameliorates γ-secretase activity by 1.65-fold ($P = .02, N = 3$) (Figures 3(d) and 3(e)). These data are consistent with those obtained with phenanthroline, and suggest that adding Ca^{2+} and Mg^{2+}metal ions facilitate γ-secretase cleavage of APP substrate.

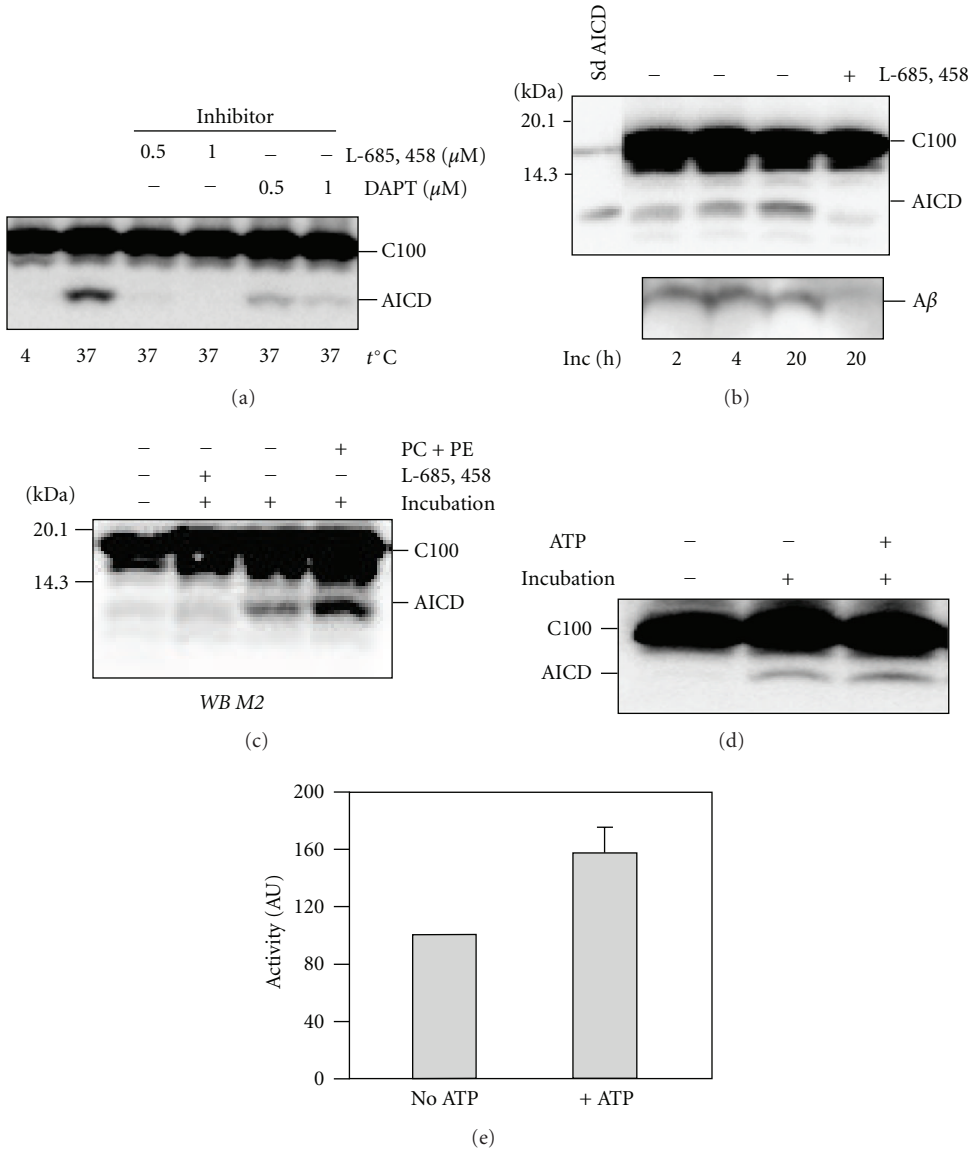


Figure 1: Characterization of γ-secretase *in vitro* assay with guinea pig brain enzyme. ~1 μg of purified recombinant C100-3XFLAG was incubated with 2.75 μg of CHAPSO solubilised membranes of guinea pig brain in 20 mM HEPES buffer, pH 7.3, plus 3 mM $CaCl_2$, 3 mM $MgCl_2$ and 150 mM KCl. The reactions were stopped by adding Laemmli buffer, boiled, and separated on Tris-Tricine gels. M2 (anti-FLAG) antibody and WO2 (anti-Aβ 1–16) were used for western blot detection. (a) Production of an AICD fragment in the assay is inhibited by the γ-secretase inhibitors, L-685,458 and DAPT. (b) Both AICD and Aβ are produced in the reaction and inhibited by L-685,458. AICD signal increases in a time dependent manner over 20 h. Aβ signal is increased at 4 h compared to 2 h, but decreased at 20 h, possibly due to degradation. (Inc, incubation at 37°C). (c) Positive effect of phospholipids on AICD production in the γ-secretase assay. PC, phosphatidylcholine; PE, phosphatidylethanolamine. (d) Positive effect of ATP on the γ-secretase assay. ATP was added 1.25 mM concentration. (e) Quantitation of three separate experiments using GeneTools Syngene software shows ~1.6 fold enhancement of γ-secretase activity in the presence of 1.25 mM ATP. The error bars represent SEM.

3.4. Ca^{2+} and Mg^{2+} Facilitate γ-Secretase Activity. To define which of Ca^{2+} and Mg^{2+} ions were important for γ-secretase activity, we tested the effect of EGTA that has much greater affinity for calcium than magnesium. In the reactions carried out with guinea pig or mouse γ-secretase activity, there were similar levels of AICD produced in the presence of EGTA than in the presence of EDTA (Figures 4(a) and 4(b)), suggesting that Ca^{2+}ions are required for the enhancement of γ-secretase activity. When, reactions were prepared in the presence of 2 mM EDTA and increasing concentrations of $CaCl_2$ or $MgCl_2$ were added, both Ca and Mg ions individually restored AICD production to levels achieved with the Ca and Mg buffer (Figure 4(c)). However, when Mg ions were absent in the reaction, adding 3 mM calcium resulted in precipitation of the C100 substrate. Therefore, both calcium and magnesium are required for optimal assay conditions.

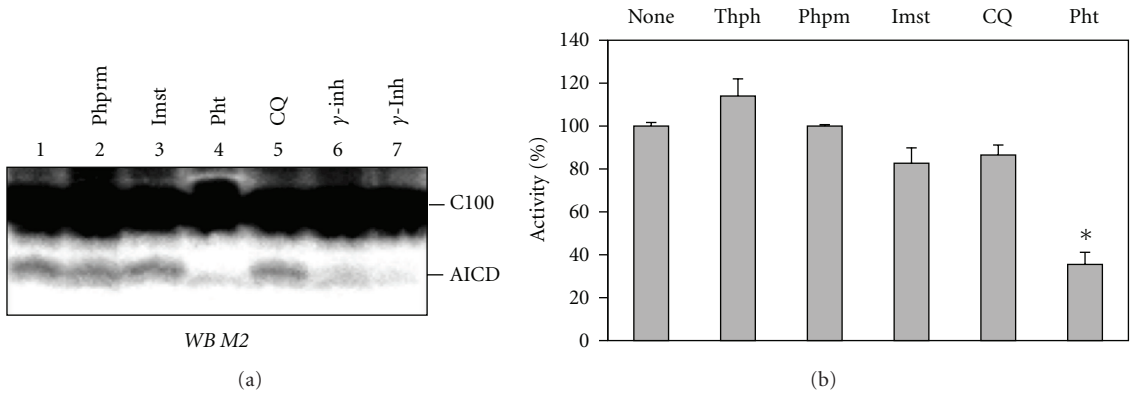


Figure 2: Effect of metalloprotease inhibitors, and metal chelators on γ-secretase. Chelators and inhibitors were added as DMSO solutions (final DMSO concentration 2.5%) to the guinea pig brain γ-secretase/C100-3XFLAG reactions. (a) Representative western blot of γ-secretase assay in the presence of various inhibitors. Phpm, phosphoramidon; Ilmst, ilomastat; Pht, phenanthroline; CQ, clioquinol; γ-inh, L-685,458, used at 10 nM (lane 6) and 100 nM (lane 7). (b) Effect of metalloprotease inhibitors on the γ-secretase assay. Activity is expressed as the % of substrate converted into AICD, as determined from band density analysis with GeneTools software. The error bars represent SEM. Inhibitor concentrations were selected as follows: thiorphan, 250 nM; phosphoramidon, 25 μM; ilomastat, 250 nM; phenanthroline, 5 mM; clioquinol, 100 μM.

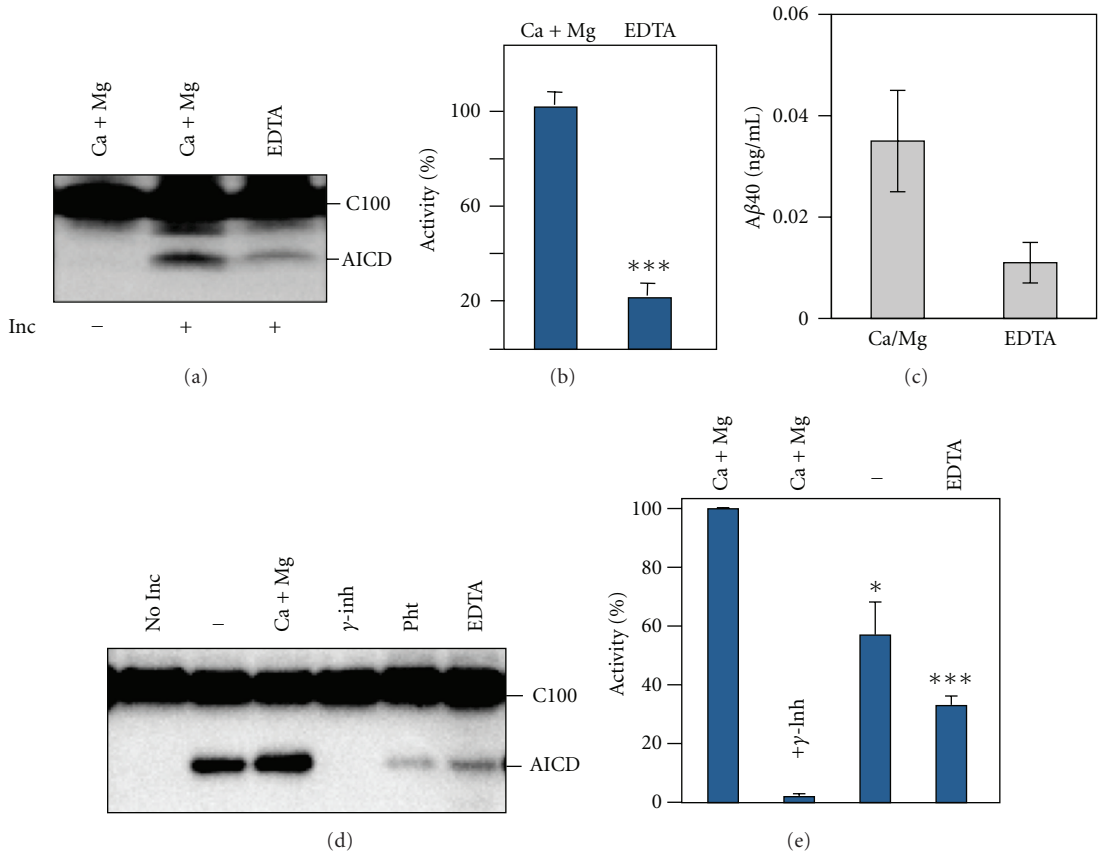


Figure 3: Effect of EDTA and phenanthroline on γ-secretase activity. (a) Guinea pig γ-secretase activity was assayed on C100-3XFLAG in alternate buffer conditions (2 mM EDTA or 3 mM $CaCl_2$ and 3 mM $MgCl_2$). (b) Quantitative analysis of five separate experiments shows AICD production in the presence of EDTA was 28% of that produced in the presence of $CaCl_2$, and $MgCl_2$. (c) Quantitative analysis of Aβ40 by sandwich DELFIA shows that Aβ production in the presence of EDTA is lower than in the presence of $CaCl_2$, and $MgCl_2$. (d) γ-Secretase activity extracted from SH-SY5Y membranes was assayed in buffer conditions as indicated. Phenanthroline (Pht) concentration was 5 mM. L-685,458 was 0.5 μM. (e) Quantitative analysis of three blots corresponding to assays carried out as in (d) The error bars represent SEM.

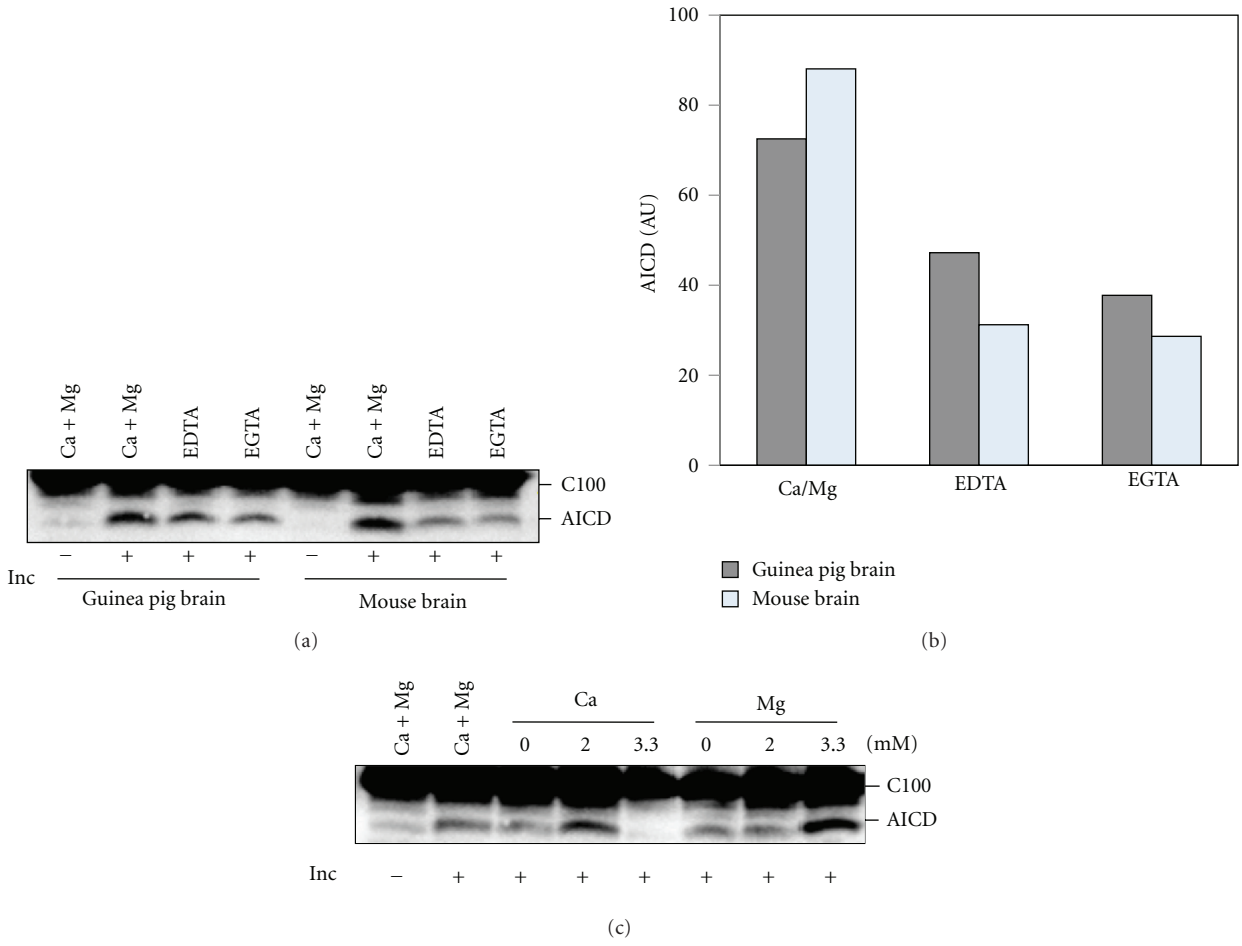


FIGURE 4: Effect of Ca^{2+} and Mg^{2+} on γ-secretase activity. (a) The effects of EDTA (2 mM) and EGTA (2 mM) were compared in assays carried out with γ-secretase preparations from guinea pig or mouse brains. (b) Quantitative analysis of the blot shown in (a). (c) γ-secretase assay with guinea pig brain activity was carried out in HEPES buffer plus 150 mM KCl, in the presence of increasing $CaCl_2$ or $MgCl_2$ concentrations as indicated.

3.5. Calcium and Magnesium Ions Stabilize a High Molecular Weight γ-Secretase Complex. To determine if calcium and magnesium ions had an effect on the size and stability of γ-secretase complexes, SH-SY5Y membrane-extracts diluted with 20 mM HEPES, and 150 mM KCl (pH 7.3) to a CHAPSO concentration of 0.5% were incubated in the presence of either 2 mM EDTA, 2 mM $MgCl_2$, or 2 mM $CaCl_2$, and analyzed by BN-PAGE and western blotting for PS1 (Figure 5(a)). In HEPES buffer, with no added calcium or magnesium, and in the presence of EDTA or EGTA, PS1 was found mostly associated with a 350 kDa complex, and a minor amount with a 450 kDa complex. In the incubations with Mg^{2+}, with or without ATP, presenilin was found associated with a ~450 kDa complex and also, in a small proportion with a 1,000 kDa complex. In the presence of Ca^{2+}, PS1 was found mostly associated with the ~450 kDa complex. These data suggest that Ca^{2+} and Mg^{2+} ions contribute to stabilization of γ-secretase complexes, possibly in association with binding partners. Further analysis of PS1 γ-secretase complex sizes by gel filtration on a Superose-6 column (Figure 5(b)) showed that, in the presence of Mg^{2+} ions, most PS1 immunoreactivity is recovered in a symmetrical peak in fractions corresponding to ~1,000 kDa. In the presence of EDTA, the peak of PS1 immunoreactivity was less sharp and broader, suggesting decreased complex stability.

4. Discussion

Biometal ions and metalloenzymes play an important role in the metabolism of APP and Aβ, and the pharmaco-modulation of copper levels in the brain represents a promising therapeutic approach to treat AD [18]. Little is known about the effect of metal ions on γ-secretase activity. We found that the copper-selective chelator, clioquinol did not significantly alter AICD production, suggesting that copper is not directly involved in γ-secretase cleavage of the APP substrate. This finding is consistent with our previous report that CQ does not alter production of Aβ in CHO-APP cells [28]. The zinc chelators, thiorphan and phosphoramidon, and the α-secretase inhibitor, ilomastat, showed no significant effect on the γ-secretase reaction.

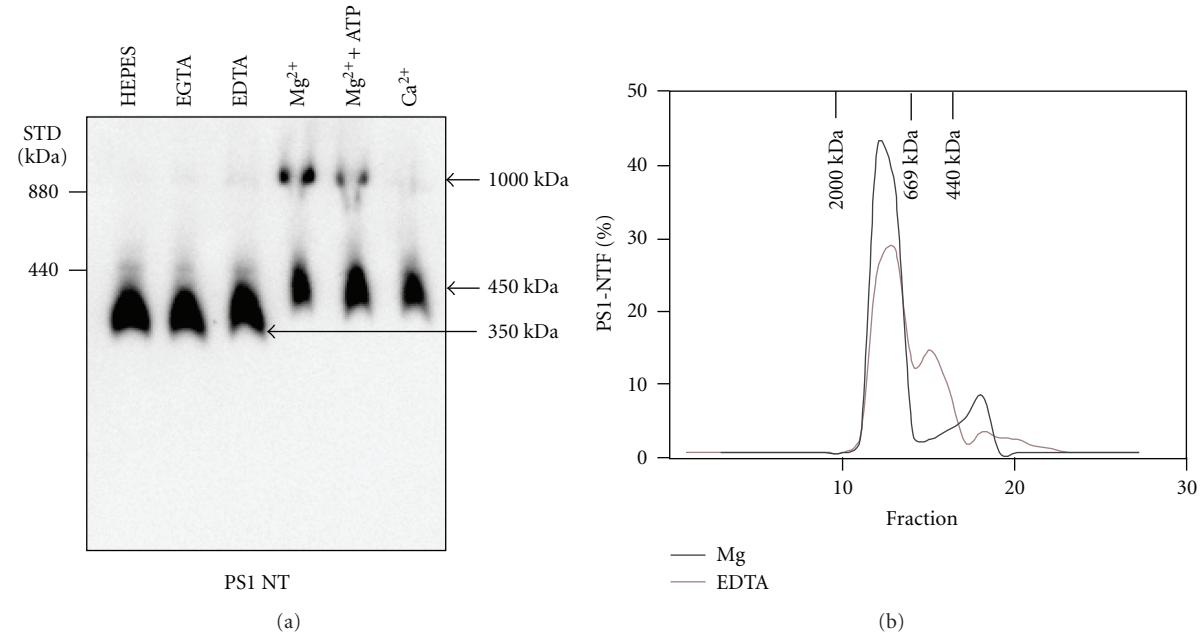


Figure 5: Effect of Ca^{2+} and Mg^{2+} on the size of γ-secretase complexes analyzed by BNPAGE and size-exclusion chromatography. 10 μg samples of protein extract from SHSY5Y membranes were incubated with HEPES buffer, pH 7.3, with or without additives, as indicated. EGTA and EDTA concentrations were 2 mM. $CaCl_2$ and $MgCl_2$ concentrations were 3 mM. (a) The incubations were mixed with BN-PAGE sample buffer and electrophoresed on 3–8% Tris-acetate gels. Proteins were transferred to PVDF membrane and the blot probed with anti-PS1 antibody 98/1. (b) SH-SY5Y membranes (200 μg per experiment) were extracted with 0.5% CHAPSO in buffer containing either 2 mM EDTA or 3 mM $MgCl_2$. The samples were chromatographed on superose-6 size-exclusion column and eluted fractions were analysed by western blotting with PS1 N-terminal antibody 98/1. Western blot signals were quantified and plotted against fraction numbers.

In contrast, γ-secretase activity was reduced in the presence of EDTA and phenanthroline. EGTA had an effect comparable to that of EDTA, indicating the possible involvement of calcium ions in the assay. Adding back calcium or magnesium to incubations carried out in the presence of EDTA showed that both enhanced AICD production.

In vitro γ-secretase assays described in the literature are carried out in alternative conditions, either in buffers containing EDTA [42, 46], or in buffers supplemented with Ca^{2+} and Mg^{2+} [39, 40, 43–45]. The γ-secretase assays based on Aβ detection are usually carried out in the presence of EDTA, probably to prevent metal-dependent self-aggregation of the peptide that would interfere with antibody capture in immunoassays, whereas the assays based on western blot detection of AICD have been preferably carried out in the presence of calcium and magnesium ions. Parallel incubations in EDTA buffer and in buffer supplemented with $CaCl_2$, and $MgCl_2$, revealed that AICD production was lower by 70–78% in the presence of EDTA than in the presence of Ca^{2+} and Mg^{2+}. Aβ levels, detected by ELISA (Figure 3(c)), were also higher in the presence of Ca^{2+} and Mg^{2+}, suggesting that the same effect of these ions applies to Aβ and AICD production.

To investigate whether calcium and magnesium ions influence the stability of γ-secretase complex, we used both BN-PAGE analysis and gel filtration. These techniques have been previously used to demonstrate that γ-secretase activity associates with high molecular weight complexes [44, 47]. In the present paper, fractionation of γ-secretase preparations by BN-PAGE suggested that Ca^{2+} and Mg^{2+} ions stabilized association of PS1 with high molecular weight complexes of 1,000 and 450 kDa, compared to ~ 350 kDa complexes observed in the presence of EDTA and EGTA. When incubations with EDTA and without Ca^{2+} or Mg^{2+}were treated with a cross-linker prior to BN-PAGE, the ~1,000 complex was also detected in these incubations (data not shown), suggesting that this 1,000 kDa form was normally present but was less stable through electrophoresis in the absence of Ca^{2+} and Mg^{2+} ions, and without cross-linking. Size-exclusion chromatography also suggested that Mg^{2+} ions stabilized HMW γ-secretase complexes. Although only the four subunits, PS, nicastrin, Aph1, and Pen-2 are required for γ-secretase activity and formation of a complex of ~350 kDa, many proteins and other molecules such as phospholipid and ATP have also been shown to associate with γ-secretase complexes and to modulate their activity [48]. For instance, a recent paper reports that PION protein modulates the specificity of γ-secretase towards APP [49]. Three-dimensional reconstruction of the γ-secretase structure from electron micrographs of purified γ-secretase has shown the presence of two weak-density lateral regions [50] and it is tempting to speculate that these could accommodate metal ions. Magnesium ions form complexes with ATP and could mediate the interaction between ATP and presenilin [40]. Alternatively, calcium and magnesium

may play an accessory role by ordering membrane phospholipids. Indeed, it has been reported that substituting EDTA-containing buffer for a buffer supplemented with calcium and magnesium help stabilize and isolate lipid rafts [51]. γ-Secretase components associate with lipid rafts, cholesterol-rich membrane regions where high γ-secretase-specific activity has been detected [52, 53]. Furthermore, experimental evidence indicates that lipids are important for γ-secretase activity [44]. Therefore, calcium and magnesium may facilitate movement of membrane domains and substrate access to the γ-secretase active site. Structural analysis by solid-state NMR and electron microscopy has demonstrated that cations modulate phospholipid bicelle size and that $MgCl_2$ and $CaCl_2$ stabilize large diameter (500 Å) bicelle discs [54]. Thus, the increase in molecular mass of γ-secretase that was observed when adding divalent cations may be consistent with the association of a larger number of detergent and phospholipid molecules with the γ-secretase complex.

Considering that Aβ has been shown to form membrane pores and disrupt neuronal calcium homeostasis by increasing Ca^{2+} influx [55], elevated Aβ could contribute to increased intraneuronal $[Ca^{2+}]$ that might in turn increase γ-secretase activity and Aβ production. Thus, it will be important to clarify how calcium and magnesium tune γ-secretase activity as this may have potential implications in AD pathogenesis.

5. Conclusions

Our study indicates that copper and zinc chelators have no direct effect on γ-secretase activity *in vitro*. It also demonstrates that chelators of broad specificity decrease cleavage of APP C100 substrate. Furthermore, it shows that Ca^{2+} and Mg^{2+} ions facilitate AICD production and contribute to stabilizing HMW γ-secretase complexes. This finding suggests that Ca^{2+} and Mg^{2} mediate molecular associations that modulate γ-secretase activity.

Abbreviations

Aβ: amyloid-β peptide
AICD: APP intracellular domain
APP: amyloid-β precursor protein
ATP: adenosine 5′-triphosphate
BN-PAGE: blue native polyacrylamide gel electrophoresis
C99: 99 amino acid carboxyl terminal fragment of APP
CHAPSO: [3-[(3-cholamidopropyl)dimethylammonio-]-2-hydroxy-1-propanesulfonate]
CTF: C-terminal fragment
CQ: 5-chloro7-iodo-8-hydroxyquinoline, or clioquinol
DMSO: dimethyl sulfoxide
DSP: dithiobis[succinimidylpropionate]
EGTA: ethylene glycol-bis[2-aminoethyl ether]-N,N,N′,N′-tetraacetic acid
HEPES: N-(2-hydroxyethyl)piperazine-N′-2-ethanesulfonic acid
MMP: matrix metalloprotease
PC: phosphatidylcholine
PE: phosphotidyl-ethanolamine
PS: presenilin
PVDF: polyvinylidene fluoride
SDS: sodium dodecylsulfate.

Acknowledgments

This study was supported in part by the Australian NHMRC (project Grants 400073 and 566520 to G. Evin and J. G. Culvenor). The authors thank Barbara Solchenberger for technical help with some parts of the study and Katrina Laughton for Aβ ELISA.

References

[1] G. Evin, M. F. Sernee, and C. L. Masters, "Inhibition of γ-secretase as a therapeutic intervention for Alzheimer's disease: prospects, limitations and strategies," *CNS Drugs*, vol. 20, no. 5, pp. 351–372, 2006.

[2] J. Kang, H.-G. Lemaire, A. Unterbeck et al., "The precursor of Alzheimer's disease amyloid A4 protein resembles a cell-surface receptor," *Nature*, vol. 325, no. 6106, pp. 733–736, 1987.

[3] R. Vassar, "BACE1: the β-secreiase enzyme in Alzheimer's disease," *Journal of Molecular Neuroscience*, vol. 23, no. 1-2, pp. 105–113, 2004.

[4] A. Weidemann, S. Eggert, F. B. M. Reinhard et al., "A novel ε-cleavage within the transmembrane domain of the Alzheimer amyloid precursor protein demonstrates homology with Notch processing," *Biochemistry*, vol. 41, no. 8, pp. 2825–2835, 2002.

[5] X. Cao and T. C. Südhof, "A transcriptivety active complex of APP with Fe65 and histone acetyltransferase Tip60," *Science*, vol. 293, no. 5527, pp. 115–120, 2001.

[6] G. Zhao, M. Z. Cui, G. Mao et al., "γ-cleavage is dependent on ζ-cleavage during the proteolytic processing of amyloid precursor protein within its transmembrane domain," *Journal of Biological Chemistry*, vol. 280, no. 45, pp. 37689–37697, 2005.

[7] Y. Qi-Takahara, M. Morishima-Kawashima, Y. Tanimura et al., "Longer forms of amyloid β protein: implications for the mechanism of intramembrane cleavage by γ-secretase," *Journal of Neuroscience*, vol. 25, no. 2, pp. 436–445, 2005.

[8] G. Evin and A. Weidemann, "Biogenesis and metabolism of Alzheimer's disease Aβ amyloid peptides," *Peptides*, vol. 23, no. 7, pp. 1285–1297, 2002.

[9] T. Iwatsubo, "The γ-secretase complex: machinery for intramembrane proteolysis," *Current Opinion in Neurobiology*, vol. 14, no. 3, pp. 379–383, 2004.

[10] B. De Strooper, P. Saftig, K. Craessaerts et al., "Deficiency of presenilin-1 inhibits the normal cleavage of amyloid precursor protein," *Nature*, vol. 391, no. 6665, pp. 387–390, 1998.

[11] M. S. Wolfe, W. Xia, B. L. Ostaszewski, T. S. Diehl, W. T. Kimberly, and D. J. Selkoe, "Two transmembrane aspartates in presenilin-1 required for presenilin endoproteolysis and γ-secretase activity," *Nature*, vol. 398, no. 6727, pp. 513–517, 1999.

[12] A. I. Bush, G. Multhaup, R. D. Moir et al., "A novel zinc(II) binding site modulates the function of the βA4 amyloid protein precursor of Alzheimer's disease," *Journal of Biological Chemistry*, vol. 268, no. 22, pp. 16109–16112, 1993.

[13] A. I. Bush, W. H. Pettingell, M. D. Paradis, and R. E. Tanzi, "Modulation of Aβ adhesiveness and secretase site cleavage by zinc," *Journal of Biological Chemistry*, vol. 269, no. 16, pp. 12152–12158, 1994.

[14] L. Hesse, D. Beher, C. L. Masters, and G. Multhaup, "The βA4 amyloid precursor protein binding to copper," *FEBS Letters*, vol. 349, no. 1, pp. 109–116, 1994.

[15] G. Multhaup, A. Schlicksupp, L. Hesse et al., "The amyloid precursor protein of Alzheimer's disease in the reduction of copper(II) to copper(I)," *Science*, vol. 271, no. 5254, pp. 1406–1409, 1996.

[16] A. R. White, G. Multhaup, F. Maher et al., "The Alzheimer's disease amyloid precursor protein modulates copper-induced toxicity and oxidative stress in primary neuronal cultures," *Journal of Neuroscience*, vol. 19, no. 21, pp. 9170–9179, 1999.

[17] X. Huang, C. S. Atwood, M. A. Hartshorn et al., "The Aβ peptide of Alzheimer's disease directly produces hydrogen peroxide through metal ion reduction," *Biochemistry*, vol. 38, no. 24, pp. 7609–7616, 1999.

[18] P. J. Crouch, A. R. White, and A. I. Bush, "The modulation of metal bio-availability as a therapeutic strategy for the treatment of Alzheimer's disease," *FEBS Journal*, vol. 274, no. 15, pp. 3775–3783, 2007.

[19] R. A. Cherny, C. S. Atwood, M. E. Xilinas et al., "Treatment with a copper-zinc chelator markedly and rapidly inhibits β-amyloid accumulation in Alzheimer's disease transgenic mice," *Neuron*, vol. 30, no. 3, pp. 665–676, 2001.

[20] D. J. Selkoe, "Clearing the brain's amyloid cobwebs," *Neuron*, vol. 32, no. 2, pp. 177–180, 2001.

[21] A. Mukherjee, E. S. Song, M. Kihiko-Ehmann et al., "Insulysin hydrolyzes amyloid β peptides to products that are neither neurotoxic nor deposit on amyloid plaques," *Journal of Neuroscience*, vol. 20, no. 23, pp. 8745–8749, 2000.

[22] V. Chesneau, K. Vekrellis, M. R. Rosner, and D. J. Selkoe, "Purified recombinant insulin-degrading enzyme degrades amyloid β-protein but does not promote its oligomerization," *Biochemical Journal*, vol. 351, no. 2, pp. 509–516, 2000.

[23] D. Edbauer, M. Willem, S. Lammich, H. Steiner, and C. Haass, "Insulin-degrading enzyme rapidly removes the β-amyloid precursor protein intracellular domain (AICD)," *Journal of Biological Chemistry*, vol. 277, no. 16, pp. 13389–13393, 2002.

[24] W. Farris, S. Mansourian, Y. Chang et al., "Insulin-degrading enzyme regulates the levels of insulin, amyloid β-protein, and the β-amyloid precursor protein intracellular domain in vivo," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 100, no. 7, pp. 4162–4167, 2003.

[25] N. Iwata, S. Tsubuki, Y. Takaki et al., "Identification of the major Aβ-degrading catabolic pathway in brain parenchyma: suppression leads to biochemical and pathological deposition," *Nature Medicine*, vol. 6, no. 2, pp. 143–150, 2000.

[26] R. A. Marr, H. Guan, E. Rockenstein et al., "Neprilysin regulates amyloid β peptide levels," *Journal of Molecular Neuroscience*, vol. 22, no. 1-2, pp. 5–11, 2004.

[27] K. Shirotani, S. Tsubuki, N. Iwata et al., "Neprilysin degrades both amyloid β peptides 1-40 and 1-42 most rapidly and efficiently among thiorphan- and phosphoramidon-sensitive endopeptidases," *Journal of Biological Chemistry*, vol. 276, no. 24, pp. 21895–21901, 2001.

[28] A. R. White, T. Du, K. M. Laughton et al., "Degradation of the Alzheimer disease amyloid β-peptide by metal-dependent up-regulation of metalloprotease activity," *Journal of Biological Chemistry*, vol. 281, no. 26, pp. 17670–17680, 2006.

[29] K. J. Yin, J. R. Cirrito, P. Yan et al., "Matrix metalloproteinases expressed by astrocytes mediate extracellular amyloid-β peptide catabolism," *Journal of Neuroscience*, vol. 26, no. 43, pp. 10939–10948, 2006.

[30] P. Yan, X. Hu, H. Song et al., "Matrix metalloproteinase-9 degrades amyloid-β fibrils in vitro and compact plaques in situ," *Journal of Biological Chemistry*, vol. 281, no. 34, pp. 24566–24574, 2006.

[31] S. Lammich, E. Kojro, R. Postina et al., "Constitutive and regulated α-secretase cleavage of Alzheimer's amyloid precursor protein by a disintegrin metalloprotease," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 96, no. 7, pp. 3922–3927, 1999.

[32] B. Angeletti, K. J. Waldron, K. B. Freeman et al., "BACE1 cytoplasmic domain interacts with the copper chaperone for superoxide dismutase-1 and binds copper," *Journal of Biological Chemistry*, vol. 280, no. 18, pp. 17930–17937, 2005.

[33] D. E. Hoke, J. L. Tan, N. T. Ilaya et al., "In vitro γ-secretase cleavage of the Alzheimer's amyloid precursor protein correlates to a subset of presenilin complexes and is inhibited by zinc," *FEBS Journal*, vol. 272, no. 21, pp. 5544–5557, 2005.

[34] A. J. George, R. M. D. Holsinger, C. A. McLean et al., "APP intracellular domain is increased and soluble Aβ is reduced with diet-induced hypercholesterolemia in a transgenic mouse model of Alzheimer disease," *Neurobiology of Disease*, vol. 16, no. 1, pp. 124–132, 2004.

[35] G. Evin, L. D. Canterford, D. E. Hoke, R. A. Sharples, J. G. Culvenor, and C. L. Masters, "Transition-state analogue γ-secretase inhibitors stabilize a 900 kDa presenilin/nicastrin complex," *Biochemistry*, vol. 44, no. 11, pp. 4332–4341, 2005.

[36] J. G. Culvenor, G. Evin, M. A. Cooney et al., "Presenilin 2 expression in neuronal cells: induction during differentiation of embryonic carcinoma cells," *Experimental Cell Research*, vol. 255, no. 2, pp. 192–206, 2000.

[37] M. S. Shearman, D. Beher, E. E. Clarke et al., "L-685,458, an aspartyl protease transition state mimic, is a potent inhibitor of amyloid β-protein precursor γ-secretase activity," *Biochemistry*, vol. 39, no. 30, pp. 8698–8704, 2000.

[38] H. F. Dovey, V. John, J. P. Anderson et al., "Functional γ-secretase inhibitors reduce β-amyloid peptide levels in brain," *Journal of Neurochemistry*, vol. 76, no. 1, pp. 173–181, 2001.

[39] P. C. Fraering, W. Ye, J. M. Strub et al., "Purification and characterization of the human γ-secretase complex," *Biochemistry*, vol. 43, no. 30, pp. 9774–9789, 2004.

[40] J. D. J. Wrigley, I. Schurov, E. J. Nunn et al., "Functional overexpression of γ-secretase reveals protease-independent trafficking functions and a critical role of lipids for protease activity," *Journal of Biological Chemistry*, vol. 280, no. 13, pp. 12523–12535, 2005.

[41] P. C. Fraering, W. Ye, M. J. LaVoie, B. L. Ostaszewski, D. J. Selkoe, and M. S. Wolfe, "γ-secretase substrate selectivity can be modulated directly via interaction with a nucleotide-binding site," *Journal of Biological Chemistry*, vol. 280, no. 51, pp. 41987–41996, 2005.

[42] D. Beher, M. Fricker, A. Nadin et al., "In vitro characterization of the presenilin-dependent γ-secretase complex using a novel affinity ligand," *Biochemistry*, vol. 42, no. 27, pp. 8133–8142, 2003.

[43] S. Shah, S. F. Lee, K. Tabuchi et al., "Nicastrin functions as a γ-secretase-substrate receptor," *Cell*, vol. 122, no. 3, pp. 435–447, 2005.

[44] Y. M. Li, M. T. Lai, M. Xu et al., "Presenilin 1 is linked with γ-secretase activity in the detergent solubilized state," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 97, no. 11, pp. 6138–6143, 2000.

[45] W. T. Kimberly, W. P. Esler, W. Ye et al., "Notch and the amyloid precursor protein are cleaved by similar γ-secretase(s)," *Biochemistry*, vol. 42, no. 1, pp. 137–144, 2003.

[46] G. Tian, S. V. Ghanekar, D. Aharony et al., "The mechanism of γ-secretase: multiple inhibitor binding sites for transition state analogs and small molecule inhibitors," *Journal of Biological Chemistry*, vol. 278, no. 31, pp. 28968–28975, 2003.

[47] T. Ogura, K. Mio, I. Hayashi et al., "Three-dimensional structure of the ^{3}-secretase complex," *Biochemical and Biophysical Research Communications*, vol. 343, no. 2, pp. 525–534, 2006.

[48] T. Wakabayashi, K. Craessaerts, L. Bammens et al., "Analysis of the γ-secretase interactome and validation of its association with tetraspanin-enriched microdomains," *Nature Cell Biology*, vol. 11, no. 11, pp. 1340–1346, 2009.

[49] G. He, W. Luo, P. Li et al., "γ-secretase activating protein is a therapeutic target for Alzheimer's disease," *Nature*, vol. 467, no. 7311, pp. 95–98, 2010.

[50] V. K. Lazarov, P. C. Fraering, W. Ye, M. S. Wolfe, D. J. Selkoe, and H. Li, "Electron microscopic structure of purified, active γ-secretase reveals an aqueous intramembrane chamber and two pores," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 103, no. 18, pp. 6889–6894, 2006.

[51] J. L. Macdonald and L. J. Pike, "A simplified method for the preparation of detergent-free lipid rafts," *Journal of Lipid Research*, vol. 46, no. 5, pp. 1061–1067, 2005.

[52] S. Wahrle, P. Das, A. C. Nyborg et al., "Cholesterol-dependent γ-secretase activity in buoyant cholesterol-rich membrane microdomains," *Neurobiology of Disease*, vol. 9, no. 1, pp. 11–23, 2002.

[53] Y. Urano, I. Hayashi, N. Isoo et al., "Association of active γ-secretase complex with lipid rafts," *Journal of Lipid Research*, vol. 46, no. 5, pp. 904–912, 2005.

[54] A. Arnold, T. Labrot, R. Oda, and E. J. Dufourc, "Cation modulation of bicelle size and magnetic alignment as revealed by solid-state NMR and electron microscopy," *Biophysical Journal*, vol. 83, no. 5, pp. 2667–2680, 2002.

[55] D. H. Small, R. Gasperini, A. J. Vincent, A. C. Hung, and L. Foa, "The role of Aβ-induced calcium dysregulation in the pathogenesis of Alzheimer's disease," *Journal of Alzheimer's Disease*, vol. 16, no. 2, pp. 225–233, 2009.

SAGE-Hindawi Access to Research
International Journal of Alzheimer's Disease
Volume 2011, Article ID 231595, 6 pages
doi:10.4061/2011/231595

Research Article

Ceruloplasmin/Transferrin Ratio Changes in Alzheimer's Disease

Rosanna Squitti,[1,2] Carlo Salustri,[3] Mariacristina Siotto,[2] Mariacarla Ventriglia,[1,2] Fabrizio Vernieri,[2] Domenico Lupoi,[4] Emanuele Cassetta,[1] and Paolo M. Rossini[2,5]

[1] *Department of Neuroscience, AFaR—Fatebenefratelli Hospital, 00186 Rome, Italy*
[2] *Department of Neurology, Campus Bio-Medico University, 00128 Rome, Italy*
[3] *Institute of Cognitive Sciences and Technologies (CNR), Fatebenefratelli Hospital, 00186 Rome, Italy*
[4] *Department of Radiology, Fatebenefratelli Hospital, 00186 Rome, Italy*
[5] *Department of Imaging, San Raffaele Cassino, Via G. Di Biasio 1, 03043 Cassino(FR), Italy*

Correspondence should be addressed to Rosanna Squitti, rosanna.squitti@afar.it

Received 24 September 2010; Accepted 22 November 2010

Academic Editor: Anthony R. White

Copyright © 2011 Rosanna Squitti et al. This is an open access article distributed under the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original work is properly cited.

The link between iron and Alzheimer's disease (AD) has been mainly investigated with a focus on the local accumulation of this metal in specific areas of the brain that are critical for AD. In the present study, we have instead looked at systemic variations of markers of iron metabolism. We measured serum levels of iron, ceruloplasmin, and transferrin and calculated the transferrin saturation and the ceruloplasmin to transferrin ratio (Cp/Tf). Cp/Tf and transferrin saturation increased in AD patients. Cp/Tf ratios also correlated positively with peroxide levels and negatively with serum iron concentrations. Elevated values of ceruloplasmin, peroxides, and Cp/Tf inversely correlated with MMSE scores. Isolated medial temporal lobe atrophy positively correlated with Cp/Tf and negatively with serum iron. All these findings indicate that the local iron accumulation found in brain areas critical for AD should be viewed in the frame of iron systemic alterations.

1. Introduction

Alzheimer's disease (AD) is a heterogeneous and progressive neurodegenerative disorder representing the most common cause of dementia in the elderly. The disease results from a complex interaction between predisposing genes, biochemical variables, and environmental factors [1]. The variant E4 of the APOE gene (APOE4), for example, is widely recognized to contribute to the risk of developing the more common late-onset AD [2], while mutations in the genes that encode presenilin 1 and 2 and the amyloid precursor protein (APP) are known to be causative factors of a small percentage of familial and early-onset AD cases.

There is also total agreement on the existence of a link between AD and oxidative stress phenomena triggered by transition metals. However, these phenomena have been generally viewed as originating locally within specific brain areas, and autonomously, that is, independently of systemic influences. In this frame, authors have reported enhanced iron concentrations in AD brains, both in autopsy brain tissues and in cerebrospinal fluid (CSF) [3, 4], especially in the basal ganglia (in vivo: [5, 6] autopsy: [7]), in the hippocampus (in vivo: [6, 8]; autopsy: [9, 10]), neocortex (autopsy: [7, 9, 11]), and around the senile plaques of amyloid beta (Aβ) or the neurofibrillary tangles commonly found in AD brains [12–15]. Authors have also reported altered local concentrations of specific proteins regulating iron levels, such as ceruloplasmin [16–18], transferrin [7, 19], and ferritin [19].

Recently, a wider and somewhat complementary view has emerged suggesting a relationship of AD with systemic, rather than autonomous, changes of metal metabolisms. Some pilot studies based on this view have focused primarily on the genes of hemochromatosis (HFE) and of transferrin, since they play a key role in iron homeostasis, but investigations on circulating markers of iron metabolism are still relatively scanty.

In the present study, we focused on this aspect and studied systemic variations of markers of iron metabolism, such as serum iron, ceruloplasmin, transferrin, transferrin saturation, and ratio of ceruloplasmin to transferrin (Cp/Tf), in a sample of AD patients and in one of healthy elderly subjects.

2. Subjects and Methods

Forty-nine AD patients (mean age 75.6) and 46 cognitively normal individuals (mean age 71.2) were included in the study (see Table 1). The two samples were slightly different in sex and age; consequently, when appropriate, all statistical analyses were adjusted for these two confounders. The AD patient sample consisted of individuals with a clinical diagnosis of probable AD based on NINCDS-ADRDA criteria [20] and with a Mini-Mental State Examination (MMSE) [21] score of 25 or less. All AD patients underwent neurologic, neuroimaging (magnetic resonance imaging: MRI or computed tomography: CT), and neuropsychological evaluation, as well as routine laboratory tests. Average disease duration (from symptom onset) was 27 (range 6–96) months.

The control sample consisted of elderly volunteers with no clinical evidence of neurologic or psychiatric disease.

Twelve AD patients had either poor quality MRI or reasons not to take it (e.g., metal prostheses). In these cases, a CT scan was taken to rule out other causes of dementia. Six AD patients did not complete the entire battery of neuropsychological tests. The same AD cohort had been investigated in a previous study [22].

All controls underwent a neurologic examination and MMSE, but only 12 consented to MRI.

Criteria for exclusion of both patients and controls were conditions known to affect metal metabolism and biological variables of oxidative stress, for example, diabetes mellitus, inflammatory diseases, Hodgkin's disease, recent history of heart or respiratory failure, chronic liver or renal failure, malignant tumors, and alcohol abuse, assessed by tests including complete blood count, erythrocyte sedimentation rate (ESR), cholesterol, triglycerides, ferritin and fibrinogen levels, serum protein electrophoresis, renal function (creatinine, blood urea nitrogen), fasting glucose, electrolytes, vitamin B12, and folic acid and uric acid levels; thyroid function tests (thyroxin, triiodothyronine, and thyrotropin levels); liver enzymes (transaminases), cardiac enzymes (creatine phosphokinase, lactate dehydrogenase), bilirubin levels; serology for syphilis, and urine analysis.

Patients and controls with abnormal values of thyroid, liver, kidney, and cardiac functions were also excluded from the study.

The study was approved by the local IRB, and all participants or legal guardians gave written, informed consent.

2.1. Biochemical and Molecular Investigations. Sera from fasting blood samples were collected in the morning and rapidly stored at –80°C. Methods for measuring biological variables of metals and oxidative stress are described in detail elsewhere [23]. Briefly, hydroperoxide content was assessed by d-ROMs test (Diacron, Italy) and expressed in arbitrary units (U.CARR), 1 U.CARR corresponding to 0.08 mg/100 mL of hydrogen peroxide. Normal range was between 230 and 310 U.CARR [23]. Transferrin and ceruloplasmin were measured by immunoturbidimetric assays (Roche, Diagnostic, Germany) utilizing a goat antihuman transferrin antibody in TRIS/HCl buffer and a rabbit antihuman ceruloplasmin antibody in phosphate buffer and iron using Ferene [20, 21].

All biochemical measures were automated on a Hitachi 912 analyser (Roche Diagnostics) and performed in duplicate. APOE genotyping was performed according to established methods [24].

Table 1: Characteristics of the study groups.

	AD patients	Controls	Overall significance
Number of subjects	49	46	
Sex M/F	11/38	25/21	$\chi^2 = 10.15$; df = 1 $P = .001$
Age (years) Mean (SD)	75.6 (7.7)	71.2 (10.8)	t test; $P = .023$
ApoE $\varepsilon 4$ allele frequency (%)	19.6	4	Pearson's = 6.4 $P = .012$
MMSE Mean (SD)	19 (3.9)	28.2 (1.2)	t test, $P < .001$
Disease duration (months) Median (min-max)	27 (6–96)		

2.2. MRI Evaluation. Brain MRI was performed using a 1.5 Tesla superconductor magnet. The imaging protocol consisted of axial T2 W double Spin Echo (SE) sequences and T1 W SE images in axial, coronal, and sagittal planes, with 5 mm slice thickness and intersection gap = 0.5 mm. MR images were evaluated by two experienced neuroradiologists, blind to the patients' diagnoses and laboratory results, with an agreement of about 95%. Atrophy and white matter lesions were graded following standardized visual rating scales on plain MRI [25–27]. The degree of medial temporal lobe atrophy (MTA) was evaluated with a ranking procedure and validated by linear measurements of the medial temporal lobe including the hippocampal formation and surrounding spaces occupied by CSF, following standardized criteria (five-point rating scale of MTA) [26]. Generalized brain atrophy (ventricular and sulcal atrophy) was rated as present (=1) or absent (=0; global atrophy). The visual rating scale of white matter changes included the anatomical distribution as well as the severity of the lesions. Based on anatomical distribution, a distinction was made between areas of periventricular hyperintensities (PVH) (caps and rims) and deeper hyperintensities (including frontal, parieto-occipital, and temporal white matter hyperintensities: DWMH, basal

ganglia hyperintensities: BGH, and infratentorial hyperintensities: ITH). Large vessel cortical infarcts in the anterior, posterior, and medial cerebral artery were also evaluated. Presence of mass lesions and lobar hemorrhages was an exclusion criteria from the study [27, 28].

2.3. Ultrasonographic Examination of the Cerebral Vessels. The carotid and vertebral arteries were studied by means of color-coded duplex ultrasonography (7.5 MHz probe; Acuson, Aspen, USA) according to standardized criteria [29]. Detailed description of the sonographic procedures has been previously reported [30]. Particular attention was paid to detecting both presence and degree of stenosis of atherosclerotic plaques in the carotid and vertebral arteries. The intima and media thickness (IMT) from the distal portion of the CCA was investigated in detail in each patient according to previous studies [31]. Intracranial vessels were also examined by transcranial Doppler (TCD Multidop T TCD-DWL, Germany), according to previously described methods [32].

2.4. Statistical Analyses. AD and controls were described in terms of main demographic, genetic, and cognitive characteristics and statistically compared with either T- or χ^2-square tests. Both age and sex effects were considered in the statistical analyses. Correlation analyses between biological variables, MMSE scores, MRI atrophy, and vascular measurements (Spearman's rho) were performed. A P value less than .05 was considered significant in all statistical analyses. The t test was used when the homogeneity of variances could be assumed. When the variances were statistically heterogeneous, the Games-Howell procedure was applied.

We used the SPSS 11.5 for Windows statistical software package (SPSS Inc., Chicago).

3. Results

The two groups differed for MMSE scores ($P < .001$). No differences for fasting glucose, vitamin B12, folic acid and uric acid levels, ESR, bilirubin levels, cholesterol, triglycerides, albumin, complete blood count, and hypertension were detected between the two groups (data not shown). APOE $\varepsilon 4$ allele was more frequent in AD patients (Table 1).

None among the biological variables under study correlated with age, with the exception of ceruloplasmin ($r = 0.355$, $P = .01$) and peroxides ($r = 0.215$, $P = .039$), which both increased with age (these two variables were corrected for the age effect before entering the statistical analyses). No variations on sex effect were found in our data.

Table 2 reports the values of serum iron, ceruloplasmin, transferrin, transferrin saturation, and Cp/Tf in AD patients and controls. Transferrin saturation ($P = .033$) and Cp/Tf ($P < .001$) resulted increased in AD patients compared to controls (Table 2).

Cp/Tf correlated positively with peroxide levels ($r = 0.560$, $P < .001$) and negatively with serum iron concentrations ($r = -0.417$, $P < .001$). Elevated values of ceruloplasmin, peroxides, and Cp/Tf inversely correlated with MMSE scores (Table 3). Correlation analysis from the Doppler examination revealed that no AD patient had abnormal flow velocity in the cerebral arteries examined by Duplex sonography and TCD (results not shown). Moreover, none of the biochemical variables assayed correlated with ultrasound indicators of cerebrovascular burden, namely, presence of carotid plaque or stenosis, except for the IMT values that correlated with APOE $\varepsilon 4$ genotype (Spearman rho $= 0.36$; $P < .01$) confirming what was previously reported [20]. Analyses of MRI data showed that presence of supratentorial atrophy, (enlarged sulci, widened lateral, and third ventricles), and atrophy of the temporal lobes (reduction in hippocampal volume), was prominent in AD subjects, while control subjects' values were consistent with those found in normal aging. Among MRI measurements and serum oxidative-iron markers values, there was a negative correlation between isolated medial temporal atrophy (MTA) and serum iron and a positive one between Cp/Tf and MTA (Table 3). White matter changes and deep-seated ischemic changes did not correlate with any metal marker as well as peroxides.

Table 2: Serum levels of the investigated variables in AD patients and controls (means (SD)).

	AD patients ($n = 49$)	Controls ($n = 46$)	t test
Iron (ng/dL)	69.3 (27.79)	76.7 (23.6)	$P = .209$
Ceruloplasmin (mg/dL)	29.9 (5.8)	26.5 (4.6)	$P = .017$
Peroxides (U.CARR)	349 (63)	310 (56)	$P = .005$
Transferrin (g/L)	2.5 (0.52)	2.7 (0.35)	$P = .232$
Transferrin saturation (%)	33.9 (9.6)	30.5 (4.1)	$P = .03$
Cp/Tf	12.4 (3.1)	10 (2.1)	$P = .001$

After correction for age effect.

4. Discussion

The results of our comparison between AD patients and healthy controls show that major markers of circulating iron status, such as ceruloplasmin, Cp/Tf, transferrin saturation, and peroxides levels, are abnormal in AD patients. This finding demonstrates that systemic alterations of iron metabolism accompany the disease and indicate that the role attributed by existing literature to local iron accumulations in brain areas critical for AD should be rather viewed in the frame of a wider systemic alteration. In this scenario, antioxidant systems play of course a role. In the present AD study, as we did previously with stroke patients [33], we used the Cp/Tf ratio to represent the functionality of one of these systems, the Cp-Tf system [31], which is otherwise difficult to monitor biochemically [31].

Table 3: Correlations between outcomes of MMSE, MTA evaluation, and biological variables under study.

Spearman's rho correlations	MMSE	MTA
Iron	rho = 0.121 P = .364 n = 58	rho = −0.393 P = **.008** n = 45
Ceruloplasmin	rho = −0.265 P = **.040** n = 60	rho = 0.184 P = .225 n = 45
Peroxides	rho = −0.329 P = **.008** n = 64	rho = 0.184 P = .211 n = 48
Transferrin	rho = 0.113 P = .372 n = 65	rho = −0.269 P = .065 n = 48
Transferrin saturation	rho = −0.120 P = .336 n = 66	rho = 0.280 P = .051 n = 49
Ceruloplasmin/transferrin ratio	rho = −0.373 P = **.004** n = 59	rho = 0.300 P = **.048** n = 44

After correction for sex and age.

In the Cp-Tf system, ceruloplasmin catalyzes the oxidation of ferrous iron (Fe^{2+}) into ferric iron (Fe^{3+}), by way of the following chemical reaction:

$$4\,Fe^{2+} + 4\,H^{+} + O_2 \longrightarrow 4\,Fe^{3+} + 2\,H_2O. \quad (1)$$

This is generally viewed as a "good" reaction both because its byproduct is innocuous water and because, once transformed into Fe^{3+}, iron binds to transferrin by which it is transported to cells. Ceruloplasmin-catalyzed iron oxidation and removal from circulation by transferrin is a very important process since it reduces the amount of Fe^{2+} available for participation in Fenton's reactions, whose byproducts are instead extremely vicious. In fact, ferrous iron can also convert hydrogen peroxide into the highly reactive radicals $OH^{\bullet}$ and $OOH^{\bullet}$:

$$Fe^{2+} + H_2O_2 \longrightarrow Fe^{3+} + OH^{\bullet} + OH^{-} \quad (2)$$

which, in turn, proceeds as follows:

$$Fe^{3+} + H_2O_2 \longrightarrow Fe^{2+} + OOH^{\bullet} + H^{+}. \quad (3)$$

In other words, if it were not oxidized and removed by the coordinated ceruloplasmin and transferrin actions, iron would cyclically produce highly reactive radicals, which the circulating blood would then distribute all over the organism. Nonetheless, it is important to keep in mind that if, on one hand, this mechanism avoids or reduces production of lethal oxidative species in general circulation and allows the vital transport of iron to cells, on the other hand, when iron moves into the cells, as it could be the case for neurons, it can still cause brain damage if a concomitant neuronal iron defective efflux does occur [34]. Evidence of a defective iron efflux from neurons, linked to APP, was reported in several studies on cell cultures and APP knockout mice (APP−/−), in which APP expression is experimentally suppressed [34, 35]. The ablation of APP, which is normally expressed both in the brain and in a limited number of non-neural tissues as platelets, liver, kidney, and heart [34, 35], produces metal disbalance in these organs and tissues. In particular, some authors [35] demonstrated an increase of copper concentrations in the liver (80%) and in the brain (40%) in APP−/− mice. Other authors [34] demonstrated that APP ablation causes an iron increase of 26% in the brain, 31% in the liver, and 15% in the kidney. They also showed that, like ceruloplasmin, APP catalytically oxidizes Fe^{2+} [34]. All together, these studies demonstrate that APP plays a role in copper and iron mobilization, indicating that its abnormalities in AD have either a systemic effect on those metals' homeostasis or a local effect on neuronal metal efflux. Our current results of increased brain atrophy (MTA scores) in AD patients are in support to this concept, as MTA scores associate to either decreasing levels of serum iron or increased values of Cp/Tf ratios. Both these results appear coherent with the picture of an abnormal activation of the Cp-Tf system in AD, the subtraction of iron from general circulation, and its probable internalization into neural cells. This eventually could cause neuronal death via generation of oxidative stress in Aβ transition metal(s) reactions [2].

Finally, in the same direction go also our results on MMSE scores, which worsen with increasing Cp/Tf ratios.

Ceruloplasmin increase and transferrin decrease concentrations were identified in diverse pathological conditions as an important defense mechanism reflecting the body's resistance to an oxidant insult [36]. This is the case, for example, in severe preeclampsia, where women have high levels of lipid peroxidation and serum ceruloplasmin and low levels of transferrin [37, 38], in acute stroke, where the Cp/Tf correlates with the severity of the clinical status [33], or in experimental hypercholesterolemia [36].

Ceruloplasmin increase and transferrin decrease concentrations are also reported as a sign of inflammation in a number of conditions spanning from cardiac to psychiatric. In fact, these proteins are acute phase reactants (positive and negative, resp.), produced by the liver together with several other proteins.

Another protein affecting iron homeostasis is HFE, a membrane protein which controls iron absorption by regulating the affinity of transferrin receptors on cell membrane [39]. Specific mutations of the HFE gene cause hemochromatosis, that is, an increased absorption of dietary iron and its consequent over deposition in tissues and organs [40]. Recently, a number of studies have shown various relationships between AD and mutations of the HFE, as well as the transferrin, genes but reported data are controversial and far from univocal interpretation [41–46]. In a very recent study of ours, we found that the synergy of altered markers of iron status (iron levels, transferrin, transferrin saturation, ferritin, and Cp/Tf ratio) and mutations of the HFE gene can increase the probability of developing AD (submitted).

Overall, Cp-Tf antioxidant system represents the serum capacity to sequester exchangeable metals (copper, iron) [36]

which make a labile metal pool prone to partake in reactions generating oxidative damage [47, 48]. Previous studies of altered levels of this labile metal pool in AD, especially referred to a serum increase of "free" copper, that is, the quantity of copper which is not bound to ceruloplasmin, sustain this concept [49, 50]. Support to this notion comes also from a study of our laboratory showing lower cortical responses in depressed patients who presented lower transferrin and higher free copper serum levels [51].

As a whole the data presented are in agreement with the past key works by both Loeffer's [16] and Castellani's [17] groups, who reported higher ceruloplasmin levels in brain areas critical for AD. However, the relationship between systemic processes and local effects in the brain is the main focus of our laboratory's work in progress.

Conflict of Interests

All authors have no conflict of interests.

Acknowledgments

This study was partially supported by the following Grants: (i) Italian Health Department: "Profilo Biologico e Genetico della Disfunzione dei Metalli nella Malattia di Alzheimer e nel "Mild Cognitive Impairment" (RF 2006 conv.58); (ii) Programma Strategico 2007 conv. PS39, "Ricerca Corrente" grants IRCCS Brescia, Italy; (iii) ERA-Net NEURON (JTC 2008 nEUROsyn), AFaR Foundation—Osp. Fatebenefratelli; (iv) "AAMS l gioco sicuro."

References

[1] M. D. Kirkitadze and A. Kowalska, "Molecular mechanisms initiating amyloid β-fibril formation in Alzheimer's disease," *Acta Biochimica Polonica*, vol. 52, no. 2, pp. 417–423, 2005.

[2] D. Blacker, J. L. Haines, L. Rodes et al., "ApoE-4 and age at onset of Alzheimer's disease: the NIMH genetics initiative," *Neurology*, vol. 48, no. 1, pp. 139–147, 1997.

[3] M. Lavados, M. Guillón, M. C. Mujica, L. E. Rojo, P. Fuentes, and R. B. Maccioni, "Mild cognitive impairment and Alzheimer patients display different levels of redox-active CSF iron," *Journal of Alzheimer's Disease*, vol. 13, no. 2, pp. 225–232, 2008.

[4] M. A. Smith, X. Zhu, M. Tabaton et al., "Increased iron and free radical generation in preclinical Alzheimer disease and mild cognitive impairment," *Journal of Alzheimer's Disease*, vol. 19, no. 1, pp. 363–372, 2010.

[5] G. Bartzokis and T. A. Tishler, "MRI evaluation of basal ganglia ferritin iron and neurotoxicity in Alzheimer's and Huntingon's disease," *Cellular and Molecular Biology*, vol. 46, no. 4, pp. 821–833, 2000.

[6] B. Ding, KE. M. Chen, H. W. Ling et al., "Correlation of iron in the hippocampus with MMSE in patients with alzheimer's disease," *Journal of Magnetic Resonance Imaging*, vol. 29, no. 4, pp. 793–798, 2009.

[7] D. A. Loeffler, J. R. Connor, P. L. Juneau et al., "Transferrin and iron in normal, Alzheimer's disease, and Parkinson's disease brain regions," *Journal of Neurochemistry*, vol. 65, no. 2, pp. 710–724, 1995.

[8] J. F. Schenck, E. A. Zimmerman, Z. Li et al., "High-field magnetic resonance imaging of brain iron in Alzheimer disease," *Topics in Magnetic Resonance Imaging*, vol. 17, no. 1, pp. 41–50, 2006.

[9] C. R. Cornett, W. R. Markesbery, and W. D. Ehmann, "Imbalances of trace elements related to oxidative damage in Alzheimer's disease brain," *NeuroToxicology*, vol. 19, no. 3, pp. 339–346, 1998.

[10] M. A. Deibel, W. D. Ehmann, and W. R. Markesbery, "Copper, iron, and zinc imbalances in severely degenerated brain regions in Alzheimer's disease: possible relation to oxidative stress," *Journal of the Neurological Sciences*, vol. 143, no. 1-2, pp. 137–142, 1996.

[11] D. J. Dedman, A. Treffry, J. M. Candy et al., "Iron and aluminium in relation to brain ferritin in normal individuals and Alzheimer's-disease and chronic renal-dialysis patients," *Biochemical Journal*, vol. 287, no. 2, pp. 509–514, 1992.

[12] J. R. Connor, B. S. Snyder, J. L. Beard, R. E. Fine, and E. J. Mufson, "Regional distribution of iron and iron-regulatory proteins in the brain in aging and Alzheimer's disease," *Journal of Neuroscience Research*, vol. 31, no. 2, pp. 327–335, 1992.

[13] P. F. Good, D. P. Perl, L. M. Bierer, and J. Schmeidler, "Selective accumulation of aluminum and iron in the neurofibrillary tangles of Alzheimer's disease: a laser microprobe (LAMMA) study," *Annals of Neurology*, vol. 31, no. 3, pp. 286–292, 1992.

[14] K. Jellinger, W. Paulus, I. Grundke-Iqbal, P. Riederer, and M. B. H. Youdim, "Brain iron and ferritin in Parkinson's and Alzheimer's diseases," *Journal of Neural Transmission: Parkinson's Disease and Dementia Section*, vol. 2, no. 4, pp. 327–340, 1990.

[15] C. M. Morris, J. M. Kerwin, and J. A. Edwardson, "Non-haem iron histochemistry of the normal and Alzheimer's disease hippocampus," *Neurodegeneration*, vol. 3, no. 4, pp. 267–275, 1994.

[16] D. A. Loeffler, P. A. LeWitt, P. L. Juneau et al., "Increased regional brain concentrations of ceruloplasmin in neurodegenerative disorders," *Brain Research*, vol. 738, no. 2, pp. 265–274, 1996.

[17] R. J. Castellani, M. A. Smith, A. Nunomura, P. L. R. Harris, and G. Perry, "Is increased redox-active iron in Alzheimer disease a failure of the copper-binding protein ceruloplasmin?" *Free Radical Biology and Medicine*, vol. 26, no. 11-12, pp. 1508–1512, 1999.

[18] J. R. Connor, P. Tucker, M. Johnson, and B. Snyder, "Ceruloplasmin levels in the human superior temporal gyrus in aging and Alzheimer's disease," *Neuroscience Letters*, vol. 159, no. 1-2, pp. 88–90, 1993.

[19] J. R. Connor, S. L. Menzies, S. M. ST. Martin, and E. J. Mufson, "A histochemical study of iron, transferrin, and ferritin in Alzheimer's diseased brains," *Journal of Neuroscience Research*, vol. 31, no. 1, pp. 75–83, 1992.

[20] G. McKhann, D. Drachman, and M. Folstein, "Clinical diagnosis of Alzheimer's disease: report of the NINCDS-ADRDA work group under the auspices of Department of Health and Human Services Task Force on Alzheimer's disease," *Neurology*, vol. 34, no. 7, pp. 939–944, 1984.

[21] M. F. Folstein, S. E. Folstein, and P. R. McHugh, ""Mini mental state". A practical method for grading the cognitive state of patients for the clinician," *Journal of Psychiatric Research*, vol. 12, no. 3, pp. 189–198, 1975.

[22] R. Squitti, P. Pasqualetti, G. Dal Forno et al., "Excess of serum copper not related to ceruloplasmin in Alzheimer disease," *Neurology*, vol. 64, no. 6, pp. 1040–1046, 2005.

[23] R. Squitti, P. M. Rossini, E. Cassetta et al., "D-penicillamine reduces serum oxidative stress in Alzheimer's disease patients," *European Journal of Clinical Investigation*, vol. 32, no. 1, pp. 51–59, 2002.

[24] J. E. Hixson and D. T. Vernier, "Restriction isotyping of human apolipoprotein E by gene amplification and cleavage with HhaI," *Journal of Lipid Research*, vol. 31, no. 3, pp. 545–548, 1990.

[25] P. Scheltens, D. Leys, F. Barkhof et al., "Atrophy of medial temporal lobes on MRI in "probable" Alzheimer's disease and normal ageing: diagnostic value and neuropsychological correlates," *Journal of Neurology Neurosurgery and Psychiatry*, vol. 55, no. 10, pp. 967–972, 1992.

[26] P. Scheltens, L. J. Launer, F. Barkhof, H. C. Weinstein, and W. A. Van Gool, "Visual assessment of medial temporal lobe atrophy on magnetic resonance imaging: interobserver reliability," *Journal of Neurology*, vol. 242, no. 9, pp. 557–560, 1995.

[27] P. Scheltens, T. Erkinjunti, D. Leys et al., "White matter changes on CT and MRI: an overview of visual rating scales," *European Neurology*, vol. 39, no. 2, pp. 80–89, 1998.

[28] T. Erkinjuntti, D. Inzitari, L. Pantoni et al., "Research criteria for subcortical vascular dementia in clinical trials," *Journal of Neural Transmission, Supplement*, no. 59, pp. 23–30, 2000.

[29] E. Bartels, *Color-Coded Duplex Ultrasonography of the Cerebral Vessels: Atlas and Manual*, Schattauer, Stuttgart, Germany, 1999.

[30] R. Squitti, D. Lupoi, P. Pasqualetti et al., "Elevation of serum copper levels in Alzheimer's disease," *Neurology*, vol. 59, no. 8, pp. 1153–1161, 2002.

[31] D. H. O'Leary, J. F. Polak, R. A. Kronmal, T. A. Manolio, G. L. Burke, and S. K. Wolfson Jr., "Carotid-artery intima and media thickness as a risk factor for myocardial infarction and stroke in older adults," *New England Journal of Medicine*, vol. 340, no. 1, pp. 14–22, 1999.

[32] R. Aaslid, T. M. Markwalder, and H. Nornes, "Noninvasive transcranial Doppler ultrasound recording of flow velocity in basal cerebral arteries," *Journal of Neurosurgery*, vol. 57, no. 6, pp. 769–774, 1982.

[33] C. Altamura, R. Squitti, P. Pasqualetti et al., "Ceruloplasmin/Transferrin system is related to clinical status in acute stroke," *Stroke*, vol. 40, no. 4, pp. 1282–1288, 2009.

[34] J. A. Duce, A. Tsatsanis, M. A. Cater et al., "Iron-export ferroxidase activity of β-amyloid precursor protein is inhibited by zinc in Alzheimer's disease," *Cell*, vol. 142, no. 6, pp. 857–867, 2010.

[35] A. R. White, R. Reyes, J. F. B. Mercer et al., "Copper levels are increased in the cerebral cortex and liver of APP and APLP2 knockout mice," *Brain Research*, vol. 842, no. 2, pp. 439–444, 1999.

[36] A. V. Kozlov, V. I. Sergienko, I. A. Vladimirov, and O. A. Azizova, "The antioxidant system of transferrin-ceruloplasmin in experimental hypercholesterolemia," *Byulleten Eksperimentalnoi Biologii i Meditsiny*, vol. 98, no. 12, pp. 668–671, 1984.

[37] H. Aksoy, S. Taysi, K. Altinkaynak, E. Bakan, N. Bakan, and Y. Kumtepe, "Antioxidant potential and transferrin, ceruloplasmin, and lipid peroxidation levels in women with preeclampsia," *Journal of Investigative Medicine*, vol. 51, no. 5, pp. 284–287, 2003.

[38] Z. Serdar, E. Gür, and O. Develioğlu, "Serum iron and copper status and oxidative stress in severe and mild preeclampsia," *Cell Biochemistry and Function*, vol. 24, no. 3, pp. 209–215, 2006.

[39] R. E. Fleming, M. C. Migas, C. C. Holden et al., "Transferrin receptor 2: continued expression in mouse liver in the face of iron overload and in hereditary hemochromatosis," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 97, no. 5, pp. 2214–2219, 2000.

[40] J. R. Burdo, D. A. Antonetti, E. B. Wolpert, and J. R. Connor, "Mechanisms and regulation of transferrin and iron transport in a model blood-brain barrier system," *Neuroscience*, vol. 121, no. 4, pp. 883–890, 2003.

[41] B. Z. Alizadeh, O. T. Njajou, M. R. Millán, A. Hofman, M. M. Breteler, and C. M. van Duijn, "HFE variants, APOE and Alzheimer's disease: findings from the population-based Rotterdam Study," *Neurobiology of Aging*, vol. 30, no. 2, pp. 330–332, 2009.

[42] L. Blázquez, D. De Juan, J. Ruiz-Martínez et al., "Genes related to iron metabolism and susceptibility to Alzheimer's disease in Basque population," *Neurobiology of Aging*, vol. 28, no. 12, pp. 1941–1943, 2007.

[43] D. Berlin, G. Chong, H. Chertkow, H. Bergman, N. A. Phillips, and H. M. Schipper, "Evaluation of HFE (hemochromatosis) mutations as genetic modifiers in sporadic AD and MCI," *Neurobiology of Aging*, vol. 25, no. 4, pp. 465–474, 2004.

[44] K. J. H. Robson, D. J. Lehmann, V. L. C. Wimhurst et al., "Synergy between the C2 allele of transferrin and the C282Y allele of the haemochromatosis gene (HFE) as risk factors for developing Alzheimer's disease," *Journal of Medical Genetics*, vol. 41, no. 4, pp. 261–265, 2004.

[45] M. Sampietro, L. Caputo, A. Casatta et al., "The hemochromatosis gene affects the age of onset of sporadic Alzheimer's disease," *Neurobiology of Aging*, vol. 22, no. 4, pp. 563–568, 2001.

[46] D. J. Lehmann, M. Worwood, R. Ellis et al., "Iron genes, iron load and risk of Alzheimer's disease," *Journal of medical genetics*, vol. 43, no. 10, article e52, 2006.

[47] B. Halliwell, "Free radicals and metal ions in health and disease," *Proceedings of the Nutrition Society*, vol. 46, no. 1, pp. 13–26, 1987.

[48] S. J. Van Rensburg, J. Van Zyl, D. Hon et al., "Biochemical model for inflammation of the brain: the effect of iron and transferrin on monocytes and lipid peroxidation," *Metabolic Brain Disease*, vol. 19, no. 1-2, pp. 97–112, 2004.

[49] R. Scacchi, L. De Bernardini, E. Mantuano, L. M. Donini, T. Vilardo, and R. M. Corbo, "Apolipoprotein E (APOE) allele frequencies in late-onset sporadic Alzheimer's disease (AD), mixed dementia and vascular dementia: lack of association of ε4 allele with AD in Italian octogenarian patients," *Neuroscience Letters*, vol. 201, no. 3, pp. 231–234, 1995.

[50] J. C. De la Torre, "Alzheimer disease as a vascular disorder: nosological evidence," *Stroke*, vol. 33, no. 4, pp. 1152–1162, 2002.

[51] C. Salustri, F. Tecchio, F. Zappasodi et al., "Cortical excitability and rest activity properties in patients with depression," *Journal of Psychiatry and Neuroscience*, vol. 32, no. 4, pp. 259–266, 2007.

SAGE-Hindawi Access to Research
International Journal of Alzheimer's Disease
Volume 2011, Article ID 720658, 9 pages
doi:10.4061/2011/720658

Review Article

Iron and Mechanisms of Neurotoxicity

Gabriela A. Salvador, Romina M. Uranga, and Norma M. Giusto

Instituto de Investigaciones Bioquímicas Bahía Blanca, Universidad Nacional del Sur y Consejo Nacional de Investigaciones Científicas y Técnicas, 8000 Bahía Blanca, Argentina

Correspondence should be addressed to Gabriela A. Salvador, salvador@criba.edu.ar

Received 13 September 2010; Accepted 10 November 2010

Academic Editor: Anthony R. White

Copyright © 2011 Gabriela A. Salvador et al. This is an open access article distributed under the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original work is properly cited.

The accumulation of transition metals (e.g., copper, zinc, and iron) and the dysregulation of their metabolism are a hallmark in the pathogenesis of several neurodegenerative diseases. This paper will be focused on the mechanism of neurotoxicity mediated by iron. This metal progressively accumulates in the brain both during normal aging and neurodegenerative processes. High iron concentrations in the brain have been consistently observed in Alzheimer's (AD) and Parkinson's (PD) diseases. In this connection, metalloneurobiology has become extremely important in establishing the role of iron in the onset and progression of neurodegenerative diseases. Neurons have developed several protective mechanisms against oxidative stress, among them, the activation of cellular signaling pathways. The final response will depend on the identity, intensity, and persistence of the oxidative insult. The characterization of the mechanisms mediating the effects of iron-induced increase in neuronal dysfunction and death is central to understanding the pathology of a number of neurodegenerative disorders.

1. Introduction

The so-called "biometals" (e.g., iron, copper, or zinc) are known to play a fundamental role in numerous essential metabolic processes, thus being considered as essential for life. Metal ion homeostasis is maintained through highly regulated mechanisms of uptake, storage, and secretion [1]. A specific set of transporters functions in each cellular compartment to provide a strict balance of transport activities across their membranes. Nonbound copper and iron are potentially harmful mainly due to their redox activities. Normally, under healthy conditions, these metal ions are bound to ligands (e.g., transferrin, ceruloplasmin), and they are not found as free species. However, the release of free ionic or exchangeable zinc and copper has been reported in the synaptic cleft. In addition, zinc is also being increasingly involved in several cellular reactions like calcium, and it has been proposed as a new class of second messenger.

A loss or an abnormal metal homeostasis might cause cellular death or severe dysfunction, and it has been recognized as a triggering factor for different neurodegenerative disorders such as Alzheimer's (AD), Parkinson's (PD), and Huntington's (HD) diseases as well as amyotrophic lateral sclerosis (ALS) [2–6]. Although the etiology of these diseases is still largely unknown, oxidative damage mediated by metals has been thought to be a significant contributor since metals such as iron, aluminum, zinc, and copper have been observed to be dysregulated and/or increased in AD brains and prone to generate a pro-oxidative environment [7–11]. Taking into account the great amount of information regarding the role of transition metals in cell biology, this paper will be mainly focused on the role of iron in neurodegeneration.

Loss of iron may cause neurological disease, and, in opposition, its accumulation or abnormal interaction with cellular components such as proteins, lipids, or nucleic acids may also contribute to neurodegenerative disorders. The intracellular pool of free iron, the labile iron pool (LIP), has been well established to modulate the expression of various proteins, including the amyloid precursor protein (APP) [12, 13].

In the brain, the movement of metals across the blood-brain barrier is highly regulated, and there is no passive flux of metals from the circulation to the brain [1, 14].

While iron, copper, and zinc are being increasingly implicated in interactions with the major protein components of neurodegenerative diseases, this is not merely due to increased (e.g., toxicological) exposure to metals but rather because of a breakdown in the homeostatic mechanisms that compartmentalize and regulate metals [7].

The ability of iron to accept and donate electrons can lead to the formation of reactive nitrogen and oxygen species (the latter named "ROS" in this paper) which may trigger the oxidative attack of tissue components, therefore contributing to disease and perhaps aging itself [15, 16]. Increasing age is the main risk factor associated with the appearance of neurodegenerative diseases. Several studies in animals and humans have reported a rise in brain iron as a function of ageing [17, 18]. The vulnerability of the brain to abnormal iron regulation has been demonstrated by the relationship between the failure of ferroxidases, ceruloplasmin [19, 20], ferritin [18, 21, 22], and frataxin [23, 24], iron accumulation (IA), and the onset of neurodegenerative diseases.

2. Iron Accumulation in Neurodegenerative Diseases

The accumulation of transition metals in the nervous system is a common observation in different neurodegenerative diseases that support a role of metals in these disorders [25, 26]. Particularly, iron homeostasis has shown to be altered [26, 27]. Excessive iron deposition has been reported to occur in the central nervous system (CNS) in a number of neurodegenerative pathologies such as AD, PD, ALS, and neuroferritinopathies, among others [4, 28–33].

ALS is a neurodegenerative disorder characterized by progressive paralysis of skeletal muscles and degeneration of motor neurons in the spinal cord, brainstem, and cortex. High levels of iron in the CNS of both familial and sporadic forms of ALS have been reported [32, 34, 35]. However, neither the mechanisms underlying iron accumulation nor its complete role in the pathogenesis of the disease are clear.

Neuroferritinopathies, like neurodegeneration with brain iron accumulation (NBIA), are defined as extrapyramidal disorders [33] characterized by radiographic evidence of focal iron accumulation in the brain [36]. These diseases are progressive movement disorders caused by nucleotide insertions in exon 4 of the ferritin light chain gene [37]. These patients show low levels of serum ferritin [22] and abundant spherical inclusions in the brain, skin, kidney, liver, and muscle [33] that are positive for iron, ferritin, and ubiquitin staining [38].

Moreover, abnormal accumulation of iron is also considered to be involved in the pathogenesis of myelin diseases such as multiple sclerosis (MS). Histochemical studies have shown that abnormal iron deposits are observed in reactive microglia, axons, neurons, and oligodendrocytes in patients with MS [39, 40]. Indeed, ferritin levels are increased in the CNS of mice with experimental autoimmune encephalomyelitis, an animal model of MS [41], and in the cerebrospinal fluid of MS patients [42, 43]. Since the synthesis of ferritin can reduce toxic ferrous iron (Fe^{2+}), the elevated level of ferritin in autoimmune encephalomyelitis mice and MS patients is considered to be cytoprotective [40].

Although iron has been demonstrated to have a potential role in many diseases of the CNS, this paper will focus mainly on the information regarding the role of iron in AD. It is well known that transition metals provoke oxidative stress by generating ROS through the Fenton reaction, thus causing brain lipid peroxidation [44] and protein oxidation [45, 46]. Interestingly, not only has iron been involved in lipid and protein oxidation but also in DNA damage. It has been shown that iron is able to oxidize DNA bases, and it has been suggested that the accumulation of this transition metal observed in some neurodegenerative disorders could act by both increasing oxidative genome damage and also preventing its repair [47]. Iron itself has been related to neurotoxicity, and its accumulation, mainly in the hippocampus and cortex, has been observed to occur before AD lesions are detectable. Moreover, it has been also demonstrated to accumulate both in AD senile plaques [48] and in amyloid deposits in AβPP2576 transgenic mouse model of AD [49]. Interestingly, Aβ insoluble aggregates have been shown to be dissolved by metal chelators [50].

Oxidative stress is considered to be the earliest change in the pathogenesis of AD, and high levels of oxidative stress have been demonstrated to occur in the clinical precursor of AD, known as mild cognitive impairment (MCI) [51, 52]. Coincidently, increased iron levels were found both in the cortex and cerebellum from the preclinical AD/MCI cases. Moreover, iron concentrations have been found to be increased in the bilateral hippocampus, parietal cortex, frontal white matter, putamen, caudate nucleus, thalamus, red nucleus, substantia nigra, and dentate nucleus subregions of patients with diagnosed AD and in normal elderly patients [53, 54]. It is important to note that these brain iron concentrations, particularly those in the parietal cortex at the early stages of AD, have been found to positively correlate with the severity of patients' cognitive impairment [53]. Although extensive evidence links the dysregulation of iron homeostasis and AD, relatively little is known about the resulting forms of iron that accumulate in the brain. Numerous techniques have been developed in order to characterize, locate, and quantify iron species and iron-containing compounds in AD. For example, the use of iron fluorescence together with synchrotron X-ray absorption spectroscopy showed *in situ* iron accumulations containing high concentrations of ferritin and magnetite in AD brain tissue sections [55].

Alterations in iron metabolism with age have been described, and they may involve iron uptake and release, storage, and intracellular metabolism [56–59]. Although some issues remain unclear, it is well known that the dyshomeostasis of brain iron metabolism is one of the initial events that trigger neuronal death in some neurodegenerative disorders [60–63]. Existing evidence shows that these mechanisms may well be altered by the ageing process with increased (IA) in the brain as the final outcome [64–66]. Age-induced IA has shown to be a consequence of the accumulation of different iron-containing molecules in different brain regions known to be particularly affected in disorders such

as AD and PD [18, 56]. Cellular studies have shown that iron is specifically accumulated in microglia and astrocytes in the cerebral cortex, cerebellum, substantia nigra, and hippocampus, and it is believed that this metal ion would be involved in the neuroinflammation observed in AD and PD [28]. The mechanism underlying IA in the brain is unclear yet. However, one hypothesis holds that it is the blood brain barrier dysfunction that is responsible for the exudation of serum components, with iron among them [67]. Another hypothesis with strong experimental support proposes that IA is a consequence of the dysregulation of proteins that govern metal homeostasis. Among candidates, it has been demonstrated that the iron regulatory proteins (IREG) participate in neuronal IA. Increased expression of IREG1 has been related with neuronal survival during IA [68]. In addition, IREG2 knockout (Ireb2(−/−)) mice develop IA in white matter tracts and nuclei in different brain areas and display neurodegeneration signs in Purkinje cells [69]. Mutations in the divalent metal transporter 1 (DMT1) have been shown to impair iron transport and to protect rodents against neurotoxins like 6-hydroxydopamine, supporting a critical role for DMT1 in iron-mediated neurodegeneration [70]. Mitochondrial IA, loss of iron-sulfur cluster-containing enzymes, and increased oxidative damage are known to occur in yeast and mouse frataxin-depleted mutants as well as in tissues and cell lines from Friedrich's ataxia (FRDA) patients, suggesting that frataxin may be involved in the export of iron from the mitochondria, the synthesis of iron-sulfur clusters, and/or the protection from oxidative damage [71]. The use of Deferiprone (DFP, a chelator in clinical use for treating iron overload) in FRDA cells has been found to reduce the mitochondrial LIP increased by frataxin deficiency [72]. A new recently reported mitochondrial ferritin (MtFt) specifically expresses in high energy-consuming cells, including neurons. The overexpression of MtFt has been observed to lead to a cytosolic iron deficiency and to significantly prevent the alteration of iron redistribution and, consequently, neuronal toxicity induced by 6-hydrodopamine [69].

Although the existing data clearly show a relationship between iron metabolism, aging, and neurodegeneration, more and deeper studies are needed to completely understand the role of this transition metal in the onset and progression of neurodegenerative diseases and neurological age-related disorders.

3. Iron Interaction with Amyloid Beta (Aβ) Peptide

Interestingly, IA, as well as oxidative stress in AD brains, has been linked to altered Aβ deposition. It is well known that Aβ accumulates in senile plaques in AD, and it has also been demonstrated to participate in a positive feedback loop, where oxidative stress leads to increased Aβ generation, and, conversely, the mechanism of Aβ polymerization generates oxidative stress which in turn enhances Aβ production [73]. Additionally, Aβ has been characterized as a metalloprotein able to bind transition metals (e.g., zinc, iron, copper) via 3 histidine (positions 6, 13, and 14) and 1 tyrosine (position 10) residues located in the hydrophilic N-terminal part of the peptide [74, 75], and in so doing Aβ would prevent these potentially redox-active ions from causing oxidative stress. Notably, the redox potential of iron is significantly attenuated by Aβ, supporting a neuroprotective chelating role for Aβ in AD pathogenesis [76, 77]. This particular feature of Aβ could, at least in part, explain the enrichment of these transition metals in AD plaques [48]. It has been shown that not only ROS production induces Aβ aggregation but also its ability to bind metal ions as well. Augmented iron concentrations and oxidative stress have been found to correlate with changes in the concentration of both soluble and deposited Aβ [78]. Interestingly, the metal-dependent generation of ROS by Aβ may be a good target for therapeutics. For example, chelation therapy with desferrioxamine and clioquinol (which are iron and copper/zinc chelators, resp.) has shown to induce clinical improvement in patients with AD [79, 80]. Moreover, coincubation of Aβ from postmortem AD brains with metal chelators has shown to dissolve Aβ deposits [81]. In addition, both animal and human studies with clioquinol have been found to reverse Aβ deposition, improve cognition, general behavior and health, and lower plasma Aβ levels [79, 82, 83].

Taken together, all these data clearly demonstrate that a deeper understanding of the metal-related mechanisms operating in neurodegenerative disorders such as AD is needed, since it may provide insights into new therapeutic approaches.

4. Neuronal Signaling during Iron-Induced Neurotoxicity

Neurons have developed several mechanisms in response to oxidative injury; one of them is the activation of signaling pathways that promote death or survival. The extent and duration of the oxidative insult as well as the cell type injured are crucial factors in determining which pathways are activated, their prevalence, and, in consequence, the final cellular fate [84, 85]. In this aspect, metal-induced oxidative stress has been implicated as the triggering factor of several protective and proapoptotic signaling pathways in neurons [7, 86].

Synapses are sites where the first manifestations of neurodegenerative processes are likely to appear. Their vulnerability to iron-induced oxidative stress has been largely demonstrated by the presence of membrane lipid peroxidation, impairment of membrane ion-motive ATPases, glucose and glutamate transport, and mitochondrial function [87]. In this regard, several key components of signaling pathways like extracellular signal-regulated kinase (ERK), phosphoinositide 3-kinase (PI3K), Akt, and glycogen synthase kinase 3β (GSK3β) are activated in situ in isolated synaptic endings exposed to iron-induced oxidative injury [88, 89]. Moreover, several key biochemical events that are known to occur in intact neurons undergoing apoptosis (i.e., exposure of phosphatidylserine on the plasma membrane surface, activation of caspase-3, mitochondrial calcium uptake, and ROS accumulation) also occur in isolated synaptosomes

exposed to iron-induced oxidative stress [90]. Synapse loss, a key event in neurodegenerative disorders, might also involve in situ apoptotic cascades that might occur before, or independently of, neuronal death. This assumption is supported by the appearance of degenerative morphological changes in synapses preceding amyloid deposition and neuronal degeneration [91]. However, mechanisms whereby apoptotic events triggered by iron-induced oxidative stress in synapses propagate to the cell body remain unknown.

Some cellular signaling pathways are clearly linked to enhanced survival, while others are associated with cell death. Hence, it has been suggested that the balance between the magnitude of ERK and Jun kinase (JNK) activation is key to determining survival. While this idea is still generally accepted, more recent evidence suggests that ERK can exert apoptotic influences, and JNK can exert antiapoptotic influences during the cellular response to oxidative stress.

The presence of iron and Aβ provokes a marked decrease in protein kinase C isoforms, reduced Akt serine/threonine kinase activity, Bcl-2-associated death promoter (BAD) phosphorylation, and enhanced p38 mitogen-activated protein kinase (MAPK) and caspase-9 and caspase-3 activation [92–94]. In isolated synaptic endings, the coincubation with iron and Aβ triggers the activation of Akt and ERK signaling in an oxidation-dependent manner [95]. The phosphorylation and subsequent inhibition of GSK3β mediated by AMP-activated protein kinase (AMPK) contributes to protecting mitochondria against iron-catalyzed oxidative stress [96]. In AD, abnormal activation of GSK3β pathway might play an important role in neurodegeneration, and compounds such as lithium that modulate GSK3β activity have been shown to reduce Aβ production and tau phosphorylation in APP transgenic mice [97].

Deferoxamine, a known iron chelator, has been shown to block all the proapoptotic signaling events triggered by Aβ-Fe. Moreover, Aβ alone has been shown not to activate proapoptotic signaling, thus demonstrating that apoptotic cell death can be only triggered by the presence of iron in vitro [92–94].

Iron has been found to be required for long-term potentiation in hippocampal CA1 neurons, and it is known to participate in the stimulation of calcium release through ROS produced via the Fenton reaction triggering the stimulation of the ERK signaling pathway. These results support a coordinated action between iron and calcium in synaptic plasticity and raise the possibility that elevated iron levels may contribute to neuronal degeneration through excessive intracellular calcium increase caused by iron-induced oxidative stress [98].

As previously mentioned, iron (alone or in combination with Aβ) is able to activate different signaling cascades. The activation of these signaling pathways is, in most cases, a necessary upstream event for the activation of several transcription factors (TFs). These TFs regulate the expression of specific genes that participate in cellular events such as survival or death. Nuclear factor kappa B (NF-κB) plays crucial roles in cellular resistance to oxidants and survival. Although the knowledge of NF-κB gene targets in neurons is limited, it has been demonstrated that this TF can promote their survival and regulate synaptic plasticity [99]. The involvement of NF-κB in the inhibition of apoptosis has been well established [100]. NF-κB plays a central role in the induction of neuroprotective antiapoptotic gene products, such as MnSOD and Bcl-2 that are known to contribute to ischemic tolerance [101]. Activation of NF-κB has also been associated with increased resistance of neurons to apoptosis induced by iron exposure [102]. Levels of p65 immunoreactivity have been reported to be increased in neurons and astrocytes associated with Aβ plaques in the brains of AD patients, suggesting an increased NF-κB activation [103]. Exposure of cultured neurons to Aβ or a secreted form of amyloid precursor protein (sAPP) has shown to induce NF-κB activation, thus suggesting a role for proteolytic products of APP in NF-κB activation in AD [104]. Levels of NF-κB activity have been reported to be increased in cholinergic neurons in the basal forebrain of AD patients [105]. Others have established a correlation between increased NF-κB activity and COX-2 gene transcription in brain regions affected in AD patients [106]. In addition, the inhibition of NF-κB transcriptional activity results in increased vulnerability of neurons to death induced by Aβ [107].

Hypoxia-inducible factor (HIF) is a TF that regulates the expression of more than 60 genes. The expression of genes relevant to iron metabolism such as ceruloplasmin, transferrin receptor, transferrin, and heme-oxygenase 1 has been shown to be regulated by HIF [108–111]. Iron chelation therapy that reduces the size of LIP has been reported to induce the activity of this TF. Moreover, a new multifunctional nontoxic, brain permeable iron chelator, M30, has shown to activate the HIF-1alpha signaling pathway in rat primary culture of cortical cells. M30 has also been found to increase the expression levels of the transcripts of brain-derived neurotrophic factor (BDNF) and growth-associated protein-43 (GAP-43). In connection with AD, M30 has been reported to enhance the levels of phospho-Akt (Ser473) and phospho-GSK3β (Ser9) and to attenuate Tau phosphorylation [112].

The activator protein-1 (AP-1) is another redox-sensitive TF. AP-1 is known to participate in critical cellular processes such as proliferation, differentiation, and survival. Strong evidence supports the involvement of AP-1 in oxidative stress signaling in neurons. In rat cortical neurons and astrocytes, H_2O_2 has been demonstrated to activate MAPK cascade [113]. Upstream of AP-1, c-Jun-N-terminal kinase (JNK), and p38 (two stress-related MAPK) has also shown to be activated by increases in the intracellular levels of oxidants [114–116]. Both MAPK and AP-1 are implicated in normal physiological functions of the brain. c-Jun, a component of AP-1, has been recently attributed a dual role: it is believed to mediate neurodegeneration and cell death as well as participate in plasticity and repair mechanisms. Moreover, upregulation of iron regulatory proteins and DMT-1 isoforms after neuronal injury induced by kainate has been found to be modulated by AP-1 in rat hippocampus [117].

Activation of JNK signaling in neurons has shown to increase stress resistance and to extend life span by the

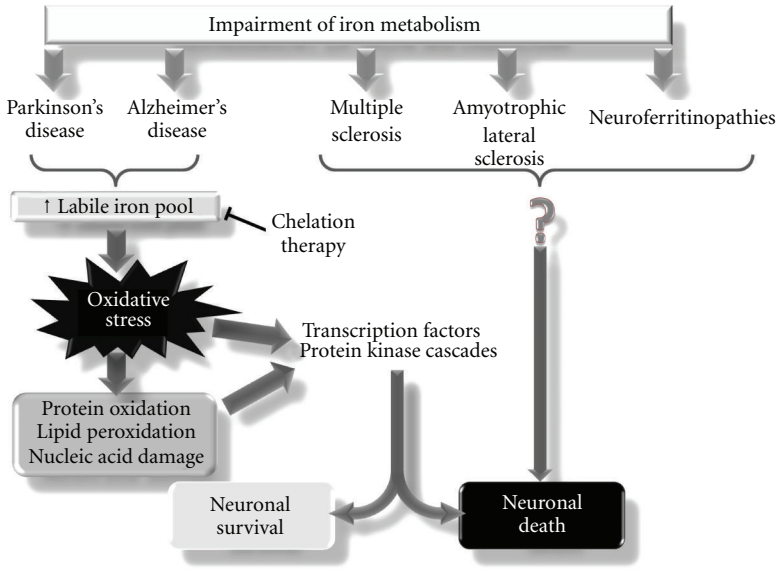


Figure 1: Relationship between the impairment of iron metabolism and neurodegenerative diseases. Impaired iron metabolism is a hallmark in several neurodegenerative diseases such as Parkinson's (PD) and Alzheimer's (AD) diseases, multiple sclerosis (MS), amyotrophic lateral sclerosis (ALS), and neuroferritinopathies. In the case of PD and AD, iron has been shown to play a key role in neuronal fate: depending on the extent and intensity of the oxidative stress caused by the increase in the labile iron pool, it affects transcriptional activity and signaling cascades that could participate in neuronal survival or death. Although a role for iron has also been observed in MS, ALS, and neuroferritinopathies, the molecular events that lead to neuronal death are not fully understood.

activation of the forkhead transcription factor (FOXO) family in Drosophila [118]. Recent studies have suggested that MST1 mediates oxidative stress-induced neuronal cell death by phosphorylating the transcription factor FOXO3 at serine 207, a site that is conserved in other FOXO family members [119, 120]. All these data support the hypothesis that FOXO signaling extends life span via amelioration of oxidative damage and mitochondrial dysfunction in neurons. However, to date, there is no link between FOXO signaling and iron-induced oxidative stress in neurons. Understanding signal transduction networks that participate in iron-induced neurotoxicity constitutes one essential objective for the discovery of new drugs and treatments aimed at the improvement and delay of AD symptoms.

5. Concluding Remarks

In this paper, we summarize the latest knowledge about the role of iron in neurodegeneration processes. Iron, a redox-active transition metal, has been proposed as an important contributing factor to the neuropathology of Alzheimer's disease. Even though increasing evidence points towards iron participation in oxidative stress events and protein aggregation, we are still far from totally comprehending the role of this transition metal in the onset and progression of neurodegenerative disorders (Figure 1). The advancement in this field will be fundamental for the establishment of new therapies intended for neuronal protection during iron mismanagement conditions.

References

[1] E. Mills, X. P. Dong, F. Wang, and H. Xu, "Mechanisms of brain iron transport: insight into neurodegeneration and CNS disorders," *Future Medicinal Chemistry*, vol. 2, no. 1, pp. 51–64, 2010.

[2] J. R. Connor and S. A. Benkovic, "Iron regulation in the brain: histochemical, biochemical, and molecular considerations," *Annals of Neurology*, vol. 32, pp. S51–S61, 1992.

[3] G. Liu, W. Huang, R. D. Moir et al., "Metal exposure and Alzheimer's pathogenesis," *Journal of Structural Biology*, vol. 155, no. 1, pp. 45–51, 2006.

[4] T. A. Rouault, "Systemic iron metabolism: a review and implications for brain iron metabolism," *Pediatric Neurology*, vol. 25, no. 2, pp. 130–137, 2001.

[5] M. B. H. Youdim, M. Fridkin, and H. Zheng, "Bifunctional drug derivatives of MAO-B inhibitor rasagiline and iron chelator VK-28 as a more effective approach to treatment of brain ageing and ageing neurodegenerative diseases," *Mechanisms of Ageing and Development*, vol. 126, no. 2, pp. 317–326, 2005.

[6] D. B. Kell, "Towards a unifying, systems biology understanding of large-scale cellular death and destruction caused by poorly liganded iron: Parkinson's, Huntington's, Alzheimer's, prions, bactericides, chemical toxicology and others as examples," *Archives of Toxicology*, vol. 84, no. 11, pp. 825–889, 2010.

[7] K. J. Barnham and A. I. Bush, "Metals in Alzheimer's and Parkinson's Diseases," *Current Opinion in Chemical Biology*, vol. 12, no. 2, pp. 222–228, 2008.

[8] L. Bica, P. J. Crouch, R. Cappai, and A. R. White, "Metallo-complex activation of neuroprotective signalling pathways as

a therapeutic treatment for Alzheimer's disease," *Molecular BioSystems*, vol. 5, no. 2, pp. 134–142, 2009.

[9] Y. Christen, "Oxidative stress and Alzheimer disease," *American Journal of Clinical Nutrition*, vol. 71, no. 2, pp. 621S–629S, 2000.

[10] G. Perry, L. M. Sayre, C. S. Atwood et al., "The role of iron and copper in the aetiology of neurodegenerative disorders: therapeutic implications," *CNS Drugs*, vol. 16, no. 5, pp. 339–352, 2002.

[11] P. Zatta, R. Lucchini, S. J. Van Rensburg, and A. Taylor, "The role of metals in neurodegenerative processes: aluminum, manganese, and zinc," *Brain Research Bulletin*, vol. 62, no. 1, pp. 15–28, 2003.

[12] H. -H. Cho, C. M. Cahill, C. R. Vanderburg et al., "Selective translational control of the Alzheimer amyloid precursor protein transcript by iron regulatory protein-1," *Journal of Biological Chemistry*, vol. 285, no. 41, pp. 31217–31232, 2010.

[13] J. Frackowiak, A. Potempska, and B. Mazur-Kolecka, "Formation of amyloid-β oligomers in brain vascular smooth muscle cells transiently exposed to iron-induced oxidative stress," *Acta Neuropathologica*, vol. 117, no. 5, pp. 557–567, 2009.

[14] D. R. Richardson and P. Ponka, "The molecular mechanisms of the metabolism and transport of iron in normal and neoplastic cells," *Biochimica et Biophysica Acta*, vol. 1331, no. 1, pp. 1–40, 1997.

[15] B. Halliwell, "The wanderings of a free radical," *Free Radical Biology and Medicine*, vol. 46, no. 5, pp. 531–542, 2009.

[16] B. Halliwell, "Role of free radicals in the neurodegenerative diseases: therapeutic implications for antioxidant treatment," *Drugs and Aging*, vol. 18, no. 9, pp. 685–716, 2001.

[17] Y. A. Ke and Z. M. Qian, "Brain iron metabolism: neurobiology and neurochemistry," *Progress in Neurobiology*, vol. 83, no. 3, pp. 149–173, 2007.

[18] L. Zecca, M. B. H. Youdim, P. Riederer, J. R. Connor, and R. R. Crichton, "Iron, brain ageing and neurodegenerative disorders," *Nature Reviews Neuroscience*, vol. 5, no. 11, pp. 863–873, 2004.

[19] Y. J. Suh and S. David, "Age-related changes in iron homeostasis and cell death in the cerebellum of ceruloplasmin-deficient mice," *Journal of Neuroscience*, vol. 26, no. 38, pp. 9810–9819, 2006.

[20] S. J. Texel, X. Xu, and Z. L. Harris, "Ceruloplasmin in neurodegenerative diseases," *Biochemical Society Transactions*, vol. 36, part 6, pp. 1277–1281, 2008.

[21] A. V. Alekseenko, T. V. Waseem, and S. V. Fedorovich, "Ferritin, a protein containing iron nanoparticles, induces reactive oxygen species formation and inhibits glutamate uptake in rat brain synaptosomes," *Brain Research*, vol. 1241, pp. 193–200, 2008.

[22] P. F. Chinnery, D. E. Crompton, D. Birchall et al., "Clinical features and natural history of neuroferritinopathy caused by the FTL1 460InsA mutation," *Brain*, vol. 130, no. 1, pp. 110–119, 2007.

[23] O. Gakh, T. Bedekovics, S. F. Duncan, D. Y. Smith, D. S. Berkholz, and G. Isaya, "Normal and Friedreich ataxia cells express different isoforms of frataxin with complementary roles in iron-sulfur cluster assembly," *Journal of Biological Chemistry*. In press.

[24] M. C. Mantovan, A. Martinuzzi, F. Squarzanti et al., "Exploring mental status in Friedreich's ataxia: a combined neuropsychological, behavioral and neuroimaging study," *European Journal of Neurology*, vol. 13, no. 8, pp. 827–835, 2006.

[25] D. Berg and M. B. Youdim, "Role of iron in neurodegenerative disorders," *Topics in Magnetic Resonance Imaging*, vol. 17, no. 1, pp. 5–17, 2006.

[26] D. T. Dexter, P. Jenner, A. H.V. Schapira, and C. D. Marsden, "Alterations in levels of iron, ferritin, and other trace metals in neurodegenerative diseases affecting the basal ganglia. The Royal Kings and Queens Parkinson's Disease Research Group," *Annals of Neurology*, vol. 32, supplement, pp. S94–S100, 1992.

[27] D. A. Simmons, M. Casale, B. Alcon, N. Pham, N. Narayan, and G. Lynch, "Ferritin accumulation in dystrophic microglia is an early event in the development of Huntington's Disease," *GLIA*, vol. 55, no. 10, pp. 1074–1084, 2007.

[28] W. Y. Ong and A. A. Farooqui, "Iron, neuroinflammation, and Alzheimer's disease," *Journal of Alzheimer's Disease*, vol. 8, no. 2, pp. 183–200, 2005.

[29] C. Quintana, S. Bellefqih, J. Y. Laval et al., "Study of the localization of iron, ferritin, and hemosiderin in Alzheimer's disease hippocampus by analytical microscopy at the subcellular level," *Journal of Structural Biology*, vol. 153, no. 1, pp. 42–54, 2006.

[30] T. A. Rouault, "Iron on the brain," *Nature Genetics*, vol. 28, no. 4, pp. 299–300, 2001.

[31] C. N. Roy and N. C. Andrews, "Recent advances in disorders of iron metabolism: mutations, mechanisms and modifiers," *Human Molecular Genetics*, vol. 10, no. 20, pp. 2181–2186, 2001.

[32] Y. Imon, S. Yamaguchi, Y. Yamamura et al., "Low intensity areas observed on T2-weighted magnetic resonance imaging of the cerebral cortex in various neurological diseases," *Journal of the Neurological Sciences*, vol. 134, supplement 1, pp. 27–32, 1995.

[33] J. Burn and P. F. Chinnery, "Neuroferritinopathy," *Seminars in Pediatric Neurology*, vol. 13, no. 3, pp. 176–181, 2006.

[34] E. J. Kasarskis, L. Tandon, M. A. Lovell, and W. D. Ehmann, "Aluminum, calcium, and iron in the spinal cord of patients with sporadic amyotrophic lateral sclerosis using laser microprobe mass spectroscopy: a preliminary study," *Journal of the Neurological Sciences*, vol. 130, no. 2, pp. 203–208, 1995.

[35] H. Oba, T. Araki, K. Ohtomo et al., "Amyotrophic lateral sclerosis: T2 shortening in motor cortex at MR imaging," *Radiology*, vol. 189, no. 3, pp. 843–846, 1993.

[36] A. Gregory and S. J. Hayflick, "Neurodegeneration with brain iron accumulation," *Folia Neuropathologica*, vol. 43, no. 4, pp. 286–296, 2005.

[37] S. Levi, A. Cozzi, and P. Arosio, "Neuroferritinopathy: a neurodegenerative disorder associated with L-ferritin mutation," *Best Practice and Research: Clinical Haematology*, vol. 18, no. 2, pp. 265–276, 2005.

[38] A. R. J. Curtis, C. Fey, C. M. Morris et al., "Mutation in the gene encoding ferritin light polypeptide causes dominant adult-onset basal ganglia disease," *Nature Genetics*, vol. 28, no. 4, pp. 350–354, 2001.

[39] S. M. Levine, "Iron deposits in multiple sclerosis and Alzheimer's disease brains," *Brain Research*, vol. 760, no. 1-2, pp. 298–303, 1997.

[40] S. M. LeVine and A. Chakrabarty, "The role of iron in the pathogenesis of experimental allergic encephalomyelitis

and multiple sclerosis," *Annals of the New York Academy of Sciences*, vol. 1012, pp. 252–266, 2004.

[41] A. Chakrabarty, M. R. Emerson, and S. M. LeVine, "Heme oxygenase-I in SJL mice with experimental allergic encephalomyelitis," *Multiple Sclerosis*, vol. 9, no. 4, pp. 372–381, 2003.

[42] S. M. LeVine, S. G. Lynch, C. -N. Ou, M. J. Wulser, E. Tam, and N. Boo, "Ferritin, transferrin and iron concentrations in the cerebrospinal fluid of multiple sclerosis patients," *Brain Research*, vol. 821, no. 2, pp. 511–515, 1999.

[43] C. Sfagos, A. C. Makis, A. Chaidos et al., "Serum ferritin, transferrin and soluble transferrin receptor levels in multiple sclerosis patients," *Multiple Sclerosis*, vol. 11, no. 3, pp. 272–275, 2005.

[44] L. M. Sayre, D. A. Zelasko, P. L. R. Harris, G. Perry, R. G. Salomon, and M. A. Smith, "4-Hydroxynonenal-derived advanced lipid peroxidation end products are increased in Alzheimer's disease," *Journal of Neurochemistry*, vol. 68, no. 5, pp. 2092–2097, 1997.

[45] M. A. Smith, M. Tabaton, and G. Perry, "Early contribution of oxidative glycation in Alzheimer disease [2]," *Neuroscience Letters*, vol. 217, no. 2-3, pp. 210–211, 1996.

[46] M. A. Smith, P. L. R. Harris, L. M. Sayre, and G. Perry, "Iron accumulation in Alzheimer disease is a source of redox-generated free radicals," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 94, no. 18, pp. 9866–9868, 1997.

[47] M. L. Hegde, P. M. Hegde, L. M.F. Holthauzen, T. K. Hazra, K. S.J. Rao, and S. Mitra, "Specific inhibition of NEIL-initiated repair of oxidized base damage in human genome by copper and iron: potential etiological linkage to neurodegenerative diseases," *Journal of Biological Chemistry*, vol. 285, no. 37, pp. 28812–28825, 2010.

[48] M. A. Lovell, J. D. Robertson, W. J. Teesdale, J. L. Campbell, and W. R. Markesbery, "Copper, iron and zinc in Alzheimer's disease senile plaques," *Journal of the Neurological Sciences*, vol. 158, no. 1, pp. 47–52, 1998.

[49] M. F. Falangola, S. -P. Lee, R. A. Nixon, K. Duff, and J. A. Helpern, "Histological co-localization of iron in Aβ plaques of PS/APP transgenic mice," *Neurochemical Research*, vol. 30, no. 2, pp. 201–205, 2005.

[50] R. A. Cherny, J. T. Legg, C. A. McLean et al., "Aqueous dissolution of Alzheimer's disease Aβ amyloid deposits by biometal depletion," *Journal of Biological Chemistry*, vol. 274, no. 33, pp. 23223–23228, 1999.

[51] D. A. Butterfield, M. L. Bader Lange, and R. Sultana, "Involvements of the lipid peroxidation product, HNE, in the pathogenesis and progression of Alzheimer's disease," *Biochimica et Biophysica Acta*, vol. 1801, no. 8, pp. 924–929, 2010.

[52] M. A. Smith, X. Zhu, M. Tabaton et al., "Increased iron and free radical generation in preclinical Alzheimer disease and mild cognitive impairment," *Journal of Alzheimer's Disease*, vol. 19, no. 1, pp. 363–372, 2010.

[53] W. -Z. Zhu, W. -D. Zhong, W. Wang et al., "Quantitative MR phase-corrected imaging to investigate increased brain iron deposition of patients with Alzheimer disease," *Radiology*, vol. 253, no. 2, pp. 497–504, 2009.

[54] E. V. Sullivan, E. Adalsteinsson, T. Rohlfing, and A. Pfefferbaum, "Relevance of iron deposition in deep gray matter brain structures to cognitive and motor performance in healthy elderly men and women: exploratory findings," *Brain Imaging and Behavior*, vol. 3, no. 2, pp. 167–175, 2009.

[55] J. F. Collingwood, A. Mikhaylova, M. Davidson et al., "In situ characterization and mapping of iron compounds in Alzheimer's disease tissue," *Journal of Alzheimer's Disease*, vol. 7, no. 4, pp. 267–272, 2005.

[56] G. Bartzokis, T. A. Tishler, IL. S. Shin, PO. H. Lu, and J. L. Cummings, "Brain ferritin iron as a risk factor for age at onset in neurodegenerative diseases," *Annals of the New York Academy of Sciences*, vol. 1012, pp. 224–236, 2004.

[57] W. A. Cass, R. Grondin, A. H. Andersen et al., "Iron accumulation in the striatum predicts aging-related decline in motor function in rhesus monkeys," *Neurobiology of Aging*, vol. 28, no. 2, pp. 258–271, 2007.

[58] J. R. Connor, B. S. Snyder, J. L. Beard, R. E. Fine, and E. J. Mufson, "Regional distribution of iron and iron-regulatory proteins in the brain in aging and Alzheimer's disease," *Journal of Neuroscience Research*, vol. 31, no. 2, pp. 327–335, 1992.

[59] W. R. W. Martin, F. Q. Ye, and P. S. Allen, "Increasing striatal iron content associated with normal aging," *Movement Disorders*, vol. 13, no. 2, pp. 281–286, 1998.

[60] G. Casadesus, M. A. Smith, X. Zhu et al., "Alzheimer disease: evidence for a central pathogenic role of iron-mediated reactive oxygen species," *Journal of Alzheimer's Disease*, vol. 6, no. 2, pp. 165–169, 2004.

[61] D. Kaur and J. Andersen, "Does cellular iron dysregulation play a causative role in Parkinson's disease?" *Ageing Research Reviews*, vol. 3, no. 3, pp. 327–343, 2004.

[62] YA. Ke and Z. M. Qian, "Iron misregulation in the brain: a primary cause of neurodegenerative disorders," *Lancet Neurology*, vol. 2, no. 4, pp. 246–253, 2003.

[63] M. Thomas and J. Jankovic, "Neurodegenerative disease and iron storage in the brain," *Current Opinion in Neurology*, vol. 17, no. 4, pp. 437–442, 2004.

[64] G. Bartzokis, P. H. Lu, T. A. Tishler et al., "Prevalent iron metabolism gene variants associated with increased brain ferritin iron in healthy older men," *Journal of Alzheimer's Disease*, vol. 20, no. 1, pp. 333–341, 2010.

[65] A. S. Dornelles, V. A. Garcia, M. N. M. De Lima et al., "mRNA expression of proteins involved in iron homeostasis in brain regions is altered by age and by iron overloading in the neonatal period," *Neurochemical Research*, vol. 35, no. 4, pp. 564–571, 2010.

[66] X. Xu, Q. Wang, and M. Zhang, "Age, gender, and hemispheric differences in iron deposition in the human brain: an in vivo MRI study," *NeuroImage*, vol. 40, no. 1, pp. 35–42, 2008.

[67] T. Morita, Y. Mizutani, M. Sawada, and A. Shimada, "Immunohistochemical and ultrastructural findings related to the blood-brain barrier in the blood vessels of the cerebral white matter in aged dogs," *Journal of Comparative Pathology*, vol. 133, no. 1, pp. 14–22, 2005.

[68] P. Aguirre, N. Mena, V. Tapia, M. Arredondo, and M. T. Núñez, "Iron homeostasis in neuronal cells: a role for IREG1," *BMC Neuroscience*, vol. 6, article 3, 2005.

[69] Z. -H. Shi, G. Nie, X. -L. Duan et al., "Neuroprotective mechanism of mitochondrial ferritin on 6-hydroxydopamine-induced dopaminergic cell damage: implication for neuroprotection in Parkinson's disease," *Antioxidants and Redox Signaling*, vol. 13, no. 6, pp. 783–796, 2010.

[70] J. Salazar, N. Mena, S. Hunot et al., "Divalent metal transporter 1 (DMT1) contributes to neurodegeneration in animal models of Parkinson's disease," *Proceedings of the*

National Academy of Sciences of the United States of America, vol. 105, no. 47, pp. 18578–18583, 2008.

[71] H. Puccio, D. Simon, M. Cossée et al., "Mouse models for Friedreich ataxia exhibit cardiomyopathy, sensory nerve defect and Fe-S enzyme deficiency followed by intramitochondrial iron deposits," *Nature Genetics*, vol. 27, no. 2, pp. 181–186, 2001.

[72] OR. Kakhlon, W. Breuer, A. Munnich, and Z. I. Cabantchik, "Iron redistribution as a therapeutic strategy for treating diseases of localized iron accumulation," *Canadian Journal of Physiology and Pharmacology*, vol. 88, no. 3, pp. 187–196, 2010.

[73] L. Zhang, B. Zhao, D. T. Yew, J. W. Kusiak, and G. S. Roth, "Processing of Alzheimer's amyloid precursor protein during HO-induced apoptosis in human neuronal cells," *Biochemical and Biophysical Research Communications*, vol. 235, no. 3, pp. 845–848, 1997.

[74] C. S. Atwood, R. D. Moir, X. Huang et al., "Dramatic aggregation of alzheimer by Cu(II) is induced by conditions representing physiological acidosis," *Journal of Biological Chemistry*, vol. 273, no. 21, pp. 12817–12826, 1998.

[75] C. S. Atwood, R. C. Scarpa, X. Huang et al., "Characterization of copper interactions with Alzheimer amyloid β peptides: identification of an attomolar-affinity copper binding site on amyloid β1-42," *Journal of Neurochemistry*, vol. 75, no. 3, pp. 1219–1233, 2000.

[76] C. A. Rottkamp, C. S. Atwood, J. A. Joseph, A. Nunomura, G. Perry, and M. A. Smith, "The state versus amyloid-β: the trial of the most wanted criminal in Alzheimer disease," *Peptides*, vol. 23, no. 7, pp. 1333–1341, 2002.

[77] L. M. Sayre, G. Perry, P. L. R. Harris, Y. Liu, K. A. Schubert, and M. A. Smith, "In situ oxidative catalysis by neurofibrillary tangles and senile plaques in Alzheimer's disease: a central role for bound transition metals," *Journal of Neurochemistry*, vol. 74, no. 1, pp. 270–279, 2000.

[78] C. S. Atwood, M. E. Obrenovich, T. Liu et al., "Amyloid-β: a chameleon walking in two worlds: a review of the trophic and toxic properties of amyloid-β," *Brain Research Reviews*, vol. 43, no. 1, pp. 1–16, 2003.

[79] C. W. Ritchie, A. I. Bush, A. Mackinnon et al., "Metal-protein attenuation with Iodochlorhydroxyquin (Clioquinol) targeting Aβ amyloid deposition and toxicity in alzheimer disease: a pilot phase 2 clinical trial," *Archives of Neurology*, vol. 60, no. 12, pp. 1685–1691, 2003.

[80] C. A. Perez, Y. Tong, and M. Guo, "Iron chelators as potential therapeutic agents for Parkinson's disease," *Current Bioactive Compounds*, vol. 4, no. 3, pp. 150–158, 2008.

[81] R. A. Cherny, K. J. Barnham, T. Lynch et al., "Chelation and intercalation: complementary properties in a compound for the treatment of Alzheimer's disease," *Journal of Structural Biology*, vol. 130, no. 2-3, pp. 209–216, 2000.

[82] R. A. Cherny, C. S. Atwood, M. E. Xilinas et al., "Treatment with a copper-zinc chelator markedly and rapidly inhibits β-amyloid accumulation in Alzheimer's disease transgenic mice," *Neuron*, vol. 30, no. 3, pp. 665–676, 2001.

[83] B. Regland, W. Lehmann, I. Abedini et al., "Treatment of Alzheimer's disease with clioquinol," *Dementia and Geriatric Cognitive Disorders*, vol. 12, no. 6, pp. 408–414, 2001.

[84] A. Nunomura, P. I. Moreira, H. G. Lee et al., "Neuronal death and survival under oxidative stress in Alzheimer and Parkinson diseases," *CNS and Neurological Disorders—Drug Targets*, vol. 6, no. 6, pp. 411–423, 2007.

[85] R. B. Petersen, A. Nunomura, H. G. Lee et al., "Signal transduction cascades associated with oxidative stress in Alzheimer's disease," *Journal of Alzheimer's Disease*, vol. 11, no. 2, pp. 143–152, 2007.

[86] G. A. Salvador, "Iron in neuronal function and dysfunction," *BioFactors*, vol. 36, no. 2, pp. 103–110, 2010.

[87] Z. H. Guo and M. P. Mattson, "Neurotrophic factors protect cortical synaptic terminals against amyloid- and oxidative stress-induced impairment of glucose transport, glutamate transport and mitochondrial function," *Cerebral Cortex*, vol. 10, no. 1, pp. 50–57, 2000.

[88] R. M. Uranga, M. V. Mateos, N. M. Giusto, and G. A. Salvador, "Activation of phosphoinositide-3 kinase/Akt pathway by $FeSO_4$ in rat cerebral cortex synaptic endings," *Journal of Neuroscience Research*, vol. 85, no. 13, pp. 2924–2932, 2007.

[89] R. M. Uranga, N. M. Giusto, and G. A. Salvador, "Iron-induced oxidative injury differentially regulates PI3K/Akt/GSK3β pathway in synaptic endings from adult and aged rats," *Toxicological Sciences*, vol. 111, no. 2, pp. 331–344, 2009.

[90] M. P. Mattson, "Apoptotic and anti-apoptotic synaptic signaling mechanisms," *Brain Pathology*, vol. 10, no. 2, pp. 300–312, 2000.

[91] S. T. DeKosky, S. W. Scheff, and S. D. Styren, "Structural correlates of cognition in dementia: quantification and assessment of synapse change," *Neurodegeneration*, vol. 5, no. 4, pp. 417–421, 1996.

[92] F. Kuperstein and E. Yavin, "Pro-apoptotic signaling in neuronal cells following iron and amyloid beta peptide neurotoxicity," *Journal of Neurochemistry*, vol. 86, no. 1, pp. 114–125, 2003.

[93] F. Kuperstein, A. Brand, and E. Yavin, "Amyloid Aβ preconditions non-apoptotic signals in vivo and protects fetal rat brain from intrauterine ischemic stress," *Journal of Neurochemistry*, vol. 91, no. 4, pp. 965–974, 2004.

[94] M. P. Mattson, "NF-κB in the survival and plasticity of neurons," *Neurochemical Research*, vol. 30, no. 6-7, pp. 883–893, 2005.

[95] R. M. Uranga, N. M. Giusto, and G. A. Salvador, "Effect of transition metals in synaptic damage induced by amyloid beta peptide," *Neuroscience*, vol. 170, no. 2, pp. 381–389, 2010.

[96] S. H. Choi, Y. W. Kim, and S. G. Kim, "AMPK-mediated GSK3β inhibition by isoliquiritigenin contributes to protecting mitochondria against iron-catalyzed oxidative stress," *Biochemical Pharmacology*, vol. 79, no. 9, pp. 1352–1362, 2010.

[97] E. Rockenstein, M. Torrance, A. Adame et al., "Neuroprotective effects of regulators of the glycogen synthase kinase-3β signaling pathway in a transgenic model of Alzheimer's disease are associated with reduced amyloid precursor protein phosphorylation," *Journal of Neuroscience*, vol. 27, no. 8, pp. 1981–1991, 2007.

[98] C. Hidalgo and M. T. Núñez, "Calcium, iron and neuronal function," *IUBMB Life*, vol. 59, no. 4-5, pp. 280–285, 2007.

[99] A. M. Adamo and P. I. Oteiza, "Zinc deficiency and neurodevelopment: the case of neurons," *BioFactors*, vol. 36, no. 2, pp. 117–124, 2010.

[100] M. P. Mattson, C. Culmsee, Z. Yu, and S. Camandola, "Roles of nuclear factor κB in neuronal survival and plasticity," *Journal of Neurochemistry*, vol. 74, no. 2, pp. 443–456, 2000.

[101] T. Toyoda, N. F. Kassell, and K. S. Lee, "Induction of ischemic tolerance and antioxidant activity by brief focal ischemia," *NeuroReport*, vol. 8, no. 4, pp. 847–851, 1997.

[102] G. W. Glazner, S. Camandola, and M. P. Mattson, "Nuclear factor-κB mediates the cell survival-promoting action of activity-dependent neurotrophic factor peptide-9," *Journal of Neurochemistry*, vol. 75, no. 1, pp. 101–108, 2000.

[103] K. Terai, A. Matsuo, and P. L. McGeer, "Enhancement of immunoreactivity for NF-κB in the hippocampal formation and cerebral cortex of Alzheimer's disease," *Brain Research*, vol. 735, no. 1, pp. 159–168, 1996.

[104] K. Z. Bourne, D. C. Ferrari, C. Lange-Dohna, S. Roßner, T. G. Wood, and J. R. Perez-Polo, "Differential regulation of BACE1 promoter activity by nuclear factor-κB in neurons and glia upon exposure to β-amyloid peptides," *Journal of Neuroscience Research*, vol. 85, no. 6, pp. 1194–1204, 2007.

[105] F. Boissière, S. Hunot, B. Faucheux et al., "Nuclear translocation of NF-κB in cholinergic neurons of patients with Alzheimer's disease," *NeuroReport*, vol. 8, no. 13, pp. 2849–2852, 1997.

[106] W. J. Lukiw and N. G. Bazan, "Strong nuclear factor-κB-DNA binding parallels cyclooxygenase-2 gene transcription in aging and in sporadic Alzheimer's disease superior temporal lobe neocortex," *Journal of Neuroscience Research*, vol. 53, no. 5, pp. 583–592, 1998.

[107] B. Kaltschmidt, M. Uherek, H. Wellmann, B. Volk, and C. Kaltschmidt, "Inhibition of NF-κB potentiates amyloid β-mediated neuronal apoptosis," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 96, no. 16, pp. 9409–9414, 1999.

[108] D. W. Lee and J. K. Andersen, "Role of HIF-1 in iron regulation: potential therapeutic strategy for neurodegenerative disorders," *Current Molecular Medicine*, vol. 6, no. 8, pp. 883–893, 2006.

[109] F. Martin, T. Linden, D. M. Katschinski et al., "Copper-dependent activation of hypoxia-inducible factor (HIF)-1: implications for ceruloplasmin regulation," *Blood*, vol. 105, no. 12, pp. 4613–4619, 2005.

[110] L. Tacchini, E. Gammella, C. De Ponti, S. Recalcati, and G. Cairo, "Role of HIF-1 and NF-κB transcription factors in the modulation of transferrin receptor by inflammatory and anti-inflammatory signals," *Journal of Biological Chemistry*, vol. 283, no. 30, pp. 20674–20686, 2008.

[111] D. Wang, L. -H. Wang, Y. Zhao, Y. -P. Lu, and L. Zhu, "Hypoxia regulates the ferrous iron uptake and reactive oxygen species level via divalent metal transporter 1 (DMT1) exon1B by hypoxia-inducible factor-1," *IUBMB Life*, vol. 62, no. 8, pp. 629–636, 2010.

[112] Y. Avramovich-Tirosh, O. Bar-Am, T. Amit, M. B.H. Youdim, and O. Weinreb, "Up-regulation of hypoxia-inducible factor (hif)-1α and hif-target genes in cortical neurons by the novel multifunctional iron chelator anti-alzheimer drug, M30," *Current Alzheimer Research*, vol. 7, no. 4, pp. 300–306, 2010.

[113] M. Scortegagna, Z. Galdzicki, S. I. Rapoport, and I. Hanbauer, "Activator protein-1 DNA binding activation by hydrogen peroxide in neuronal and astrocytic primary cultures of trisomy-16 and diploid mice," *Molecular Brain Research*, vol. 73, no. 1-2, pp. 144–150, 1999.

[114] L. Aimo and P. I. Oteiza, "Zinc deficiency increases the susceptibility of human neuroblastoma cells to lead-induced activator protein-1 activation," *Toxicological Sciences*, vol. 91, no. 1, pp. 184–191, 2006.

[115] W. M. Toone, B. A. Morgan, and N. Jones, "Redox control of AP-1-like factors in yeast and beyond," *Oncogene*, vol. 20, no. 19, pp. 2336–2346, 2001.

[116] L. Benvenisti-Zarom, J. Chen-Roetling, and R. F. Regan, "Inhibition of the ERK/MAP kinase pathway attenuates heme oxygenase-1 expression and heme-mediated neuronal injury," *Neuroscience Letters*, vol. 398, no. 3, pp. 230–234, 2006.

[117] E. Huang, W. -Y. Ong, M. -L. Go, and J. R. Connor, "Upregulation of iron regulatory proteins and divalent metal transporter-1 isoforms in the rat hippocampus after kainate induced neuronal injury," *Experimental Brain Research*, vol. 170, no. 3, pp. 376–386, 2006.

[118] K. S. Lee, K. Iijima-Ando, K. Iijima et al., "JNK/FOXO-mediated neuronal expression of fly homologue of peroxiredoxin II reduces oxidative stress and extends life span," *Journal of Biological Chemistry*, vol. 284, no. 43, pp. 29454–29461, 2009.

[119] M. K. Lehtinen, Z. Yuan, P. R. Boag et al., "A Conserved MST-FOXO Signaling Pathway Mediates Oxidative-Stress Responses and Extends Life Span," *Cell*, vol. 125, no. 5, pp. 987–1001, 2006.

[120] Z. Yuan, M. K. Lehtinen, P. Merlo, J. Villén, S. Gygi, and A. Bonni, "Regulation of neuronal cell death by MST1-FOXO1 signaling," *Journal of Biological Chemistry*, vol. 284, no. 17, pp. 11285–11292, 2009.

SAGE-Hindawi Access to Research
International Journal of Alzheimer's Disease
Volume 2011, Article ID 971021, 10 pages
doi:10.4061/2011/971021

Review Article

The Role of Zinc in Alzheimer's Disease

Nicole T. Watt, Isobel J. Whitehouse, and Nigel M. Hooper

Institute of Molecular and Cellular Biology, Faculty of Biological Sciences, University of Leeds, Clarendon Way, Leeds LS2 9JT, UK

Correspondence should be addressed to Nigel M. Hooper, n.m.hooper@leeds.ac.uk

Received 14 September 2010; Accepted 9 November 2010

Academic Editor: Anthony R. White

Copyright © 2011 Nicole T. Watt et al. This is an open access article distributed under the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original work is properly cited.

Zinc, the most abundant trace metal in the brain, has numerous functions, both in health and in disease. Zinc is released into the synaptic cleft of glutamatergic neurons alongside glutamate from where it interacts and modulates NMDA and AMPA receptors. In addition, zinc has multifactorial functions in Alzheimer's disease (AD). Zinc is critical in the enzymatic nonamyloidogenic processing of the amyloid precursor protein (APP) and in the enzymatic degradation of the amyloid-β (Aβ) peptide. Zinc binds to Aβ promoting its aggregation into neurotoxic species, and disruption of zinc homeostasis in the brain results in synaptic and memory deficits. Thus, zinc dyshomeostasis may have a critical role to play in the pathogenesis of AD, and the chelation of zinc is a potential therapeutic approach.

1. Introduction

Alzheimer's disease (AD) is the most prevalent form of dementia, which affects more than 37 million people worldwide, with an estimated cost of $422 billion in 2009 [1, 2]. Moreover, the incidence of the illness and the prospect of an aging population will result in rising social and economic demands. AD is characterised by the presence of extracellular amyloid plaques and intracellular neurofibrillary tangles within the afflicted brain, which cause neuronal loss in the neocortex, hippocampus, and basal forebrain, leading to progressive cognitive and behavioural decline [3]. Zinc, in addition to copper and iron, has been shown to be involved in AD. Here, we review the current literature relating to neuronal zinc metabolism and the way in which zinc can modulate normal brain activity. We discuss also the contribution of zinc to the formation, aggregation, and degradation of the amyloid-β (Aβ) peptide and the contribution of zinc to the pathogenesis of AD.

2. Physiological Role of Zinc in the Brain

As the most abundant trace metal in the brain, zinc is found tightly associated with numerous proteins conferring either structural or catalytic properties upon them [4]. However, a significant amount of loosely bound, chelatable zinc can be found sequestered in presynaptic vesicles forming a subpopulation of "zinc enriched" (ZEN) neurones [5, 6], which co-release zinc with the neurotransmitter glutamate upon excitation. The majority of these "gluzincergic" neurones [7] have their cell bodies located in either the cerebral cortex or the limbic structures of the forebrain [8], and so an extensive network uniting limbic and cerebrocortical functions is created [9]. This connection between zinc and glutamatergic neurotransmission allows the ion to modulate the overall excitability of the brain and also influence synaptic plasticity [10].

The identification of synaptic zinc was first made by McLardy over fifty years ago who identified that a band of zinc dithizonate staining correlated with hippocampal mossy fibre axons [7]. Since then, many more cerebrocortical pathways have been identified which contain zinc-rich synaptic vesicles; indeed nearly 50% of the glutamatergic synapses are actually "gluzincergic" in some parts of the cerebral cortex. Significantly, only small amounts of chelatable zinc can be determined in glutamatergic pathways which originate outside the cerebral cortex or limbic systems.

Despite this extensive network of zinc-containing neurons, little is known about how zinc homeostasis is maintained within the neuron. There are two families of zinc transporters: the ZnT family, which act to decrease intracellular zinc concentrations by exporting zinc from the cytoplasm to the lumen of organelles or the extracellular

space, and the ZIP family, which import the metal from the extracellular space or organellar lumen into the cytoplasm [11]. Whilst many of the transporters have particular distribution patterns, only ZnT3 expression is restricted to the brain and the testis [12]. It is located in the vesicular membrane [13] and is necessary to transport zinc from the cytoplasm into the synaptic vesicle of the neuron. The vesicular concentration of zinc correlates with the amount of ZnT3 present [14]. Targeted disruption of ZnT3 in a mouse model resulted in a complete lack of chelatable zinc [15].

A number of approaches have been taken to confirm that zinc is coreleased with glutamate from the presynaptic bouton during neuronal excitation: imaging of zinc in boutons before and after stimulation [16], analytical detection of zinc released into perfusates [17], and direct imaging of released zinc using fluorescent extracellular probes [18, 19]. This latter approach has provided the most definitive results. An early study employed a reporter construct whose fluorescence properties changed upon zinc binding. Stimulation of organotypic cultures from rat hippocampus produced a cloud of green fluorescence as the released zinc bound an apometalloenzyme confirming the release of zinc from the culture [17]. A later study by Quinta-Ferreira and colleagues [20] demonstrated a release of zinc with each pulse of an action potential. Whilst there is now no doubt that zinc is released during synaptic activity, there is little consensus on the amount or duration of its existence in the synaptic cleft [21].

Following an intense burst of neuronal activity, the release of glutamate and postsynaptic membrane depolarisation open a variety of zinc-permeable ion channels which contribute to removing the ion from the extracellular fluid. These include N-methyl-D-aspartate (NMDA) channels and calcium permeable α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid (AMPA)/kainate channels. The consequences of zinc acting on these receptors are diverse and demonstrate the significance zinc has in modulating fast excitatory glutamatergic transmission. Zinc can act to either enhance or depress synaptic activity with varying degrees of potency [21].

The most studied interaction is zinc with NMDA receptors (NMDAR). Initially, zinc was thought to selectively inhibit NMDAR-mediated neuronal activity by inducing a voltage-independent noncompetitive inhibition that decreased the probability of the channel being open [22–24]. A voltage-dependent inhibition of NMDAR could be observed at higher concentrations of zinc (>20 μM) and was believed to be due to binding of the cation within the channel pore [25]. With the cloning of NMDAR subunits, it was confirmed that zinc could cause both voltage-independent and voltage-dependent inhibition [26]. The exceptional sensitivity of the GluN2A subunit towards zinc suggests that even contaminating ions found in routine laboratory solutions are sufficient to cause inhibition [27]. Significantly, despite being responsible for inhibitory effects at NMDAR, it has also been shown that NMDAR activation may provide a route of influx for zinc contributing to toxic effects of exposure [28] (Figure 1).

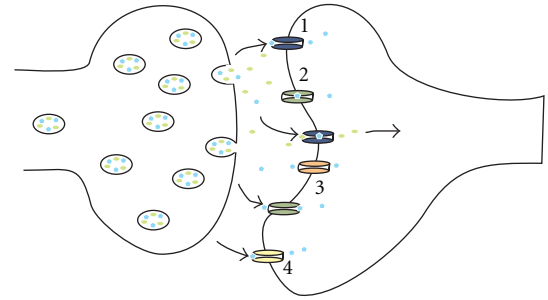


Figure 1: Multiple mechanisms for zinc uptake following synaptic release. Zinc and glutamate are released from a "gluzincergic" synapse during neurotransmission. The actions of glutamate, alongside membrane depolarisation, open a number of zinc-permeable channels on the postsynaptic membrane which clear the ion from the extracellular fluid. (1) AMPA receptors; (2) NMDA receptors; (3) voltage-gated calcium channels; (4) TRPM7. (blue pentagons) zinc ion and (green ellipses) glutamate molecule. For simplicity, zinc-permeable channels are only shown on the postsynaptic membrane.

Whilst it is thought that zinc exposure generally attenuates NMDAR-mediated neurotoxicity, zinc has been shown to potentiate AMPAR-mediated toxicity at suggested physiological concentrations (50 μM). Originally, it was proposed that the toxic effect was due to zinc influx via voltage-gated calcium channels [29], with Lin et al. [30] showing that desensitisation of AMPAR would explain such an enhancement. Subsequently, it has been demonstrated that zinc can carry currents directly via AMPAR, mostly via the calcium-permeable subtype [31] (Figure 1). At high supraphysiological doses (1 mM), zinc has been shown to inhibit AMPAR [21, 32]. A few studies have also looked directly at zinc-mediated inhibition of voltage-gated calcium channels, as their proximity to vesicular release sites on the presynaptic membrane suggests they could interact [33, 34]. Most recently, neurotoxicity has been attributed to transient receptor potential metastatin 7 (TRPM7) channel activation resulting in increased intracellular zinc [35] (Figure 1).

The significance of synaptically released zinc centres on the amount that is released into the synaptic cleft upon excitation. Some authors argue that the amount of zinc (10–100 μM) released following an action potential arriving at the glutamatergic bouton is high enough to bring about the voltage-dependent inhibition of NMDAR and that there would be no spillover onto neighbouring cells [36, 37]. Other authors disagree, suggesting that the zinc concentration would be sufficient to affect nearby cells [38]. It has also been demonstrated that the zinc level (low nM) is only sufficient to block the voltage-independent component of NMDAR activity [39]. Alternatively, it could be that there is little or no diffusible zinc released, supporting the notion that zinc behaves in a "tonic" mode. Kay and Tóth [40] proposed that zinc is exocytosed from the presynaptic membrane and that instead of diffusing into the extracellular space it remains tightly bound to an as-yet unidentified presynaptic component. This would create a "veneer" [41] of zinc ions

which would build up with synaptic activity or erode with quiescence to modulate plasticity.

Thus, the implication is that there are three different groups of zinc signals. First, "synaptic zinc" acts as a conventional neurotransmitter, is contained within presynaptic vesicles, and is released upon excitation and binds to a variety of receptors on the postsynaptic membrane. The downstream consequence of receptor binding is one of tonic modulation of glutamatergic excitatory synapses. The second type is similar to calcium signalling and occurs in conjunction with synaptic zinc signalling. A transmembrane flux of "synaptic zinc" from the extracellular space passes through post-synaptic zinc-permeable ion channels. The third is "intracellular zinc signalling" whereby existing intracellular stores are released [7]. This class is difficult to define and as yet has not been identified in neurons but has been demonstrated in mast cells [42].

Therefore, zinc can be classified as an endogenous modulator of synaptic transmission. It is found in synaptic vesicles, released upon excitation, and has multiple synaptic targets. The significant inhibitory effect of zinc on NMDAR, alongside the crucial function of NMDAR in both neurophysiology and pathophysiology, advocates a vital role for zinc in both healthy and diseased brains [21].

3. Role of Zinc in APP Processing

The most prominent lesions in the brain of AD sufferers are the amyloid or "senile" plaques, which predominantly consist of Aβ peptides derived from the proteolytic processing of the amyloid precursor protein (APP). APP is an ubiquitously expressed glycosylated transmembrane protein with a large N-terminal extracellular domain, a single hydrophobic transmembrane domain, and a small C-terminal cytoplasmic domain. A specific and saturable binding site for zinc (K_D = 750 nM) has been reported in the cysteine-rich region on the ectodomain of APP [44, 45]. It is hypothesised that zinc could have a role in sustaining the adhesiveness of APP during cell-cell and cell-matrix interactions [46, 47]. APP can be processed by one of two pathways: the amyloidogenic pathway, leading to the production of Aβ, and the non-amyloidogenic pathway (Figure 2(a)), reviewed in [48].

In the amyloidogenic pathway, APP is sequentially cleaved by the aspartyl protease, β-site APP-cleaving enzyme 1 (BACE1) forming the secreted APPβ (sAPPβ) fragment and a membrane bound C-terminal fragment of 99 amino acids (C99). The C99 fragment is then further processed by the γ-secretase complex into APP intracellular domain (AICD) and Aβ peptides, predominantly 40 and 42 amino acids in length. It is these aggregation-prone Aβ peptides which form oligomeric and fibrillar structures which deposit in the brain and over time cause AD. The γ-secretase complex comprises four components: presenilin (PS) 1 or 2, nicastrin (Nct), presenilin enhancer 2 (PEN-2), and anterior pharynx defective-1 (Aph-1) [49]. sAPPβ is involved in the pruning of synapses during the development of central and peripheral neurons [50] and AICD is known to be a transcription factor for several genes, including the upregulation of the Aβ-degrading enzyme, neprilysin (NEP) [51].

The predominant APP-processing pathway in healthy brain is the nonamyloidogenic pathway where APP is cleaved by the α-secretase within the Aβ region forming the secreted APPα (sAPPα) fragment and the membrane-bound C-terminal fragment of 83 amino acids (C83) (Figure 2(a)). C83 is subsequently cleaved by the γ-secretase complex generating AICD and p3 (Figure 2(a)). α-secretase activity is attributed to the disintegrin and metalloprotease (ADAM) family of zinc metalloproteases (reviewed in [48, 52]). The ADAMs, along with the matrix metalloproteases (MMPs), are members of the metzincin clan of metalloproteases as they have a long zinc-binding consensus sequence *H*EBX*H*XBGBX*H* (*H*, histidine, zinc ligand; E, catalytic glutamate; G, glycine; B, bulky apolar residue; X, any amino acid) which contains three zinc ligands [53]. Structurally, the catalytic domain is globular and divided into two subdomains, with the active site cleft running between the two [54]. The defining feature of the metzincins is the conserved methionine residue which creates a 1,4-β-turn (Met-turn), creating the catalytic cleft [53]. The catalytic zinc atom sits at the bottom of the groove between the subdomains, with the subsites in the groove determining specificity for particular amino acid sequences in the substrate (reviewed in [52]). A number of enzymes in this family, namely, ADAM9, 10, 17 (also known as TNF-α converting enzyme, TACE) and 19, are known to exert α-secretase activity, but it is unclear which enzyme or enzymes are responsible for the α-cleavage of APP *in vivo* [55–58]. ADAM10 appears to be the predominant enzyme as overexpression of functional ADAM10 in AD transgenic mice led to an increase in sAPPα and reduced Aβ production, plaque deposition, and cognitive deficits [59]. Although fibroblasts from ADAM10$^{-/-}$ mice showed no deficiency in α-secretase activity, probably due to compensation by ADAM17 [60], a recent detailed study has provided strong evidence that ADAM10 is the physiologically relevant constitutive α-secretase in primary neurons [61].

4. Role of Zinc in Aβ Degradation

In a healthy brain, the relatively small amount of Aβ-constitutively being produced is rendered safe by Aβ degrading enzymes. The steady state levels of Aβ synthesis and clearance in cerebrospinal fluid (CSF) are 7.6% and 8.3% per hour, respectively [62]. A large number of candidate Aβ-degrading enzymes have been identified, with the majority being zinc metalloproteases. These include NEP, insulin-degrading enzyme (IDE), endothelin-converting enzyme (ECE) 1 and 2, MMP2, 3, and 9, and PreP (reviewed in [43]). NEP, ECE1 and 2, and MMPs have the conserved zinc-binding motif *H*EXX*H* (*H*, histidine, zinc ligand; E, catalytic glutamate; X, any amino acid), and IDE and its homolog PreP are both inverzincins, as they contain the inverted zinc-binding motif *H*XXE*H*.

NEP appears to be the dominant Aβ protease [63–66], and is capable of degrading monomeric and oligomeric Aβ [63, 67] (Figure 2(b)). NEP knockout mice have significantly

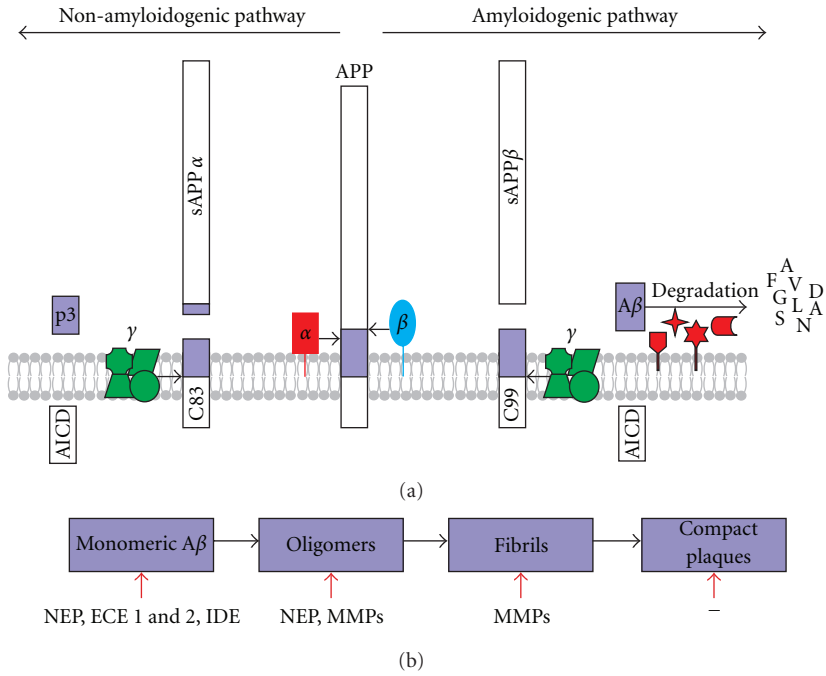


Figure 2: Zinc metalloprotease activity in APP processing. (a) In the amyloidogenic pathway, the transmembrane APP is cleaved first by β-secretase (β) to form the soluble sAPPβ and the membrane-bound C99, which in turn is cleaved by γ-secretase (γ) to form AICD and the amyloidogenic Aβ peptides. The Aβ peptides are degraded by a number of zinc metalloproteases including NEP and IDE. In the nonamyloidogenic pathway, APP is cleaved first by the α-secretase (α) (zinc metalloprotease) to form the soluble sAPPα and the membrane bound C83, which is then cleaved by γ-secretase (γ) to form p3 and AICD. Zinc metalloproteases depicted in red. Representative amino acids from the degradation of Aβ are shown in single letter code. (b) Action of zinc metalloproteases on monomeric and aggregated forms of Aβ (modified from [43]). Aβ: Amyloid β; AICD: APP intracellular domain; APP: Amyloid precursor protein; ECE: Endothelin-converting enzyme; IDE: Insulin degrading enzyme; MMP: Matrix metalloprotease; NEP: Neprilysin.

elevated Aβ levels [68, 69] and more importantly, human postmortem tissue analysis suggests that NEP expression is inversely related to the extent of AD pathology [70–72]. IDE is predominantly a cytosolic enzyme but has been found extracellularly [73] and can only degrade monomeric Aβ and not oligomers (Figure 2(b)), although it did lower the net production of oligomers by cultured cells [74]. Even though there are conflicting data regarding IDE and AD [75], IDE knockout mice have elevated levels of endogenous Aβ [76, 77]. There are two isoforms of ECE, 1 and 2, both of which have similar catalytic activity, with ECE 1 being the predominant isoform within the brain [65, 78]. However, both ECEs are primarily localised to endothelial cells but have been detected in neurons and glial [78]. The MMPs, specifically MMP2 and MMP9, degrade fibrillar Aβ [79], whereas PreP degrades monomeric Aβ. However, PreP is confined to mitochondria, so it would therefore have limited action on the majority of Aβ which is extracellular [80].

5. Zinc Binding to Aβ

High concentrations of zinc (up to 1 mM) have also been found within amyloid plaques [81], which is thought to have been released from glutamatergic synapses [82]. Aggregation of the Aβ peptides can be rapidly induced in the presence of zinc ions under physiological conditions *in vitro* [83, 84]

Figure 3: Aβ peptide sequence with potential zinc binding sites highlighted. Histidine residues are shown in blue and other potential zinc-binding sites are shown in pink.

and studies on AD tissues show that zinc colocalised with Aβ deposits [85]. The metal binding site for zinc on Aβ is the same as the copper binding site and lies within the N-terminal hydrophilic region, the first 16 residues [86] (Figure 3). The majority of studies on zinc binding to Aβ have utilised truncated peptides of 16 (Aβ1–16) and 28 (Aβ1–28) amino acids, as the binding region is situated within these sequences [86]. Aβ1–16 does not aggregate under moderate conditions [87–90], making it an ideal model system, and Aβ1–28 can undergo aggregation and fibril formation, but at significantly reduced rates, to the full-length peptides [91]. Zinc binds to Aβ in a 1 : 1 stoichiometry with a mononuclear binding site [84, 86–90, 92–94]. NMR studies have shown that all three histidine residues (residues 6, 13 and 14; see Figure 3) are involved in zinc binding [87, 88, 90, 93, 95], with confirmation from mutational studies [96, 97]. Other possible ligands have also been reported as zinc coordination classically involves four to six ligands [91]. The candidate ligands are Asp1 [91, 93], Arg5 [94],

Ser 8 [93], Glu11 [90], and Tyr10 [95] (Figure 3) however, Tyr10 has been ruled out [89] and Arg5 has been deemed highly unlikely [91]. The carboxyl side chain of Glu11 is a zinc ligand [88, 90] however, Asp1 is considered the most attractive zinc ligand, either through its N-terminal amino group and/or its side-chain carboxylate group [87, 88, 91, 93]. Raman spectroscopy has shown zinc binding to the Nτ site of histidine side chains in senile plaques taken from AD brains [98], but it is unclear if zinc only binds to free Aβ peptides that subsequently aggregate or whether zinc binds to Aβ in preformed plaques.

The reported apparent binding constants (^{a}Kd) of zinc to Aβ peptides vary from 1 to 300 μM (reviewed in [91]). The published ^{a}Kd values vary greatly due to the *in vitro* conditions (e.g., buffer composition, pH), Aβ fragment, and experimental method. The high ^{a}Kd (20–300 μM) values come from tyrosine fluorescence experiments however, even these are contentious and hard to reproduce [99–101], and any change in tyrosine fluorescence could be due to Aβ aggregation rather than zinc binding [101]. Discounting the tyrosine fluorescence measurements, the most likely apparent ^{a}Kd value for zinc binding to Aβ peptides is a range of 1–20 μM [91]. The binding affinity of zinc for preformed Aβ fibrils is approximately the same as of the peptides with a ^{a}Kd of 1–20 μM [89, 101].

6. Role of Zinc in Alzheimer's Disease

Numerous studies have looked to address whether zinc levels change with AD progression. It has been shown that there is a significant increase in serum [102] and hippocampal [5] zinc in AD patients compared to age-matched controls. Jiménez-Jiménez et al. [103] demonstrated a significant decrease in CSF zinc but could find no difference in serum zinc levels between AD and age-matched controls. A decrease in serum zinc has also been reported, though it is possible that some of the AD patients included in one study were malnourished [104, 105]. Overall, there is currently no consensus on what happens to zinc concentrations in AD subjects though much of the discrepancy could be put down to differences in patient allocation, sample type, postmortem interval, or type of analysis used.

Alternatively, a redistribution of zinc could be sufficient to promote disease progression. Lovell and coworkers [106, 107] have mapped the expression levels of a number of the ZnT zinc transporters in AD. ZnT-1, 4, and 6 were all found to show increased expression in early stages of disease, though ZnT-1 expression was decreased during mild cognitive impairment [106, 107]. Although it is unknown whether increased expression necessarily correlates with increased activity, these changes in transporter level could result in modified subcellular zinc concentrations. An increase in ZnT6 would lead to an increase in zinc in the TGN [108] which could reduce α-secretase activity [83].

It is well established that amyloid plaques contain increased concentrations of copper, iron, and zinc [98, 109]. Whilst copper and iron appear to be primarily responsible for the toxicity of Aβ via oxidative-stress-type mechanisms [109, 110], zinc has a crucial role in Aβ aggregation which is the most well-established contribution that zinc may have in AD pathogenesis. Whilst the concentration of zinc required for fibrillisation to occur is contentious, with concentrations differing by 100-fold being suggested, zinc is an unequivocal partner in the process [83, 111, 112]. In 2006, Dong and co-workers were able to show that zinc could control the rate of self-assembly of the Aβ peptides and go on to regulate the amyloid morphology via specific coordination sites [113]. Furthermore, it has been demonstrated that zinc can spontaneously coordinate both intra-and intermolecular bridging between two peptides to promote Aβ aggregation [114] and that synaptic zinc promotes Aβ oligomer formation and their accumulation at excitatory synapses [115].

Studies with synthetic Aβ showed that chelation chemistry could help solubilise amyloid plaques, with depletion of zinc having a more marked effect on extracting Aβ than depletion of copper [116]. Oral treatment with 5-chloro-7-iodo-8-hydroxyquinoline (Clioquinol CQ) in Tg2576 mice resulted in a 49% reduction in cortical amyloid deposition [117]. Although CQ has a fairly low affinity (nM) for both copper and zinc, it was still able to release the ions from the Aβ binding site [118]. A pilot phase II trial in humans showed a decrease in cognitive decline and a reduction in plasma $A\beta_{1-42}$ in moderately severe AD compared with placebo control [119]. It has been suggested that although CQ may chelate copper and zinc from metallated Aβ and promote disaggregation, it may not completely holt the aggregation process [120]. A second generation chelator (PBT-2), with improved blood brain barrier penetration, has just completed a phase II clinical trial in early AD with promising results showing good tolerance, a reduction in CSF Aβ and neuropsychological testing [121].

Recently, it has been shown that zinc can also accelerate the aggregation of a Tau peptide under reducing conditions [122]. Zinc inhibited the formation of intramolecular disulphide bonds but promoted intermolecular bonds between key cysteine residues. Furthermore, zinc exposure has been shown to increase the phosphorylation of PI3K and MAPK-dependent pathways which are key players in Tau modifications [123].

The essential requirement for ZnT3 in loading zinc into synaptic vesicles would suggest that this transporter could have a major impact on zinc signalling in the neuron, even regulating cognitive function. Whilst a lack of zinc signalling in brain slices from $ZnT3^{-/-}$ mice confirmed the vesicular origin of the released zinc, the mice failed to express a cognitive phenotype. Initial studies detailed a 20% reduction in total zinc level and a loss of histochemically reactive zinc in the synaptic vesicle; however, there was no impairment of spatial learning, memory, or sensorimotor function [124]. The implication being that the vesicular zinc is not required for cognitive function or that compensatory mechanisms made up for the deficits. However, a follow-up study demonstrated marked differences in learning and memory when an older (6 month) cohort of mice was used [125], suggesting that the lack of effect in the previous study was due to the young age (6–10 weeks) of the mice and highlights the importance of aging (the most significant risk

factor) when modelling AD pathology. The results obtained from the older ZnT3$^{-/-}$ cohort established a requirement for zinc in memory function and the maintenance of synaptic health upon aging. Adlard and colleagues proposed that β-amyloid pathology could cause cognitive impairment by trapping zinc within plaques rather than via a directly toxic mechanism [125]. The zinc immobilisation by amyloid would then have similar consequences as a loss of ZnT3 activity with a loss of zinc-dependent synaptic modulation promoting cognitive decline

An alternative approach to minimising the consequences of released zinc could be to promote mechanisms which enhance reuptake. Recently, it has been shown that the cellular form of the prion protein (PrP^C) is an evolutionary descendent of the ZIP family of divalent metal transporters. In particular, ZIPs 5, 6, and 10 were found to have a "prion-like" domain with significant structural similarity. As both PrP^C and the ZIPs bind divalent metal ions via histidine-rich motifs contained with N-terminal repeating sequences, this could suggest that PrP^C is involved in zinc sensing, scavenging, or transport [126]. In agreement with that possibility, we have shown that PrP^C promotes zinc uptake (Watt et al., unpublished). Ensuring efficient clearance of extracellular zinc from the synaptic cleft via PrP^C would exploit an existing physiological process. Furthermore, enhancing zinc uptake would help prevent its ability to contribute to the synaptic targeting of Aβ oligomers, thus preserving synaptic function [115] and maintaining the proposed ferroxidase activity of APP [127]. As PrP^C levels decrease with age and in sporadic AD [128], it is possible that zinc is cleared less efficiently from the synaptic cleft enhancing aggregation of Aβ and inhibiting APP ferroxidase activity to promote a pro-oxidative environment. This would suggest that preserving PrP^C function during AD could provide multifactorial benefits, an inhibition of BACE1 which would reduce Aβ formation [129] and ensure efficient clearance of zinc from the synaptic cleft to prevent aggregation of Aβ peptides, as well as provide protection against oxidative stress [130, 131].

7. Conclusions

It is clear that zinc not only plays critical roles in the structural and functional integrity of many proteins, but that it also modulates the activity of glutamatergic synapses and indeed may act as a neurotransmitter in its own right. Several of the enzymes involved in processing APP and Aβ are zinc metalloproteases, with an essential requirement for zinc in their catalytic activity. Zinc binds to Aβ, promoting its aggregation and thereby modulating its neurotoxicity. Although zinc dyshomeostasis may contribute to the development of AD, further work is required to clarify the molecular and cellular mechanisms affected by zinc under both normal and disease situations.

Acknowledgments

The authors gratefully acknowledge the financial support of the Medical Research Council. N. T. Watt and I. J. Whitehouse contributed equally to this work.

References

[1] C. Mount and C. Downton, "Alzheimer disease: progress or profit?" *Nature Medicine*, vol. 12, no. 7, pp. 780–784, 2006.

[2] A. Wimo, B. Winblad, and L. Jönsson, "The worldwide societal costs of dementia: estimates for 2009," *Alzheimer's and Dementia*, vol. 6, no. 2, pp. 98–103, 2010.

[3] D. J. Selkoe, "Alzheimer's disease: genes, proteins, and therapy," *Physiological Reviews*, vol. 81, no. 2, pp. 741–766, 2001.

[4] N. M. Hooper, "The biological roles of zinc and families of zinc metalloproteases," in *Zinc Metalloproteases in Health and Disease*, N. M. Hooper, Ed., pp. 1–21, Talor & Francis, London, UK, 1996.

[5] G. Danscher, K. B. Jensen, C. J. Frederickson et al., "Increased amount of zinc in the hippocampus and amygdala of Alzheimer's diseased brains: a proton-induced X-ray emission spectroscopic analysis of cryostat sections from autopsy material," *Journal of Neuroscience Methods*, vol. 76, no. 1, pp. 53–59, 1997.

[6] C. J. Frederickson and G. Danscher, "Zinc-containin neurons in hippocampus and related CNS structures," *Progress in Brain Research*, vol. 83, pp. 71–84, 1990.

[7] C. J. Frederickson and A. I. Bush, "Synaptically released zinc: physiological functions and pathological effects," *BioMetals*, vol. 14, no. 3-4, pp. 353–366, 2001.

[8] L. Slomianka, G. Danscher, and C. J. Frederickson, "Labeling of the neurons of origin of zinc-containing pathways by intraperitoneal injections of sodium selenite," *Neuroscience*, vol. 38, no. 3, pp. 843–854, 1990.

[9] J. Y. Koh, "Endogenous zinc in neurological diseases," *Journal of Clinical Neurology*, vol. 1, pp. 121–133, 2005.

[10] Y. Tian, Z. Yang, and T. Zhang, "Zinc ion as modulator effects on excitability and synaptic transmission in hippocampal CA1 neurons in Wistar rats," *Neuroscience Research*, vol. 68, no. 3, pp. 167–175, 2010.

[11] L. A. Lichten and R. J. Cousins, "Mammalian zinc transporters: nutritional and physiologic regulation," *Annual Review of Nutrition*, vol. 29, pp. 153–176, 2009.

[12] R. J. McMahon and R. J. Cousins, "Mammalian zinc transporters," *Journal of Nutrition*, vol. 128, no. 4, pp. 667–670, 1998.

[13] R. D. Palmiter, T. B. Cole, C. J. Quaife, and S. D. Findley, "ZnT-3, a putative transporter of zinc into synaptic vesicles," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 93, no. 25, pp. 14934–14939, 1996.

[14] D. H. Linkous, J. M. Flinn, J. Y. Koh et al., "Evidence that the ZNT3 protein controls the total amount of elemental zinc in synaptic vesicles," *Journal of Histochemistry and Cytochemistry*, vol. 56, no. 1, pp. 3–6, 2008.

[15] T. B. Cole, H. J. Wenzel, K. E. Kafer, P. A. Schwartzkroin, and R. D. Palmiter, "Elimination of zinc from synaptic vesicles in the intact mouse brain by disruption of the ZnT3 gene," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 96, no. 4, pp. 1716–1721, 1999.

[16] Y. Li, C. J. Hough, S. W. Suh, J. M. Sarvey, and C. J. Frederickson, "Rapid translocation of Zn^{2+} from presynaptic terminals into postsynaptic hippocampal neurons after physiological stimulation," *Journal of Neurophysiology*, vol. 86, no. 5, pp. 2597–2604, 2001.

[17] R. B. Thompson, W. O. Whetsell Jr., B. P. Maliwal, C. A. Fierke, and C. J. Frederickson, "Fluorescence microscopy of stimulated Zn(II) release from organotypic cultures of mammalian hippocampus using a carbonic anhydrase-based

biosensor system," *Journal of Neuroscience Methods*, vol. 96, no. 1, pp. 35–45, 2000.

[18] C. J. Frederickson, J. Y. Koh, and A. I. Bush, "The neurobiology of zinc in health and disease," *Nature Reviews Neuroscience*, vol. 6, no. 6, pp. 449–462, 2005.

[19] C. Bastian and Y. V. Li, "Fluorescence imaging study of extracellular zinc at the hippocampal mossy fiber synapse," *Neuroscience Letters*, vol. 419, no. 2, pp. 119–124, 2007.

[20] M. E. Quinta-Ferreira, C. M. Matias, M. Arif, and J. C. Dionísio, "Measurement of presynaptic zinc changes in hippocampal mossy fibers," *Brain Research*, vol. 1026, no. 1, pp. 1–10, 2004.

[21] P. Paoletti, A. M. Vergnano, B. Barbour, and M. Casado, "Zinc at glutamatergic synapses," *Neuroscience*, vol. 158, no. 1, pp. 126–136, 2009.

[22] S. Peters, J. Koh, and D. W. Choi, "Zinc selectively blocks the action of N-methyl-D-aspartate on cortical neurons," *Science*, vol. 236, no. 4801, pp. 589–593, 1987.

[23] G. L. Westbrook and M. L. Mayer, "Micromolar concentrations of Zn^{2+} antagonize NMDA and GABA responses of hippocampal neurons," *Nature*, vol. 328, no. 6131, pp. 640–643, 1987.

[24] C. W. Christine and D. W. Choi, "Effect of zinc on NMDA receptor-mediated channel currents in cortical neurons," *Journal of Neuroscience*, vol. 10, no. 1, pp. 108–116, 1990.

[25] P. Legendre and G. L. Westbrook, "The inhibition of single N-methyl-D-aspartate-activated channels by zinc ions on cultured rat neurones," *Journal of Physiology*, vol. 429, pp. 429–449, 1990.

[26] N. Chen, A. Moshaver, and L. A. Raymond, "Differential sensitivity of recombinant N-methyl-D-aspartate receptor subtypes to zinc inhibition," *Molecular Pharmacology*, vol. 51, no. 6, pp. 1015–1023, 1997.

[27] P. Paoletti, P. Ascher, and J. Neyton, "High-affinity zinc inhibition of NMDA NR1-NR2A receptors," *Journal of Neuroscience*, vol. 17, no. 15, pp. 5711–5725, 1997.

[28] J. Y. Koh, "Zinc toxicity on cultured cortical neurons: involvement of N-methyl-D-aspartate receptors," *Neuroscience*, vol. 60, no. 4, pp. 1049–1057, 1994.

[29] J. H. Weiss, D. M. Hartley, J. Y. Koh, and D. W. Choi, "AMPA receptor activation potentiates zinc neurotoxicity," *Neuron*, vol. 10, no. 1, pp. 43–49, 1993.

[30] D. D. Lin, A. S. Cohen, and D. A. Coulter, "Zinc-induced augmentation of excitatory synaptic currents and glutamate receptor responses in hippocampal CA3 neurons," *Journal of Neurophysiology*, vol. 85, no. 3, pp. 1185–1196, 2001.

[31] Y. Jia, J. M. Jeng, S. L. Sensi, and J. H. Weiss, "Zn^{2+} currents are mediated by calcium-permeable AMPA/kainate channels in cultured murine hippocampal neurones," *Journal of Physiology*, vol. 543, no. 1, pp. 35–48, 2002.

[32] A. Mathie, G. L. Sutton, C. E. Clarke, and E. L. Veale, "Zinc and copper: pharmacological probes and endogenous modulators of neuronal excitability," *Pharmacology and Therapeutics*, vol. 111, no. 3, pp. 567–583, 2006.

[33] J. Magistretti, L. Castelli, V. Taglietti, and F. Tanzi, "Dual effect of Zn^{2+} on multiple types of voltage-dependent Ca currents in rat palaeocortical neurons," *Neuroscience*, vol. 117, no. 2, pp. 249–264, 2003.

[34] H. S. Sun, K. Hui, D. W. K. Lee, and Z. P. Feng, "Zn^{2+} sensitivity of high- and low-voltage activated calcium channels," *Biophysical Journal*, vol. 93, no. 4, pp. 1175–1183, 2007.

[35] K. Inoue, D. Branigan, and Z. G. Xiong, "Zinc-induced neurotoxicity mediated by transient receptor potential melastatin 7 channels," *The Journal of Biological Chemistry*, vol. 285, no. 10, pp. 7430–7439, 2010.

[36] P. Molnár and J. V. Nadler, "Synaptically-released zinc inhibits N-methyl-D-aspartate receptor activation at recurrent mossy fiber synapses," *Brain Research*, vol. 910, no. 1-2, pp. 205–207, 2001.

[37] K. Vogt, J. Mellor, G. Tong, and R. Nicoll, "The actions of synaptically released zinc at hippocampal mossy fiber synapses," *Neuron*, vol. 26, no. 1, pp. 187–196, 2000.

[38] S. Ueno, M. Tsukamoto, T. Hirano et al., "Mossy fiber Zn^{2+} spillover modulates heterosynaptic N-methyl-D-aspartate receptor activity in hippocampal CA3 circuits," *Journal of Cell Biology*, vol. 158, no. 2, pp. 215–220, 2002.

[39] Y. M. Lu, F. A. Taverna, R. Tu et al., "Endogenous Zn^{2+} is required for the induction of long-term potentiation at rat hippocampal mossy fiber-CA3 synapses," *Synapse*, vol. 38, no. 2, pp. 187–197, 2000.

[40] A. R. Kay and K. Tóth, "Influence of location of a fluorescent zinc probe in brain slices on its response to synaptic activation," *Journal of Neurophysiology*, vol. 95, no. 3, pp. 1949–1956, 2006.

[41] A. R. Kay, "Evidence for chelatable zinc in the extracellular space of the hippocampus, but little evidence for synaptic release of Zn," *Journal of Neuroscience*, vol. 23, no. 17, pp. 6847–6855, 2003.

[42] S. Yamasaki, K. Sakata-Sogawa, A. Hasegawa et al., "Zinc is a novel intracellular second messenger," *Journal of Cell Biology*, vol. 177, no. 4, pp. 637–645, 2007.

[43] M. A. Leissring, "The AβCs of Aβ-cleaving proteases," *The Journal of Biological Chemistry*, vol. 283, no. 44, pp. 29645–29649, 2008.

[44] A. I. Bush, G. Multhaup, R. D. Moir et al., "A novel zinc(II) binding site modulates the function of the βA4 amyloid protein precursor of Alzheimer's disease," *The Journal of Biological Chemistry*, vol. 268, no. 22, pp. 16109–16112, 1993.

[45] A. I. Bush, W. H. Pettingell, M. De Paradis, R. E. Tanzi, and W. Wasco, "The amyloid β-protein precursor and its mammalian homologues. Evidence for a zinc-modulated heparin-binding superfamily," *The Journal of Biological Chemistry*, vol. 269, no. 43, pp. 26618–26621, 1994.

[46] G. Multhaup, A. I. Busha, P. Pollweina, and C. L. Masters, "Interaction between the zinc(II) and the heparin binding site of the Alzheimer's disease βA4 amyloid precursor protein (APP)," *FEBS Letters*, vol. 355, no. 2, pp. 151–154, 1994.

[47] G. Multhaup, T. Ruppert, A. Schlicksupp et al., "Copper-binding amyloid precursor protein undergoes a site-specific fragmentation in the reduction of hydrogen peroxide," *Biochemistry*, vol. 37, no. 20, pp. 7224–7230, 1998.

[48] V. W. Chow, M. P. Mattson, P. C. Wong, and M. Gleichmann, "An overview of APP processing enzymes and products," *Neuromolecular Medicine*, vol. 12, no. 1, pp. 1–12, 2010.

[49] D. Edbauer, E. Winkler, J. T. Regula, B. Pesold, H. Steiner, and C. Haass, "Reconstitution of γ-secretase activity," *Nature Cell Biology*, vol. 5, no. 5, pp. 486–488, 2003.

[50] A. Nikolaev, T. McLaughlin, D. D. M. O'Leary, and M. Tessier-Lavigne, "APP binds DR6 to trigger axon pruning and neuron death via distinct caspases," *Nature*, vol. 457, no. 7232, pp. 981–989, 2009.

[51] Ł. P. Słomnicki and W. Leśniak, "A putative role of the Amyloid Precursor Protein Intracellular Domain (AICD) in transcription," *Acta Neurobiologiae Experimentalis*, vol. 68, no. 2, pp. 219–228, 2008.

[52] D. R. Edwards, M. M. Handsley, and C. J. Pennington, "The ADAM metalloproteinases," *Molecular Aspects of Medicine*, vol. 29, no. 5, pp. 258–289, 2009.

[53] W. Bode, F. X. Gomis-Ruth, and W. Stockler, "Astacins, serralysins, snake venom and matrix metalloproteinases exhibit identical zinc-binding environments (HEXXHXXGXXH and Met-turn) and topologies and should be grouped into a common family, the ' metzincins' ," *FEBS Letters*, vol. 331, no. 1-2, pp. 134–140, 1993.

[54] F. X. Gomis-Rüth, "Structural aspects of the metzincin clan of metalloendopeptidases," *Applied Biochemistry and Biotechnology Part B*, vol. 24, no. 2, pp. 157–202, 2003.

[55] T. M. J. Allinson, E. T. Parkin, A. J. Turner, and N. M. Hooper, "ADAMs family members as amyloid precursor protein alpha-secretases," *Journal of Neuroscience Research*, vol. 74, no. 3, pp. 342–352, 2003.

[56] M. Asai, C. Hattori, B. Szabó et al., "Putative function of ADAM9, ADAM10, and ADAM17 as APP α-secretase," *Biochemical and Biophysical Research Communications*, vol. 301, no. 1, pp. 231–235, 2003.

[57] F. Fahrenholz, S. Gilbert, E. Kojro, S. Lammich, and R. Postina, "α-Secretase activity of the disintegrin metalloprotease ADAM 10: influences of domain structure," *Annals of the New York Academy of Sciences*, vol. 920, pp. 215–222, 2000.

[58] C. Tanabe, N. Hotoda, N. Sasagawa, A. Sehara-Fujisawa, K. Maruyama, and S. Ishiura, "ADAM19 is tightly associated with constitutive Alzheimer's disease APP α-secretase in A172 cells," *Biochemical and Biophysical Research Communications*, vol. 352, no. 1, pp. 111–117, 2007.

[59] R. Postina, A. Schroeder, I. Dewachter et al., "A disintegrin-metalloproteinase prevents amyloid plaque formation and hippocampal defects in an Alheizmer disease mouse model," *Journal of Clinical Investigation*, vol. 113, no. 10, pp. 1456–1464, 2004.

[60] D. Hartmann, B. de Strooper, L. Serneels et al., "The disintegrin/metalloprotease ADAM 10 is essential for Notch signalling but not for α-secretase activity in fibroblasts," *Human Molecular Genetics*, vol. 11, no. 21, pp. 2615–2624, 2002.

[61] P.-H. Kuhn, H. Wang, B. Dislich et al., "ADAM10 is the physiologically relevant, constitutive α-secretase of the amyloid precursor protein in primary neurons," *EMBO Journal*, vol. 29, no. 17, pp. 3020–3032, 2010.

[62] R. J. Bateman, L. Y. Munsell, J. C. Morris, R. Swarm, K. E. Yarasheski, and D. M. Holtzman, "Human amyloid-β synthesis and clearance rates as measured in cerebrospinal fluid in vivo," *Nature Medicine*, vol. 12, no. 7, pp. 856–861, 2006.

[63] S. S. El-Amouri, H. Zhu, J. Yu, R. Marr, I. M. Verma, and M. S. Kindy, "Neprilysin: an enzyme candidate to slow the progression of Alzheimer's disease," *American Journal of Pathology*, vol. 172, no. 5, pp. 1342–1354, 2008.

[64] L. B. Hersh and D. W. Rodgers, "Neprilysin and amyloid beta peptide degradation," *Current Alzheimer Research*, vol. 5, no. 2, pp. 225–231, 2008.

[65] J. S. Miners, S. Baig, J. Palmer, L. E. Palmer, P. G. Kehoe, and S. Love, "Aβ-degrading enzymes in Alzheimer's disease," *Brain Pathology*, vol. 18, no. 2, pp. 240–252, 2008.

[66] E. R. L. C. Vardy, A. J. Catto, and N. M. Hooper, "Proteolytic mechanisms in amyloid-β metabolism: therapeutic implications for Alzheimer's disease," *Trends in Molecular Medicine*, vol. 11, no. 10, pp. 464–472, 2005.

[67] H. Kanemitsu, T. Tomiyama, and H. Mori, "Human neprilysin is capable of degrading amyloid β peptide not only in the monomeric form but also the pathological oligomeric form," *Neuroscience Letters*, vol. 350, no. 2, pp. 113–116, 2003.

[68] J. Apelt, K. Ach, and R. Schliebs, "Aging-related down-regulation of neprilysin, a putative β-amyloid-degrading enzyme, in transgenic Tg2576 Alzheimer-like mouse brain is accompanied by an astroglial upregulation in the vicinity of β-amyloid plaques," *Neuroscience Letters*, vol. 339, no. 3, pp. 183–186, 2003.

[69] W. Farris, S. G. Schütz, J. R. Cirrito et al., "Loss of neprilysin function promotes amyloid plaque formation and causes cerebral amyloid angiopathy," *American Journal of Pathology*, vol. 171, no. 1, pp. 241–251, 2007.

[70] H. Akiyama, H. Kondo, K. Ikeda, M. Kato, and P. L. McGeer, "Immunohistochemical localization of neprilysin in the human cerebral cortex: inverse association with vulnerability to amyloid β-protein (Aβ) deposition," *Brain Research*, vol. 902, no. 2, pp. 277–281, 2001.

[71] E. A. Eckman, S. K. Adams, F. J. Troendle et al., "Regulation of steady-state β-amyloid levels in the brain by neprilysin and endothelin-converting enzyme but not angiotensin-converting enzyme," *The Journal of Biological Chemistry*, vol. 281, no. 41, pp. 30471–30478, 2006.

[72] E. Hellström-Lindahl, R. Ravid, and A. Nordberg, "Age-dependent decline of neprilysin in Alzheimer's disease and normal brain: inverse correlation with Aβ levels," *Neurobiology of Aging*, vol. 29, no. 2, pp. 210–221, 2008.

[73] W. C. Duckworth, R. G. Bennett, and F. G. Hamel, "A direct inhibitory effect of insulin on a cytosolic proteolytic complex containing insulin-degrading enzyme and multicatalytic proteinase," *The Journal of Biological Chemistry*, vol. 269, no. 40, pp. 24575–24580, 1994.

[74] K. Vekrellis, Z. Ye, W. Q. Qiu et al., "Neurons regulate extracellular levels of amyloid β-protein via proteolysis by insulin-degrading enzyme," *Journal of Neuroscience*, vol. 20, no. 5, pp. 1657–1665, 2000.

[75] S. Wang, R. Wang, L. Chen, D. A. Bennett, D. W. Dickson, and D.-S. Wang, "Expression and functional profiling of neprilysin, insulin-degrading enzyme, and endothelin-converting enzyme in prospectively studied elderly and Alzheimer's brain," *Journal of Neurochemistry*, vol. 115, no. 1, pp. 47–57, 2010.

[76] M. C. Leal, V. B. Dorfman, A. F. Gamba et al., "Plaque-associated overexpression of insulin-degrading enzyme in the cerebral cortex of aged transgenic Tg2576 mice with Alzheimer pathology," *Journal of Neuropathology and Experimental Neurology*, vol. 65, no. 10, pp. 976–987, 2006.

[77] B. C. Miller, E. A. Eckman, K. Sambamurti et al., "Amyloid-β peptide levels in brain are inversely correlated with insulysin activity levels in vivo," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 100, no. 10, pp. 6221–6226, 2003.

[78] A. P. Davenport, R. E. Kuc, and J. W. Mockridge, "Endothelin-converting enzyme in the human vasculature: evidence for differential conversion of big endothelin-3 by endothelial and smooth-muscle cells," *Journal of Cardiovascular Pharmacology*, vol. 31, supplement 1, pp. S1–S3, 1998.

[79] K. J. Yin, J. R. Cirrito, P. Yan et al., "Matrix metalloproteinases expressed by astrocytes mediate extracellular amyloid-β peptide catabolism," *Journal of Neuroscience*, vol. 26, no. 43, pp. 10939–10948, 2006.

[80] A. Falkevall, N. Alikhani, S. Bhushan et al., "Degradation of the amyloid β-protein by the novel mitochondrial

peptidasome, PreP," *The Journal of Biological Chemistry*, vol. 281, no. 39, pp. 29096–29104, 2006.

[81] M. A. Lovell, J. D. Robertson, W. J. Teesdale, J. L. Campbell, and W. R. Markesbery, "Copper, iron and zinc in Alzheimer's disease senile plaques," *Journal of the Neurological Sciences*, vol. 158, no. 1, pp. 47–52, 1998.

[82] J. Y. Lee, T. B. Cole, R. D. Palmiter, S. W. Suh, and J. Y. Koh, "Contribution by synaptic zinc to the gender-disparate plaque formation in human Swedish mutant APP transgenic mice," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 99, no. 11, pp. 7705–7710, 2002.

[83] A. I. Bush, W. H. Pettingell, G. Multhaup et al., "Rapid induction of Alzheimer Aβ amyloid formation by zinc," *Science*, vol. 265, no. 5177, pp. 1464–1467, 1994.

[84] K. H. Lim, Y. K. Kim, and Y. T. Chang, "Investigations of the molecular mechanism of metal-induced Aβ (1–40) amyloidogenesis," *Biochemistry*, vol. 46, no. 47, pp. 13523–13532, 2007.

[85] L. M. Miller, Q. Wang, T. P. Telivala, R. J. Smith, A. Lanzirotti, and J. Miklossy, "Synchrotron-based infrared and X-ray imaging shows focalized accumulation of Cu and Zn co-localized with β-amyloid deposits in Alzheimer's disease," *Journal of Structural Biology*, vol. 155, no. 1, pp. 30–37, 2006.

[86] S. A. Kozin, S. Zirah, S. Rebuffat, G. Hui Bon Hoa, and P. Debey, "Zinc binding to Alzheimer's Aβ(1-16) peptide results in stable soluble complex," *Biochemical and Biophysical Research Communications*, vol. 285, no. 4, pp. 959–964, 2001.

[87] Y. Mekmouche, Y. Coppel, K. Hochgräfe et al., "Characterization of the Zn^{II} binding to the peptide amyloid-β^{1-16} linked to Alzheimer's disease," *ChemBioChem*, vol. 6, no. 9, pp. 1663–1671, 2005.

[88] C. D. Syme and J. H. Viles, "Solution H NMR investigation of Zn^{2+} and Cd^{2+} binding to amyloid-beta peptide (Aβ) of Alzheimer's disease," *Biochimica et Biophysica Acta*, vol. 1764, no. 2, pp. 246–256, 2006.

[89] C. Talmard, A. Bouzan, and P. Faller, "Zinc binding to amyloid-β: isothermal titration calorimetry and Zn competition experiments with Zn sensors," *Biochemistry*, vol. 46, no. 47, pp. 13658–13666, 2007.

[90] S. Zirah, S. A. Kozin, A. K. Mazur et al., "Structural changes of region 1-16 of the Alzheimer disease amyloid β-peptide upon zinc binding and in vitro aging," *The Journal of Biological Chemistry*, vol. 281, no. 4, pp. 2151–2161, 2006.

[91] P. Faller and C. Hureau, "Bioinorganic chemistry of copper and zinc ions coordinated to amyloid-β peptide," *Dalton Transactions*, no. 7, pp. 1080–1094, 2009.

[92] C. Talmard, L. Guilloreau, Y. Coppel, H. Mazarguil, and P. Faller, "Amyloid-beta peptide forms monomeric complexes with Cu^{II} and Zn^{II} prior to aggregation," *ChemBioChem*, vol. 8, no. 2, pp. 163–165, 2007.

[93] J. Danielsson, R. Pierattelli, L. Banci, and A. Gräslund, "High-resolution NMR studies of the zinc-binding site of the Alzheimer's amyloid β-peptide," *FEBS Journal*, vol. 274, no. 1, pp. 46–59, 2007.

[94] S. Zirah, S. Rebuffat, S. A. Kozin et al., "Zinc binding properties of the amyloid fragment Aβ(1–16) studied by electrospray-ionization mass spectrometry," *International Journal of Mass Spectrometry*, vol. 228, no. 2-3, pp. 999–1016, 2003.

[95] C. C. Curtain, F. Ali, I. Volitakis et al., "Alzheimer's disease amyloid-beta binds copper and zinc to generate an allosterically ordered membrane-penetrating structure containing superoxide dismutase-like subunits," *The Journal of Biological Chemistry*, vol. 276, no. 23, pp. 20466–20473, 2001.

[96] S. T. Liu, G. Howlett, and C. J. Barrow, "Histidine-13 is a crucial residue in the zinc ion-induced aggregation of the Aβ peptide of Alzheimer's disease," *Biochemistry*, vol. 38, no. 29, pp. 9373–9378, 1999.

[97] D. S. Yang, J. McLaurin, K. Qin, D. Westaway, and P. E. Fraser, "Examining the zinc binding site of the amyloid-β peptide," *European Journal of Biochemistry*, vol. 267, no. 22, pp. 6692–6698, 2000.

[98] J. Dong, C. S. Atwood, V. E. Anderson et al., "Metal binding and oxidation of amyloid-β within isolated senile plaque cores: raman microscopic evidence," *Biochemistry*, vol. 42, no. 10, pp. 2768–2773, 2003.

[99] W. Garzon-Rodriguez, A. K. Yatsimirsky, and C. G. Glabe, "Binding of Zn(II), Cu(II), and Fe(II) ions to Alzheimer's Aβ peptide studied by fluorescence," *Bioorganic and Medicinal Chemistry Letters*, vol. 9, no. 15, pp. 2243–2248, 1999.

[100] F. Ricchelli, D. Drago, B. Filippi, G. Tognon, and P. Zatta, "Aluminum-triggered structural modifications and aggregation of β-amyloids," *Cellular and Molecular Life Sciences*, vol. 62, no. 15, pp. 1724–1733, 2005.

[101] V. Tõugu, A. Karafin, and P. Palumaa, "Binding of zinc(II) and copper(II) to the full-length Alzheimer's amyloid-β peptide," *Journal of Neurochemistry*, vol. 104, no. 5, pp. 1249–1259, 2008.

[102] L. L. Rulon, J. D. Robertson, M. A. Lovell, M. A. Deibel, W. D. Ehmann, and W. R. Markesbery, "Serum zinc levels and Alzheimer's disease," *Biological Trace Element Research*, vol. 75, no. 1–3, pp. 79–85, 2000.

[103] F. J. Jiménez-Jiménez, J. A. Molina, M. V. Aguilar et al., "Cerebrospinal fluid levels of transition metals in patients with Parkinson's disease," *Journal of Neural Transmission*, vol. 105, no. 4-5, pp. 497–505, 1998.

[104] C. Jeandel, M. B. Nicolas, F. Dubois, F. Nabet-Belleville, F. Penin, and G. Cuny, "Lipid peroxidation and free radical scavengers in Alzheimer's disease," *Gerontology*, vol. 35, no. 5-6, pp. 275–282, 1989.

[105] L. Baum, I. H. S. Chan, S. K. K. Cheung et al., "Serum zinc is decreased in Alzheimer's disease and serum arsenic correlates positively with cognitive ability," *BioMetals*, vol. 23, no. 1, pp. 173–179, 2010.

[106] M. A. Lovell, J. L. Smith, S. Xiong, and W. R. Markesbery, "Alterations in zinc transporter protein-1 (ZnT-1) in the brain of subjects with mild cognitive impairment, early, and late-stage Alzheimer's disease," *Neurotoxicity Research*, vol. 7, no. 4, pp. 265–271, 2005.

[107] J. L. Smith, S. Xiong, W. R. Markesbery, and M. A. Lovell, "Altered expression of zinc transporters-4 and -6 in mild cognitive impairment, early and late Alzheimer's disease brain," *Neuroscience*, vol. 140, no. 3, pp. 879–888, 2006.

[108] M. A. Lovell, "A potential role for alterations of zinc and zinc transport proteins in the progression of alzheimer's disease," *Journal of Alzheimer's Disease*, vol. 16, no. 3, pp. 471–483, 2009.

[109] M. A. Smith, P. L. R. Harris, L. M. Sayre, and G. Perry, "Iron accumulation in Alzheimer disease is a source of redox-generated free radicals," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 94, no. 18, pp. 9866–9868, 1997.

[110] D. A. Butterfield, T. Reed, S. F. Newman, and R. Sultana, "Roles of amyloid β-peptide-associated oxidative stress and brain protein modifications in the pathogenesis of Alzheimer's disease and mild cognitive impairment," *Free Radical Biology and Medicine*, vol. 43, no. 5, pp. 658–677, 2007.

[111] A. Clements, D. Allsop, D. M. Walsh, and C. H. Williams, "Aggregation and metal-binding properties of mutant forms of the amyloid Aβ peptide of Alzheimer's disease," *Journal of Neurochemistry*, vol. 66, no. 2, pp. 740–747, 1996.

[112] W. P. Esler, E. R. Stimson, J. M. Jennings, J. R. Ghilardi, P. W. Mantyh, and J. E. Maggio, "Zinc-induced aggregation of human and rat β-amyloid peptides in vitro," *Journal of Neurochemistry*, vol. 66, no. 2, pp. 723–732, 1996.

[113] J. Dong, J. E. Shokes, R. A. Scott, and D. G. Lynn, "Modulating amyloid self-assembly and fibril morphology with Zn(II)," *Journal of the American Chemical Society*, vol. 128, no. 11, pp. 3540–3542, 2006.

[114] Y. Miller, B. Ma, and R. Nussinov, "Zinc ions promote Alzheimer aβ aggregation via population shift of polymorphic states," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 107, no. 21, pp. 9490–9495, 2010.

[115] A. Deshpande, H. Kawai, R. Metherate, C. G. Glabe, and J. Busciglio, "A role for synaptic zinc in activity-dependent aβ oligomer formation and accumulation at excitatory synapses," *Journal of Neuroscience*, vol. 29, no. 13, pp. 4004–4015, 2009.

[116] R. A. Cherny, J. T. Legg, C. A. McLean et al., "Aqueous dissolution of Alzheimer's disease Aβ amyloid deposits by biometal depletion," *The Journal of Biological Chemistry*, vol. 274, no. 33, pp. 23223–23228, 1999.

[117] R. A. Cherny, C. S. Atwood, M. E. Xilinas et al., "Treatment with a copper-zinc chelator markedly and rapidly inhibits β-amyloid accumulation in Alzheimer's disease transgenic mice," *Neuron*, vol. 30, no. 3, pp. 665–676, 2001.

[118] C. Opazo, S. Luza, V. L. Villemagne et al., "Radioiodinated clioquinol as a biomarker for β-amyloid: Zn^{2+} complexes in Alzheimer's disease," *Aging Cell*, vol. 5, no. 1, pp. 69–79, 2006.

[119] C. W. Ritchie, A. I. Bush, A. Mackinnon et al., "Metal-protein attenuation with iodochlorhydroxyquin (clioquinol) targeting Abeta amyloid deposition and toxicity in Alzheimer disease: a pilot phase 2 clinical trial," *Archives of Neurology*, vol. 60, no. 12, pp. 1685–1691, 2003.

[120] A. M. Mancino, S. S. Hindo, A. Kochi, and M. H. Lim, "Effects of clioquinol on metal-triggered amyloid-beta aggregation revisited," *Inorganic Chemistry*, vol. 48, no. 20, pp. 9596–9598, 2009.

[121] L. Lannfelt, K. Blennow, H. Zetterberg et al., "Safety, efficacy, and biomarker findings of PBT2 in targeting Aβ as a modifying therapy for Alzheimer's disease: a phase IIa, double-blind, randomised, placebo-controlled trial," *The Lancet Neurology*, vol. 7, no. 9, pp. 779–786, 2008.

[122] Z. Y. Mo, Y. Z. Zhu, H. L. Zhu, J. B. Fan, J. Chen, and Y. Liang, "Low micromolar zinc accelerates the fibrillization of human Tau via bridging of Cys-291 and Cys-322," *The Journal of Biological Chemistry*, vol. 284, no. 50, pp. 34648–34657, 2009.

[123] W. L. An, C. Bjorkdahl, R. Liu, R. F. Cowburn, B. Winblad, and J. J. Pei, "Mechanism of zinc-induced phosphorylation of p70 S6 kinase and glycogen synthase kinase 3β in SH-SY5Y neuroblastoma cells," *Journal of Neurochemistry*, vol. 92, no. 5, pp. 1104–1115, 2005.

[124] T. B. Cole, A. Martyanova, and R. D. Palmiter, "Removing zinc from synaptic vesicles does not impair spatial learning, memory, or sensorimotor functions in the mouse," *Brain Research*, vol. 891, no. 1-2, pp. 253–265, 2001.

[125] P. A. Adlard, J. M. Parncutt, D. I. Finkelstein, and A. I. Bush, "Cognitive loss in zinc transporter-3 knock-out mice: a phenocopy for the synaptic and memory deficits of Alzheimer's disease?" *Journal of Neuroscience*, vol. 30, no. 5, pp. 1631–1636, 2010.

[126] G. Schmitt-Ulms, S. Ehsani, J. C. Watts, D. Westaway, and H. Wille, "Evolutionary descent of prion genes from the ZIP family of metal Ion transporters," *PLoS ONE*, vol. 4, no. 9, Article ID e7208, 2009.

[127] J. A. Duce, A. Tsatsanis, M. A. Cater et al., "Iron-export ferroxidase activity of beta-amyloid precursor protein is inhibited by zinc in Alzheimer's disease," *Cell*, vol. 142, no. 6, pp. 857–867, 2010.

[128] I. J. Whitehouse, C. Jackson, A. J. Turner, and N. M. Hooper, "Prion protein is reduced in aging and in sporadic but not in familial Alzheimer's Disease," *The Journal of Alzheimer's Disease*, vol. 22, pp. 1023–1031, 2010.

[129] E. T. Parkin, N. T. Watt, I. Hussain et al., "Cellular prion protein regulates β-secretase cleavage of the Alzheimer's amyloid precursor protein," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 104, no. 26, pp. 11062–11067, 2007.

[130] N. T. Watt, D. R. Taylor, A. Gillott, D. A. Thomas, W. S. S. Perera, and N. M. Hooper, "Reactive oxygen species-mediated β-clevage of the prion protein in the cellular response to oxidative stress," *The Journal of Biological Chemistry*, vol. 280, no. 43, pp. 35914–35921, 2005.

[131] C. L. Haigh, S. C. Drew, M. P. Boland et al., "Dominant roles of the polybasic proline motif and copper in the PrP23-89-mediated stress protection response," *Journal of Cell Science*, vol. 122, no. 10, pp. 1518–1528, 2009.

SAGE-Hindawi Access to Research
International Journal of Alzheimer's Disease
Volume 2011, Article ID 491597, 8 pages
doi:10.4061/2011/491597

Review Article

Insight into Glutamate Excitotoxicity from Synaptic Zinc Homeostasis

Atsushi Takeda

Department of Medical Biochemistry, School of Pharmaceutical Sciences, University of Shizuoka, Global COE, 52-1 Yada, Suruga-ku, Shizuoka 422-8526, Japan

Correspondence should be addressed to Atsushi Takeda, takedaa@u-shizuoka-ken.ac.jp

Received 7 September 2010; Accepted 5 November 2010

Academic Editor: Anthony R. White

Copyright © 2011 Atsushi Takeda. This is an open access article distributed under the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original work is properly cited.

Zinc is released from glutamatergic (zincergic) neuron terminals in the hippocampus, followed by the increase in Zn^{2+} concentration in the intracellular (cytosol) compartment, as well as that in the extracellular compartment. The increase in Zn^{2+} concentration in the intracellular compartment during synaptic excitation is mainly due to Zn^{2+} influx through calcium-permeable channels and serves as Zn^{2+} signaling as well as the case in the extracellular compartment. Synaptic Zn^{2+} homeostasis is important for glutamate signaling and altered under numerous pathological processes such as Alzheimer's disease. Synaptic Zn^{2+} homeostasis might be altered in old age, and this alteration might be involved in the pathogenesis and progression of Alzheimer's disease; Zinc may play as a key-mediating factor in the pathophysiology of Alzheimer's disease. This paper summarizes the role of Zn^{2+} signaling in glutamate excitotoxicity, which is involved in Alzheimer's disease, to understand the significance of synaptic Zn^{2+} homeostasis in the pathophysiology of Alzheimer's disease.

1. Introduction

Over 300 proteins require zinc for their functions in microorganisms, plants, and animals. Zinc powerfully influences cell division and differentiation [1]. Zinc is essential for brain growth and its function [2, 3]. Zinc concentration in the adult brain reaches approximately 200 μM [4]. Extracellular zinc concentration in the adult brain is estimated to be less than 1 μM [5]. Zinc concentration in the cerebrospinal fluid (CSF) is approximately 0.15 μM [6], while that in the plasma is approximately 15 μM. Zinc transport from the plasma to the cerebrospinal fluid is strictly regulated by the brain-barrier system, that is, the blood-CSF barrier. The blood-CSF barrier, in addition to the blood-brain barrier, is involved in zinc homeostasis in the brain [7, 8]. Zinc is relatively concentrated in the hippocampus and amygdala [9, 10]. The biological half-life of zinc is relatively long in theses two areas (hippocampus, 28 days; amygdala, 42 days). Zinc homeostasis in the brain is closely associated with neurological diseases including Alzheimer's disease [11–13] and may be spatiotemporally altered in their pathogenesis and progression.

Approximately 90% of the total brain zinc exists as zinc metalloproteins. The rest mainly exists in the presynaptic vesicles and is histochemically reactive as revealed by Timm's sulfide-silver staining method [14]. Histochemically reactive zinc is released along with neuronal activity; there is a large number of evidence on zincergic neurons that sequester zinc in the presynaptic vesicles and release it in a calcium- and impulse-dependent manner [15–18]. In the rat brain, Timm's stain is hardly observed just after the birth, and its intensity increases with brain development [19, 20], indicating that histochemically reactive zinc is involved in not only brain growth but also brain function. However, impairment of spatial learning, memory, or sensorimotor functions is not observed in zinc transporter-3-null mice, which lack the histochemically reactive zinc in synaptic vesicles [21]. Zinc transporter-3 is involved to zinc transport into synaptic vesicles. Therefore, physiological significance of histochemically reactive zinc in neuronal activity is still poorly understood.

The hippocampus plays an important role in learning, memory, and recognition of novelty [22]. The hippocampus

receives major input from the entorhinal cortex via the perforant pathway, the dentate granule cells project to the CA3 pyramidal cells via the mossy fibers, and the CA3 pyramidal cells project to the CA1 pyramidal cells via the Schaffer collaterals. The three pathways are glutamatergic (zincergic), and terminals of them are stained by Timm's method [23]. Zinc concentration in the presynaptic vesicles is the highest in the giant boutons of hippocampal mossy fibers. All giant boutons of mossy fibers contain zinc in the presynaptic vesicles, while approximately 45% of Schaffer collateral/commissural pathway is zinc-positive [24]. It has been reported that histochemically reactive zinc serves as an endogenous neuromodulator 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 [25, 26]. The zinc may participate in synaptic plasticity such as long-term potentiation (LTP) and long-term depression (LTD) that is believed as the mechanism of learning and memory [27–29].

The exact chemical form of histochemically reactive zinc is unknown. The zinc released in the extracellular space is estimated to serve in free form (Zn^{2+}) [30]. The basal Zn^{2+} concentrations are extremely low in both the extracellular ($\sim 10^{-8}$ M) and intracellular (cytosol) ($<10^{-9}$ M) compartments [31, 32]. Zn^{2+} concentration increases in both compartments by excitation of zincergic neurons [33] and serves for signaling [34, 35]. However, the extracellular and intracellular concentrations of Zn^{2+} reached after synaptic excitation are obscure. Other organelles such as the mitochondria and the endoplasmic reticulum including the cytoplasm may participate in the increase in cytosolic Zn^{2+} [36–38]. The mechanisms on Zn^{2+} homeostasis in both compartments remain to be clarified [39, 40].

Zn^{2+} signaling is required for brain function, while alteration of Zn^{2+} homeostasis may modify glutamate excitotoxicity, which is involved in Alzheimer's disease. This paper summarizes the role of Zn^{2+} signaling in glutamate excitotoxicity to understand the significance of zinc as a key-mediating factor in the pathophysiology of Alzheimer's disease.

2. Modulation of Glutamate Signaling by Zinc

ZnAF-2 is a membrane-impermeable zinc indicator and has a low K_d value of 2.7 nM for zinc, and its fluorescence is minimally changed in the presence of calcium, magnesium, cadmium, nickel, or other heavy metals [41]. ZnAF-2 DA, a diacetylated form of ZnAF-2, is taken up by cells and hydrolyzed to ZnAF-2, which cannot permeate the cell membrane. These two indicators make possible an observation of Zn^{2+} dynamics in extracellular and intracellular compartments. Zn^{2+} released from zincergic neuron terminals is immediately retaken up by the same terminals during tetanic stimulation and also taken up into postsynaptic neurons [33, 35]. Calcium cannels such as calcium-permeable AMPA/kainate receptors are involved in Zn^{2+} influx during synaptic excitation [5, 31, 33, 35, 42] (Figure 1). Because kainate receptors are abundantly expressed in mossy fibers, they might be involved in zinc influx into mossy fiber terminals [43].

Quinta-Ferreira and Matias [44, 45] report that Ca^{2+} influx into mossy fibers by tetanic stimulation is inhibited by endogenous zinc. In the CA3 and CA1, furthermore, Zn^{2+} released from zincergic neuron terminals suppresses the increase in Ca^{2+} influx into the presynaptic terminals after tetanic stimulation, followed by negative modulation of the presynaptic activity (exocytosis) (Figure 2) [33, 35]. In an experiment using synaptosomal fraction from rat hippocampal CA3, Zn^{2+} inhibits glutamate release via activation of presynaptic ATP-dependent potassium (K_{ATP}) channels [46]. Zn^{2+} released from zincergic neuron terminals may serve for negative feedback mechanisms against glutamate release in both the extracellular and intracellular compartments (Figure 2).

3. Crosstalk of Zn^{2+} Signaling to Ca^{2+} Signaling in Glutamate Excitotoxicity

In both the extracellular and the intracellular compartments, it is possible that zinc signaling plays a neuroprotective role against glutamate-induced excitotoxicity [46, 47]. Activation of presynaptic kainate receptors is involved in the release of zinc and glutamate from mossy fibers [48, 49], and astrocytes also release glutamate [50]. Loss of astrocyte glutamate homeostasis is a prerequisite for the excitotoxic cascade, a phenomenon that is becoming recognized in an increasing number of neurological disorders [51]. The significance of zinc release in excess excitation of mossy fibers is examined by regional delivery of glutamate (1 mM) to the stratum lucidum, in which mossy fibers exist. Zn^{2+} may negatively modulate Ca^{2+} mobilization in CA3 pyramidal cells under the delivery [52]. Intracellular Ca^{2+} mobilization via group I metabotropic glutamate receptor activation can be also negatively modulated by Zn^{2+} signaling in CA3 pyramidal cells [34]. These findings suggest that Zn^{2+}can protectively act on glutamate excitotoxicity via crosstalk to Ca^{2+} signaling.

In contrast, excess of intracellular Zn^{2+} is potentially neurotoxic as well as excess of intracellular Ca^{2+} [53–60] (Figure 2). The origin of the toxic zinc is a matter of debate and seems to be not only the extracellular compartment but also the intracellular compartment [61]. The exact borderline of intracellular Zn^{2+} level between physiological regulation and pathological effects remains poorly defined as discussed later. Côté et al. [62] report that the neurotoxic and neuroprotective actions of Zn^{2+} depend on its concentration and that this dual action is cell type specific. Lavoie et al. [63] report that intracellular zinc chelator influences hippocampal neuronal excitability in rats. Furthermore, chelation of endogenous zinc by CaEDTA causes a significant increase in ischemic cell death in hippocampal slice cultures [46]. In an in vivo microdialysis experiment, the increase in extracellular glutamate concentration induced with high 100 mM KCl was significantly enhanced in the presence of 1 mM CaEDTA in both the control and zinc-deficient rats [64]. These findings indicate that Zn^{2+} released from zincergic neurons may

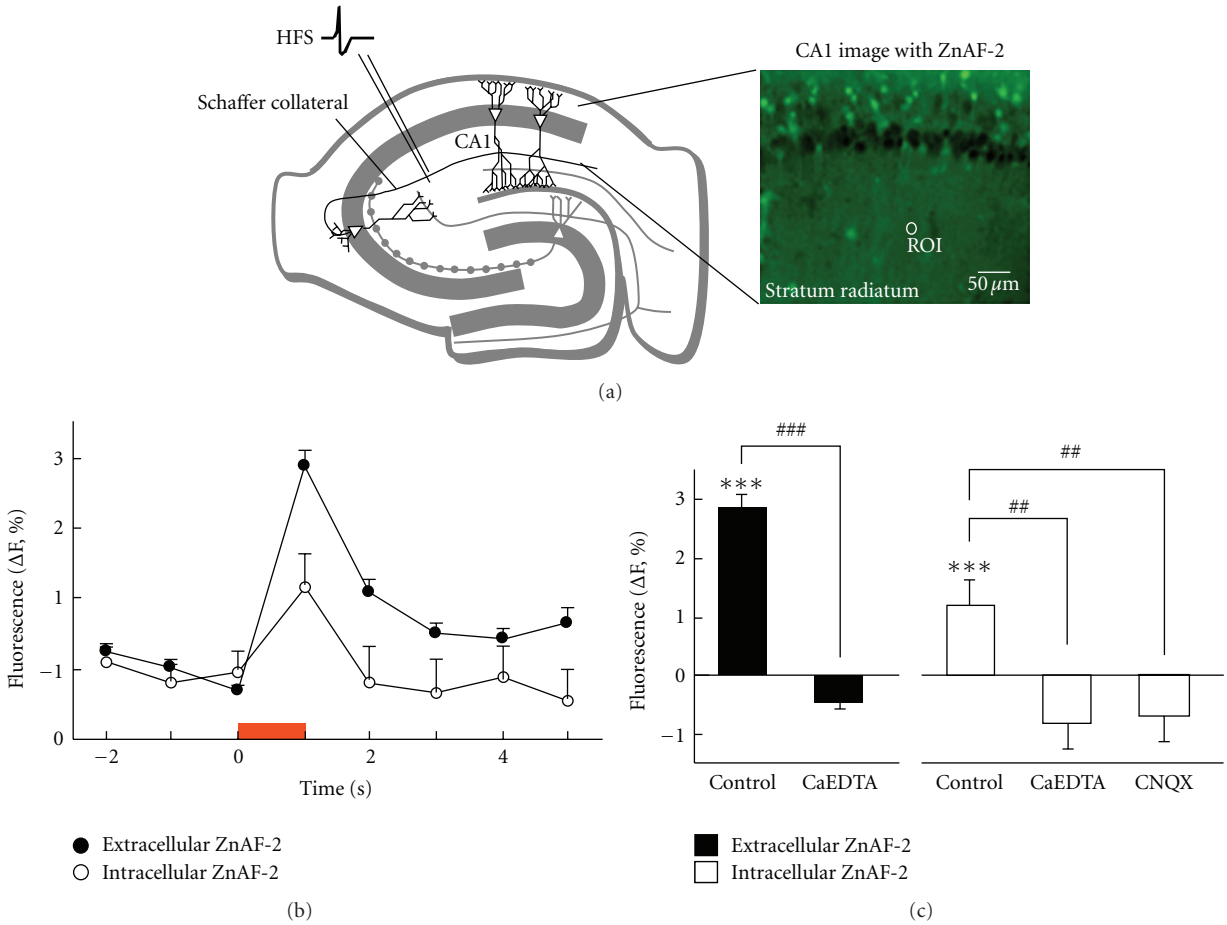


Figure 1: Changes in zinc signals in the extracellular and intracellular compartments in the hippocampal CA1 during tetanic stimulation. (a) Hippocampal illustration and CA1 image with ZnAF-2. (b) High-frequency stimulation (HSF, 200 Hz, 1 s) was delivered to the Schaffer collaterals in hippocampal slices stained with ZnAF-2 or ZnAF-2DA. The circle (around 10 μm in diameter) shown in Figure 1(a) is a representative example of the region of interest. The data represents the changed rate (%) in fluorescent signals to the basal fluorescent signal before the stimulation, which is expressed as 100%. The red bar indicates the period of electrical stimulation. (c) Tetanic stimulation (200 Hz, 1 s) was delivered to the Schaffer collaterals in hippocampal slices immersed in ACSF (control), 1 mM CaEDTA in ACSF, or 10 μM CNQX in ACSF. The data represents the changed rate (%) in fluorescent signal during tetanic stimulation to the basal fluorescent signal before the stimulation, which is expressed as 100%. $^{***}P < .001$, versus the basal level before the stimulation; $^{\#\#}P < .01$, $^{\#\#\#}P < .001$, versus the control (stimulated in ACSF). This data is cited from the paper published by Journal of Neuroscience Research, 2007 [35].

reduce glutamate release under pathological condition and protect hippocampal cells from the excitotoxicity (Figure 2).

4. Dietary Zinc Deficiency and Glutamate Excitotoxicity

Extracellular glutamate concentration is estimated to be around 2 μM in the brain, while glutamate concentration in the synaptic vesicles is markedly high (~100 mM) [65]. Excessive activation of glutamate receptors by excess of extracellular glutamate leads to a number of deleterious consequences, including impairment of calcium buffering, generation of free radicals, activation of the mitochondrial permeability transition, and secondary excitotoxicity [66, 67]. 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 [68–70]. The hippocampus is susceptible to glutamate excitotoxicity, is enriched with glucocorticoid receptors [71], and is a major target of glucocorticoids. Glucocorticoids may potentiate glutamate excitotoxicity, followed by the increase in neuronal death [72].

Dietary zinc deficiency readily decreases serum zinc level in mice and rats, while it increases serum corticosterone level through the increased hypothalamic-pituitary-adrenal (HPA) axis activity [73]. Brain zinc concentration is hardly decreased by zinc deficiency, while both histochemically reactive zinc and extracellular zinc in the brain are susceptible to chronic zinc deficiency [64, 74–76] (Figure 3). Excitability of zincergic neurons is potentially changed in cooperation with corticosterone under zinc deficiency [27]. Thus, the increased secretion of corticosterone might be

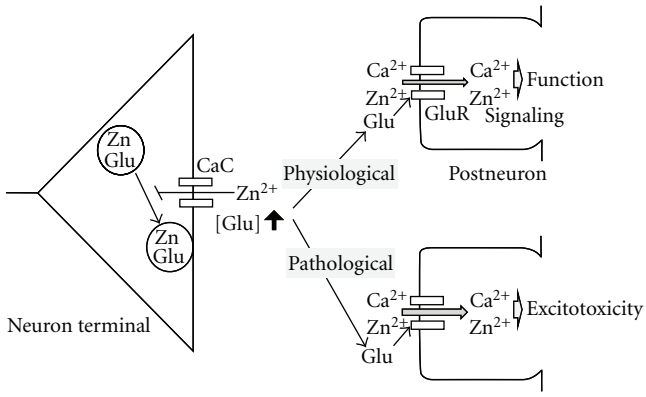


FIGURE 2: Zn^{2+} signaling and glutamate excitotoxicity. Zinc released from zincergic neuron terminals is immediately taken up into presynaptic and postsynaptic neurons through calcium-permeable channels (CaC and GluR). In presynaptic neurons, zinc negatively modulates exocytosis. The negative modulation by zinc may protectively serve for postsynaptic neurons under pathological conditions that are linked with glutamate excitotoxicity.

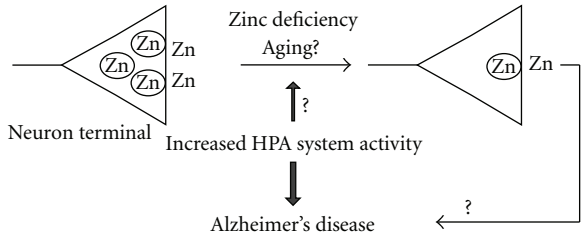


FIGURE 3: Histochemically reactive zinc level and its relation to the pathogenesis of Alzheimer's disease. Zinc deficiency can reduce histochemically reactive zinc levels, which are estimated to be susceptible to aging. Zinc deficiency, as well as aging, seems to be a risk factor for Alzheimer's disease.

associated with the decrease in histochemically reactive zinc and extracellular zinc under zinc deficiency. The increase in extracellular glutamate induced by 100 mM KCl is potentiated under zinc deficiency [64, 76]. Kainate and NMDA-induced seizures are potentiated in young mice and rats after 4-week zinc deprivation, which decreases histochemically reactive zinc [74, 77], and hippocampal cell death, which is induced by treatment with kainate, is increased under zinc deficiency [78]. These findings suggest that endogenous zinc, especially histochemically reactive zinc, has a protective action against glutamate excitotoxicity. The neurological symptoms associated with glutamate excitotoxicity may be aggravated by zinc deficiency.

Neuritic plaques, a pathological hallmark of Alzheimer's disease, are composed of β-amyloid that is precipitated by zinc released from zincergic neurons [79–81]. Glutamate excitotoxicity is associated with pathophysiology of Alzheimer's disease [67]. Glutamatergic signaling is compromised by β-amyloid-induced modulation of synaptic glutamate receptors in specific brain regions, paralleling early cognitive deficits [82]. Dietary zinc deficiency significantly increases total plaque volume in APP/PS1 mice, a transgenic mouse model of Alzheimer's disease, suggesting that zinc deficiency is a risk factor for Alzheimer's disease [83]. Interestingly, no obvious changes in histochemically reactive zinc levels are observed in zinc-deficient APP/PS1 mice. It is possible that the HPA axis activity in APP/PS1 mice is potentiated by zinc deficiency, like the case of normal mice and rats. Serum glucocorticoids are associated with the clearance of amyloid-beta peptide [84]. Thus, it seems to be important to study the participation of glucocorticoids in the β-amyloid plaque formation and degradation.

5. Zinc Homeostasis and Glutamate Excitotoxicity in Old Age

Zinc concentration in the brain remains constant in aged animals [85] and humans [4], whereas serum zinc level is significantly lower in aged animals than in young animals [86] and decreases with age in humans [87]. Histochemically reactive zinc levels are also lower in aged animals than in adult animals [88, 89]. Zinc transporter-3 expression, which is correlated with histochemically reactive zinc levels, is decreased with aging [90]. Thus, it is possible that histochemically reactive zinc levels are reduced in normal aging in humans [12, 90] (Figure 3). On the other hand, serum glucocorticoid concentration is significantly higher in aged animals [91]. The selective increase in the nocturnal levels of cortisol is observed in aged humans [92]. The increase in serum glucocorticoid level elicits some common changes in both aging and zinc deficiency. In addition to the decrease in serum zinc, the increase in the basal levels of intracellular Ca^{2+} and modification of Ca^{2+} signaling is observed in both aged [93, 94] and zinc-deficient [73, 77, 95] animals. It is likely that glucocorticoids influence the dynamics of both zinc signal and calcium signal and that the increased glucocorticoid secretion is associated with dysfunctions in zinc deficiency and aging that may increase the risk of diseases [28]. Aged animals and human might be more susceptible to glutamate excitotoxicity that is potentiated in zinc-deficient animals.

Insulin-degrading enzyme is a candidate protease in the clearance of amyloid-beta peptide from the brain and its levels are decreased in Alzheimer's disease. Insulin-degrading enzyme activity is known to be inhibited by glucocorticoid. Serum cortisol is associated with the clearance of amyloid-beta peptide [81] and the progression in subjects with Alzheimer-type dementia [96, 97]. Correlations have been reported between increases in HPA system activity and dementia severity or hippocampal volume loss in individuals with probable Alzheimer's disease [96]. On the other hand, serum zinc is decreased in progression of Alzheimer's disease [98]. Because zinc participates in amyloid-beta plaque deposition [79–81, 99], this metal may play as a key-mediating factor in the pathophysiology of Alzheimer's disease [100, 101]. Adlard et al. [90] report that cognitive loss is observed in 6-month-old zinc transporter-3-null mice, but not in 3-month-old zinc transporter-3-null mice. Cognitive impairment is age-dependent in zinc transporter-3-null mice, suggesting that long-term lack of synaptic zinc is implicated in the pathology leading to Alzheimer's disease

(Figure 3). Because zinc transporter-3 expression is reduced in the brain with Alzheimer's disease [90], it is possible that histochemical reactive zinc level is reduced in progression of Alzheimer's disease and that this reduction participates in its pathophysiology. In contrast, histochemically reactive zinc levels are not significantly changed in zinc-deficient APP/PS1 mice as described above [83]. Cognitive loss is potentially observed prior to the decrease in histochemically reactive zinc in zinc-deficient rats [102]. Judging from these data, it is likely that the increase in HPA axis activity participates in the pathogenesis and progression of Alzheimer's disease (Figure 3). This increase might be associated with the decrease in histochemically reactive zinc levels.

The basal (resting) level of histochemical reactive zinc/Zn^{2+} is estimated to be pico- to nanomolar in the cytosolic compartment ($8.1 < -\log[Zn^{2+}]_{\text{“free”}} < 10$) [103–105]. The synaptic vesicles serve as a large pool of histochemical reactive zinc in zincergic neurons. Other organelles such as the mitochondria and the endoplasmic reticulum might generally serve as the pool of histochemical reactive zinc in neurons and glia cells [36, 106]. Metallothioneins are also pools of Zn^{2+} [37, 38, 107]. On the other hand, extracellular zinc concentration after tetanic stimulation is estimated to range between 10 and 100 μM, because the low-affinity site ($IC_{50} \approx 20\,\mu M$ at -40 mV) of NMDA receptors is bound by zinc as an NMDA receptor blocker [108]. Hippocampal LTP is multifunctionally modulated in the presence of 5 μM $ZnCl_2$ [43, 109–111], suggesting that the concentration of endogenous zinc reaches very low micromolar concentrations in the extracellular compartment during the LTP induction. Judging from this estimation, it is possible that zinc signal transiently increases to more than 100 times of the basal level in the cytosolic compartment. Zn^{2+} might potentially reach submicromolar concentrations ($-\log[Zn^{2+}]_{\text{“free”}} < 6$) under pathological conditions [105].

In conclusion, the analysis on the relationship between Zn^{2+} dynamics and glutamatergic (zincergic) neuron activity in the brain in process of aging may be useful to find out the strategy to prevent neurodegenerative disorders such as Alzheimer's disease [112].

References

[1] B. L. Vallee and K. H. Falchuk, "The biochemical basis of zinc physiology," *Physiological Reviews*, vol. 73, no. 1, pp. 79–118, 1993.

[2] H. H. Sandstead, C. J. Frederickson, and J. G. Penland, "History of zinc as related to brain function," *Journal of Nutrition*, vol. 130, no. 2, pp. 496S–502S, 2000.

[3] S. C. Burdette and S. J. Lippard, "Meeting of the minds: metalloneurochemistry," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 100, no. 7, pp. 3605–3610, 2003.

[4] W. R. Markesbery, W. D. Ehmann, M. Alauddin, and T. I. M. Hossain, "Brain trace element concentrations in aging," *Neurobiology of Aging*, vol. 5, no. 1, pp. 19–28, 1984.

[5] J. H. Weiss, S. L. Sensi, and J. Y. Koh, "Zn^{2+}: a novel ionic mediator of neural injury in brain disease," *Trends in Pharmacological Sciences*, vol. 21, no. 10, pp. 395–401, 2000.

[6] C. O. Hershey, L. A. Hershey, and A. Varnes, "Cerebrospinal fluid trace element content in dementia: clinical, radiologic, and pathologic correlations," *Neurology*, vol. 33, no. 10, pp. 1350–1353, 1983.

[7] A. Takeda, "Movement of zinc and its functional significance in the brain," *Brain Research Reviews*, vol. 34, no. 3, pp. 137–148, 2000.

[8] A. Takeda, "Zinc homeostasis and functions of zinc in the brain," *BioMetals*, vol. 14, no. 3-4, pp. 343–351, 2001.

[9] A. Takeda, T. Akiyama, J. Sawashita, and S. Okada, "Brain uptake of trace metals, zinc and manganese, in rats," *Brain Research*, vol. 640, no. 1-2, pp. 341–344, 1994.

[10] A. Takeda, J. Sawashita, and S. Okada, "Biological half-lives of zinc and manganese in rat brain," *Brain Research*, vol. 695, no. 1, pp. 53–58, 1995.

[11] M. Capasso, J. M. Jeng, M. Malavolta, E. Mocchegiani, and S. L. Sensi, "Zinc dyshomeostasis: a key modulator of neuronal injury," *Journal of Alzheimer's Disease*, vol. 8, no. 2, pp. 93–108, 2005.

[12] E. Mocchegiani, C. Bertoni-Freddari, F. Marcellini, and M. Malavolta, "Brain, aging and neurodegeneration: role of zinc ion availability," *Progress in Neurobiology*, vol. 75, no. 6, pp. 367–390, 2005.

[13] J. P. Bressler, L. Olivi, J. H. Cheong, Y. Kim, A. Maerten, and D. Bannon, "Metal transporters in intestine and brain: their involvement in metal-associated neurotoxicities," *Human and Experimental Toxicology*, vol. 26, no. 3, pp. 221–229, 2007.

[14] C. J. Frederickson, "Neurobiology of zinc and zinc-containing neurons," *International review of neurobiology*, vol. 31, pp. 145–238, 1989.

[15] C. J. Frederickson and A. I. Bush, "Synaptically released zinc: physiological functions and pathological effects," *BioMetals*, vol. 14, no. 3-4, pp. 353–366, 2001.

[16] C. J. Frederickson and D. W. Moncrieff, "Zinc-containing neurons," *Biological Signals*, vol. 3, no. 3, pp. 127–139, 1994.

[17] J. Qian and J. L. Noebels, "Visualization of transmitter release with zinc fluorescence detection at the mouse hippocampal mossy fibre synapse," *Journal of Physiology*, vol. 566, no. 3, pp. 747–758, 2005.

[18] C. J. Frederickson, L. J. Giblin III, B. Rengarajan et al., "Synaptic release of zinc from brain slices: factors governing release, imaging, and accurate calculation of concentration," *Journal of Neuroscience Methods*, vol. 154, no. 1-2, pp. 19–29, 2006.

[19] T. Valente and C. Auladell, "Developmental expression of ZnT3 in mouse brain: correlation between the vesicular zinc transporter protein and chelatable vesicular zinc (CVZ) cells. Glial and neuronal CVZ cells interact," *Molecular and Cellular Neuroscience*, vol. 21, no. 2, pp. 189–204, 2002.

[20] T. Valente, C. Auladell, and J. Pérez-Clausell, "Postnatal development of zinc-rich terminal fields in the brain of the rat," *Experimental Neurology*, vol. 174, no. 2, pp. 215–229, 2002.

[21] T. B. Cole, A. Martyanova, and R. D. Palmiter, "Removing zinc from synaptic vesicles does not impair spatial learning, memory, or sensorimotor functions in the mouse," *Brain Research*, vol. 891, no. 1-2, pp. 253–265, 2001.

[22] C. M. Bird and N. Burgess, "The hippocampus and memory: insights from spatial processing," *Nature Reviews Neuroscience*, vol. 9, no. 3, pp. 182–194, 2008.

[23] C. J. Frederickson and G. Danscher, "Zinc-containin neurons in hippocampus and related CNS structures," *Progress in Brain Research*, vol. 83, pp. 71–84, 1990.

[24] C. B. Sindreu, H. Varoqui, J. D. Erickson, and J. Pérez-Clausell, "Boutons containing vesicular zinc define a subpopulation of synapses with low AMPAR content in rat hippocampus," *Cerebral Cortex*, vol. 13, no. 8, pp. 823–829, 2003.

[25] T. G. Smart, X. Xie, and B. J. Krishek, "Modulation of inhibitory and excitatory amino acid receptor ion channels by zinc," *Progress in Neurobiology*, vol. 42, no. 3, pp. 393–441, 1994.

[26] L. Besser, E. Chorin, I. Sekler et al., "Synaptically released zinc triggers metabotropic signaling via a zinc-sensing receptor in the hippocampus," *Journal of Neuroscience*, vol. 29, no. 9, pp. 2890–2901, 2009.

[27] A. Takeda and H. Tamano, "Insight into zinc signaling from dietary zinc deficiency," *Brain Research Reviews*, vol. 62, no. 1, pp. 33–44, 2009.

[28] A. Takeda and H. Tamano, "Zinc signaling through glucocorticoid and glutamate signaling in stressful circumstances," *Journal of Neuroscience Research*, vol. 88, no. 14, pp. 3002–3010, 2010.

[29] A. S. Nakashima and R. H. Dyck, "Zinc and cortical plasticity," *Brain Research Reviews*, vol. 59, no. 2, pp. 347–373, 2009.

[30] C. J. Frederickson, J. Y. Koh, and A. I. Bush, "The neurobiology of zinc in health and disease," *Nature Reviews Neuroscience*, vol. 6, no. 6, pp. 449–462, 2005.

[31] S. L. Sensi, L. M. T. Canzoniero, S. P. Yu et al., "Measurement of intracellular free zinc in living cortical neurons: routes of entry," *Journal of Neuroscience*, vol. 17, no. 24, pp. 9554–9564, 1997.

[32] C. J. Frederickson, L. J. Giblin, A. Krezel et al., "Concentrations of extracellular free zinc (pZn) in the central nervous system during simple anesthetization, ischemia and reperfusion," *Experimental Neurology*, vol. 198, no. 2, pp. 285–293, 2006.

[33] A. Minami, N. Sakurada, S. Fuke et al., "Inhibition of presynaptic activity by zinc released from mossy fiber terminals during tetanic stimulation," *Journal of Neuroscience Research*, vol. 83, no. 1, pp. 167–176, 2006.

[34] A. Takeda, S. Fuke, A. Minami, and N. Oku, "Role of zinc influx via AMPA/kainate receptor activation in metabotropic glutamate receptor-mediated calcium release," *Journal of Neuroscience Research*, vol. 85, no. 6, pp. 1310–1317, 2007.

[35] A. Takeda, S. Fuke, W. Tsutsumi, and N. Oku, "Negative modulation of presynaptic activity by zinc released from schaffer collaterals," *Journal of Neuroscience Research*, vol. 85, no. 16, pp. 3666–3672, 2007.

[36] S. L. Sensi, D. Ton-That, P. G. Sullivan et al., "Modulation of mitochondrial function by endogenous Zn^{2+} pools," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 100, no. 10, pp. 6157–6162, 2003.

[37] W. Maret, "Zinc coordination environments in proteins as redox sensors and signal transducers," *Antioxidants and Redox Signaling*, vol. 8, no. 9-10, pp. 1419–1441, 2006.

[38] A. Krezel, Q. Hao, and W. Maret, "The zinc/thiolate redox biochemistry of metallothionein and the control of zinc ion fluctuations in cell signaling," *Archives of Biochemistry and Biophysics*, vol. 463, no. 2, pp. 188–200, 2007.

[39] V. Frazzini, E. Rockabrand, E. Mocchegiani, and S. L. Sensi, "Oxidative stress and brain aging: is zinc the link?" *Biogerontology*, vol. 7, no. 5-6, pp. 307–314, 2006.

[40] Y. Qin, D. Thomas, C. P. Fontaine, and R. A. Colvin, "Mechanisms of Zn^{2+} efflux in cultured cortical neurons," *Journal of Neurochemistry*, vol. 107, no. 5, pp. 1304–1313, 2008.

[41] T. Hirano, K. Kikuchi, Y. Urano, and T. Nagano, "Improvement and biological applications of fluorescent probes for zinc, ZnAFs," *Journal of the American Chemical Society*, vol. 124, no. 23, pp. 6555–6562, 2002.

[42] Y. Jia, J. M. Jeng, S. L. Sensi, and J. H. Weiss, "Zn^{2+} currents are mediated by calcium-permeable AMPA/kainate channels in cultured murine hippocampal neurones," *Journal of Physiology*, vol. 543, no. 1, pp. 35–48, 2002.

[43] M. Ando, N. Oku, and A. Takeda, "Zinc-mediated attenuation of hippocampal mossy fiber long-term potentiation induced by forskolin," *Neurochemistry International*, vol. 57, no. 5, pp. 608–614, 2010.

[44] M. E. Quinta-Ferreira and C. M. Matias, "Hippocampal mossy fiber calcium transients are maintained during long-term potentiation and are inhibited by endogenous zinc," *Brain Research*, vol. 1004, no. 1-2, pp. 52–60, 2004.

[45] M. E. Quinta-Ferreira and C. M. Matias, "Tetanically released zinc inhibits hippocampal mossy fiber calcium, zinc and synaptic responses," *Brain Research*, vol. 1047, no. 1, pp. 1–9, 2005.

[46] V. Bancila, I. Nikonenko, Y. Dunant, and A. Bloc, "Zinc inhibits glutamate release via activation of pre-synaptic K_{ATP} channels and reduces ischaemic damage in rat hippocampus," *Journal of Neurochemistry*, vol. 90, no. 5, pp. 1243–1250, 2004.

[47] E. Cohen-Kfir, W. Lee, S. Eskandari, and N. Nelson, "Zinc inhibition of γ-aminobutyric acid transporter 4 (GAT4) a link between excitatory and inhibitory neurotransmission," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 102, no. 17, pp. 6154–6159, 2005.

[48] S. E. Lauri, Z. A. Bortolotto, R. Nistico et al., "A role for Ca^{2+} stores in kainate receptor-dependent synaptic facilitation and LTP at mossy fiber synapses in the hippocampus," *Neuron*, vol. 39, no. 2, pp. 327–341, 2003.

[49] A. Rodríguez-Moreno and T. S. Sihra, "Presynaptic kainate receptor facilitation of glutamate release involves protein kinase A in the rat hippocampus," *Journal of Physiology*, vol. 557, no. 3, pp. 733–745, 2004.

[50] S. Koizumi, K. Fujishita, and K. Inoue, "Regulation of cell-to-cell communication mediated by astrocytic ATP in the CNS," *Purinergic Signalling*, vol. 1, no. 3, pp. 211–217, 2005.

[51] A. M. D. J. Domingues, M. Taylor, and R. Fern, "Glia as transmitter sources and sensors in health and disease," *Neurochemistry International*, vol. 57, no. 4, pp. 359–366, 2010.

[52] A. Takeda, A. Minami, N. Sakurada, S. Nakajima, and N. Oku, "Response of hippocampal mossy fiber zinc to excessive glutamate release," *Neurochemistry International*, vol. 50, no. 2, pp. 322–327, 2007.

[53] J. Y. Koh, S. W. Suh, B. J. Gwag, Y. Y. He, C. Y. Hsu, and D. W. Choi, "The role of zinc in selective neuronal death after transient global cerebral ischemia," *Science*, vol. 272, no. 5264, pp. 1013–1016, 1996.

[54] D. W. Choi and J. Y. Koh, "Zinc and brain injury," *Annual Review of Neuroscience*, vol. 21, pp. 347–375, 1998.

[55] J. M. Lee, G. J. Zipfel, and D. W. Choi, "The changing landscape of ischaemic brain injury mechanisms," *Nature*, vol. 399, no. 6738, pp. A7–A14, 1999.

[56] S. L. Sensi and J. M. Jeng, "Rethinking the excitotoxic ionic millieu: the emerging role of Zn^{2+} in ischemic neuronal injury," *Current Molecular Medicine*, vol. 4, no. 2, pp. 87–111, 2004.

[57] K. M. Noh, H. Yokota, T. Mashiko, P. E. Castillo, R. S. Zukin, and M. V. L. Bennett, "Blockade of calcium-permeable AMPA receptors protects hippocampal neurons against global schemia-induced death," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 102, no. 34, pp. 12230–12235, 2005.

[58] S. W. Suh, E. T. Gum, A. M. Hamby, P. H. Chan, and R. A. Swanson, "Hypoglycemic neuronal death is triggered by glucose reperfusion and activation of neuronal NADPH oxidase," *Journal of Clinical Investigation*, vol. 117, no. 4, pp. 910–918, 2007.

[59] M. Hershfinkel, K. Kandler, M. E. Knoch et al., "Intracellular zinc inhibits KCC2 transporter activity," *Nature Neuroscience*, vol. 12, no. 6, pp. 725–727, 2009.

[60] K. He and E. Aizenman, "ERK signaling leads to mitochondrial dysfunction in extracellular zinc-induced neurotoxicity," *Journal of Neurochemistry*, vol. 114, no. 2, pp. 452–461, 2010.

[61] J. Y. Lee, T. B. Cole, R. D. Palmiter, and J. Y. Koh, "Accumulation of zinc in degenerating hippocampal neurons of ZnT3-null mice after seizures: evidence against synaptic vesicle origin," *The Journal of Neuroscience*, vol. 20, no. 11, p. RC79, 2000.

[62] A. Côté, M. Chiasson, M. R. Peralta III, K. Lafortune, L. Pellegrini, and K. Tóth, "Cell type-specific action of seizure-induced intracellular zinc accumulation in the rat hippocampus," *Journal of Physiology*, vol. 566, no. 3, pp. 821–837, 2005.

[63] N. Lavoie, M. R. Peralta III, M. Chiasson et al., "Extracellular chelation of zinc does not affect hippocampal excitability and seizure-induced cell death in rats," *Journal of Physiology*, vol. 578, no. 1, pp. 275–289, 2007.

[64] A. Takeda, H. Tamano, H. Itoh, and N. Oku, "Attenuation of abnormal glutamate release in zinc deficiency by zinc and Yokukansan," *Neurochemistry International*, vol. 53, no. 6–k8, pp. 230–235, 2008.

[65] B. S. Meldrum, "Glutamate as a neurotransmitter in the brain: review of physiology and pathology," *Journal of Nutrition*, vol. 130, no. 4, pp. 1007S–1015S, 2000.

[66] N. C. Danbolt, "Glutamate uptake," *Progress in Neurobiology*, vol. 65, no. 1, pp. 1–105, 2001.

[67] X. X. Dong, Y. Wang, and Z. H. Qin, "Molecular mechanisms of excitotoxicity and their relevance to pathogenesis of neurodegenerative diseases," *Acta Pharmacologica Sinica*, vol. 30, no. 4, pp. 379–387, 2009.

[68] D. W. Choi and S. M. Rothman, "The role of glutamate neurotoxicity in hypoxic-ischemic neuronal death," *Annual Review of Neuroscience*, vol. 13, pp. 171–182, 1990.

[69] S. A. Lipton and P. A. Rosenberg, "Mechanisms of disease: excitatory amino acids as a final common pathway for neurologic disorders," *New England Journal of Medicine*, vol. 330, no. 9, pp. 613–622, 1994.

[70] T. P. Obrenovitch and J. Urenjak, "Altered glutamatergic transmission in neurological disorders: from high extracellular glutamate to excessive synaptic efficacy," *Progress in Neurobiology*, vol. 51, no. 1, pp. 39–87, 1997.

[71] M. Kawata, K. Yuri, H. Ozawa et al., "Steroid hormones and their receptors in the brain," *Journal of Steroid Biochemistry and Molecular Biology*, vol. 65, no. 1-6, pp. 273–280, 1998.

[72] L. P. Reagan and B. S. McEwen, "Controversies surrounding glucocorticoid-mediated cell death in the hippocampus," *Journal of Chemical Neuroanatomy*, vol. 13, no. 3, pp. 149–167, 1997.

[73] A. Takeda, H. Tamano, F. Kan, H. Itoh, and N. Oku, "Anxiety-like behavior of young rats after 2-week zinc deprivation," *Behavioural Brain Research*, vol. 177, no. 1, pp. 1–6, 2007.

[74] A. Takeda, M. Hirate, H. Tamano, D. Nisibaba, and N. Oku, "Susceptibility to kainate-induced seizures under dietary zinc deficiency," *Journal of Neurochemistry*, vol. 85, no. 6, pp. 1575–1580, 2003.

[75] A. Takeda, H. Itoh, H. Tamano, and N. Oku, "Responsiveness to kainate in young rats after 2-week zinc deprivation," *BioMetals*, vol. 19, no. 5, pp. 565–572, 2006.

[76] A. Takeda, M. Hirate, H. Tamano, and N. Oku, "Release of glutamate and GABA in the hippocampus under zinc deficiency," *Journal of Neuroscience Research*, vol. 72, no. 4, pp. 537–542, 2003.

[77] A. Takeda, H. Itoh, A. Nagayoshi, and N. Oku, "Abnormal Ca mobilization in hippocampal slices of epileptic animals fed a zinc-deficient diet," *Epilepsy Research*, vol. 83, no. 1, pp. 73–80, 2009.

[78] A. Takeda, H. Tamano, A. Nagayoshi, K. Yamada, and N. Oku, "Increase in hippocampal cell death after treatment with kainate in zinc deficiency," *Neurochemistry International*, vol. 47, no. 8, pp. 539–544, 2005.

[79] A. I. Bush, W. H. Pettingell, G. Multhaup et al., "Rapid induction of Alzheimer Aβ amyloid formation by zinc," *Science*, vol. 265, no. 5177, pp. 1464–1467, 1994.

[80] J. Y. Lee, T. B. Cole, R. D. Palmiter, S. W. Suh, and J. Y. Koh, "Contribution by synaptic zinc to the gender-disparate plaque formation in human Swedish mutant APP transgenic mice," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 99, no. 11, pp. 7705–7710, 2002.

[81] S. W. Suh, K. B. Jensen, M. S. Jensen et al., "Histochemically-reactive zinc in amyloid plaques, angiopathy, and degenerating neurons of Alzheimer's diseased brains," *Brain Research*, vol. 852, no. 2, pp. 274–278, 2000.

[82] K. Parameshwaran, M. Dhanasekaran, and V. Suppiramaniam, "Amyloid beta peptides and glutamatergic synaptic dysregulation," *Experimental Neurology*, vol. 210, no. 1, pp. 7–13, 2008.

[83] M. Stoltenberg, A. I. Bush, G. Bach et al., "Amyloid plaques arise from zinc-enriched cortical layers in APP/PS1 transgenic mice and are paradoxically enlarged with dietary zinc deficiency," *Neuroscience*, vol. 150, no. 2, pp. 357–369, 2007.

[84] J. J. Kulstad, P. J. McMillan, J. B. Leverenz et al., "Effects of chronic glucocorticoid administration on insulin-degrading enzyme and amyloid-beta peptide in the aged macaque," *Journal of Neuropathology and Experimental Neurology*, vol. 64, no. 2, pp. 139–146, 2005.

[85] A. Takeda, N. Sakurada, S. Kanno, M. Ando, and N. Oku, "Vulnerability to seizures induced by potassium dyshomeostasis in the hippocampus in aged rats," *Journal of Health Science*, vol. 54, no. 1, pp. 37–42, 2008.

[86] E. Mocchegiani, R. Giacconi, C. Cipriano, E. Muti, N. Gasparini, and M. Malavolta, "Are zinc-bound metallothionein isoforms (I+II and III) involved in impaired thymulin production and thymic involution during ageing?" *Immunity and Ageing*, vol. 1, article no. 5, 2004.

[87] M. Idei, K. Miyake, Y. Horiuchi et al., "Serum zinc concentration decreases with age and is associated with anemia in middle-aged and elderly people," *Rinsho Byori*, vol. 58, no. 3, pp. 205–210, 2010.

[88] A. Ricci, M. T. Ramacci, O. Ghirardi, and F. Amenta, "Age-related changes of the mossy fibre system in rat hippocampus: effect of long term acetyl-L-carnitine treatment," *Archives of Gerontology and Geriatrics*, vol. 8, no. 1, pp. 63–71, 1989.

[89] P. Barili, C. Fringuelli, A. Ricci, I. Rossodivita, and M. Sabbatini, "Age-related changes of sulphide-silver staining in the rat hippocampus," *Mechanisms of Ageing and Development*, vol. 99, no. 2, pp. 83–94, 1997.

[90] P. A. Adlard, J. M. Parncutt, D. I. Finkelstein, and A. I. Bush, "Cognitive loss in zinc transporter-3 knock-out mice: a phenocopy for the synaptic and memory deficits of Alzheimer's disease?" *Journal of Neuroscience*, vol. 30, no. 5, pp. 1631–1636, 2010.

[91] P. W. Landfield and J. C. Eldridge, "Evolving aspects of the glucocorticoid hypothesis of brain aging: hormonal modulation of neuronal calcium homeostasis," *Neurobiology of Aging*, vol. 15, no. 4, pp. 579–588, 1994.

[92] E. Ferrari, D. Casarotti, B. Muzzoni et al., "Age-related changes of the adrenal secretory pattern: possible role in pathological brain aging," *Brain Research Reviews*, vol. 37, no. 1–3, pp. 294–300, 2001.

[93] J. M. Billard, "Ageing, hippocampal synaptic activity and magnesium," *Magnesium Research*, vol. 19, no. 3, pp. 199–215, 2006.

[94] T. C. Foster, "Calcium homeostasis and modulation of synaptic plasticity in the aged brain," *Aging Cell*, vol. 6, no. 3, pp. 319–325, 2007.

[95] H. Tamano, F. Kan, M. Kawamura, N. Oku, and A. Takeda, "Behavior in the forced swim test and neurochemical changes in the hippocampus in young rats after 2-week zinc deprivation," *Neurochemistry International*, vol. 55, no. 7, pp. 536–541, 2009.

[96] J. G. Csernansky, H. Dong, A. M. Fagan et al., "Plasma cortisol and progression of dementia in subjects with Alzheimer-type dementia," *American Journal of Psychiatry*, vol. 163, no. 12, pp. 2164–2169, 2006.

[97] H. Dong and J. G. Csernansky, "Effects of stress and stress hormones on amyloid-beta protein and plaque deposition," *Journal of Alzheimer's Disease*, vol. 18, no. 2, pp. 459–469, 2009.

[98] J. Dong, J. D. Robertson, W. R. Markesbery, and M. A. Lovell, "Serum zinc in the progression of Alzheimer's disease," *Journal of Alzheimer's Disease*, vol. 15, no. 3, pp. 443–450, 2008.

[99] A. Deshpande, H. Kawai, R. Metherate, C. G. Glabe, and J. Busciglio, "A role for synaptic zinc in activity-dependent aβ oligomer formation and accumulation at excitatory synapses," *Journal of Neuroscience*, vol. 29, no. 13, pp. 4004–4015, 2009.

[100] M. P. Cuajungco and K. Y. Fagét, "Zinc takes the center stage: its paradoxical role in Alzheimer's disease," *Brain Research Reviews*, vol. 41, no. 1, pp. 44–56, 2003.

[101] S. Vasto, G. Candore, F. Listì et al., "Inflammation, genes and zinc in Alzheimer's disease," *Brain Research Reviews*, vol. 58, no. 1, pp. 96–105, 2008.

[102] A. Takeda, H. Tamano, M. Tochigi, and N. Oku, "Zinc homeostasis in the hippocampus of zinc-deficient young adult rats," *Neurochemistry International*, vol. 46, no. 3, pp. 221–225, 2005.

[103] R. A. Colvin, A. I. Bush, I. Volitakis et al., "Insights into Zn^{2+} homeostasis in neurons from experimental and modeling studies," *American Journal of Physiology*, vol. 294, no. 3, pp. C726–C742, 2008.

[104] W. Maret, "Molecular aspects of human cellular zinc homeostasis: redox control of zinc potentials and zinc signals," *BioMetals*, vol. 22, no. 1, pp. 149–157, 2009.

[105] A. Krezel and W. Maret, "Thionein/metallothionein control Zn(II) availability and the activity of enzymes," *Journal of Biological Inorganic Chemistry*, vol. 13, no. 3, pp. 401–409, 2008.

[106] C. J. Stork and Y. V. Li, "Zinc release from thapsigargin/IP3-sensitive stores in cultured cortical neurons," *Journal of Molecular Signaling*, vol. 5, article no. 5, 2010.

[107] W. Maret and Y. Li, "Coordination dynamics of zinc in proteins," *Chemical Reviews*, vol. 109, no. 10, pp. 4682–4707, 2009.

[108] K. Vogt, J. Mellor, G. Tong, and R. Nicoll, "The actions of synaptically released zinc at hippocampal mossy fiber synapses," *Neuron*, vol. 26, no. 1, pp. 187–196, 2000.

[109] A. Takeda, S. Kanno, N. Sakurada, M. Ando, and N. Oku, "Attenuation of hippocampal mossy fiber long-term potentiation by low micromolar concentrations of zinc," *Journal of Neuroscience Research*, vol. 86, no. 13, pp. 2906–2911, 2008.

[110] A. Takeda, S. Fuke, M. Ando, and N. Oku, "Positive modulation of long-term potentiation at hippocampal CA1 synapses by low micromolar concentrations of zinc," *Neuroscience*, vol. 158, no. 2, pp. 585–591, 2009.

[111] A. Takeda, H. Iwaki, M. Ando, K. Itagaki, M. Suzuki, and N. Oku, "Zinc differentially acts on components of long-term potentiation at hippocampal CA1 synapses," *Brain Research*, vol. 1323, pp. 59–64, 2010.

[112] J. A. Duce and A. I. Bush, "Biological metals and Alzheimer's disease: implications for therapeutics and diagnostics," *Progress in Neurobiology*, vol. 92, no. 1, pp. 1–18, 2010.

SAGE-Hindawi Access to Research
International Journal of Alzheimer's Disease
Volume 2011, Article ID 607543, 11 pages
doi:10.4061/2011/607543

Review Article

Lead, Manganese, and Methylmercury as Risk Factors for Neurobehavioral Impairment in Advanced Age

Bernard Weiss

Department of Environmental Medicine, School of Medicine and Dentistry, University of Rochester, Rochester, NY 14642, USA

Correspondence should be addressed to Bernard Weiss, bernard_weiss@urmc.rochester.edu

Received 13 September 2010; Revised 28 October 2010; Accepted 24 November 2010

Academic Editor: Peter Faller

Copyright © 2011 Bernard Weiss. This is an open access article distributed under the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original work is properly cited.

Contamination of the environment by metals is recognized as a threat to health. One of their targets is the brain, and the adverse functional effects they induce are reflected by neurobehavioral assessments. Lead, manganese, and methylmercury are the metal contaminants linked most comprehensively to such disorders. Because many of these adverse effects can appear later in life, clues to the role of metals as risk factors for neurodegenerative disorders should be sought in the exposure histories of aging populations. A review of the available literature offers evidence that all three metals can produce, in advanced age, manifestations of neurobehavioral dysfunction associated with neurodegenerative disease. Among the critical unresolved questions is timing; that is, during which periods of the lifespan, including early development, do environmental exposures lay the foundations for their ultimate effects?

1. Introduction

In soliciting contributions for this special issue of the *International Journal of Alzheimer's Disease*, the editors featured the metals copper, zinc, iron, and aluminum. Except perhaps for aluminum, none of the four are considered to be major environmental hazards for neurodegenerative disorders. The three metals addressed in this paper provide a contrasting view. Lead, organic mercury, and manganese are identified as environmental risk factors for such disorders because of the scale of their distribution. It is worth reviewing how they achieved this status because such an appraisal may offer a useful model for the ways in which we evaluate the heath risks of other metals as well as those discussed here.

Environmental neurotoxicology adopted a view of the coupling of metal exposure and disease determined by its early connections to risk assessment. It approached its evaluations from angles dictated by legislation and regulation, especially after passage, by the US Congress, of the Toxic Substances Control Act (TSCA), signed into law in 1976.

As practiced by the US Environmental Protection Agency, and, in fact, by its counterparts in other nations, its primary task is to determine the health risks of exposure to environmental agents, quantify them, and then prescribe exposure standards that offer an adequate margin of safety. Although economic impacts are to be considered, typically, because exposures to environmental chemicals offer no health benefits, the health risks assume priority and exposure standards are sought that offer a robust margin of safety. The standards are not formulated specifically to protect against clinical disorders such as Alzheimer's disease. Instead, the margin of safety is designed to provide a buffer against even minimal adverse effects. The exposure criterion is designated as the Reference Dose (RfD) and is defined as "An estimate, with uncertainty spanning perhaps an order of magnitude, of a daily oral exposure to the human population (including sensitive subgroups) that is likely to be without an appreciable risk of deleterious effects during a lifetime." This definition is pertinent to disease etiology because it underscores the developing consensus that neurodegenerative disorders may originate in events and circumstances that antedate, even by decades, their overt clinical manifestations. Further, the first indications of such a progression may take the form of subtle, insidious, minimal toxic manifestations that foreshadow the emergence of clinically apparent disorders [1].

Long before the concept of environmental protection arose, along with its regulatory machinery, all three metals

Table 1: Age-specific incidence rates for Alzheimer's disease. Mean estimates are the averages of the smoothed age-specific incidence curves from these studies [2].

Age, years	Estimate
60	0.08
65	0.17
70	0.35
75	0.71
80	1.44
85	2.92
90	5.95
95	12.10

had been identified as neurotoxicants. Lead and mercury had been acknowledged as poisons even in ancient texts. Manganese came to be viewed as a neurological poison in the 19th century. The new principles of environmental protection changed the equation to a focus on early detection of potential adverse effects.

The connection between the principles and practices of environmental protection and the role of metals in neurodegenerative disorders may seem superficially remote. It is, in fact, one model for how we might formulate research on that question and how to convert that research into practice. That model emphasizes the early stages of the progression to a clinical entity.

The perspective adopted by this paper adheres to that approach. One way in which it might be depicted graphically appears in Figure 1. It was formulated originally [3] to show how even a small shift in the IQ distribution of a population exposed to elevated levels of lead can create disproportionate consequences at the extremes of the distribution. As shown there, a 5% lowering of the mean (5 IQ points on standardized tests) leads to a 57% increase in the proportion of the population classified as "retarded," a definition based on the usual criterion of an IQ of 70. With a mean of 100, the usual standardized mean, and a standard deviation (SD) of 15, as on the Stanford-Binet, an IQ of 70 lies two SDs below the mean.

Figure 1 is only a model for how to depict a specific measure. A similar chart could be made for a component of cognitive function such as memory. On a population basis, most aspects of cognitive function decline with age. According to the analysis of Hedden and Gabrieli [4], these include inductive reasoning, spatial orientation, perceptual speed, and verbal memory. Vocabulary remains intact or improves. Drag and Bieliauskas [5] also noted that vocabulary remains resistant to aging and may even improve, but that both long-term and short-term memory and processing speed decline. All show distributions of scores that fit the model in Figure 1.

Assume, provisionally, that for a selected age group, we plot a distribution of an ability, such as memory, as in Figure 1. With progressively older age groups, the mean of the distribution will shift to lower scores, which means that an increasingly greater proportion of that population will fall within what we might define as a *zone of impairment*. We might define such a zone as that proportion two SDs or more below the mean of a criterion group such as adults 25–45 years of age. With a typical normal or bell-shaped distribution, the older the age group is, the greater will be the impact of a specific shift in the mean, such as one caused by an environmental exposure because a greater total percentage of the age group will fall into the impairment zone. Put another way, the exposure is not a direct cause of the disease; rather, it shifts the population's cognitive performance toward scores that are characteristic of an older population.

Neurodegenerative diseases follow a pattern, as exemplified by Alzheimer's disease (AD), in which prevalence increases with age. Brookmeyer et al. [2] calculated age-specific incidence rates for AD from a body of data provided by four studies. Their mean estimates are the averages of the smoothed age-specific incidence curves from these studies. Their predicted percentages of cases per age group appear in Table 1. Hebert et al. [6], based on their studies of three Chicago populations, show striking changes in the proportions of individuals within three age groups (65–74, 75–84, and >85) in the scores attained on the Mini-Mental State Examination (MMSE), with a sharp rise in the severe category in patients over 85 years of age. Thus, both diminished cognitive function and AD follow an equivalent age pattern. Were MMSE score distributions to be shifted downward in an exposed population by, say, 5%, we could legitimately interpret the exposure as a risk factor for AD.

In the following discussions of lead, manganese, and mercury I will review data indicating that, at environmentally relevant levels, such exposures augment the expected decline in neurobehavioral function associated with aging. I will use these data to argue that such exposures then constitute risk factors for neurodegenerative disease states. That is, they would not be labeled as the primary sources of these diseases. Rather, (1) they elevate the likelihood that latent factors, such as genetic predispositions, will find overt expression or (2) they cause the clinical form of the disease to appear earlier in the lifespan. For example, the onset of clinically diagnosed AD is often preceded by a transitional phase termed *mild cognitive impairment (MCI)*. It describes a condition in which neuropsychological function, particularly memory, is compromised, but not enough to meet the criteria for a clinical diagnosis of AD, recognizing that the ultimate criterion for such a diagnosis is neuropathology. If an environmental exposure itself is able to magnify MCI, it would be considered a risk factor for clinically manifested AD as well as a condition that diminishes the individual's quality of life.

2. Lead

Although identified as a neurotoxicant as long ago as the second century BCE, its effects on neurobehavioral development have generated the greatest volume of research during the past five decades. Effects on adults occupy only a small proportion of the contemporary literature, and effects during advanced age an even smaller proportion.

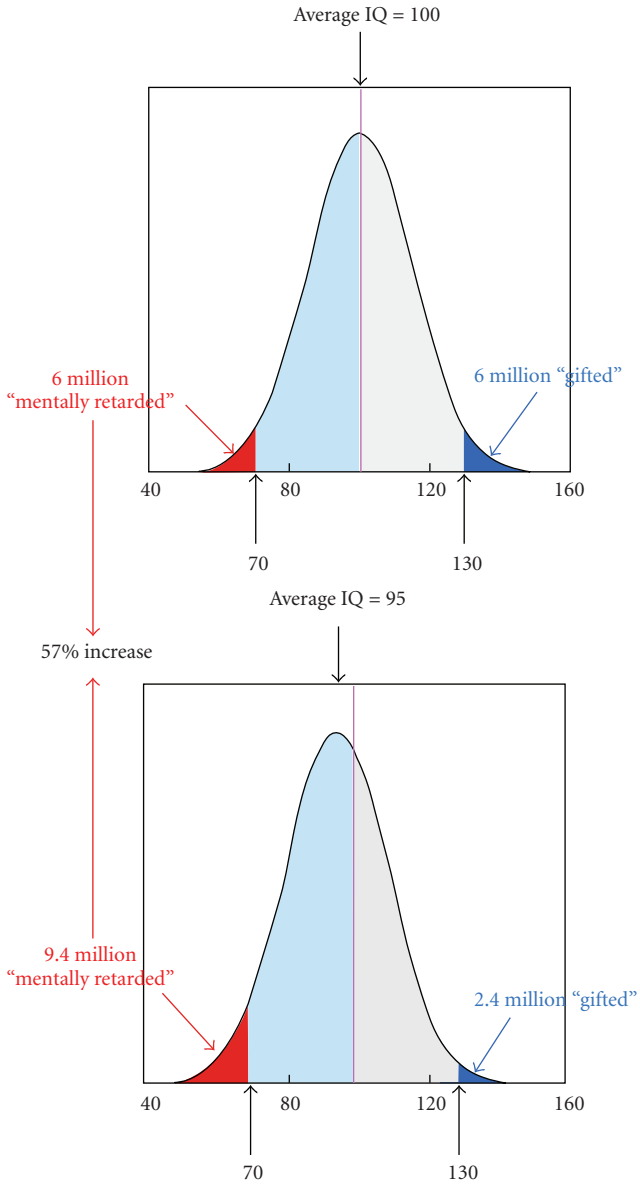


Figure 1: Showing the consequences of a shift in the population IQ of 5 points (5%) in a population of 100 million. In most US school districts, an IQ of 70 or lower (2 SDs below the mean) mandates remedial action. (Based on Weiss [3]).

That imbalance is slowly undergoing a shift, one propelled by increasing recognition of the health risks posed to a ballooning population of the elderly. Among the risks arousing the greatest concerns are those bearing on cognitive function. Other clinical entities such as osteoporosis and hypertension can be treated with drugs, which is a useful property given that lead is a risk factor for hypertension, coupled with the connections between hypertension and multi-infarct dementia as well as with cognitive decline. Cognitive dysfunction itself, at this stage of knowledge, lies mostly outside the realm of pharmacotherapy.

The US Centers for Disease Control and Prevention (CDC) has set a value of 10 micrograms of lead per deciliter

Table 2: Signs of manganese poisoning.

(i) Impaired coordination
(ii) Abnormal gait
(iii) Abnormal laughter
(iv) Expressionless face
(v) Weakness
(vi) Bradykinesia
(vii) Somnolence
(viii) Dysarthria
(ix) Difficulty walking
(x) Clumsiness
(xi) Lack of balance
(xii) Muscle pains
(xiii) Diminished leg power

of blood (μg/dL) as the level at which it recommends that public health actions be initiated for young children because of the recognized vulnerability of the developing brain. This value is the latest in a progression of exposure criteria that have evolved in parallel with accumulating information about the scope of lead neurotoxicity and the low levels at which neurotoxicity is observed. Gilbert and Weiss [7], however, have argued that this value is too high and that 2 μg/dL is a more reasonable figure given that no discernible threshold for lead toxicity has been established.

What can be gleaned from the current lead literature is that older adults appear to be as vulnerable as children to lead neurotoxicity. Muldoon et al. [8] measured neuropsychological function in a cohort of elderly women and found that levels as low as 8 μg/dL were significantly associated with poorer cognitive function. As they noted, "even a slight decrement in cognition would have a large public health impact due to the large number of elderly at risk."

Subsequent studies have relied more on measures of skeletal lead than of blood lead because the half-life of lead in the latter is about 35 days while the half-life in the skeleton, depending on the assay location, can be as much as two decades. Skeletal measures, based on X-ray fluorescence (XRF) methods, have the virtue, in older adults, of providing a historical and cumulative index of exposure [9], a considerable advantage because lead is so embedded in the environment that epidemiological studies cannot readily distinguish the effects of recent from past exposures. In one study [10], which surveyed nearly 1,000 adults in Baltimore, Maryland, performance scores on a variety of neuropsychological tests were found to be associated with tibia lead concentrations (language, processing speed, eye-hand coordination, executive functioning, verbal memory and learning, and visual memory). The mean blood lead level at the time of testing was 3.2 μg/dL.

In another study of an elderly population in Boston [11], the investigators measured bone lead in about 1,100 men at various times between 1991 and 1999. In this population, performance over this period on a battery of neuropsychological tests worsened as bone lead increased. The most robust effects were reflected by performance and

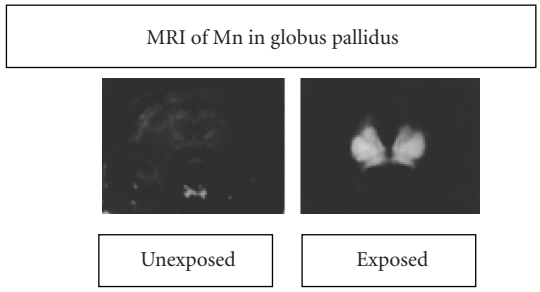


Figure 2: Magnetic resonance images of two monkeys, one exposed to inhaled manganese and one contol. They show that manganese achieves high concentrations in the global pallidus. Based on Newland and Weiss [12].

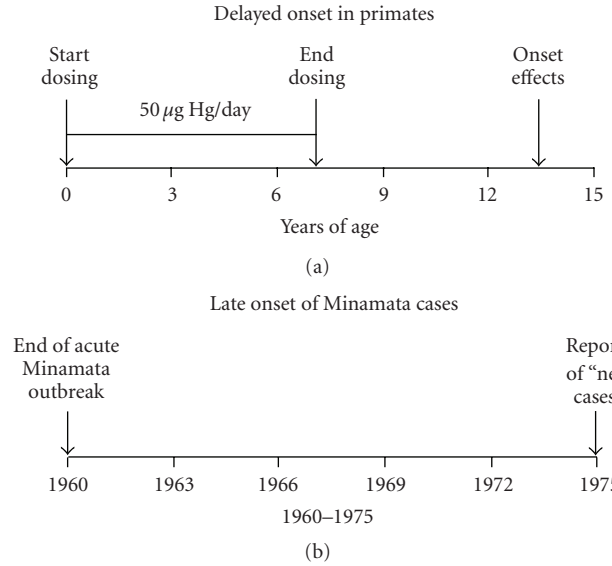


Figure 3: Depicting the phenomenon of delayed onset of neurotoxicity with methylmercury in nonnuman primates (a) and (b) in the Minamata population [18, 19].

reaction time scores on visuospatial/visuomotor tests. Here, mean blood lead values lay in the range of 5 μg/dL. The authors, in describing the rationale for their study and its implications for AD, commented as follows: "Mild cognitive impairment is more prevalent than AD and is receiving increasing attention, not only as a possible intermediate stage on the path to AD, but as an important deficit in its own right." The argument of Gilbert and Weiss [7] that acceptable lead levels be reduced applies to adults as well as children.

Because women had been underrepresented in lead studies, Weuve et al. [13] chose a population from the Nurses Health Study, which began in 1976. Over 90% of the participants have continued to participate, completing mail questionnaires every two years. Nearly 600 women took part in the current study and ranged in age from 47 to 74. They underwent cognitive testing and bone and blood lead assessment. Remarkably, mean blood lead values lay in the 2-3 μg/dL range. As noted by the authors, current values do not reflect the much higher exposures that prevailed in the past. Not until 1971 did the US Congress pass the Lead-Based Paint Poisoning Prevention Act, and not until 1986 was the phase out of lead from gasoline completed. As a result of this action, mean US blood-lead levels declined by 78 percent from 1978 to 1991. As noted earlier, however, the legacy of earlier environmental lead exposures remains implanted in the individual, either visibly in the skeleton, or epigenetically [14] or covertly, as silent neural damage that is not revealed until the compensatory capacity of the brain has been eroded by aging [15].

Generally, cognitive assessment occurred five years following the exposure assessments. The tests were conducted by trained nurses and administered by telephone interviews. The assessment battery consisted primarily of memory tests. As in the aging study in men, the concentration of lead in tibia bone was associated with worse overall cognitive performance. The authors translated this decrement into an age effect as follows: "Specifically, the average decrement in cognitive test scores we observed for each SD increase in tibia lead corresponded to the decrement in scores we observed for each 3-year increase in age among women in our study." Put another way, lead exposure induced premature aging, in essence shifting the score distribution to lower values.

Most neuropsychological tests are based on the needs of clinical practice and were designed to assist in the diagnosis in individual patients. Performance testing adopts a different point of view, one stemming from the needs of laboratory investigators, whose aim is to determine the effects of some experimental procedures on a specific behavior in a population sample. Proceeding from this standpoint, van Wijngaarden et al. [16] assessed a group of healthy subjects 55–67 years of age on a battery of behavioral tests derived from procedures originally developed in the animal laboratory. These procedures, part of the Cambridge Neuropsychological Test Automated Battery (CANTAB), were selected because they had been shown to reflect the kinds of impairment characteristic of AD. They included delayed matching-to-sample (DMS), paired associate learning (PAL), and spatial recognition memory (SRM). In addition, the subjects completed the Montreal Cognitive Assessment (MoCA) as an index of MCI.

As in other recent studies, the authors relied on XRF of bone to capture an index of cumulative lead exposure. And, following conventional practice, they corrected for a number of covariates that might serve as confounders such as age, smoking, alcohol use, body mass index (BMI), and others. In this pilot sample of 47 subjects, the clearest associations with exposure were seen with the DMS procedure, which calls upon the subject to select, after a variable delay, one of four complex visual patterns that matches a single test pattern shown before the delay. A similar task revealed deficits in nonhuman primates exposed chronically to manganese [17]. The other two CANTAB tests (SRM and PAL) also showed significant associations with bone lead, but not as conspicuously as the DMS procedure. The MoCA inventory showed only a weak association.

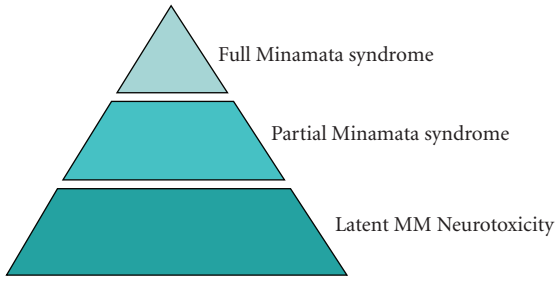


Figure 4: Schematic of scope of neurotoxic signs stemming from exposure to methylmercury. The full syndrome, as in Table 3, occurs with massive exposures. The primary current question, a population question, arises from the possible occurrence of subtle neuropsychological deficits that would only be evident with systematic testing; that is, latent effects.

Table 3: Scope of methylmercury poisoning experienced in Japan beginning in the 1950s. This syndrome became known as Minamata Disease. Later, when the effects of prenatal exposure became evident, a new category, Fetal Minamata Disease, was established.

SENSORY
(i) Paresthesia (numbness and tingling)
(ii) Pain in limbs
(iii) Visual disturbances (field constriction)
(iv) Hearing disturbances
(v) Asterognosis (discrimination by touch)
MOTOR
(i) Disturbances of gait
(ii) Weakness, leg unsteadiness, falling
(iii) Thick, slurred speed (dysarthria)
(iv) Tremor
OTHER
Headaches, rashes, "mental disturbance"

The message conveyed by these recent publications tells us that historical and cumulative as well as current lead exposure must be considered a risk factor for neurobehavioral dysfunction, including a decline in cognitive performance. Translating these findings into the model depicted in Figure 1, it will be seen that lead exposure shifts the test score distribution toward lower values. The result is that a larger proportion of the population falls into the range of markedly diminished cognitive function—a basic criterion for dementia. Or, as noted earlier, because population indices of cognitive function also decline with age, it shifts the scores toward more advanced ages; that is, premature aging.

One other aspect of these data warrants comment, although we have no direct evidence of their impact in human populations. Environmental health specialists now view as a principle the notion that toxic exposures during early development create the foundation for adult disease. A substantial literature in neurotoxicology testifies to the validity of that principle for neurobehavioral function. Recent research links developmental lead exposure to elevated deposits of β-amyloid deposits in both aged rats [20] and monkeys [21], leading the authors to propose a two-stage model for the genesis of dementia. Stage one is triggered by a condition such as lead exposure, which inscribes an epigenetic footprint in the form of altered patterns of DNA methylation. Stage two occurs late in life, possibly a result of further stress, including diminished brain compensatory capacity. Acting in concert, this combination of stressors is postulated to evoke the pathogenic processes that underlie dementia. Whether and how such a sequence eventually results in impaired performance on behavioral tests late in life is a question that warrants further exploration.

3. Manganese

Manganese (Mn) has been recognized as a neurotoxic metal for over 150 years. Exposure to high levels of inhaled manganese, as in miners working in an environment laden with dust from manganese ore (MnO_2), leads to a constellation of psychological and motor symptoms depicted in Table 2. The psychological changes are known as "locura manganica" in the South American manganese mines where manganism is endemic. Many of the motor and postural signs are consistent with those of Parkinson's disease (PD), an overlap that has led to debates about whether manganese neurotoxicity is truly a form of PD. Most observers agree that the movement disorders induced by manganese exposure and idiopathic Parkinsonism differ in fundamental respects. PD patients typically walk with shuffling gait. In manganism, patients may walk with an almost theatrical high-stepping gait. Dopamine replacement can attenuate PD signs; it has little effect on manganism. In PD, degeneration is seen primarily in the substantia nigra; in manganism, it is seen primarily in the globus pallidus.

Because manganese is paramagnetic, it can be localized in the brain by magnetic resonance imaging (MRI). Figure 2 shows two images, one from a monkey exposed by inhalation and one from a control subject [12]. The figure shows the tendency of inhaled manganese to collect in the globus pallidus. A similar pattern of distribution occurs in humans as well [22].

The overtly observable manifestations of overt manganese poisoning have obscured the more subtle, more widespread consequences of low-level exposures, much as the overt signs of Parkinson's disease have tended to eclipse its neuropsychological accompaniments such as cognitive impairment and depression. In parallel, at lower levels of exposure than those associated with mining, such as those experienced by welders exposed to manganese fumes from welding rods, or to fumes by workers in ferromanganese-processing plants, or in areas where air or water levels are elevated, the manifestations are far less dramatic. They do not rise to the level of clinical detection. Instead, they require the application of neuropsychological tests.

Two aspects of manganese neurotoxicity render it a more difficult question to resolve than is the case with lead and mercury. First, it is an essential element. It is required to maintain health as well as to support growth and development. It is present in our tissues and is necessary for

certain biochemical reactions. Environmental levels cannot be reduced to zero. Second, perhaps because it is an essential element, no unambiguous biological marker, of the kind we have for lead and mercury, is available. Blood Mn seems to be the best commonly available index of exposure.

From the standpoint of general population risk, the main questions posed by manganese exposure lie more in the realm of neuropsychological function than in the realm of motor function. It must be recognized, however, that the two realms are intertwined. Both spatially and functionally, the brain is highly interactive. Simply because the globus pallidus is part of the basal ganglia does not isolate it from cognitive pathways. For example, McNab and Klingberg [23] demonstrated, with fMRI, how working memory depends on pallidal and frontal cortical cooperation, so to speak.

Inhalation is the predominant mode of exposure leading to neurotoxicity. For chronically exposed individuals, the latency to clinical manganism is highly variable, dependent on ambient concentrations, particle size, and individual susceptibility. In some instances, it apparently surfaces long after exposure has ceased. Because incipient poisoning may be expressed primarily by subjective symptoms such as mood changes, a connection to manganese is easily overlooked. This is the rationale for neuropsychological testing. As in other neurotoxic syndromes, systematic testing is more sensitive than the typical neurological examination. It is why the cognitive effects of manganese neurotoxicity were virtually ignored until the last two decades. They were submerged by the more blatant signs such as the motor abnormalties and the striking emotional displays such as hysterical laughing and weeping. More recently, however, we have begun to realize that cognitive deficits remain and may even worsen, years after exposure has ceased [24]. Perhaps even more worrisome, cognitive and associated measures of neurobehavioral function have been documented in children [25]. Such losses, we now recognize, may persist for a lifetime, perhaps lying dormant until advanced age.

The cognitive deficiencies embrace a variety of measures. They include diminished attention, reduced scores on tests of working memory, lower scores on intelligence tests, impaired learning, and slowed response speed. The recent literature expands these findings, as exemplified by two recent papers.

Diminished neuropsychological function (such as attention and memory) is detectable at about the same exposure levels as the motor deficits typically thought of as most characteristic of manganism. Bowler et al. [24] examined 47 welders and 42 controls on a variety of tests: the Wechsler Adult Intelligence Scale (WAIS-III), the Wechsler Memory Scale (WMS-III), the Boston Naming Test, the Wide Range Achievement Test (WRAT-3), Cancellation H, Trail Making Tests A and B, Auditory Consonant Trigrams, Stroop (color naming) test, Rey-Osterreith, Animal Naming, Controlled Oral Word Association (COWAT), Test of Memory Malingering, Rey 15-item, Fingertapping, Grooved Pegboard, Dynamometer, Visual Attention Test, and the Lanthony d-15 Color Vision test. They saw diminished performance, among the welders, in motor skills, visuomotor tracking speed and information processing, working memory, verbal skills (COWAT), delayed memory, and visuospatial skills.

Concentrations of manganese in the globus pallidus are correlated with neuropsychological test performance. Chang et al. [22] administered a battery of such tests to subjects who had also been evaluated by MRI. To 43 asymptomatic male welders and 29 age- and sex-matched healthy controls, they administered the following tests: Digit symbol, auditory verbal learning test (delayed recall), complex figure test (copy and immediate recall), digit span, verbal fluency test, Stroop test, grooved pegboard, finger tapping, and frequency dispersion and harmonic index of tremor. They also assessed olfactory function and mood. Welders attained lower scores than controls on several cognitive tests, but, most interesting, multiple regression analyses revealed pallidal index to be a better predictor of performance in welders than blood Mn. (Pallidal index was defined as the ratio of the signal intensity of the globus pallidus to that of the subcortical frontal white matter in axial T1-weighted MRI planes.).

Nonhuman primates are viewed as the most appropriate animal model for studies of manganese neurotoxicity because of their similarities to humans in brain anatomy and neurobehavioral function. Two recent publications indicate the depth and extent of understanding that can be achieved by making use of these characteristics.

Schneider et al. [17] trained Cynomologous monkeys on three memory tasks: spatial working memory (delayed position choice), nonspatial working memory (delayed match-to-sample), and reference memory (pattern discrimination). Once trained, they were placed on a regimen of intravenous manganese sulfate injections over a period of about 230 days. When tested at the end of this period, the treated animals displayed mild deficits in spatial memory, greater deficits in nonspatial memory, and no deficits in reference memory. By analyzing regional Mn concentrations, the experimenters determined a significant inverse relationship between working memory task performance and Mn levels.

In a direct quest for associations between manganese exposure and Alzheimer's disease, Guilarte [26] conducted a gene array analysis of frontal cortex tissue from the same animals. Of the 61 upregulated genes, the most highly elevated was Amyloid-β Precursor-like Protein 1 (APLP1), a member of the Amyloid Precursor Protein (APP) family. Along with this finding, immunochemistry showed the presence of Amyloid-β plaques, an unexpected finding given that the subjects were only 6–8 years of age. In addition, immunochemistry revealed the presence of α-synuclein aggregates, which have been linked to PD as well as AD. These findings, then, link the Mn-induced β-amyloid deposits to impaired memory function in a species generally considered to be an especially useful model for extrapolation to humans.

Because manganese exposure seems primarily to occur via inhalation, I would be remiss in not observing current data about the mechanics of ambient particulates. They have received considerable attention as risk factors for cardiovascular disease. We are now aware that the smallest particles, termed ultrafine or nanoparticles, may directly enter the central nervous system as they travel along the olfactory nerve. In rats exposed to manganese oxide ultrafine particles for 12 days, Elder et al. [27] found these particles

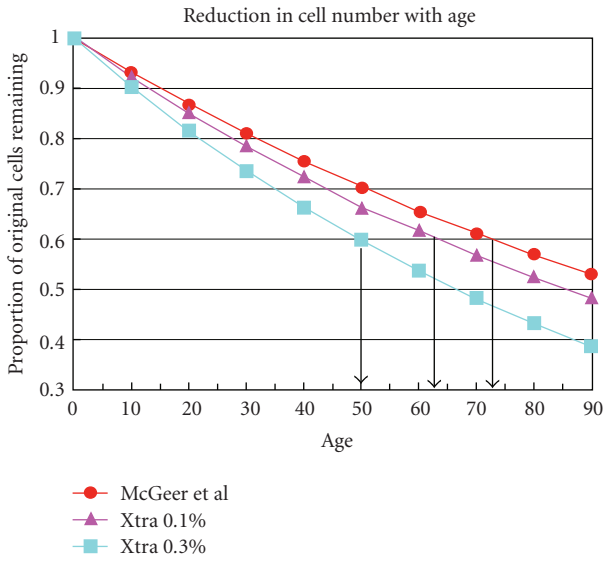


Figure 5: Substantia nigra cell populations diminish with age, as shown by McGeer et al. [31]. At about age 72, about 40% of the cells have been lost. If the decline is accelerated by 0.1% per year, such a loss would occur at about age 64.

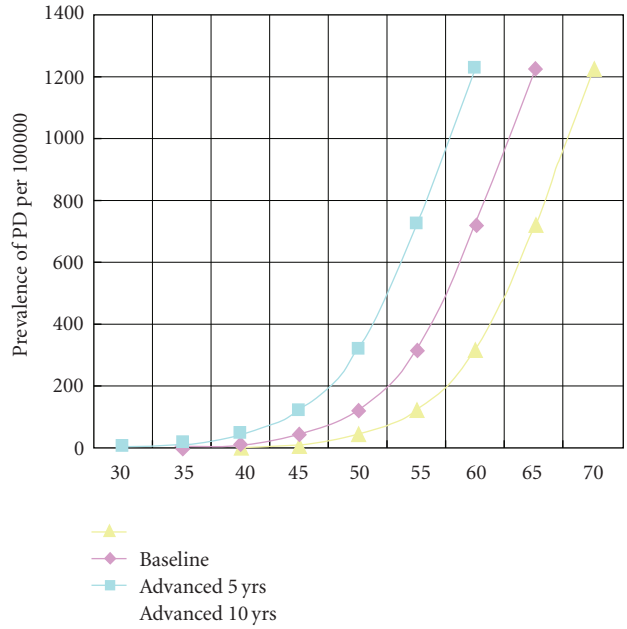


Figure 6: The chart demonstrates how the age-adjusted functions of a disorder such as Parkinson's disease are modified by an acceleration of cell loss as depicted in Figure 5. At age 65 years, the prevalence without acceleration is, based on available data, about 700/100,000. If advanced by 5 years, the prevalence would be 1,200/100,000.

translocating into the striatum, frontal cortex, and cerebellum. This is an exposure pathway that needs considerably more attention from the standpoint of neurodegenerative disease [28, 29].

4. Methylmercury

An ecological disaster in Japan provided one of the earliest signals about how chemical contamination of the environment could engender major threats to human health, particularly brain function. Perhaps even more than Rachel Carson's *Silent Spring*, which lamented the disappearance of bird song [30], it framed its message in images of deformed children and crippled adults. The chemical contaminant was methylmercury and the site was in Japan, on the island of Kyushu, in the small fishing village of Minamata. There, beginning in the 1950s, the inhabitants began to suffer from a strange neurological disease traced to methylmercury contamination of fish and shellfish from Minamata bay. The first cases were sporadic, but, with time, more and more cases became evident and finally led to an investigation that indicted mercury dumped into the bay by a chemical factory. The clinical signs of methylmercury poisoning are listed in Table 3.

The story has been told many times, but none as graphically as in the photographic essay by E. Smith and A. Smith [32]. Since then, methylmercury has been the subject of several thousand scientific papers and still presents numerous mysteries [33].

The overwhelming proportion of publications dealing with methylmercury neurotoxicity is devoted to early brain development [34, 35]. Although one of the earliest contributions to methylmercury research reported that perinatal exposure in mice could induce adverse consequences that might erupt eventually in advanced age [36], that portion of the lifespan has still received relatively little attention.

The paucity of this literature fails to match the scope of the problem. We are exposed to methylmercury almost exclusively through fish consumption. One exception was the mass poisoning in Iraq in 1971-1972, but its source was grain treated with a methylmercury fungicide [37, 38]. Exposures therefore tend to continue through a lifetime in those whose diet includes fish. Even as a consequence of developmental exposure alone, we must address the possibility that exposures early in life may produce undetected, latent, or "silent" damage that emerges only when the functional capacities of the nervous system are challenged by other conditions, such as aging [39, 40] as was noted earlier for the two-stage (or two-hit) model for lead. Figure 3 shows two findings bearing on this question. First, that for many years after the first cases were documented, new cases continued to appear, possibly because of the combination of aging and silent damage. Second, in nonhuman primates exposed during early development to lead [18] and methylmercury [19], a long period elapsed before neurotoxic effects made an overt appearance.

The most serious gap is in our understanding of methylmercury's possible effects on cognitive function during aging lies in the domain of neuropsychological assessment. Perhaps the absence of such data can be ascribed to the most conspicuous signs of poisoning in adults; that is, those listed in Table 3. They are so flagrant that more subtle outcomes, such as cognitive dysfunction, were

not pursued. No neuropsychologists were enlisted in the numerous studies conducted by Japanese physicians and scientists in the years following the onset of the poisoning. Surveys of elderly Japanese in areas marked by elevated exposures, as determined by biomarkers such as hair, or less precisely, by geographic location, have tended to focus on endpoints, such as paresthesias and ataxia, that are part of a conventional neurological examination [41, 42]. In two studies that evaluated subjective complaints, however, "forgetfulness," a cognitive term, emerged as a common complaint [43, 44].

Once systematic neuropsychological assessments were undertaken, however, they uncovered evidence of impairment. Carta et al. [45] compared a group of 22 adult male tuna fish eaters with 22 controls, both about 50 years of age, on a neurobehavioral test battery. The tuna eaters exhibited much higher levels of organic mercury in blood and hair. Two cognitive tests differentiated the two groups statistically, digit-symbol and color word reaction time, while overall performance was inversely correlated with biomarker levels.

Chang et al. [46] took advantage of a Taiwanese population living in the vicinity of an abandoned chlor-alkali factory; such factories typically use big tanks of elemental mercury as an electrode. Fish in the area were heavily contaminated because of previous mercury waste discharges into the adjacent river where, as in most waterways, microorganisms in bottom sediment convert the inorganic mercury into the methyl species. Measures of total and organic mercury in blood indicated somewhat elevated levels compared to US residents, but close to those of residents of the Faroe Islands and the Republic of Seychelles, both sites of large prospective studies of methylmercury effects on brain development [34, 35]. For the neurobehavioral assessments, the authors relied upon the Cognitive Abilities Screening Instrument (CASI) and the Mini-Mental State Exam (MMSE). They then divided the population in two: high blood and low blood methylmercury values. The two groups achieved significantly different scores on the CASI and MMSE, with the low ones superior. Although all the subtests showed better scores by those with low values, three subtests yielded significant differences on their own: remote memory, recent memory, and mental manipulation.

The published literature contains other studies pointing in the same direction. For example, Yokoo et al. [47], studying an Amazonian population, found correlations between hair concentrations of methylmercury with performance on several neuropsychological tests. It seems, then, even with this small group of studies, that adult brains are not as resistant to methylmercury neurotoxicity, compared to developing brains, as had been believed. Chronic exposure, combined with the diminished performance associated with aging, may account for this unexpected sensitivity.

All three studies present the same conundrum. As with all fish-consuming populations, unless they are followed from conception onwards, it becomes virtually impossible to differentiate the effects of current exposure from those occurring early in development.

The laboratory animal literature supports the view that advanced age is a period during which the damage inflicted by developmental or chronic exposure may first emerge. Spyker [36] showed a variety of adverse effects such as obesity, kyphosis, and impaired locomotion in old mice exposed prenatally. Newland et al. [48, 49] found that that the adult consequences of developmental exposure in rats can be reflected in how precisely subjects adjust to new behavioral demands. In nonhuman primates, Rice [19] and Burbacher et al. [50] found enduring effects of developmental exposure on coordination and sensory function in monkeys that appeared only many years after cessation of dosing.

The Minamata progression, from its first focus on clinical poisoning to determining who was actually a victim to the more recent question of subtle neuropsychological effects, can be depicted by the pyramid in Figure 4, which actually represents the kind of questions evoked by environmental contaminants in general. It is often the case that only after we see obvious signs of adverse effects do we begin to probe further and to search for the predecessors of such effects.

5. Discussion

In 1956, in the midst of the continuing controversy over the Minamata disaster, the Japanese government asked Kumamoto University, located nearby, to undertake studies of the outbreak. The researchers affirmed the connection between the outbreak of the disease and methylmercury contamination resulting from waste discharges into Minamata Bay by the Chisso chemical plant. The question of compensation for victims then arose: who was injured and what was the evidence? In 1973, the *Research Committee on Minamata Disease of Kumamoto University* described its dilemma in assigning compensation in these terms as follows:

> "...the problem about the relationship between a small amount of methylmercury pollution for a long period and its accumulation in the brain still remains obscure...Subclinical Minamata disease was sometimes revealed by detectable symptoms during the aging process...The subclinical Minamata disease...could be called a delayed type of Minamata disease in aged people."

This statement highlights the issues posed in this paper. Neurodegenerative disorders such as Alzheimer's disease are disorders of aging, and the primary risk factor, except for the familial form, is age. To what extent their etiology and, perhaps more narrowly, their rate of progression or latency to clinical emergence is fostered by environmental contaminants is all but unknown at present. We can, however, note how aging intersects with neurotoxicant exposure [51]. Many manifestations of neurotoxicity, such as impaired sensory acuity, mimic the natural course of aging. Some adverse effects may arise for the first time in advanced age because it is a period of declining compensatory margins. With aging, for example, synaptic density decreases [52, 53]. Attrition of nerve cells also occurs naturally as the brain ages but is also

accompanied by dendritic sprouting in those that remain. Damage incurred earlier in life, as in mild polio infections that seemed to fade, may emerge late in life in the form of the postpolio syndrome. Could parallel processes be induced by neurotoxicants such as methylmercury?

(i) Early development, it now seems clear, is not the only life stage during which the brain exhibits intensified responses to the adverse effects of chemicals. Vulnerability to toxic processes rises again late in life; recovery from damage is sluggish, and pharmacokinetic and metabolic variables change with aging in ways that recapitulate the imperfect defenses deployed by the immature organism.

(ii) "Aging" is not a mechanistic explanation. Events occurring during life must account for the changes. Older brains are already high-maintenance properties, so that exposure to substances with neurotoxic properties, such as certain metals, may accelerate the process or exploit its dwindling capacities to resist their effects. From this vantage point, toxicants can act in three ways to depress function during advanced age; they may interfere with brain development, leaving a legacy of diminished redundancy not apparent until it is further compromised during aging; they may hasten the progressive erosion of function observed with certain abilities; they may exert greater effects in the aging brain because the aging nervous system has already undergone a reduction in its ability to withstand toxic challenges.

These arguments can be illustrated by Figures 5 and 6. Figure 5 is based on data from McGeer et al. [31], which trace the decline in substantia nigra cell number that occurs with aging. Using the same rationale as that of Weiss and Simon [39], it compares the McGeer data with two models of rate of decline. One shows an additional acceleration of 1% per year, the other one of 3% per year. At the 1% rate, remaining cell number shows a fall of 40% at about age 64 while the baseline rate of decline would not reach that level until age 72.

Figure 6 converts these speculative projections into their public health implications. It plots the age-indexed prevalence of Parkinson's disease for a baseline population against one that has been advanced by 5 years and another that has been advanced by 10 years. At age 65, the prevalence comes to about 700/100,000 for the baseline population. For the population advanced by 5 years, the figure becomes about 1,200/100,000. This phenomenon is what happens with disorders whose prevalence increases with age [54].

Figures 5 and 6 convey the core message of this paper. It misleads us to conceive of environmental contaminants such as metals as *causes* of neurodegenerative diseases such as AD. To truly establish such a classification requires data from several biological levels ranging from epidemiology to molecular mechanisms. With the three metals surveyed here, however, the available information is sturdy enough that we can label them as potential contributors or risk factors for the onset or progression of the disorder. Given the paucity of information we now possess about the etiology of neurodegenerative disorders, they warrant further investigation about their influence on aging.

Acknowledgment

Preparation supported in part by research Grant no. RC2 ES018736 and Center Grant no. ES01247 from the National Institute of Environmental Health Sciences.

References

[1] D. K. Lahiri, N. H. Zawia, N. H. Greig, K. Sambamurti, and B. Maloney, "Early-life events may trigger biochemical pathways for Alzheimer's disease: the "LEARn" model," *Biogerontology*, vol. 9, no. 6, pp. 375–379, 2008.

[2] R. Brookmeyer, S. Gray, and C. Kawas, "Projections of Alzheimer's disease in the United States and the public health impact of delaying disease onset," *American Journal of Public Health*, vol. 88, no. 9, pp. 1337–1342, 1998.

[3] B. Weiss, "Neurobehavioral toxicity as a basis for risk assessment," *Trends in Pharmacological Sciences*, vol. 9, no. 2, pp. 59–62, 1988.

[4] T. Hedden and J. D. E. Gabrieli, "Insights into the ageing mind: a view from cognitive neuroscience," *Nature Reviews Neuroscience*, vol. 5, no. 2, pp. 87–96, 2004.

[5] L. L. Drag and L. A. Bieliauskas, "Contemporary review 2009: cognitive aging," *Journal of Geriatric Psychiatry and Neurology*, vol. 23, no. 2, pp. 75–93, 2010.

[6] L. E. Hebert, P. A. Scherr, J. L. Bienias, D. A. Bennett, and D. A. Evans, "Alzheimer disease in the US population: prevalence estimates using the 2000 census," *Archives of Neurology*, vol. 60, no. 8, pp. 1119–1122, 2003.

[7] S. G. Gilbert and B. Weiss, "A rationale for lowering the blood lead action level from 10 to 2 μg/dL," *NeuroToxicology*, vol. 27, no. 5, pp. 693–701, 2006.

[8] S. B. Muldoon, J. A. Cauley, L. H. Kuller et al., "Effects of blood lead levels on cognitive function of older women," *Neuroepidemiology*, vol. 15, no. 2, pp. 62–72, 1996.

[9] H. Hu, R. Shih, S. Rothenberg, and B. S. Schwartz, "The epidemiology of lead toxicity in adults: measuring dose and consideration of other methodologic issues," *Environmental Health Perspectives*, vol. 115, no. 3, pp. 455–462, 2007.

[10] R. A. Shih, T. A. Glass, K. Bandeen-Roche et al., "Environmental lead exposure and cognitive function in community-dwelling older adults," *Neurology*, vol. 67, no. 9, pp. 1556–1562, 2006.

[11] M. G. Weisskopf, S. P. Proctor, R. O. Wright et al., "Cumulative lead exposure and cognitive performance among elderly men," *Epidemiology*, vol. 18, no. 1, pp. 59–66, 2007.

[12] M. C. Newland and B. Weiss, "Persistent effects of manganese on effortful responding and their relationship to manganese accumulation in the primate globus pallidus," *Toxicology and Applied Pharmacology*, vol. 113, no. 1, pp. 87–97, 1992.

[13] J. Weuve, S. A. Korrick, M. A. Weisskopf et al., "Cumulative exposure to lead in relation to cognitive function in older women," *Environmental Health Perspectives*, vol. 117, no. 4, pp. 574–580, 2009.

[14] D. K. Lahiri, B. Maloney, and N. H. Zawia, "The LEARn model: an epigenetic explanation for idiopathic neurobiological diseases," *Molecular Psychiatry*, vol. 14, no. 11, pp. 992–1003, 2009.

[15] B. Weiss, T. W. Clarkson, and W. Simon, "Silent latency periods in methylmercury poisoning and in neurodegenerative disease," *Environmental Health Perspectives*, vol. 110, supplement 5, pp. 851–854, 2002.

[16] E. van Wijngaarden, J. R. Campbell, and D. A. Cory-Slechta, "Bone lead levels are associated with measures of memory impairment in older adults," *NeuroToxicology*, vol. 30, no. 4, pp. 572–580, 2009.

[17] J. S. Schneider, E. Decamp, K. Clark, C. Bouquio, T. Syversen, and T. R. Guilarte, "Effects of chronic manganese exposure on working memory in non-human primates," *Brain research*, vol. 1258, pp. 86–95, 2009.

[18] D. C. Rice and K. F. Karpinski, "Lifetime low-level lead exposure produces deficits in delayed alternation in adult monkeys," *Neurotoxicology and Teratology*, vol. 10, no. 3, pp. 207–214, 1988.

[19] D. C. Rice, "Evidence for delayed neurotoxicity produced by methylmercury," *NeuroToxicology*, vol. 17, no. 3-4, pp. 583–596, 1996.

[20] MD. R. Basha, M. Murali, H. K. Siddiqi et al., "Lead (Pb) exposure and its effect on APP proteolysis and Aβ aggregation," *FASEB Journal*, vol. 19, no. 14, pp. 2083–2084, 2005.

[21] J. Wu, MD. R. Basha, B. Brock et al., "Alzheimer's Disease (AD)-like pathology in aged monkeys after infantile exposure to environmental metal lead (Pb): evidence for a developmental origin and environmental link for AD," *Journal of Neuroscience*, vol. 28, no. 1, pp. 3–9, 2008.

[22] Y. Chang, Y. Kim, S. T. Woo et al., "High signal intensity on magnetic resonance imaging is a better predictor of neurobehavioral performances than blood manganese in asymptomatic welders," *NeuroToxicology*, vol. 30, no. 4, pp. 555–563, 2009.

[23] F. McNab and T. Klingberg, "Prefrontal cortex and basal ganglia control access to working memory," *Nature Neuroscience*, vol. 11, no. 1, pp. 103–107, 2008.

[24] R. M. Bowler, S. Gysens, E. Diamond, S. Nakagawa, M. Drezgic, and H. A. Roels, "Manganese exposure: neuropsychological and neurological symptoms and effects in welders," *NeuroToxicology*, vol. 27, no. 3, pp. 315–326, 2006.

[25] G. A. Wasserman, X. Liu, F. Parvez et al., "Water manganese exposure and children's intellectual function in Araihazar, Bangladesh," *Environmental Health Perspectives*, vol. 114, no. 1, pp. 124–129, 2006.

[26] T. R. Guilarte, "APLP1, Alzheimer's-like pathology and neurodegeneration in the frontal cortex of manganese-exposed non-human primates," *NeuroToxicology*, vol. 31, no. 5, pp. 572–574, 2010.

[27] A. Elder, R. Gelein, V. Silva et al., "Translocation of inhaled ultrafine manganese oxide particles to the central nervous system," *Environmental Health Perspectives*, vol. 114, no. 8, pp. 1172–1178, 2006.

[28] M. L. Block and L. Calderón-Garcidueñas, "Air pollution: mechanisms of neuroinflammation and CNS disease," *Trends in Neurosciences*, vol. 32, no. 9, pp. 506–516, 2009.

[29] L. Calderón-Garcidueñas, A. C. Solt, C. Henríquez-Roldán et al., "Long-term air pollution exposure is associated with neuroinflammation, an altered innate immune response, disruption of the blood-brain barrier, ultrafine particulate deposition, and accumulation of amyloid β-42 and α-synuclein in children and young adults," *Toxicologic Pathology*, vol. 36, no. 2, pp. 289–310, 2008.

[30] R. Carson, *Silent Spring*, Houghton Mifflin, New York, NY, USA, 1962.

[31] P. L. McGeer, S. Itagaki, H. Akiyama, and E. G. McGeer, "Rate of cell death in parkinsonism indicates active neuropathological process," *Annals of Neurology*, vol. 24, no. 4, pp. 574–576, 1988.

[32] E. Smith and A. Smith, *Minamata*, Apeture (Grossman), New York, NY, USA, 1975.

[33] B. Weiss, "Why methylmercury remains a conundrum 50 years after Minamata," *Toxicological Sciences*, vol. 97, no. 2, pp. 223–225, 2007.

[34] P. W. Davidson, G. J. Myers, C. Cox et al., "Methylmercury and neurodevelopment: longitudinal analysis of the Seychelles child development cohort," *Neurotoxicology and Teratology*, vol. 28, no. 5, pp. 529–535, 2006.

[35] F. Debes, E. Budtz-Jørgensen, P. Weihe, R. F. White, and P. Grandjean, "Impact of prenatal methylmercury exposure on neurobehavioral function at age 14 years," *Neurotoxicology and Teratology*, vol. 28, no. 3, pp. 363–375, 2006.

[36] J. Spyker, "Behavioral teratology and toxicology," in *Behavioral Toxicology*, B. Weiss and V. G. Laties, Eds., pp. 311–349, Plenum, New York, NY, USA, 1975.

[37] F. Bakir, S. F. Damluji, and I. Amin Zaki, "Methylmercury poisoning in Iraq: an interuniversity report," *Science*, vol. 181, no. 4096, pp. 230–241, 1973.

[38] T. W. Clarkson, L. Amin Zaki, and S. K. Al Tikriti, "An outbreak of methylmercury poisoning due to consumption of contaminated grain," *Federation Proceedings*, vol. 35, no. 12, pp. 2395–2399, 1976.

[39] B. Weiss and W. Simon, "Quantitative perspectives on the long-term toxicity of methylmercury and similar poisons," in *Behavioral Toxicology*, B. Weiss and V. G. Laties, Eds., pp. 429–435, Plenum, New York, NY, USA, 1975.

[40] B. Weiss, T. W. Clarkson, and W. Simon, "Silent latency periods in methylmercury poisoning and in neurodegenerative disease," *Environmental Health Perspectives*, vol. 110, no. 5, pp. 851–854, 2002.

[41] S. Ekino, M. Susa, T. Ninomiya, K. Imamura, and T. Kitamura, "Minamata disease revisited: an update on the acute and chronic manifestations of methyl mercury poisoning," *Journal of the Neurological Sciences*, vol. 262, no. 1-2, pp. 131–144, 2007.

[42] T. Yorifuji, T. Tsuda, S. Takao, and M. Harada, "Long-term exposure to methylmercury and neurologic signs in Minamata and neighboring communities," *Epidemiology*, vol. 19, no. 1, pp. 3–9, 2008.

[43] Y. Kinjo, H. Higashi, A. Nakano, M. Sakamoto, and R. Sakai, "Profile of subjective complaints and activities of daily living among current patients with Minamata disease after 3 decades," *Environmental Research*, vol. 63, no. 2, pp. 241–251, 1993.

[44] Y. Fukuda, K. Ushijima, T. Kitano, M. Sakamoto, and M. Futatsuka, "An analysis of subjective complaints in a population living in a methylmercury-polluted area," *Environmental Research*, vol. 81, no. 2, pp. 100–107, 1999.

[45] P. Carta, C. Flore, R. Alinovi et al., "Sub-clinical neurobehavioral abnormalities associated with low level of mercury exposure through fish consumption," *NeuroToxicology*, vol. 24, no. 4-5, pp. 617–623, 2003.

[46] J. W. Chang, M. C. Pai, H. L. Chen, H. R. Guo, H. J. Su, and C. C. Lee, "Cognitive function and blood methylmercury in adults living near a deserted chloralkali factory," *Environmental Research*, vol. 108, no. 3, pp. 334–339, 2008.

[47] E. M. Yokoo, J. G. Valente, L. Grattan, S. L. Schmidt, I. Platt, and E. K. Silbergeld, "Low level methylmercury exposure

affects neuropsychological function in adults," *Environmental Health*, vol. 2, no. 1, pp. 1–11, 2003.

[48] M. C. Newland, P. A. Reile, and J. L. Langston, "Gestational exposure to methylmercury retards choice in transition in aging rats," *Neurotoxicology and Teratology*, vol. 26, no. 2, pp. 179–194, 2004.

[49] M. C. Newland, E. M. Paletz, and M. N. Reed, "Methylmercury and nutrition: adult effects of fetal exposure in experimental models," *NeuroToxicology*, vol. 29, no. 5, pp. 783–801, 2008.

[50] T. M. Burbacher, K. S. Grant, D. B. Mayfield, S. G. Gilbert, and D. C. Rice, "Prenatal methylmercury exposure affects spatial vision in adult monkeys," *Toxicology and Applied Pharmacology*, vol. 208, no. 1, pp. 21–28, 2005.

[51] B. Weiss, "Vulnerability to pesticide neurotoxicity is a lifetime issue," *NeuroToxicology*, vol. 21, no. 1-2, pp. 67–74, 2000.

[52] H. Duan, S. L. Wearne, A. B. Rocher, A. Macedo, J. H. Morrison, and P. R. Hof, "Age-related dendritic and spine changes in corticocortically projecting neurons in macaque monkeys," *Cerebral Cortex*, vol. 13, no. 9, pp. 950–961, 2003.

[53] P. R. Hof and J. H. Morrison, "The aging brain: morphomolecular senescence of cortical circuits," *Trends in Neurosciences*, vol. 27, no. 10, pp. 607–613, 2004.

[54] B. Weiss, "Economic implications of manganese neurotoxicity," *NeuroToxicology*, vol. 27, no. 3, pp. 362–368, 2006.

SAGE-Hindawi Access to Research
International Journal of Alzheimer's Disease
Volume 2011, Article ID 208634, 9 pages
doi:10.4061/2011/208634

Research Article

New Proteins Found Interacting with Brain Metallothionein-3 Are Linked to Secretion

Issam El Ghazi, Bruce L. Martin, and Ian M. Armitage

Biochemistry, Molecular Biology and Biophysics, University of Minnesota, 6-155 Jackson Hall, 321 Church Street, SE, Minneapolis, MN 55455, USA

Correspondence should be addressed to Ian M. Armitage, armitage@msi.umn.edu

Received 19 August 2010; Revised 19 October 2010; Accepted 5 November 2010

Academic Editor: Peter Faller

Copyright © 2011 Issam El Ghazi et al. This is an open access article distributed under the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original work is properly cited.

Metallothionein 3 (MT-3), also known as growth inhibitory factor (GIF), exhibits a neuroinhibitory activity. Our lab and others have previously shown that this biological activity involves interacting protein partners in the brain. However, nothing specific is yet known about which of these interactions is responsible for the GIF activity. In this paper, we are reporting upon new proteins found interacting with MT-3 as determined through immunoaffinity chromatography and mass spectrometry. These new partner proteins—Exo84p, 14-3-3 Zeta, α and β Enolase, Aldolase C, Malate dehydrogenase, ATP synthase, and Pyruvate kinase—along with those previously identified have now been classified into three functional groups: transport and signaling, chaperoning and scaffolding, and glycolytic metabolism. When viewed together, these interactions support a proposed model for the regulation of the GIF activity of MT-3.

1. Introduction

In a comparative study, Uchida et al. found that brain extract from Alzheimer's disease (AD) patient had an enhanced neurotrophic effect when compared to normal brain extract [1]. This was subsequently attributed to the reduction of a protein originally called, growth inhibitory factor (GIF). The latter was first thought to be involved in the formation of the senile plaques, which leads to the enhanced sprouting and the exhaustion of the neurons and, therefore, increases the susceptibility of the neurons to exocitotoxic substances [2]. However, in a followup study, a correlation was found between the reduction of the GIF activity and the presence of neurofibrillary tangles and not with the senile plaques [3]. Further isolation, purification, and sequencing of the GIF revealed that it belonged to the metallothionein (MT) protein family, the third isoform isolated and purified in this family, thus the name metallothionein-3 (MT-3) [3].

MTs are a class of small (6-7 kDa) cysteine-rich (30%) proteins binding both essential (Cu^{+} and Zn^{2+}) and non-essential (Cd^{2+}, Ag^{+} and Hg^{2+}) metal ions [4]. While metal binding is very tight, there is a facile metal exchange between MTs and other metalloproteins [5]. In mammalian species, four isoforms are currently known, which are designated as MT-1, 2, 3, and 4 [6]. MT-1 and 2 are expressed in almost every organ, particularly high in the kidney and liver and both exhibit similar properties as far as metal-binding characteristics are concerned. MT-3 is expressed mainly in the brain, though some studies have reported finding MT-3 in the kidney and some cancer cells [7]. MT-4 is expressed in certain stratified tissues [8].

Since the discovery of MT-1 and MT-2, there have been numerous publications describing the biochemical characteristics and possible function of these isoforms [9]. Although the essential physiological functions remain elusive, MT's have been shown to be involved in these vital processes: (1) the modulation of free oxygen radicals and nitric oxide [10], (2) apoptosis [11], and (3) homeostasis of heavy metals [4]. All three physiological processes play a key role in several diseases [9].

The binding of metal ions occurs, specifically through cysteine thiolate bonds, in two separate domains. The N-terminal domain, called β-domain, binds 3 divalent metals in a Me_3S_9 tetrahedral coordination. The C-terminal domain,

called α-domain, binds 4 divalent metals in a Me_4S_{11} tetrahedral coordination [12, 13]. While MT-1 and MT-2 have been shown to be inducible at the transcriptional level by the very heavy metals that they bind, such as Cd, Cu, and Zn [14], MT-3 is not induced by metal ions. Indeed, MT-3 and MT-1 respond differentially to zinc deprivation of cells in culture with only MT-1 being downregulated in these cells [15]. Thus, it is reasonable to conclude that MT-3 is not involved in metal ion homeostasis, as both the MT-1 and MT-2 isoforms appears to be [4, 14].

Studies have shown that the MT-3 β-domain was sufficient to exhibit the GIF activity [16–18]. However, MT-3 by itself does not exhibit GIF activity when added to neuronal cell cultures. This activity is only expressed when combined with brain extracts [17]. From this, one can conclude that the inhibitory activity is invoked by specific interaction(s) between MT-3 and other component(s) in the brain extract. Indeed, we and others have previously described some other components in the brain interacting with MT-3 [19–23].

Immunoaffinity chromatography is a highly sensitive and selective method for the isolation of target proteins and for the identification of protein-protein interactions [20]. The results from these experiments presented in this current paper represent a significant extension with 7 new proteins positively identified interacting with MT3, along with the 5 proteins identified in the preliminary mass spectra analysis of the bands in the tryptic digested gel [21]. Selected protein binding partners identified in Lahti et al. [21] were validated using antisera available for the associated proteins. A recent paper appeared by Chung et al. [24], which showed the release of MT-1 and 2 from astrocytes and uptake in neurons in response to brain injury. The mechanism of the secretion of these two MT isoforms, however, is quite distinct from the mechanism proposed here for MT-3. In this paper, we present a detailed description of new proteins found interacting with MT-3, which leads us to propose a new model for the secretion of MT-3 from the astrocytes and uptake in neurons, thus providing the first mechanistic description of its neuroinhibitory activity.

2. Materials and Methods

2.1. Protein Extraction. Whole mouse (8–10 weeks) brains (Swiss Webster, Pel-Freez) were used for total protein extraction. The brains were homogenized at a ratio of 2 : 1 (v/w) in 10 mM MOPS, 10 mM NaCl, pH 7.0 containing 1 mM PMSF and EDTA-free protease inhibitor cocktail (Roche). The homogenate was then centrifuged at 30000 g, 4°C for 30 min. The supernatant thus obtained was used for the MT-3 protein complex purification.

2.2. Affinity Chromatography. Affi-Gel Hz (Biorad) was used to immobilize the MT-3 antibody previously purified [25]. The supernatant was applied to the column and incubated overnight. The nonbound fraction was then thoroughly washed with the homogenization buffer. The column was then rinsed with 10 mM MOPS, 150 mM NaCl pH 7.0 containing 1 mM PMSF and the protease inhibitor cocktail until no protein was eluted from the column (see Figure 1). The MT-3 protein complex was then chaotropically eluted using 10 mM MOPS, 3 M NaSCN, pH 7.0. The MT-3 protein complex was then concentrated using a spin-filter with MWCO of 3 kDa and the buffer exchanged to the homogenization buffer.

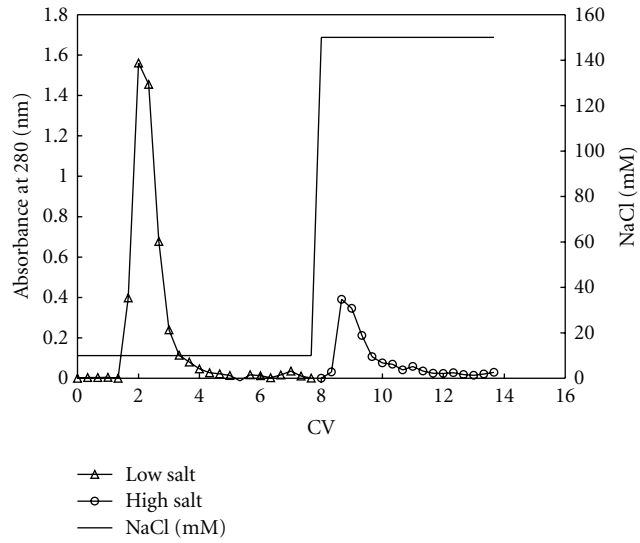


Figure 1: Washing steps for the immunoaffinity column. After loading the column was washed with different salt concentration until no protein was eluted (absorbance at 280 nm).

2.3. SDS-PAGE and In-Gel Trypsin Digestion. The MT-3 protein complex fraction was then SDS-paged using NuPAGE Novex 4–12% Bis-Tris gel and run for 30 min at 200 V using MES-SDS buffer (Invitrogen). The gel was then silver-stained using an enhanced silver stain kit (Biorad).

Protein bands were excised using the Investigator Proprep from Genomics Solutions. The digestion of the band was done according to a modified procedure [26]. Briefly, bands were excised and destained using 30 mM potassium ferricyanide and 100 mM sodium thiosulphate, washed with Milli-Q and then with 100% acetonitrile, followed by SpeedVac for 30 min. Trypsin (sequence-grade modified porcine, Promega) was then added to a final concentration of 12.5 ng/μL in ice-cold 50 mM ammonium bicarbonate containing 5 mM $CaCl_2$. The mixture was incubated on ice for 1 hour and then at 37°C overnight. The supernatant was removed and the gel slices washed once with 20 mM ammonium bicarbonate for 20 min and then pooled together. The gel slices were washed three times with 30 μL of 50% acetonitrile/5% formic acid for 20 min each, and then pooled with the previous supernatant.

The collected supernatant was then SpeedVac to near dryness and subjected to cysteine reduction and alkylation. This was done by rehydration in 10 mM DTT/100 mM NH_4HCO_3 at 56°C for 45 min. After cooling the sample to room temperature, alkylation was conducted using freshly made 55 mM iodoacetamide, 100 mM NH_4HCO_3, for 30 min at room temperature in dark.

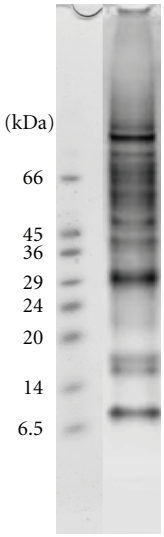


Figure 2: 4–12% SDS-PAGE of the fractions collected from the immunoaffinity chromatography of the normal mouse brain homogenate. Lane 1: Markers, Lane 2: fractions collected with 3 M NaSCN, 10 mM MOPS, pH 7.6.

2.4. Mass Spectrometry Analysis. MALDI-TOF data were collected on a Biflex III instrument (Bruker Daltonics). Full mass scans were collected, in the positive ion mode between m/z 500 and 3500, for the peptide mixtures of each sample placed on an anchor chip target. Each peak list of measured peptide masses was then used to search the National Center of Biotechnology Information (NCBI) sequence database for protein identification using Mascot (http://www.matrixscience.com/). Searches were performed allowing modifications that included methionine oxidation and carbamidomethyl addition, as well as up to 1 missed cleavage site and up to 250 ppm mass error. Only hits with $P < .05$ were considered positive hits, where P is the probability that the observed match is a random event.

MS-MS experiments were preformed on a linear ion trap (LTQ, Thermo Electron Corp., San Jose, CA) using nanoscale microcapillary reversed-phase liquid chromatography electrospray ionization tandem mass spectrometry (LC-MS/MS). Peptide mixtures were auto sampled on a Paradigm MS4 system (Michrom Bioresources, Inc., Auburn, CA). Samples were desalted and concentrated on a Paradigm Platinum Peptide Nanotrap precolumn (0.15 × 50 mm, Michrom Bioresources, Inc.) and subsequently on a fused silica microcapillary column (75 μm i. d.) packed in-house with Magic C18AQ reversed-phase material on a flow splitter (Michrom Bioresources, Inc.) at a flow rate of approximately 250 NL/min. The samples were subjected to a 60 min (10–40% ACN) gradient and directly eluted into the microcapillary column set to 2.0 kV. The LTQ was operated in the positive-ion mode using data-dependent acquisition methods initiated by a survey MS scan, which was followed by MS/MS (collision energy of 29%) on the 4 most abundant ions detected in the survey scan. M/Z values selected in the survey scan for MS/MS were excluded for subsequence MS/MS for 30 sec. The signal intensity threshold for an ion to be selected for MS/MS was set to a lower limit of 1000. Mass spectra were analyzed using BioWorks SeQuest Analysis Software package (ABI, Inc., Foster City, CA). Proteins that contained peptides with Sequest cross-correlation scores greater than 2 and contained 2 or more peptides were further examined manually.

3. Results

The analysis of the MT-3 protein complex(es) with SDS-PAGE revealed the presence of about 16 bands (Figure 2). Twelve proteins have been confidently identified thus far using MALDI-TOF and MS/MS. All the proteins reported previously to interact with MT-3 [19, 21] were reproducibly detected. New proteins found in the current study include: Exocyst complex component 8 (Exo84p), α and γ-enolase, 14-3-3 zeta, Malate dehydrogenase, Fructose-biphosphate aldolase 3, and ATP synthase (Table 1). The low MW protein region (<24 kDa) did not yield sufficiently significant scores for protein identification with high confidence. Rab3A [22, 23] was not identified although a faint band was seen in the gel with a MW of about 20 kDa. No significant protein ($P < .05$) was found for this band when searching the database, however the hit returned was for a GTP-binding protein.

To confirm the ID of the newly identified proteins, we conducted ESI-MS/MS on the trypsin digest of the bands. Results were analyzed using Sequest software. The sequence of the peptide was considered a positive hit if the cross-correlation score (xCorr) was higher than 2 and the number of matching ions higher than 60%. MS/MS results allowed not only the confirmation of the proteins previously identified by MALDI-TOF, but also the specific isoform of the proteins interacting with MT-3 (see supplemental material, Tables 2 and 3 available online at doi:10.4061/2011/208634). Moreover, the MS/MS allowed for the detection of proteins that were not confidently detected with MALDI-TOF (valosin containing protein, HSP70 glucose-regulated protein and protein 8, pyruvate kinase 3, tubulin alpha 3) each with at least two peptides and an xCorr value higher than 2.

Table 1 summarizes the regrouped results of the MALDI-TOF and MS/MS for all proteins identified and their known function. This information allowed for the classification of the partner proteins into three groups: (1) proteins that are involved in transport, (2) chaperoning/scaffolding and regulation, and (3) glucose metabolism related proteins (metabolic enzymes). A brief description of a representative protein from each group is provided below.

3.1. Group 1: Exocyst Complex Component 8 (Exo84p). Exo84p is a component of the exocyst multiprotein complex that plays an important role in targeting and docking the secretory vesicle, containing proteins and lipids, at the plasma membrane [27]. The exocyst multiprotein complex is composed of Sec3p, Sec5p, Sec6p, Sec8p, Sec10p, Sec15p, Exo70p and Exo84p [28]. The exocyst complex plays an essential role in tethering secretory vesicles to

specific domains of the plasma membrane for exocytosis [29]. Exo84p plays a key role in the organization and the polarized localization of the exocyst complex [29]. Exo84p does interact with Sec5p and/or Sec10p [27], proteins that we were not able to identify with confidence in our data. Exo84p can also interact with Ras and Ral GTPase proteins similar to Rab3a [30]. The Ral-binding domain of Exo84p adopts a plekstrin homology (PH) domain fold, which consists of a seven-stranded β-sandwich containing two orthogonal antiparallel β-sheets and an abutting C-terminal α-helix [28].

3.2. Group 2: DRP-2/CRMP2. Dihydropyrimidinase-Related Protein 2 (DRP-2) is a protein that is related to dihyropyrimidinase and is usually referred to as collapsing response mediator protein (CRMP2). Five isoforms of CRMPs exists in humans (CRMP1–CRMP5), which are approximately 70% identical to each other. There is no known enzymatic activity for the DRP-2 protein family, although it is 58% identical to the human dihydropyrimidinase that catalyzes the second step in pyrimidine degradation. DRP-2 is known to be involved in neuronal differentiation, axonal guidance and neuronal polarity. The protein is highly expressed in the developing nervous system. Overexpression of DRP-2 induces the growth of numerous axons and it is also involved in the maturation of neurites and preexisting dendrites to axons [31]. DRP-2 binds to tubulin heterodimers promoting microtubule assembly [32]. However, this binding is inactivated by the phosphorylation of Thr514 by glycogen synthase kinase 3β (GSK-3β) [33].

3.2.1. 14-3-3 Zeta. 14-3-3 proteins were first identified in 1967 during a study of the soluble acidic proteins from mammalian brains [34]. They are small proteins (~30 kDa) that form both homo- and heterodimers. They include seven isoforms in human cells. The importance of these proteins was not recognized until later when it was discovered that these proteins can activate tyrosine, tryptophan hydroxylases and Raf and can inhibit protein kinase C [35]. 14-3-3 proteins bind to specific phosphoserine or phosphothreonine motifs (RSXpSXP and RXXXpSXP) in protein targets [36]. 14-3-3 proteins can alter their target proteins using three ways: (1) conformational change, (2) physical occlusion of sequence-specific or structural protein features, and (3) scaffolding [35]. They can, thereby, prevent the interaction with other proteins, regulate the subcellular distribution of proteins or protect proteins from proteolysis [37].

14-3-3 zeta modulates the action of proteins that are involved in the cell cycle, transcriptional control, signal transduction, intracellular trafficking and regulation of ion channels [38]. Recently, 14-3-3 zeta was proposed to be a "Sweeper" of misfolded proteins [39].

3.3. Group 3: Enolase. Enolase, also called 2-phospho-D-glycerate hydrolase, is a metal-activated enzyme that catalyzes the dehydration of 2-phospho-D-glycerate to phosphoenolpyruvate (pyruvate kinase substrate) [40]. In vertebrates, three tissue-specific isoform are found: nonneuronal enolase (α), neuron-specific enolase (γ) and muscle-specific enolase (β) [41]. Enolase exists in a physiological state as a dimer, with the two monomer subunits oriented in an antiparallel manner [40]. The dimerization process depends on the two Mg^{2+} that are bound to the monomers and the divalent metal ion plays a critical role in catalysis [40]. Although Mg^{2+} is the primary activator of enolase, the enzyme also binds zinc strongly, which has a lower activity [40].

Table 1: Proteins interacting with MT-3 identified with MALDI-TOF and MS/MS, and their functions.

Protein ID	MALDI-TOF	MS/MS	Function
Exo84p	x		Transport
ATP synthase β subunit	x	x	Transport
Valosin containing protein		x	
HSP 84	x	x	Chaperoning
HSP70 glucose-regulated protein		x	Chaperoning
HSP70 protein 8		x	Chaperoning
Tubulin alpha 3		x	Scaffold
γ-Actin	x		
β-Actin	x	x	Scaffold
14-3-3 zeta	x	x	Scaffold/regulation
Pyruvate kinase 3		x	Glycolytic metabolism
γ-Enolase		x	Glycolytic metabolism
α-Enolase	x	x	Glycolytic metabolism
Creatine Kinase BB	x	x	Glycolytic metabolism
Aldolase 3		x	
Aldolase 1	x	x	Glycolytic metabolism
Malate dehydrogenase	x	x	Glycolytic metabolism
DRP-2	x	x	Neuronal growth
DRP-3		x	

Studies in yeast, vertebrates and mammalian cells have shown that α-enolase may have other functions such as thermal tolerance, growth control [42, 43], and hypoxia tolerance [44]. Enolase also functions as a cell surface receptor for plasminogen (PGn), which has a neurotrophic-like effect on neurons and enhances the neurite outgrowth of neocortical explants [42, 43, 45].

The cellular localization of α-enolase is known to be predominantly cytosolic [46] and can translocate to the plasma membrane in either the homodimeric ($\alpha\alpha$) or heterodimeric ($\alpha\gamma$) form. While the γ isoform is mainly detected in cells of neuronal origin, the α isoform is widely distributed among different tissues [47]. The $\alpha\alpha$ isoenzyme (nonneuronal) is expressed in many tissues, while the $\beta\beta$ isoenzyme occurs exclusively in muscle. The $\gamma\gamma$ isoenzyme (neuronal) is present in neurons and neuroendocrine tissues [48].

The $\alpha\gamma$ and $\gamma\gamma$ isoforms are the predominant dimers in the brain, representing the neuron-specific enolases [49].

Specifically, the γ-type enolase subunit is mainly located in neurons while α-type subunits are mostly found in glial cells [49].

4. Discussion

MT-3 by itself is not toxic nor does it inhibit the growth of neurons. Indeed, it maintains the survival of neurons in culture [50]. MT-3 knockout mice show a higher susceptibility to kainic acid induced neuronal injury, while mice overexpressing MT-3 show enhanced resistance [51]. These results suggest that MT-3 may play a neuroprotective role against toxic substances. MT-3 is abundantly expressed in astrocytes of normal brain [1, 52]. Moreover, when added to neuronal cell cultures that are exposed to amyloid-β peptides, MT-3 enhanced the resistance of the cells against the amyloid-β toxicity. This activity is now known as the MT-3 antiamyloid activity [53]. From these activities, one can conclude that MT-3 needs to be secreted into the extracellular milieu. Indeed, although MT-3 does not have any signal sequence, a 23-day-old astrocytes cell culture did secrete MT-3 into the media [50]. A recently published results [24] showed a very important difference in the secretion of MT-1 and 2 being triggered only in the presence of Zn and Interleukin-1, neither of these factors have been implicated in MT-3 secretion [50], which we propose is mediated by one or more of the specific partner protein interactions identified in this study.

The subcellular localization of MT-3 in rat brain astrocytes showed an association with organelles involved in the secretion pathway-free ribosomes, rough endoplasmic reticulum, small vesicles, the outer membrane of the mitochondria, plasma membrane, and also around the blood vessels [54]. The localization in neurons was mostly found in the axons, dendrites, the synaptic vesicles, and the postsynaptic densities [54]. It is, therefore, reasonable to hypothesize that MT-3 may well be expressed in the astrocytes. We propose that through its interaction with some of the proteins that we have identified, MT-3 is secreted to the extracellular milieu (blood vessels) and then absorbed by the neurons. Strengthening this argument is the fact that some of the proteins interacting with MT-3 are in fact involved in the transport process: Rab3a, Exo84p. Indeed, Exo84p is a subunit of the exocyst complex, which is important in intracellular trafficking. In metazoa, Exo84p has a PH domain towards its N-terminus. PH domains share little sequence conservation, but all have a common fold, which is electrostatically polarized. They are often involved in targeting proteins to the plasma membrane, with a few displaying strong specificity in lipid binding. PH domains are found in cellular signaling proteins such as serine/threonine kinase, tyrosine kinases, regulators of G-proteins, endocytotic GTPases adaptors, as well as in cytoskeletal associated molecules and in lipid-associated enzymes. Exo84p can also interact with Ras and Ral GTPase proteins similar to Rab3A [30]. Rabphilin3A, a downstream effector of Rab3A that binds the GTP-bound form of Rab3A [55], also binds 14-3-3, another protein found in the MT-3 multiprotein complex [56]. 14-3-3 has also been shown to interact with Sec23, a GTPase activating protein and a component of the Sec23p-Sec24p heterodimeric complex of the COPII vesicle coat, which is involved in ER to Golgi transport in *S. cerevisiae* [57].

Based upon the above, a model for the secretion of MT-3 is proposed. The secretion is regulated through the "mitochondrial pathway" and involves MT-3's interaction with Rab3A, Exo84p and the exocyst complex, 14-3-3 zeta, and rabphilin3A (Figure 3, Left panel). Once MT-3 is secreted, PGn and/or $\alpha\gamma$-enolase could facilitate its uptake by the neurons, which have been shown to uptake MT-1/2 [24]. Indeed, $\alpha\gamma$-enolase is located on the cell surface of a certain population of neurons and has been shown to interact with the microglia-derived PGn, which has a neurotrophic-like effect on neurons [43].

The neuronal MT-3 could then regulate the growth of the neurons through either or both of the following mechanisms: (i) Considering that MT-3 has a GIF activity, one could speculate that MT-3 might inhibit PGn. This may well be facilitated through the common interacting protein enolase. (ii) DRP-2 is known to regulate neuronal differentiation, axonal guidance and neuronal polarity. Moreover, DRP-2 is involved in the maturation of neurites and preexisting dendrites to axons, and its overexpression induces the outgrowth of numerous axons [31]. On the other hand, MT-3 suppresses the neurite extension of neurons in the early period of differentiation and suppresses cell death of more differentiated neurons [50]. Based on this, we hypothesize that through its interaction with DRP-2, MT-3 could regulate the growth of the neurons (Figure 3, right panel).

The above model conforms to the neuroactivity of MT3 and its reported reduction in AD brain tissue [52], where the above regulatory interactions would be disrupted. The trigger might well be the oxidative stress or other hallmark pathology associated with the disease: the neurofibrillary tangles or the plaques. Most proteins found in the multiprotein complex(es) with MT3 have in fact been shown to be altered in AD patients. 14-3-3 zeta is overexpressed in the temporal cortex of patient with cognitive impairment, leading to its implication in the AD neuropathology [58]. Glyceraldehyde-3-phosphate dehydrogenase, pyruvate kinase, malate dehydrogenase and 14-3-3 zeta were found to be significantly oxidized both in rat brains and in cell cultures that were exposed to amyloid-β_{42} [59, 60]. γ-enolase and malate dehydrogenase were found to be downregulated in the Tg2576 AD mouse model, whereas pyruvate kinase, α-enolase, and DRP-2 were upregulated [61]. The expression of DRP-2 in the hippocampal neurons promotes axon elongation. This latter function seems to depend upon the phosphorylation of DRP-2 by GSK-3β [31, 32, 62, 63]. 14-3-3 zeta has been proposed to be an effector of tau phosphorylation by GSK-3β and hyperphosphorylated DRP-2 has been detected within neurofibrillary tangles from the brains of Alzheimer's disease patients [62, 64]. The hyperphosphorylation of DRP-2 could inhibit the interaction with MT-3 leading to the alteration of the GIF activity of MT-3 and, therefore, to the outgrowth of the neurons. It is noteworthy, to reemphasize the positive correlation found between the GIF activity and the neurofibrillary tangles [3].

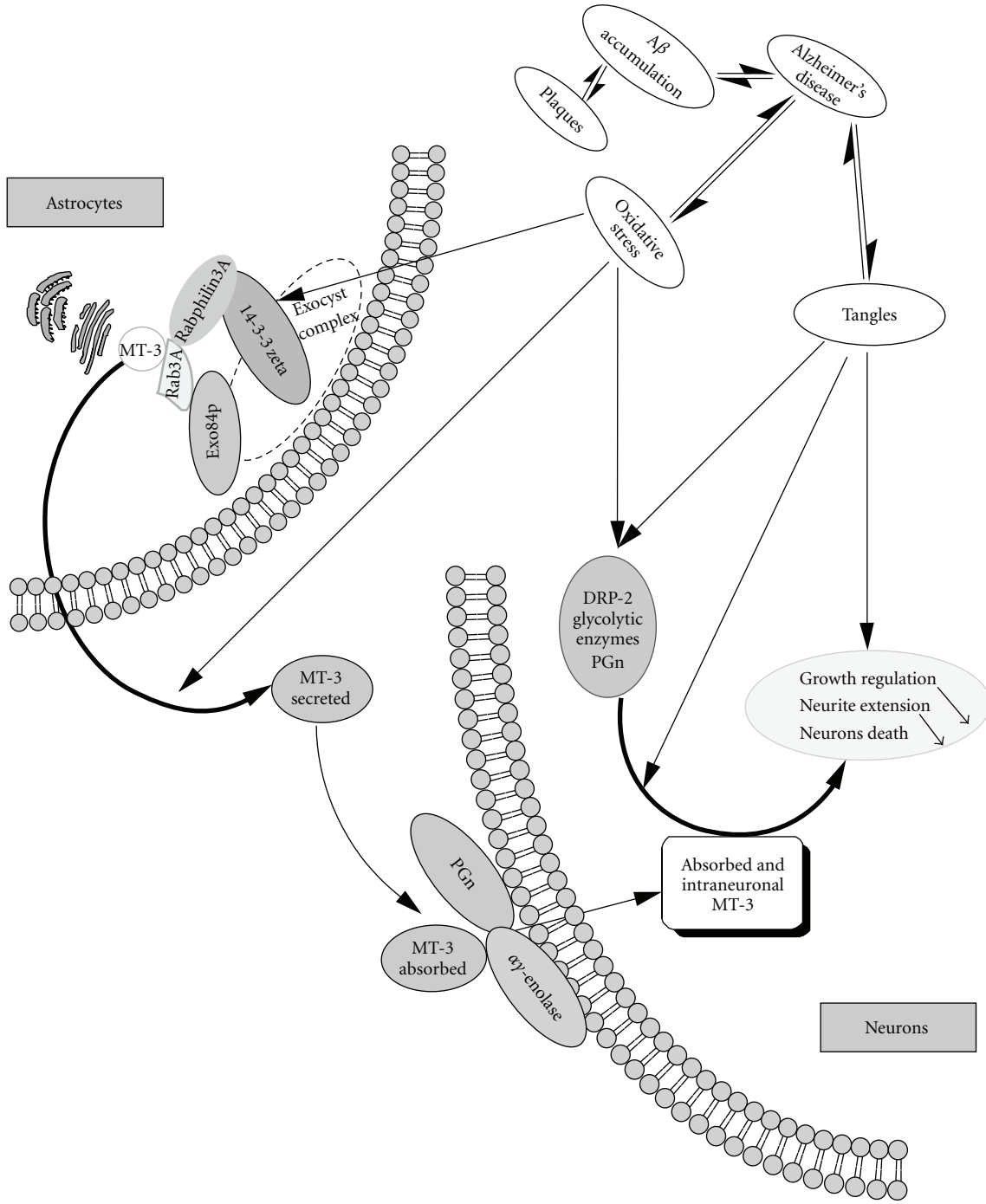


Figure 3: Schematic model of the interactions of MT-3 and its implication on the biological functions of MT-3. The hypothetical model describes the process of secretion of MT-3, from the astrocytes into the extracellular milieu, through its interaction with Rab3A, 14-3-3 zeta, Exo84p (Left panel). The uptake of MT-3 into the neurons and their growth regulation: uptake is facilitated through the interaction with the plasminogen (PGn) and enolase (either, $\alpha\gamma$ or $\gamma\gamma$). Once inside the neurons, MT-3 protects the neurons from the extension and the outgrowth through its interaction with DRP-2. In Alzheimer's disease, oxidative stress alters several proteins involved in the interaction with MT-3, therefore potentially affecting the pathway of secretion and/or the uptake.

To summarize, novel new partner proteins for MT3 have been identified. The identities of a couple of these associated proteins, 14-3-3 and Exo84p, provide a basis for a biologically relevant model for the processing of MT3. In view of the size of MT3, we favor an interaction model involving multiple interactions as opposed to single complex. The interaction model we envision is a thiol/disulfide interchange model, which could involve one or more of the many cysteines in MT3. Indeed, Rab3A, 14-3-3Z, Exo84p, α-enolase, all have cysteines that could participate in this

thiol/disulfide interchange model. In this regard, it is important to note that published work [65] has established that the beta domain of MT3 binds 4 copper leaving two cysteines free to participate in such a mechanism. This is in contrast to MT1 and 2, both of which bind either 3 zinc or 6 coppers to the beta domain leaving no free cysteines to participate in such a thiol exchange mechanism. The proposed model, along with Rab3A [22, 23], promotes the secretion of MT3 from astrocytes. Now in the extracellular milieu, MT-3 is positioned to function consistent with its reported activities: the regulation of neuronal growth and the antagonism of β-amyloid. Regulation of the growth of neurons could be mediated through two possible mechanisms: (i) direct inhibition of the PGn at the surface of neurons, or (ii) the uptake of MT3 into neurons facilitated by the interaction with enolase located in the cell surface of the neurons. Once taken up by the neurons, MT-3 could then regulate the growth of neurons through its association with DRP-2. Further studies are in progress to validate this newly proposed functional model for MT3's role in regulating neuronal growth.

Abbreviations

AD: Alzheimer's disease,
MT-3: Metallothionein-3,
GIF: Growth inhibitory factor,
Exo84p: Exocyst complex component 8,
PH: Plekstrin homology,
PGn: Plasminogen,
DRP-2: Dihydropyriminase like protein-2,
CRMP: Collapsin response mediator protein,
GSK-3β: Glycogen synthase kinase (3β).

Acknowledgments

We are very thankful to the help of Bruce Witthuhn and LeeAnn Higgins at the Proteomics Analysis Core and the Mass Spectrometry Consortium for the Life Sciences of the University of Minnesota. Partial support for this research from Minnesota Medical Foundation (MMF) and BMBB department is gratefully acknowledged.

References

[1] Y. Uchida, Y. Ihara, and M. Tomonaga, "Alzheimer's disease brain extract stimulates the survival of cerebral cortical neurons from neonatal rats," *Biochemical and Biophysical Research Communications*, vol. 150, no. 3, pp. 1263–1267, 1988.

[2] Y. Uchida and M. Tomonaga, "Neurotrophic action of Alzheimer's disease brain extract is due to the loss of inhibitory factors for survival and neurite formation of cerebral cortical neurons," *Brain Research*, vol. 481, no. 1, pp. 190–193, 1989.

[3] Y. Uchida, K. Takio, K. Titani, Y. Ihara, and M. Tomonaga, "The growth inhibitory factor that is deficient in the Alzheimer's disease brain is a 68 amino acid metallothionein-like protein," *Neuron*, vol. 7, no. 2, pp. 337–347, 1991.

[4] J. H. R. Kägi and A. Schäffer, "Biochemistry of metallothionein," *Biochemistry*, vol. 27, no. 23, pp. 8509–8515, 1988.

[5] J. D. Otvos, H. R. Engeseth, D. G. Nettesheim, and C. R. Hilt, "Interprotein metal exchange reactions of metallothionein," *Experientia. Supplementum*, vol. 52, pp. 171–178, 1987.

[6] M. Vašák and D. W. Hasler, "Metallothioneins: new functional and structural insights," *Current Opinion in Chemical Biology*, vol. 4, no. 2, pp. 177–183, 2000.

[7] S. H. Garrett, V. Phillips, S. Somji et al., "Expression of MT-3 protein in the human kidney," *Toxicology Letters*, vol. 126, pp. 69–80, 1999.

[8] C. J. Quaife, S. D. Findley, J. C. Erickson et al., "Induction of a new metallothionein isoform (MT-IV) occurs during differentiation of stratified squamous epithelia," *Biochemistry*, vol. 33, no. 23, pp. 7250–7259, 1994.

[9] C. D. Klaassen, *Metallothionein IV*, Birkhauser, Basel, Switzerland, 1999.

[10] P. K. Datta and E. A. Lianos, "Nitric oxide induces metallothionein-I gene expression in mesangial cells," *Translational Research*, vol. 148, no. 4, pp. 180–187, 2006.

[11] D. Duval, M. Trouillas, C. Thibault et al., "Apoptosis and differentiation commitment: novel insights revealed by gene profiling studies in mouse embryonic stem cells," *Cell Death and Differentiation*, vol. 13, no. 4, pp. 564–575, 2006.

[12] K. Zangger, G. Öz, J. D. Otvos, and I. M. Armitage, "Three-dimensional solution structure of mouse [Cd]-metallothionein-1 by homonuclear and heteronuclear NMR spectroscopy," *Protein Science*, vol. 8, no. 12, pp. 2630–2638, 1999.

[13] J. D. Otvos and I. M. Armitage, "Structure of the metal clusters in rabbit liver metallothionein," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 77, no. 12, pp. 7094–7098, 1980.

[14] K. Zangger and I. M. Armitage, "Metallothioneins," in *Handbook of Metalloproteins*, A. Messerschmidt, W. Bode, and M. Cygler, Eds., vol. 3, pp. 353–364, John Wiley & Sons, Chichester, UK, 2004.

[15] R. D. Palmiter, "Constitutive expression of metallothionein-III (MT-III), but not MT-I, inhibits growth when cells become zinc deficient," *Toxicology and Applied Pharmacology*, vol. 135, no. 1, pp. 139–146, 1995.

[16] Y. Uchida and Y. Ihara, "The N-terminal portion of growth inhibitory factor is sufficient for biological activity," *Journal of Biological Chemistry*, vol. 270, no. 7, pp. 3365–3369, 1995.

[17] A. K. Sewell, L. T. Jensen, J. C. Erickson, R. D. Palmiter, and D. R. Winge, "Bioactivity of metallothionein-III correlates with its novel b-domain sequence rather than metal binding properties," *Biochemistry*, vol. 34, pp. 4740–4747, 1995.

[18] Z. C. Ding, X. C. Teng, B. Cai et al., "Mutation at Glu23 eliminates the neuron growth inhibitory activity of human metallothionein-3," *Biochemical and Biophysical Research Communications*, vol. 349, no. 2, pp. 674–682, 2006.

[19] I. El Ghazi, B. L. Martin, and I. M. Armitage, "Metallothionein-3 is a component of a multiprotein complex in the mouse brain," *Experimental Biology and Medicine*, vol. 231, no. 9, pp. 1500–1506, 2006.

[20] A. M. Tokheim, I. M. Armitage, and B. L. Martin, "Development of an antiserum specific for isoform 3 of metallothionein," *Journal of Biochemical and Biophysical Methods*, vol. 63, no. 1, pp. 43–52, 2005.

[21] D. W. Lahti, J. D. Hoekman, A. M. Tokheim, B. L. Martin, and I. M. Armitage, "Identification of mouse brain proteins associated with isoform 3 of metallothionein," *Protein Science*, vol. 14, no. 5, pp. 1151–1157, 2005.

[22] Q. H. Kang, Q. L. Chen, H. W. Ren, and B. G. Ru, "Growth inhibitory factor (GIF) directly interacts with G-protein Rab3a," *Progress in Biochemistry and Biophysics*, vol. 28, no. 6, pp. 883–884, 2001.

[23] M. Knipp, G. Meloni, B. Roschitzki, and M. Vašák, "Znmetallothionein-3 and the synaptic vesicle cycle: interaction of metallothionein-3 with the small GTPase Rab3A," *Biochemistry*, vol. 44, no. 9, pp. 3159–3165, 2005.

[24] R. S. Chung, M. Penkowa, J. Dittmann et al., "Redefining the role of metallothionein within the injured brain: extracellular metallothioneins play an important role in the astrocyte-neuron response to injury," *Journal of Biological Chemistry*, vol. 283, no. 22, pp. 15349–15358, 2008.

[25] A. M. Tokheim, I. M. Armitage, and B. L. Martin, "Antiserum specific for the intact isoform-3 of metallothionein," *Journal of Biochemical and Biophysical Methods*, vol. 63, no. 1, pp. 43–52, 2005.

[26] A. Shevchenko, M. Wilm, O. Vorm, and M. Mann, "Mass spectrometric sequencing of proteins from silver-stained polyacrylamide gels," *Analytical Chemistry*, vol. 68, no. 5, pp. 850–858, 1996.

[27] W. Guo, A. Grant, and P. Novick, "Exo84p is an exocyst protein essential for secretion," *Journal of Biological Chemistry*, vol. 274, no. 33, pp. 23558–23564, 1999.

[28] G. Dong, A. H. Hutagalung, C. Fu, P. Novick, and K. M. Reinisch, "The structures of exocyst subunit Exo70p and the Exo84p C-terminal domains reveal a common motif," *Nature Structural and Molecular Biology*, vol. 12, no. 12, pp. 1094–1100, 2005.

[29] X. Zhang, A. Zajac, J. Zhang et al., "The critical role of Exo84p in the organization and polarized localization of the exocyst complex," *Journal of Biological Chemistry*, vol. 280, no. 21, pp. 20356–20364, 2005.

[30] N. I. Nicely, J. Kosak, V. De Serrano, and C. Mattos, "Crystal structures of Ral-GppNHp and Ral-GDP reveal two binding sites that are also present in Ras and Rap," *Structure*, vol. 12, no. 11, pp. 2025–2036, 2004.

[31] T. Yoshimura, Y. Kawano, N. Arimura, S. Kawabata, A. Kikuchi, and K. Kaibuchi, "GSK-3β regulates phosphorylation of CRMP-2 and neuronal polarity," *Cell*, vol. 120, no. 1, pp. 137–149, 2005.

[32] Y. Fukata, T. J. Itoh, T. Kimura et al., "CRMP-2 binds to tubulin heterodimers to promote microtubule assembly," *Nature Cell Biology*, vol. 4, no. 8, pp. 583–591, 2002.

[33] R. C. Deo, E. F. Schmidt, A. Elhabazi, H. Togashi, S. K. Burley, and S. M. Strittmatter, "Structural bases for CRMP function in plexin-dependent semaphorin3A signaling," *EMBO Journal*, vol. 23, no. 1, pp. 9–22, 2004.

[34] B. W. Moore and V. J. Perez, "Specific acidic proteins of the nervous system," in *Physiological and Biochemical Aspects of Nervous Integration*, F. D. Carlson, Ed., pp. 343–359, Prentice-Hall, Englewood Cliffs, NJ, USA, 1967.

[35] D. Bridges and G. B. Moorhead, "14-3-3 proteins: a number of functions for a numbered protein," *Science's STKE*, vol. 2005, no. 296, pp. 1–10, 2005.

[36] A. J. Muslin, J. W. Tanner, P. M. Allen, and A. S. Shaw, "Interaction of 14-3-3 with signaling proteins is mediated by the recognition of phosphoserine," *Cell*, vol. 84, no. 6, pp. 889–897, 1996.

[37] P. Beguin, R. N. Mahalakshmi, K. Nagashima et al., "14-3-3 and calmodulin control subcellular distribution of Kir/Gem and its regulation of cell shape and calcium channel activity," *Journal of Cell Science*, vol. 118, no. 9, pp. 1923–1934, 2005.

[38] D. Berg, C. Holzmann, and O. Riess, "14-3-3 proteins in the nervous system," *Nature Reviews Neuroscience*, vol. 4, no. 9, pp. 752–762, 2003.

[39] K. Kaneko and N. S. Hachiya, "The alternative role of 14-3-3 zeta as a sweeper of misfolded proteins in disease conditions," *Medical Hypotheses*, vol. 67, no. 1, pp. 169–171, 2006.

[40] V. Pancholi, "Multifunctional α-enolase: its role in diseases," *Cellular and Molecular Life Sciences*, vol. 58, no. 7, pp. 902–920, 2001.

[41] C. C. Rider and C. B. Taylor, "Enolase isoenzymes in rat tissues. Electrophoretic, chromatographic, immunological and kinetic properties," *Biochimica et Biophysica Acta*, vol. 365, no. 1, pp. 285–300, 1974.

[42] L. A. Miles, C. M. Dahlberg, J. Plescia, J. Felez, K. Kato, and E. F. Plow, "Role of cell-surface lysines in plasminogen binding to cells: identification of α-enolase as a candidate plasminogen receptor," *Biochemistry*, vol. 30, no. 6, pp. 1682–1691, 1991.

[43] K. Nakajima, M. Hamanoue, N. Takemoto, T. Hattori, K. Kato, and S. Kohsaka, "Plasminogen binds specifically to α-enolase on rat neuronal plasma membrane," *Journal of Neurochemistry*, vol. 63, no. 6, pp. 2048–2057, 1994.

[44] R. M. Aaronson, K. K. Graven, M. Tucci, R. J. McDonald, and H. W. Farber, "Non-neuronal enolase is an endothelial hypoxic stress protein," *Journal of Biological Chemistry*, vol. 270, no. 46, pp. 27752–27757, 1995.

[45] K. Nagata, K. Nakajima, N. Takemoto, H. Saito, and S. Kohsaka, "Microglia-derived plasminogen enhances neurite outgrowth from explant cultures of rat brain," *International Journal of Developmental Neuroscience*, vol. 11, no. 2, pp. 227–237, 1993.

[46] A. Subramanian and D. M. Miller, "Mapping the functional domains involved in down-regulation of the c-myc protooncogene," *Journal of Biological Chemistry*, vol. 275, no. 8, pp. 5958–5965, 2000.

[47] A. Giallongo, D. Oliva, L. Cali, G. Barba, G. Barbieri, and S. Feo, "Structure of the human gene for alpha-enolase," *European Journal of Biochemistry*, vol. 190, no. 3, pp. 567–573, 1990.

[48] G. I. Murray, M. E. Duncan, W. T. Melvin, and J. E. Fothergill, "Immunohistochemistry of neurone specific enolase with γ subunit specific anti-peptide monoclonal antibodies," *Journal of Clinical Pathology*, vol. 46, no. 11, pp. 993–996, 1993.

[49] A. Keller, A. Berod, M. Dussaillant, N. Lamande, F. Gros, and M. Lucas, "Coexpression of alpha and gamma enolase genes in neurons of adult rat brain," *Journal of Neuroscience Research*, vol. 38, no. 5, pp. 493–504, 1994.

[50] Y. Uchida, F. Gomi, T. Masumizu, and Y. Miura, "Growth inhibitory factor prevents neurite extension and the death of cortical neurons caused by high oxygen exposure through hydroxyl radical scavenging," *Journal of Biological Chemistry*, vol. 277, no. 35, pp. 32353–32359, 2002.

[51] J. C. Erickson, G. Hollopeter, S. A. Thomas, G. J. Froelick, and R. D. Palmiter, "Disruption of the metallothionein-III gene in mice: analysis of brain zinc, behavior, and neuron vulnerability to metals, aging, and seizures," *Journal of Neuroscience*, vol. 17, no. 4, pp. 1271–1281, 1997.

[52] W. H. Yu, W. J. Lukiw, C. Bergeron, H. B. Niznik, and P. E. Fraser, "Metallothionein III is reduced in Alzheimer's disease," *Brain Research*, vol. 894, no. 1, pp. 37–45, 2001.

[53] Y. Irie and W. M. Keung, "Anti-amyloid β activity of metallothionein-III is different from its neuronal growth inhibitory activity: structure-activity studies," *Brain Research*, vol. 960, no. 1-2, pp. 228–234, 2003.

[54] M. Yamada, S. Hayashi, I. Hozumi, T. Inuzuka, S. Tsuji, and H. Takahashi, "Subcellular localization of growth inhibitory factor in rat brain: light and electron microscopic immunohistochemical studies," *Brain Research*, vol. 735, no. 2, pp. 257–264, 1996.

[55] C. Ostermeier and A. T. Brunger, "Structural basis of Rab effector specificity: crystal structure of the small G protein Rab3A complexed with the effector domain of raBphilin-3A," *Cell*, vol. 96, no. 3, pp. 363–374, 1999.

[56] L. Sun, M. A. Bittner, and R. W. Holz, "Rim, a component of the presynaptic active zone and modulator of exocytosis, binds 14-3-3 through its N terminus," *Journal of Biological Chemistry*, vol. 278, no. 40, pp. 38301–38309, 2003.

[57] M. Pozuelo Rubio, K. M. Geraghty, B. H. C. Wong et al., "14-3-3-Affinity purification of over 200 human phosphoproteins reveals new links to regulation of cellular metabolism, proliferation and trafficking," *Biochemical Journal*, vol. 379, no. 2, pp. 395–408, 2004.

[58] C. Soulie, A. Nicole, A. Delacourte, and I. Ceballos-Picot, "Examination of stress-related genes in human temporal versus occipital cortex in the course of neurodegeneration: involvement of 14-3-3 zeta in this dynamic process," *Neuroscience Letters*, vol. 365, no. 1, pp. 1–5, 2004.

[59] R. Sultana, D. Boyd-Kimball, H. F. Poon et al., "Redox proteomics identification of oxidized proteins in Alzheimer's disease hippocampus and cerebellum: an approach to understand pathological and biochemical alterations in AD," *Neurobiology of Aging*, vol. 27, no. 11, pp. 1564–1576, 2006.

[60] D. Boyd-Kimball, H. F. Poon, B. C. Lynn et al., "Proteomic identification of proteins specifically oxidized in Caenorhabditis elegans expressing human Aβ(1–42): implications for Alzheimer's disease," *Neurobiology of Aging*, vol. 27, no. 9, pp. 1239–1249, 2006.

[61] J. S. Su, S. E. Lee, H. B. Jung et al., "Profiling proteins related to amyloid deposited brain of Tg2576 mice," *Proteomics*, vol. 4, no. 11, pp. 3359–3368, 2004.

[62] A. R. Cole, A. Knebel, N. A. Morrice et al., "GSK-3 phosphorylation of the Alzheimer epitope within collapsin response mediator proteins regulates axon elongation in primary neurons," *Journal of Biological Chemistry*, vol. 279, no. 48, pp. 50176–50180, 2004.

[63] N. Inagaki, K. Chihara, N. Arimura et al., "CRMP-2 induces axons in cultured hippocampal neurons," *Nature Neuroscience*, vol. 4, no. 8, pp. 781–782, 2001.

[64] Y. Gu, N. Hamajima, and Y. Ihara, "Neurofibrillary tangle-associated collapsin response mediator protein-2 (CRMP-2) is highly phosphorylated on Thr-509, Ser-518, and Ser-522," *Biochemistry*, vol. 39, no. 15, pp. 4267–4275, 2000.

[65] G. Meloni, P. Faller, and M. Vašák, "Redox silencing of copper in metal-linked neurodegenerative disorders: reaction of Zn_7metallothionein-3 with Cu_2 ions," *Journal of Biological Chemistry*, vol. 282, no. 22, pp. 16068–16078, 2007.

SAGE-Hindawi Access to Research
International Journal of Alzheimer's Disease
Volume 2011, Article ID 542043, 9 pages
doi:10.4061/2011/542043

Review Article

Alzheimer's Disease and Metals: A Review of the Involvement of Cellular Membrane Receptors in Metallosignalling

Pavithra C. Amadoruge[1,2] and Kevin J. Barnham[1,2]

[1] *Bio21 Molecular Science and Biotechnology Institute, The University of Melbourne, 30 Flemington Road, Parkville, VIC 3010, Australia*
[2] *Department of Pathology, The University of Melbourne, and The Mental Health Research Institute, Parkville, VIC 3052, Australia*

Correspondence should be addressed to Kevin J. Barnham, kbarnham@unimelb.edu.au

Received 15 October 2010; Accepted 5 January 2011

Academic Editor: Paolo Zatta

Copyright © 2011 P. C. Amadoruge and K. J. Barnham. This is an open access article distributed under the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original work is properly cited.

Alzheimer's disease (AD) is a debilitating form of dementia. The hallmark protein associated with the disease is the amyloid beta (Aβ) peptide. Aggregation of Aβ has been shown to depend on interactions with metals. The recent studies now demonstrate that metals also play additional important roles in the disease process. Consequently, there may be benefit from modulating metal homeostasis. However, the role and subcellular location of metals within neurons is not well understood. There is growing evidence to suggest that metals can act at the site of cellular membrane receptors and affect cellular signaling by modulating the signal transduction of those receptors. The glutamatergic and cholinergic receptor systems, both well-known neurotransmitter systems affected in AD, have well-documented metal interactions, as do the tropomyosin-receptor kinase (Trk) family of receptors and the epidermal growth factor (EGF) receptor. In this paper, the metal interactions with these membrane receptor systems will be explored and thus the potential for membrane receptors as an intervention point in AD will be assessed.

1. Introduction

Alzheimer's disease (AD) is the most common form of age-related dementia [1, 2]. The key neuropathological features include extracellular amyloid beta (senile) plaques (Aβ), intracellular neurofibrillary tangles, chronic oxidative stress, and disease progression leading to cognitive decline and eventually neuronal cell loss [1, 2]. The cognitive decline observed in AD has its roots at the synapse, the space between neurons, through which they communicate. The synapse is also the site at which the Aβ peptide, the characteristic amyloid protein associated with AD, is believed to first deposit [2]. It is also the site where Aβ may interact with metals released as a consequence of glutamatergic transmission. In the recent years, growing evidence points to soluble Aβ oligomers being the toxic species [3] and whose appearance correlates with disease progression [4, 5]. It has also been hypothesized that small oligomers as opposed to Aβ fibrils induce synaptic failure [6, 7], after experiments showed Aβ oligomers to inhibit long-term potentiation (LTP) [8–10], a biochemical model of synaptic strength [11]. Furthermore, early memory loss associated with the disease has been attributed to synapse loss occurring prior to neuronal cell death [6, 7], and there are reports of a decrease in synaptic protein levels in AD [12].

2. Metals, Aging, and Alzheimer's Disease

The ability of life to utilize oxygen is dependent on the chemistry of transition metal ions. Metal ions are able to coordinate O_2 enabling transport, and the ability of transition metal ions to move between various oxidation states allows the activation and ultimately utilization of oxygen. If not properly regulated the same chemistry that allows the transport and utilization of oxygen can have the potential to generate reactive oxygen species (ROS). Metals are integral for the function of enzymes and numerous intracellular signaling proteins, and in a healthy individual, the levels of these metals are highly regulated. With normal

aging and more so in a neurodegenerative disease state, such homeostatic mechanisms are postulated to become perturbed, leading to aberrant metal-dependant enzyme function, mitochondrial dysfunction, and the production of ROS, all of which are well-known aetiologies associated with AD.

The transition metals implicated in AD include copper (Cu), zinc (Zn), and iron (Fe) [13]. These metals are generally found ligand-bound, and not as free ions. Although there do exist pools of metal that are coordinated to lower affinity ligands and as such are readily exchangeable. There is increasing evidence to suggest that Cu and Zn may exist as free ions when released into the synapse as part of the synaptic transmission process (reviewed in [1]). These metals can reach up to micromolar levels in the synaptic cleft [1], with Cu reaching 15 μM but Zn reaching up to 300 μM in the mossy fibres of the hippocampus postaction potential input [1, 14].

Aging is the main risk factor associated with all neurodegenerative diseases. Metal dyshomeostasis is an important feature of AD and this may be related to aging. There is an apparent state of intracellular Cu deficiency and an extracellular increase in Cu and Zn, possibly due to the metals binding to Aβ (reviewed in [1]). Binding of metals to Aβ can promote aggregation of the peptide with pathological consequences [15].

In the case of AD, the most common form of age-related dementia [16, 17], a state of Cu imbalance, can also lead to a dysfunction of vital cuproenzymes such as cytochrome c oxidase (COX) of the electron transport chain, as well as antioxidants such as superoxide dismutase (SOD1), resulting in oxidative stress via the generation of reactive oxygen species (ROS). Fe levels are also increased in the neuropil of the AD brain [13] and can contribute to the production of ROS and oxidative stress. Oxidative stress can ultimately result in neuronal cell dysfunction, which can lead to a lack of synaptic transmission.

There is growing evidence that suggests metals are able to act on receptors at the cell membrane with the hypothesis being that altered metal homeostasis affects cell signaling due to outside-in signal transduction. In this paper, known metal interactions with relevant membrane receptors will be discussed and potential therapeutic implications to AD will be assessed.

3. The Glutamatergic System and AD

The major neurotransmitter at excitatory synapses in the brain is glutamate. The glutamatergic system of synaptic transmission contains ionotropic and metabotropic glutamate receptors, with the latter lacking a channel for ion flux but instead glutamate binding induces a change in the intracellular domain of the receptor, allowing for intracellular signaling. Failure in glutamatergic transmission is common in most neurodegenerative diseases, including AD [18].

The ionotropic glutamate receptors are divided into N-methyl-D-aspartate (NMDA) and non-NMDA receptors with both receptor classes allowing the flux of ions whereby an electrical signal is translated into a chemical signal. The non-NMDA receptors (non-NMDARs) are α-amino-3-hydroxy-5-methyl-4-isoxazole propionate (AMPA) and kainate receptors, with AMPA receptors (AMPARs) being responsible for fast and transient synaptic transmission. The role of the kainate family of ionotropic receptors is less well understood however they are present pre- and post-synaptically and maybe involved in neuron-glia signaling [19], as well as being involved in the modulation of synaptic transmission and plasticity [20]. Activation of NMDARs by presynaptically released glutamate causes calcium (Ca^{2+}) entry which activates the Ca^{2+} dependant kinase, Ca^{2+}/calmodulin dependent-protein kinase II (CaMKII) [21]. The activated CaMKII associates with the NMDAR, leading to AMPAR phosphorylation [21–23]. This event encourages greater channel conductance of the AMPAR, and more importantly AMPAR insertion upon an LTP-inducing stimulus [21, 24, 25]. Soluble Aβ oligomers bind to NMDAR in AD [26] and induce NMDAR internalization [27]. There is further work indicating that binding of CaMKII to the NMDAR channel is required for LTP induction [28].

NMDARs are reported to interact directly with a suite of intracellular proteins, adhesion and signaling molecules, such as CaMKII, neuronal nitric oxide synthase (nNOS), and F-actin (see [29]) and indirectly influence the activation of cAMP response element-binding (CREB) and brain-derived neurotrophic factor (BDNF) (see [30]) as well as intracellular kinases such as extracellular signal regulated kinase (ERK), glycogen synthase kinase 3 (GSK3), and Akt.

There is evidence to suggest that the NMDAR interacts with amyloid peptides and their precursor proteins. For example, patch clamp recordings of primary hippocampal cultures from APP KO mice showed increased NMDAR-mediated EPSCs [31]. Aβ has both direct and indirect interactions with the receptor. It is postulated, as described earlier, that small oligomeric species are the key toxic elements in numerous amyloid diseases, such as AD [3]. These species have been shown to colocalize with the NR2B NMDAR subunit in rat hippocampal slices [32]. These species, as well as the Aβ peptide itself, interact with the NMDAR and can induce an increase in intracellular Ca^{2+}. This in turn leads to membrane permeabilisation, a common phenomena when amyloid proteins interact with the cell membrane [33–36]. Aβ can also propagate the loss of NMDARs from the cell surface [37]. A loss of NMDARs from the synapse has been found in AD brains [38, 39]. Hoey et al. also recently reported that activation of synaptic NMDARs promoted α-secretase mediated APP processing and inhibited Aβ production in mouse primary cortical neurons [40].

It has been shown that different Aβ oligomers can exert different affects. In *in vitro* studies looking at the dentate gyrus, Aβ1–40 was found to selectively increase NMDAR-mediated transmission, whereas Aβ1–42 has been shown to reduce NMDAR-mediated synaptic currents in the dentate gyrus [41, 42]. Thus it is apparent that Aβ is able to influence glutamatergic transmission via the NMDAR.

Due to the implication of NMDARs in neurodegenerative diseases, and especially AD, drugs that exploit the properties of the receptor have been developed as potential therapeutics for the disease. Excitotoxicity, caused by overstimulation of NMDARs due to excessive glutamate release, is a common cause of neuronal loss in most neurological insults including stroke as well as neurodegenerative diseases, such as AD [43]. As a result, NMDAR antagonists have become attractive therapeutics for the potential treatment of these diseases with memantine being utilized clinically, to treat AD patients.

4. The Glutamatergic System and Metals

The most extensive work done examining the glutamatergic system and transition metals is via study of the NMDAR and its metal interactions. Metals, especially Cu and Zn, have been shown to have a modulatory effect on NMDAR function within the glutamatergic system.

Zn is coreleased along with glutamate into the glutamatergic synapse [44–46], thus its role in signaling within the brain may best be assessed in its role as a neurotransmitter. It is well documented that the NMDAR possesses an inhibitory Zn binding site on the NR2 subunit [47–50]. The equilibrium dissociation constant (K_d) of Zn for the NMDAR was reported to be 13 μM [51] but almost a decade later extracellular Zn concentrations as low as 3 nM were shown to be inhibitory of NR1-NR2A containing receptors [50]. Zn inhibits the NMDAR in both a voltage-dependent and -independent manner with the affinity being higher in the latter, inferring that Zn binds at a different site on the receptor to the voltage-dependent magnesium (Mg) channel blocking site. Also, the IC_{50} of the voltage-independent Zn inhibition is 50-fold lower in NR1-NR2A containing receptors than NR1-NR2B containing receptors [50]. Zn released from excitatory synapses in the hippocampus inhibits NMDARs [52, 53]. Furthermore, synaptic Zn entry via glutamate receptors into neurons in the CA3 region of the hippocampus evokes LTP [54]. Interestingly, in the CA1 region of the rat hippocampus, synapses containing presynaptic vesicular Zn showed a decrease in postsynaptic AMPAR subunit levels whilst NMDAR levels were unchanged [55]. This implies that vesicular Zn could confer the behavior of a synapse during synaptic transmission, further cementing the modulatory role that Zn plays at the glutamatergic synapse.

A known interaction of the NMDAR with metals, is the role it plays in Cu homeostasis within the cell. Schlief and Gitlin have developed a model based on experiments in mouse hippocampal neurons [56]. Upon NMDAR activation by glutamate, Ca^{2+} enters into the cell, as previously discussed. This increase in Ca^{2+} within the cell having activated an intracellular signaling cascade induces the Menkes ATPase protein to translocate to a membrane bound compartment to generate and replenish a readily releasable pool of Cu. An increase in Ca^{2+} as a result of NMDAR activation can act upon this novel pool and cause the extracellular release of Cu. This released free Cu can then act back upon the NMDAR in a functionally negative feedback fashion, to inhibit Ca^{2+} flux and inhibit further Cu release. The Menkes ATPase protein is required for Cu efflux [56], and translocation of the protein as a result of NMDAR activation creates a link between NMDAR activation and Cu homeostasis.

Further work from Schlief et al. demonstrated that Cu treatment of hippocampal neurons showed a decrease in the elevation of intracellular Ca^{2+}, without affecting the localization or distribution of NMDARs, suggesting that Cu has a direct effect on NMDAR function [57]. As Cu^{2+} is a potent electron acceptor, it can potentially catalyze S-nitrosylation of NMDARs, resulting in a loss of secretable Cu as in the case of Menkes Disease. This then leaves the cell deficient in controlling NMDAR activation which may lead to an increase in Ca^{2+} [56]. LTP studies on the CA1 region of rat hippocampus showed reduced EPSCs by low micromolar Cu concentrations [56].

Cu is released post-synaptically [58, 59] where as Zn is believed to be co-released with glutamate, presynaptically, into the synaptic cleft [60]. Cu and Zn can reach micromolar concentrations within the synaptic cleft (see above) as compared to glutamate which can reach low millimolar concentrations after excitatory synaptic transmission [56].

These data taken together suggest that metals play an important role in modulating NMDAR function.

5. The Cholinergic System and AD

Acetylcholine (ACh) is a neurotransmitter, which is important in learning and memory networks [61, 62]. Cholinergic receptors are receptors that respond to ACh. Cholinergic receptors are divided into two categories, depending on their exogenous agonists. This includes nicotinic receptors (nAChR) whose exogenous agonist is nicotine, and muscarinic receptors (mAChR) whose exogenous agonist is muscarine. Both nAChRs and mAChRs are found in the central nervous system and in the periphery; however the neuronal subclasses of each receptor type will be discussed in this paper.

Along with hallmark pathologies associated with AD for example, presence of high levels of Aβ, there are also known deficits in the cholinergic system in the AD brain [63–65]. Brain regions highly affected in AD, as the neocortex and the hippocampus have significant changes to their cholinergic innervation [64]. There are several reports of a loss of cholinergic fibers and terminals in AD, as well as reductions in cholinergic receptors [64, 66, 67]. The activities of the two major cholinergic enzymes, choline acetyltransferase (ChAT) and acetylcholinesterase (AChE), are reported to be decreased in AD [68–70]. Reductions in ChAT activity are the greatest [71, 72] and correlate with disease severity [68, 73].

The basal forebrain is where the origins of the cholinergic neurons innervating the cortex lie. It is widely reported that there is a loss of these cholinergic basal forebrain neurons in the AD brain [69, 74, 75]. There is uncertainty however, if this neuron loss occurs as result of Aβ toxicity on the cortical cholinergic terminals causing retrograde degeneration, or if the loss of cholinergic basal forebrain neurons is a primary consequence of Aβ toxicity, with the loss of cortical cholinergic innervation being a secondary

consequence. Numerous transgenic mouse models and cell lines have been used to attempt to delineate this. In aged APP23 mice, there was a significant decrease in cortical cholinergic fiber length but no loss of cholinergic basal forebrain neurons when compared with aged-matched wild-type neurons suggesting that deficit of cortical cholinergic innervation in these mice is a local effect of Aβ which is not caused by deficit of cholinergic basal forebrain neurons [72]. A significant decrease in ChAT activity in the tissue of APP23 mice with no significant effect on AChE levels, when compared with aged-matched wild-type mice was shown. Similar results were reported by Pedersen et al. where a decrease in the activity of ChAT was observed with no effect on AChE activity in SN56 cells, a mouse cell line derived from basal forebrain cholinergic neurons [71]. They also showed Aβ1–42 suppressed the synthesis of ACh in a nontoxic manner with this reduction being prevented by cotreatment with all-*trans*-retinoic acid, a compound formerly shown to increase mRNA expression of ChAT in these cells [71], indicating that Aβ can have non toxic effects on the basal forebrain cholinergic neurons.

Aβ has also been shown to bind to neuronal α7 nicotinic ACh (α7nACh) receptors with high affinity [76, 77] which can cause a suite of toxic consequences. As previously discussed Aβ binding to the NMDAR can cause internalization of the NMDAR and inhibit LTP. Snyder et al. reported α-bungarotoxin, a specific α7nACh antagonist, to reduce Aβ-induced NMDAR internalization, suggesting that NMDAR function may be negatively affected by an Aβ-α7nACh interaction [27]. Wang et al. recently showed in synaptosomes prepared from both AD postmortem tissue as well as frontal cortex slices from postmortem tissue exposed to Aβ1–42 that S 24795, a partial α7nACh agonist, can release Aβ from the Aβ-α7nACh complex allowing for partial recovery of function of the α7nACh and the NMDAR [78]. This demonstrated that disruption of the Aβ-α7nACh interaction may be a mean of reducing pathophysiological features of AD.

6. The Cholinergic System and Metals

There are a few reports of lead (Pb), aluminium (Al), and cadmium (Cd) having an effect on the cholinergic system *in vitro* and *in vivo* [79–81].

As Pb exposure can produce poor learning and deficits in intelligence tests [82], the interaction of Pb with the cholinergic system has been studied extensively as a mean of discovering the mechanism of toxicity of Pb in the brain which causes these neurological effects.

There are conflicting reports, however, on the affect of Pb on the cholinergic system. Moingeon et al. reported that an acute *in vivo* treatment of rats with the metal inhibits ACh turnover, decreases ACh content in certain brain regions, and induces a reversible increase in mAChRs in the striatum and cortex [83]. However, this latter finding is refuted by Schulte et al., where they had found there to be no major effect of Pb on mAChRs in the frontal cortex of mouse brain [84]. This finding was supported by Gotti et al., using an *in vitro* model of both differentiated and undifferentiated cholinergic neurons [85]. They also found that Pb increased the number of nAChR binding sites, although Costa and Fox had reported that chronic Pb exposure to decrease mAChRs in the visual cortex alone of neonatal rats [79]. It is apparent that the effect that Pb has on the cholinergic system is very much dependant on the type of cholinergic receptor studied.

Al increased the number of mAChRs in cholinergically differentiated IMR32 cells, a human neuroblastoma cell line, whereas it had no effect on nAChRs, as measured by α-bungarotoxin binding sites [85]. However Johnson and Jope reported Al reduced the effects of an *in vitro* ACh agonist, carbachol [80]. In the same paper, Cd increased both the mAChR and nAChR expressions in cholinergically differentiated and undifferentiated IMR32 cells.

As discussed, there are few studies investigating the role of cholinergic receptors and metals, and of those that do, few give conclusive outcomes and did not investigate other metals associated with AD pathology such as Cu and Zn. As a result, more work is required to determine if metals could play a role in mediating the cholinergic deficits associated with AD.

7. TrkB Receptors and AD

Tropomyosin-receptor kinase (Trk) receptors are necessary for the survival, differentiation, and maturation of the developing brain [86]. Trk receptors have been shown to play a role in synaptic plasticity as well as in modulating synaptic transmission [87, 88]. Furthermore a Trk receptor family variant, the TrkB receptor, has been reported to be important for LTP in CA1 neurons [89, 90]. The endogenous agonists for TrkB receptors are brain-derived neurotrophic factor (BDNF) and neurotrophin-4/5 (NT-4/5), with BDNF being specific for TrkB receptors [91].

TrkB activation was traditionally believed to be via neurotrophin binding, inducing TrkB receptor dimerisation leading to phosphorylation of the cytoplasmic tyrosine kinase tails. This mediates an elaborate signaling cascade ultimately resulting in antiapoptotic outcomes [91, 92]. However Lee et al., showed that TrkB signaling can occur independently of neurotrophin binding, through what is known as "transactivation" by G protein-coupled receptor (GPCR) ligands for example, adenosine and PACAP. Follow-up studies revealed the role of Src kinase-tyrosine phosphorylation in intracellularly activating cell membrane and intracellular TrkB receptors [93, 94].

There are reports of the TrkB receptor-BDNF pathway being compromised in AD. In the hippocampus of AD patients, a decrease in BDNF protein levels [95] as well as a reduction in BDNF mRNA levels [96, 97] has been reported. A decrease in BDNF protein levels in the entorhinal and temporal cortex of AD suffers has also been described [98]. Abnormal TrkB expression of full length and truncated forms and altered distribution has been found in AD brains [95, 99]. Ferrer et al. reported various changes in BDNF, including truncated TrkB and full length TrkB in glial cells, in neurons with hyperphosphorylated tau tangles and

dystrophic neurons surrounding Aβ plaques from brains of individuals with severe AD [100].

Although the most widely studied Trk receptor is the TrkB receptor, recently Capsoni et al. (2010) reported that TrkA beneficially activates Aβ accumulation in a transgenic mouse model and discusses the role of proNGF, NGF, and TrkA versus p75 neurotrophin receptor (p75NTR) in AD neurodegeneration [101].

8. TrkB Receptors and Metals

There have been few studies looking into a possible role for metals in TrkB signaling. Jung et al. reported that treatment of cortical neuron cultures with micromolar Zn concentrations can robustly activate TrkB as well as kinases downstream of the receptor, such as Src, ERK, and Akt [102]. The mechanism of Zn activating TrkB was found to be an extracellular one, mediated by activation of matrix metalloproteinases (MMPs) causing release of pro-BDNF by the cells, which then gets converted to mature BDNF by extracellular MMPs [93, 102]. In support of this, recently Corona [103] reported the protective role of dietary Zn supplementation in a transgenic mouse model of AD, where Zn appeared to increase BDNF signaling by MMP activation. *In vitro*, in PC12 cells, Zn has been shown to inhibit neurite outgrowth by BDNF [104] but in cortical cultures, this inhibition was only slight, revealing that the activation by Zn overrides the potential BDNF/TrkB inhibitory effect [102]. Thus, Zn release from the glutamatergic synapse could play an important role in activity-dependant activation of TrkB.

The same group later reported that Cu too was able to activate TrkB in cortical neuron cultures in an MMP dependent fashion [105]. Cu, like Zn, was able to activate Src kinase, ERK, and Akt and increased the activity of MMP2 and MMP9, thereby catalyzing the conversion of pro BDNF to mature BDNF [105]. They proposed that if Cu is released at the synapse post-depolarisation as postulated by Hartter and Barnea then as with Zn, there maybe physiologically beneficial effects that could be mediated by both metals, such as activation of TrkB signaling [58].

Huang et al. reported for the first time the ability of Zn released by stimulated CA3 hippocampal neurons to transactivate TrkB *in vivo* via Src kinase [106]. The activated TrkB receptors then play an important role in LTP at the mossy fiber-CA synapse. As previously discussed, TrkB receptor signaling has been reported to be important in hippocampal CA1 LTP, but this study puts forward a link between Zn, TrkB, and hippocampal CA3 LTP. With LTP known to be inhibited in AD, in an Aβ-NMDAR associated manner, assessing the Zn, TrkB, and LTP link may provide an interesting opportunity for therapeutic intervention in AD.

9. Membrane Receptors, Metals, and Implications for AD

The membrane receptor systems described so far have proposed roles in AD, and therefore metal interactions with these membrane receptor families could provide beneficial modulatory and intervention points, in the pursuit of the amelioration of AD pathology. Work done within our group has shown that Cu and Zn, delivered into the cell by the metal chaperones CQ, PBT2, and CuGTSM, can cause activation of phosphatidylinositol 3-kinase (PI3K) and the consequent phosphorylation of Akt and GSK3 and the subsequent activation of MAPK (ERK), all kinases previously discussed as having antiapoptotic effects. This caused activation of MMP2 leading to extracellular Aβ degradation [107–109].

CQ and PBT2 are 8-hydroxyquinoline metal ligands. CuGTSM belongs to the metal bisthiosemicarbazone (M-BTSC) family of metal-based drugs. They are stable, of a low molecular weight, neutral and most importantly capable of crossing cell membranes [108]. Due to their versatility *in vitro*, their use has been widely adopted within our group. CuGTSM has demonstrated therapeutic effects in an AD mouse model and was found to affect cellular signalling pathways central to AD as well as the amyloid proteins, Aβ and tau [109].

Price et al. reported CQ coordinated to Cu activated epidermal growth factor receptor (EGFR) in epithelial cells and neurons [110]. This activation, by phosphorylation of EGFR, did not require EGF or TGF-α making it ligand independent. The phosphorylation was mediated by Src kinase and was specific for Cu. Interestingly however, activation of EGFR by CQ coordinated to Cu resulted in the activation of ERK only, with no effects on PI3K-Akt or JNK but still resulted in Aβ degradation by MMP activation. This would infer that activation of ERK by intracellular bioavailable metals is a necessary step in Aβ degradation, mediated by upregulation of MMP.

We later reported the EGFR activation by CuGTSM and ZnBTSCs in a glial cell line as well, but CuGTSM, in contrast to CQ coordinated to Cu, did not phosphorylate EGFR in a Src kinase-mediated manner, rather CuGTSM inhibited the activity of protein tyrosine phosphatase (PTP). CuGTSM, as with CQ coordinated to Cu, did however induce activation of PI3K-Akt-GSK3, ERK, and JNK [111].

Whilst EGFR has not directly been linked to AD, the pathway downstream of the receptor being activated by an increase in intracellular Cu and Zn involves kinases associated with AD and appears to be rather similar to TrkB signalling. Thus EGFR and its metal interactions could be an interesting area to investigate and provide clues on other possible membrane receptors affected in AD.

10. Conclusion

In conclusion, an imbalance of transition metal levels in the AD brain plays a neurotoxic role, but how and where this imbalance affects signaling are not known. However, their interactions with membrane receptors in the glutamatergic system, TrkB and EGF signaling system, and to a lesser extent in the cholinergic system infer a potentially important effect on AD through these membrane receptor systems. This may involve a combination of effects including inhibitory, modulatory or activation of membrane receptor-mediated functions that have an important role in AD. Therefore it is

likely that the link between metals and membrane receptors may offer a unique point of intervention in AD.

Acknowledgments

P. Amadoruge is the recipient of The George Hicks Postgraduate Scholarship for Dementia Prevention and Risk Reduction Research-Alzheimer's Australia Victoria, The George Hicks Foundation and Alzheimer's Australia Research. The authors would like to thank Dr. Anthony White for his help and review of this paper. This work was funded by the National Health and Medical Research Council of Australia and the Australian Research Council.

References

[1] A. I. Bush, "Drug development based on the metals hypothesis of Alzheimer's disease," *Journal of Alzheimer's Disease*, vol. 15, no. 2, pp. 223–240, 2008.

[2] R. D. Terry, E. Masliah, D. P. Salmon et al., "Physical basis of cognitive alterations in Alzheimer's disease: synapse loss is the major correlate of cognitive impairment," *Annals of Neurology*, vol. 30, no. 4, pp. 572–580, 1991.

[3] D. M. Walsh and D. J. Selkoe, "Oligomers in the brain: the emerging role of soluble protein aggregates in neurodegeneration," *Protein and Peptide Letters*, vol. 11, no. 3, pp. 213–228, 2004.

[4] Y. M. Kuo, M. R. Emmerling, C. Vigo-Pelfrey et al., "Water-soluble Aβ (N-40, N-42) oligomers in normal and Alzheimer disease brains," *Journal of Biological Chemistry*, vol. 271, no. 8, pp. 4077–4081, 1996.

[5] L. F. Lue, Y. M. Kuo, A. E. Roher et al., "Soluble amyloid β peptide concentration as a predictor of synaptic change in Alzheimer's disease," *American Journal of Pathology*, vol. 155, no. 3, pp. 853–862, 1999.

[6] W. L. Klein, G. A. Krafft, and C. E. Finch, "Targeting small A β oligomers: the solution to an Alzheimer's disease conundrum?" *Trends in Neurosciences*, vol. 24, no. 4, pp. 219–224, 2001.

[7] J. Hardy and D. J. Selkoe, "The amyloid hypothesis of Alzheimer's disease: progress and problems on the road to therapeutics," *Science*, vol. 297, no. 5580, pp. 353–356, 2002.

[8] M. P. Lambert, A. K. Barlow, B. A. Chromy et al., "Diffusible, nonfibrillar ligands derived from Aβ are potent central nervous system neurotoxins," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 95, no. 11, pp. 6448–6453, 1998.

[9] D. M. Walsh, I. Klyubin, J. V. Fadeeva et al., "Naturally secreted oligomers of amyloid β protein potently inhibit hippocampal long-term potentiation in vivo," *Nature*, vol. 416, no. 6880, pp. 535–539, 2002.

[10] H. W. Wang, J. F. Pasternak, H. Kuo et al., "Soluble oligomers of β amyloid (1–42) inhibit long-term potentiation but not long-term depression in rat dentate gyrus," *Brain Research*, vol. 924, no. 2, pp. 133–140, 2002.

[11] T. V. P. Bliss and A. R. Gardner Medwin, "Long lasting potentiation of synaptic transmission in the dentate area of the unanaesthetized rabbit following stimulation of the perforant path," *Journal of Physiology*, vol. 232, no. 2, pp. 357–374, 1973.

[12] P. H. Reddy, G. Mani, B. S. Park et al., "Differential loss of synaptic proteins in Alzheimer's disease: implications for synaptic dysfunction," *Journal of Alzheimer's Disease*, vol. 7, no. 2, pp. 103–117, 2005.

[13] M. A. Lovell, J. D. Robertson, W. J. Teesdale, J. L. Campbell, and W. R. Markesbery, "Copper, iron and zinc in Alzheimer's disease senile plaques," *Journal of the Neurological Sciences*, vol. 158, no. 1, pp. 47–52, 1998.

[14] C. J. Frederickson, M. A. Klitenick, W. I. Manton, and J. B. Kirkpatrick, "Cytoarchitectonic distribution of zinc in the hippocampus of man and the rat," *Brain Research*, vol. 273, no. 2, pp. 335–339, 1983.

[15] K. J. Barnham, C. L. Masters, and A. I. Bush, "Neurodegenerative diseases and oxidatives stress," *Nature Reviews Drug Discovery*, vol. 3, no. 3, pp. 205–214, 2004.

[16] A. A. A. B. White, "The metallobiology of Alzheimer's disease: from bench to clinic. Current medical literature," *Neurology*, vol. 22, no. 4, pp. 89–97, 2006.

[17] P. J. Crouch, K. J. Barnham, A. I. Bush, and A. R. White, "Therapeutic treatments for Alzheimer's disease based on metal bioavailability," *Drug News and Perspectives*, vol. 19, no. 8, pp. 469–474, 2006.

[18] M. R. Hynd, H. L. Scott, and P. R. Dodd, "Glutamate-mediated excitotoxicity and neurodegeneration in Alzheimer's disease," *Neurochemistry International*, vol. 45, no. 5, pp. 583–595, 2004.

[19] M. Eder, K. Becker, G. Rammes et al., "Distribution and properties of functional postsynaptic kainate receptors on neocortical layer V pyramidal neurons," *Journal of Neuroscience*, vol. 23, no. 16, pp. 6660–6670, 2003.

[20] J. Lerma, "Roles and rules of kainate receptors in synaptic transmission," *Nature Reviews Neuroscience*, vol. 4, no. 6, pp. 481–495, 2003.

[21] A. S. Leonard, I. A. Lim, D. E. Hemsworth, M. C. Horne, and J. W. Hell, "Calcium/calmodulin-dependent protein kinase II is associated with the N-methyl-D-aspartate receptor," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 96, no. 6, pp. 3239–3244, 1999.

[22] B. K. Andrásfalvy and J. C. Magee, "Changes in AMPA receptor currents following LTP induction on rat CA1 pyramidal neurones," *Journal of Physiology*, vol. 559, no. 2, pp. 543–554, 2004.

[23] V. Derkach, A. Barria, and T. R. Soderling, "Ca/calmodulin-kinase II enhances channel conductance of α-amino-3-hydroxy-5-methyl-4-isoxazolepropionate type glutamate receptors," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 96, no. 6, pp. 3269–3274, 1999.

[24] Z. Wang, J. G. Edwards, N. Riley et al., "Myosin Vb mobilizes recycling endosomes and AMPA receptors for postsynaptic plasticity," *Cell*, vol. 135, no. 3, pp. 535–548, 2008.

[25] K. U. Bayer, P. De Koninck, A. S. Leonard, J. W. Hell, and H. Schulman, "Interaction with the NMDA receptor locks CaMKII in an active conformation," *Nature*, vol. 411, no. 6839, pp. 801–805, 2001.

[26] P. N. Lacor, M. C. Buniel, P. W. Furlow et al., "Aβ oligomer-induced aberrations in synapse composition, shape, and density provide a molecular basis for loss of connectivity in Alzheimer's disease," *Journal of Neuroscience*, vol. 27, no. 4, pp. 796–807, 2007.

[27] E. M. Snyder, Y. Nong, C. G. Almeida et al., "Regulation of NMDA receptor trafficking by amyloid-β," *Nature Neuroscience*, vol. 8, no. 8, pp. 1051–1058, 2005.

[28] A. Barria and R. Malinow, "NMDA receptor subunit composition controls synaptic plasticity by regulating binding to CaMKII," *Neuron*, vol. 48, no. 2, pp. 289–301, 2005.

[29] F. Gardoni and M. Di Luca, "New targets for pharmacological intervention in the glutamatergic synapse," *European Journal of Pharmacology*, vol. 545, no. 1, pp. 2–10, 2006.

[30] G. E. Hardingham and H. Bading, "Coupling of extrasynaptic NMDA receptors to a CREB shut-off pathway is developmentally regulated," *Biochimica et Biophysica Acta*, vol. 1600, no. 1-2, pp. 148–153, 2002.

[31] C. Priller, T. Bauer, G. Mitteregger, B. Krebs, H. A. Kretzschmar, and J. Herms, "Synapse formation and function is modulated by the amyloid precursor protein," *The Journal of Neuroscience*, vol. 26, no. 27, pp. 7212–7221, 2006.

[32] A. Deshpande, H. Kawai, R. Metherate, C. G. Glabe, and J. Busciglio, "A role for synaptic zinc in activity-dependent aβ oligomer formation and accumulation at excitatory synapses," *Journal of Neuroscience*, vol. 29, no. 13, pp. 4004–4015, 2009.

[33] B. L. Kelly and A. Ferreira, "β-amyloid-induced dynamin 1 degradation is mediated by N-methyl-D-aspartate receptors in hippocampal neurons," *Journal of Biological Chemistry*, vol. 281, no. 38, pp. 28079–28089, 2006.

[34] M. Bucciantini, E. Giannoni, F. Chiti et al., "Inherent toxicity of aggregates implies a common mechanism for protein misfolding diseases," *Nature*, vol. 416, no. 6880, pp. 507–511, 2002.

[35] Y. J. Zhu, H. Lin, and R. Lal, "Fresh and nonfibrillar amyloid β protein(1-40) induces rapid cellular degeneration in aged human fibroblasts: evidence for AβP-channel-mediated cellular toxicity," *FASEB Journal*, vol. 14, no. 9, pp. 1244–1254, 2000.

[36] J. I. Kourie, "Mechanisms of amyloid β protein-induced modification in ion transport systems: implications for neurodegenerative diseases," *Cellular and Molecular Neurobiology*, vol. 21, no. 3, pp. 173–213, 2001.

[37] H. Hsieh, J. Boehm, C. Sato et al., "AMPAR removal underlies Aβ-induced synaptic depression and sendritic spine loss," *Neuron*, vol. 52, no. 5, pp. 831–843, 2006.

[38] C. I. Sze, H. Bi, B. K. Kleinschmidt-DeMasters, C. M. Filley, and L. J. Martin, "N-Methyl-D-aspartate receptor subunit proteins and their phosphorylation status are altered selectively in Alzheimer's disease," *Journal of the Neurological Sciences*, vol. 182, no. 2, pp. 151–159, 2001.

[39] A. J. Mishizen-Eberz, R. A. Rissman, T. L. Carter, M. D. Ikonomovic, B. B. Wolfe, and D. M. Armstrong, "Biochemical and molecular studies of NMDA receptor subunits NR1/2A/2B in hippocampal subregions throughout progression of Alzheimer's disease pathology," *Neurobiology of Disease*, vol. 15, no. 1, pp. 80–92, 2004.

[40] S. E. Hoey, R. J. Williams, and M. S. Perkinton, "Synaptic NMDA receptor activation stimulates α-secretase amyloid precursor protein processing and inhibits amyloid-β Production," *Journal of Neuroscience*, vol. 29, no. 14, pp. 4442–4460, 2009.

[41] J. Wu, R. Anwyl, and M. J. Rowan, "β-amyloid-(1–40) increases long-term potentiation in rat hippocampus," *European Journal of Pharmacology*, vol. 284, no. 3, pp. R1–R3, 1995.

[42] Q. S. Chen, W. Z. Wei, T. Shimahara, and C. W. Xie, "Alzheimer amyloid β-peptide inhibits the late phase of long-term potentiation through calcineurin-dependent mechanisms in the hippocampal dentate gyrus," *Neurobiology of Learning and Memory*, vol. 77, no. 3, pp. 354–371, 2002.

[43] S. A. Lipton, "Failures and successes of NMDA receptor antagonists: molecular basis for the use of open-channel blockers like memantine in the treatment of acute and chronic neurologic insults," *NeuroRx*, vol. 1, no. 1, pp. 101–110, 2004.

[44] K. Vogt, J. Mellor, G. Tong, and R. Nicoll, "The actions of synaptically released zinc at hippocampal mossy fiber synapses," *Neuron*, vol. 26, no. 1, pp. 187–196, 2000.

[45] Y. Li, C. J. Hough, S. W. Suh, J. M. Sarvey, and C. J. Frederickson, "Rapid translocation of Zn(2+) from presynaptic terminals into postsynaptic hippocampal neurons after physiological stimulation," *Journal of Neurophysiology*, vol. 86, no. 5, pp. 2597–2604, 2001.

[46] J. Qian and J. L. Noebels, "Visualization of transmitter release with zinc fluorescence detection at the mouse hippocampal mossy fibre synapse," *Journal of Physiology*, vol. 566, no. 3, pp. 747–758, 2005.

[47] Y. B. Choi and S. A. Lipton, "Identification and mechanism of action of two histidine residues underlying high-affinity Zn^{2+} inhibition of the NMDA receptor," *Neuron*, vol. 23, no. 1, pp. 171–180, 1999.

[48] P. Paoletti, F. Perin-Dureau, A. Fayyazuddin, A. Le Goff, I. Callebaut, and J. Neyton, "Molecular organization of a zinc binding N-terminal modulatory domain in a NMDA receptor subunit," *Neuron*, vol. 28, no. 3, pp. 911–925, 2000.

[49] J. Rachline, F. Perin-Dureau, A. Le Goff, J. Neyton, and P. Paoletti, "The micromolar zinc-binding domain on the NMDA receptor subunit NR2B," *Journal of Neuroscience*, vol. 25, no. 2, pp. 308–317, 2005.

[50] P. Paoletti, P. Ascher, and J. Neyton, "High-affinity zinc inhibition of NMDA NR1-NR2A receptors," *Journal of Neuroscience*, vol. 17, no. 15, pp. 5711–5725, 1997.

[51] M. L. Mayer, L. Vyklicky, and G. L. Westbrook, "Modulation of excitatory amino acid receptors by group IIB metal cations in cultured mouse hippocampal neurones," *Journal of Physiology*, vol. 415, pp. 329–350, 1989.

[52] S. Peters, J. Koh, and D. W. Choi, "Zinc selectively blocks the action of N-methyl-D-aspartate on cortical neurons," *Science*, vol. 236, no. 4801, pp. 589–593, 1987.

[53] G. L. Westbrook and M. L. Mayer, "Micromolar concentrations of Zn^{2+} antagonize NMDA and GABA responses of hippocampal neurons," *Nature*, vol. 328, no. 6131, pp. 640–643, 1987.

[54] Y. Li, C. J. Hough, C. J. Frederickson, and J. M. Sarvey, "Induction of mossy fiber → CA3 long-term potentiation requires translocation of synaptically released Zn^{2+}," *Journal of Neuroscience*, vol. 21, no. 20, pp. 8015–8025, 2001.

[55] C. B. Sindreu, H. Varoqui, J. D. Erickson, and J. Pérez-Clausell, "Boutons containing vesicular zinc define a subpopulation of synapses with low AMPAR content in rat hippocampus," *Cerebral Cortex*, vol. 13, no. 8, pp. 823–829, 2003.

[56] M. L. Schlief and J. D. Gitlin, "Copper homeostasis in the CNS: a novel link between the NMDA receptor and copper homeostasis in the hippocampus," *Molecular Neurobiology*, vol. 33, no. 2, pp. 81–90, 2006.

[57] M. L. Schlief, A. M. Craig, and J. D. Gitlin, "NMDA receptor activation mediates copper homeostasis in hippocampal neurons," *Journal of Neuroscience*, vol. 25, no. 1, pp. 239–246, 2005.

[58] D. E. Hartter and A. Barnea, "Evidence for release of copper in the brain: depolarization-induced release of newly taken-up copper," *Synapse*, vol. 2, no. 4, pp. 412–415, 1988.

[59] J. Kardos, I. Kovacs, F. Hajos, M. Kalman, and M. Simonyi, "Nerve endings from rat brain tissue release copper upon depolarization. A possible role in regulating neuronal

excitability," *Neuroscience Letters*, vol. 103, no. 2, pp. 139–144, 1989.

[60] K. J. Barnham and A. I. Bush, "Metals in Alzheimer's and Parkinson's diseases," *Current Opinion in Chemical Biology*, vol. 12, no. 2, pp. 222–228, 2008.

[61] P. Dutar, M. H. Bassant, M. C. Senut, and Y. Lamour, "The septohippocampal pathway: structure and function of a central cholinergic system," *Physiological Reviews*, vol. 75, no. 2, pp. 393–427, 1995.

[62] J. Winkler, S. T. Suhr, F. H. Gage, L. J. Thal, and L. J. Fisher, "Essential role of neocortical acetylcholine in spatial memory," *Nature*, vol. 375, no. 6531, pp. 484–487, 1995.

[63] R. T. Bartus, R. L. Dean, B. Beer, and A. S. Lippa, "The cholinergic hypothesis of geriatric memory dysfunction," *Science*, vol. 217, no. 4558, pp. 408–417, 1982.

[64] J. T. Coyle, D. L. Price, and M. R. DeLong, "Alzheimer's disease: a disorder of cortical cholinergic innervation," *Science*, vol. 219, no. 4589, pp. 1184–1190, 1983.

[65] M. Goedert, "Tau protein and the neurofibrillary pathology of Alzheimer's disease," *Trends in Neurosciences*, vol. 16, no. 11, pp. 460–465, 1993.

[66] E. K. Perry, M. Johnson, J. M. Kerwin et al., "Convergent cholinergic activities in aging and Alzheimer's disease," *Neurobiology of Aging*, vol. 13, no. 3, pp. 393–400, 1992.

[67] G. Ransmayr, P. Cervera, E. C. Hirsch, W. Berger, W. Fischer, and Y. Agid, "Alzheimer's disease: is the decrease of the cholinergic innervation of the hippocampus related to intrinsic hippocampal pathology?" *Neuroscience*, vol. 47, no. 4, pp. 843–851, 1992.

[68] L. M. Bierer, V. Haroutunian, S. Gabriel et al., "Neurochemical correlates of dementia severity in Alzheimer's disease: relative importance of the cholinergic deficits," *Journal of Neurochemistry*, vol. 64, no. 2, pp. 749–760, 1995.

[69] R. S. Jope, L. Song, and R. E. Powers, "Cholinergic activation of phosphoinositide signaling is impaired in Alzheimer's disease brain," *Neurobiology of Aging*, vol. 18, no. 1, pp. 111–120, 1997.

[70] C. J. Ladner and J. M. Lee, "Pharmacological drug treatment of Alzheimer disease: the cholinergic hypothesis revisited," *Journal of Neuropathology and Experimental Neurology*, vol. 57, no. 8, pp. 719–731, 1998.

[71] W. A. Pedersen, M. A. Kloczewiak, and J. K. Blusztajn, "Amyloid β-protein reduces acetylcholine synthesis in a cell line derived from cholinergic neurons of the basal forebrain," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 93, no. 15, pp. 8068–8071, 1996.

[72] S. Boncristiano, M. E. Calhoun, P. H. Kelly et al., "Cholinergic changes in the APP23 transgenic mouse model of cerebral amyloidosis," *Journal of Neuroscience*, vol. 22, no. 8, pp. 3234–3243, 2002.

[73] D. M. Bowen, C. B. Smith, P. White, and A. N. Davison, "Neurotransmitter related enzymes and indices of hypoxia in senile dementia and other abiotrophies," *Brain*, vol. 99, no. 3, pp. 459–496, 1976.

[74] P. J. Whitehouse, D. L. Price, and R. G. Struble, "Alzheimer's disease and senile dementia: loss of neurons in the basal forebrain," *Science*, vol. 215, no. 4537, pp. 1237–1239, 1982.

[75] O. J. M. Vogels, C. A. J. Broere, H. J. Ter Laak, H. J. Ten Donkelaar, R. Nieuwenhuys, and B. P. M. Schulte, "Cell loss and shrinkage in the nucleus basalis Meynert complex in Alzheimer's disease," *Neurobiology of Aging*, vol. 11, no. 1, pp. 3–13, 1990.

[76] H. Y. Wang, D. H. S. Lee, M. R. D'Andrea, P. A. Peterson, R. P. Shank, and A. B. Reitz, "β-amyloid binds to α7 nicotinic acetylcholine receptor with high affinity. Implications for Alzheimer's disease pathology," *Journal of Biological Chemistry*, vol. 275, no. 8, pp. 5626–5632, 2000.

[77] H. Y. Wang, D. H. S. Lee, C. B. Davis, and R. P. Shank, "Amyloid peptide Aβ(1–42) binds selectively and with picomolar affinity to α7 nicotinic acetylcholine receptors," *Journal of Neurochemistry*, vol. 75, no. 3, pp. 1155–1161, 2000.

[78] H. Y. Wang, A. Stucky, J. Liu, C. Shen, C. Trocme-Thibierge, and P. Morain, "Dissociating β-amyloid from α7 nicotinic acetylcholine receptor by a novel therapeutic agent, S 24795, normalizes α7 nicotinic acetylcholine and NMDA receptor function in Alzheimer's disease brain," *Journal of Neuroscience*, vol. 29, no. 35, pp. 10961–10973, 2009.

[79] L. G. Costa and D. A. Fox, "A selective decrease of cholinergic muscarinic receptors in the visual cortex of adult rats following developmental lead exposure," *Brain Research*, vol. 276, no. 2, pp. 259–266, 1983.

[80] G. V. W. Johnson and R. S. Jope, "Aluminum impairs glucose utilization and cholinergic activity in rat brain in vitro," *Toxicology*, vol. 40, no. 1, pp. 93–102, 1986.

[81] W. S. Webster and A. A. Valois, "The toxic effects of cadmium on the neonatal mouse CNS," *Journal of Neuropathology and Experimental Neurology*, vol. 40, no. 3, pp. 247–257, 1981.

[82] C. Winder and I. Kitchen, "Lead neurotoxicity: a review of the biochemical, neurochemical and drug induced behavioural evidence," *Progress in Neurobiology*, vol. 22, no. 1, pp. 59–87, 1984.

[83] P. Moingeon, J. M. Bidart, and G. F. Alberici, "In vivo modulation of muscarine receptors in rat brain by acute lead intoxication," *Toxicology*, vol. 31, no. 2, pp. 135–142, 1984.

[84] S. Schulte, W. E. Müller, and K. D. Friedberg, "In vivo exposure to lead does not influence muscarinic receptors in the frontal cortex of the mouse brain," *Toxicology*, vol. 93, no. 2-3, pp. 99–112, 1994.

[85] C. Gotti, D. Cabrini, E. Sher, and F. Clementi, "Effects of long-term in vitro exposure to aluminum, cadmium or lead on differentiation and cholinergic receptor expression in a human neuroblastoma cell line," *Cell Biology and Toxicology*, vol. 3, no. 4, pp. 431–440, 1987.

[86] G. R. Lewin and Y. A. Barde, "Physiology of the neurotrophins," *Annual Review of Neuroscience*, vol. 19, pp. 289–317, 1996.

[87] E. S. Levine, C. F. Dreyfus, I. B. Black, and M. R. Plummer, "Brain-derived neurotrophic factor rapidly enhances synaptic transmission in hippocampal neurons via postsynaptic tyrosine kinase receptors," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 92, no. 17, pp. 8074–8077, 1995.

[88] H. Kang and E. M. Schuman, "Long-lasting neuratrophin-induced enhancement of synaptic transmission in the adult hippocampus," *Science*, vol. 267, no. 5204, pp. 1658–1662, 1995.

[89] G. Chen, R. Kolbeck, Y. A. Barde, T. Bonhoeffer, and A. Kossel, "Relative contribution of endogenous neurotrophins in hippocampal long- term potentiation," *Journal of Neuroscience*, vol. 19, no. 18, pp. 7983–7990, 1999.

[90] A. H. Kossel, S. B. Cambridge, U. Wagner, and T. Bonhoeffer, "A caged Ab reveals an immediate/instructive effect of BDNF during hippocampal synaptic potentiation," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 98, no. 25, pp. 14702–14707, 2001.

[91] M. Barbacid, "Structural and functional properties of the TRK family of neurotrophin receptors," *Annals of the New York Academy of Sciences*, vol. 766, pp. 442–458, 1995.

[92] E. J. Huang and L. F. Reichardt, "Trk receptors: roles in neuronal signal transduction," *Annual Review of Biochemistry*, vol. 72, pp. 609–642, 2003.

[93] R. Lee, P. Kermani, K. K. Teng, and B. L. Hempstead, "Regulation of cell survival by secreted proneurotrophins," *Science*, vol. 294, no. 5548, pp. 1945–1948, 2001.

[94] F. S. Lee, R. Rajagopal, A. H. Kim, P. C. Chang, and M. V. Chao, "Activation of Trk neurotrophin receptor signaling by pituitary adenylate cyclase-activating polypeptides," *Journal of Biological Chemistry*, vol. 277, no. 11, pp. 9096–9102, 2002.

[95] B. Connor, D. Young, Q. Yan, R. L. M. Faull, B. Synek, and M. Dragunow, "Brain-derived neurotrophic factor is reduced in Alzheimer's disease," *Molecular Brain Research*, vol. 49, no. 1-2, pp. 71–81, 1997.

[96] H. S. Phillips, J. M. Hains, M. Armanini, G. R. Laramee, S. A. Johnson, and J. W. Winslow, "BDNF mRNA is decreased in the hippocampus of individuals with Alzheimer's disease," *Neuron*, vol. 7, no. 5, pp. 695–702, 1991.

[97] K. D. Murray, C. M. Gall, E. G. Jones, and P. J. Isackson, "Differential regulation of brain-derived neurotrophic factor and type II calcium/calmodulin-dependent protein kinase messenger RNA expression in Alzheimer's disease," *Neuroscience*, vol. 60, no. 1, pp. 37–48, 1994.

[98] M. Narisawa-Saito, K. Wakabayashi, S. Tsuji, H. Takahashi, and H. Nawa, "Regional specificity of alterations in NGF, BDNF and NT-3 levels in Alzheimer's disease," *NeuroReport*, vol. 7, no. 18, pp. 2925–2928, 1996.

[99] A. Salehi, J. Verhaagen, P. A. Dijkhuizen, and D. F. Swaab, "Co-localization of high-affinity neurotrophin receptors in nucleus basalis of Meynert neurons and their differential reduction in Alzheimer's disease," *Neuroscience*, vol. 75, no. 2, pp. 373–387, 1996.

[100] I. Ferrer, C. Marín, M. J. Rey et al., "BDNF and full-length and truncated TrkB expression in Alzheimer disease. Implications in therapeutic strategies," *Journal of Neuropathology and Experimental Neurology*, vol. 58, no. 7, pp. 729–739, 1999.

[101] S. Capsoni, C. Tiveron, D. Vignone, G. Amato, and A. Cattaneo, "Dissecting the involvement of tropomyosin-related kinase A and p75 neurotrophin receptor signaling in NGF deficit-induced neurodegeneration," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 107, no. 27, pp. 12299–12304, 2010.

[102] J. H. Jung, M. H. Park, S. Y. Choi, and J. Y. Koh, "Activation of the Trk signaling pathway by extracellular zinc. Role of metalloproteinases," *Journal of Biological Chemistry*, vol. 280, no. 12, pp. 11995–12001, 2005.

[103] C. E. A. Corona, "Dietary zinc supplementation of 3xTg-AD mice increases BDNF levels and prevents cognitive deficits as well as mitochondrial dysfunction," *Cell Death and Disease*, vol. 1, article e91, 2010.

[104] G. M. Ross, I. L. Shamovsky, G. Lawrance et al., "Zinc alters conformation and inhibits biological activities of nerve growth factor and related neurotrophins," *Nature Medicine*, vol. 3, no. 8, pp. 872–878, 1997.

[105] J. H. Jung, MI. H. Park, and J. Y. Koh, "Copper activates TrkB in cortical neurons in a metalloproteinase-dependent manner," *Journal of Neuroscience Research*, vol. 85, no. 10, pp. 2160–2166, 2007.

[106] Y. Z. Huang, E. Pan, Z. Q. Xiong, and J. O. McNamara, "Zinc-mediated transactivation of TrkB potentiates the hippocampal mossy fiber-CA3 pyramid synapse," *Neuron*, vol. 57, no. 4, pp. 546–558, 2008.

[107] A. R. White, T. Du, K. M. Laughton et al., "Degradation of the Alzheimer disease amyloid β-peptide by metal-dependent up-regulation of metalloprotease activity," *Journal of Biological Chemistry*, vol. 281, no. 26, pp. 17670–17680, 2006.

[108] P. S. Donnelly, A. Caragounis, T. Du et al., "Selective intracellular release of copper and zinc ions from bis(thiosemicarbazonato) complexes reduces levels of Alzheimer disease amyloid-β peptide," *Journal of Biological Chemistry*, vol. 283, no. 8, pp. 4568–4577, 2008.

[109] P. J. Crouch, W. H. Lin, P. A. Adlard et al., "Increasing Cu bioavailability inhibits Aβ oligomers and tau phosphorylation," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 106, no. 2, pp. 381–386, 2009.

[110] K. A. Price, G. Filiz, A. Caragounis et al., "Activation of epidermal growth factor receptor by metal-ligand complexes decreases levels of extracellular amyloid beta peptide," *International Journal of Biochemistry and Cell Biology*, vol. 40, no. 9, pp. 1901–1917, 2008.

[111] K. A. Price, A. Caragounis, B. M. Paterson et al., "Sustained activation of glial cell epidermal growth factor receptor by bis(thiosemicarbazonato) metal complexes is associated with inhibition of protein tyrosine phosphatase activity," *Journal of Medicinal Chemistry*, vol. 52, no. 21, pp. 6606–6620, 2009.

SAGE-Hindawi Access to Research
International Journal of Alzheimer's Disease
Volume 2011, Article ID 741974, 15 pages
doi:10.4061/2011/741974

Review Article

Melatonin and Its Agonist Ramelteon in Alzheimer's Disease: Possible Therapeutic Value

Venkatramanujam Srinivasan,[1,2] Charanjit Kaur,[3] Seithikurippu Pandi-Perumal,[4] Gregory M. Brown,[5] and Daniel P. Cardinali[6]

[1] *Sri Sathya Sai Medical Educational and Research Foundation, Prasanthi Nilayam, 40- Kovai Thirunagar, Coimbatore 641014, India*
[2] *Department of Physiology, Faculty of Medicine, Karpagam University, Eachanari, Coimbatore 641021, India*
[3] *Department of Anatomy, Yong Loo Lin School of Medicine, Block MD10, Medical Drive, National University of Singapore, Singapore 117597*
[4] *Somnogen Inc., College Street, Toronto, ON, Canada M6H 1C5*
[5] *Centre for Addiction and Mental Health, Department of Psychiatry, University of Toronto, Toronto, ON, Canada M5T 1RS*
[6] *Departmento de Docencia e Investigación, Facultad de Ciencias Médicas, Pontificia Universidad Católica Argentina, Avenida Alicia Moreau de Justo 1500, 4º piso, 1107 Buenos Aires, Argentina*

Correspondence should be addressed to Daniel P. Cardinali, danielcardinali@fibertel.com.ar

Received 21 September 2010; Revised 8 October 2010; Accepted 27 October 2010

Academic Editor: Anthony R. White

Copyright © 2011 Venkatramanujam Srinivasan et al. This is an open access article distributed under the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original work is properly cited.

Alzheimer's disease (AD) is an age-associated neurodegenerative disease characterized by the progressive loss of cognitive function, loss of memory and insomnia, and abnormal behavioral signs and symptoms. Among the various theories that have been put forth to explain the pathophysiology of AD, the oxidative stress induced by amyloid β-protein (Aβ) deposition has received great attention. Studies undertaken on postmortem brain samples of AD patients have consistently shown extensive lipid, protein, and DNA oxidation. Presence of abnormal tau protein, mitochondrial dysfunction, and protein hyperphosphorylation all have been demonstrated in neural tissues of AD patients. Moreover, AD patients exhibit severe sleep/wake disturbances and insomnia and these are associated with more rapid cognitive decline and memory impairment. On this basis, the successful management of AD patients requires an ideal drug that besides antagonizing Aβ-induced neurotoxicity could also correct the disturbed sleep-wake rhythm and improve sleep quality. Melatonin is an effective chronobiotic agent and has significant neuroprotective properties preventing Aβ-induced neurotoxic effects in a number of animal experimental models. Since melatonin levels in AD patients are greatly reduced, melatonin replacement has the potential value to be used as a therapeutic agent for treating AD, particularly at the early phases of the disease and especially in those in whom the relevant melatonin receptors are intact. As sleep deprivation has been shown to produce oxidative damage, impaired mitochondrial function, neurodegenerative inflammation, and altered proteosomal processing with abnormal activation of enzymes, treatment of sleep disturbances may be a priority for arresting the progression of AD. In this context the newly introduced melatonin agonist ramelteon can be of much therapeutic value because of its highly selective action on melatonin MT_1/MT_2 receptors in promoting sleep.

1. Introduction

Alzheimer's disease (AD), a major age-associated neurodegenerative disease, is characterized by progressive loss of cognitive function, loss of memory, impaired synaptic function, and a massive brain cell loss that ultimately results in premature death. Although the exact cause of the disease is under intense investigation, the prevailing hypothesis proposes that the deposition of amyloid β-protein- (Aβ-)containing senile plaques and of intracellular neurofibrillary tangles major etiological factors in AD [1]. Deposition of amyloid plaques causes cell death by inducing mitochondrial dysfunction and oxidative stress [2]. Aβ deposition initiates the flavoenzyme-dependent increase of hydrogen peroxide (H_2O_2) and lipid peroxides that increase free radical generation [3, 4]. Neural tissues of AD patients exhibit increased levels

of end products of peroxidation such as malondialdehyde, 4-hydroxynonenal, or carbonyls. Though Aβ contributes directly or indirectly to neuronal degeneration, its potential to cause AD depends on individual's susceptibility to Aβ-mediated toxicity [5].

Mitochondrial dysfunction plays an important role in AD and the link among impaired mitochondrial function, tau phosphorylation, and Aβ amyloidosis is increasingly recognized as a major phenomenon in AD physiopathology [2, 6, 7]. Aβ accumulation and neurofibrillary tangles composed of tau protein induce functional deficits of the respiratory chain complexes thereby resulting in mitochondrial dysfunction and oxidative stress (the "Aβ cascade hypothesis of AD"). It is interesting to note that women are more vulnerable to AD than men, presumably because the mitochondria are protected by estrogens against Aβ toxicity [8].

Indeed, aging and neurodegenerative diseases are accompanied by abnormal levels of oxidation of proteins, lipids, and nucleic acids [9–11]. Mechanisms such as chronic inflammation associated with the release of cytokines and trace element neurotoxicity have also been suggested as possible contributory factors underlying the physiopathologic events of AD [12–14]. Membrane disruption and induction of apoptosis by caspase enzymes have also been implicated [15].

In addition to cognitive and memory dysfunction, sleep-wake and other circadian rhythm dysregulation, are commonly seen in AD [16–19]. These circadian rhythm disturbances are associated with disturbed melatonin rhythmicity and decreased circulating and brain melatonin levels [20–22]. It is hypothesized that the decreased levels of melatonin, in fact, could contribute to the pathophysiology of AD in view than melatonin combines chronobiotic with effective antioxidant, anti-inflammatory, and antifibrillogenic properties [23].

Among the factors known to suppress the production of melatonin by the pineal gland, hypoxia deserves to be considered [24]. Reduced production of melatonin has been reported to occur in other ischemic conditions such as coronary artery disease or severe congestive heart failure [25–27]. Hypoxia may play a role in the pathogenesis of AD as it can induce formation of Aβ [28–30]. The role of hypoxia in potentiating AD is supported by the observation that patients suffering from cardiorespiratory disorders, cerebral ischemia or stroke are much more susceptible to development of dementias including AD [31]. It is remarkable that the daily administration of melatonin reduces the hypoxia induced Aβ generation in the rat hippocampus [32].

With this background, the replacement of brain melatonin levels has been suggested as a way arresting the progress of AD and for correcting the circadian and sleep-wake disturbances associated with the disease. As melatonin is a short-lived molecule having a limited duration of action (half life = 0.54-0–67 h [33]), analogs with a high affinity for melatonin receptors and a longer duration of action have been synthesized with a potential therapeutic efficacy to treat insomnia and psychiatric disorders like depression and bipolar affective disorder [34]. Ramelteon was the first of these molecules approved by the U.S. Food and Drug Administration to be used in the treatment of insomnia [35] and its potential use in AD together with that of melatonin is discussed in this review article.

2. Melatonin in AD

Melatonin is synthesized both in the pineal gland and in a number of peripheral organs and tissues by a process starting with tryptophan conversion to serotonin (reviewed in [36]). Serotonin is then acetylated to form N-acetylserotonin by the enzyme arylakylamine N-acetyltransferase while N-acetylserotonin is converted into melatonin by the enzyme hydroxyindole-O-methyl transferase [37, 38]. Once formed melatonin is not stored within the pineal gland it diffuses into the capillary blood and the cerebrospinal fluid (CSF) [39, 40]. CSF melatonin values are nearly 30 times higher than those in the blood; thus, the brain tissue has a higher melatonin concentration than any other tissue in the body [41].

Regional distribution of melatonin in different areas of the brain varies and early studies have shown that hypothalamic melatonin concentrations are nearly fifty times higher than in plasma [42–44]. While tissue melatonin only exhibits a moderate circadian variation, circulating melatonin exhibits most pronounced circadian rhythm with highest levels occurring at night and very low levels during daytime [36].

Circulating melatonin is metabolized mainly in the liver via hydroxylation in the C6 position by cytochrome P_{450} monooxygenases (CYP1A2;CYP1A1) [45]. It is thereafter conjugated with sulphate to form 6-sulfatoxymelatonin (aMT6S), the main metabolite of melatonin in urine. In the brain, melatonin is metabolized to kynuramine derivatives like N^1-acetyl-N^2-formyl-5-methoxykynuramine (AFMK) [46, 47]. In several tissues melatonin is also nonenzymatically metabolized to cyclic 3-hydroxy melatonin [48].

Melatonin is involved in the control of various physiological functions such as circadian rhythmicity [49, 50], sleep regulation [51, 52], immune function [53, 54], antioxidant defense [55, 56], control of reproduction [57–59], inhibition of tumor growth [60, 61], and control of human mood [62, 63]. Melatonin participates in many of these functions by acting through G-protein membrane receptors, the MT_1 and MT_2 melatonin receptors [64–66]. Nuclear melatonin receptors belonging to the RZR/ROR α receptor class have also been described [56, 67, 68]. Melatonin also acts directly on the cells without the intervention of any of these receptors by binding to intracellular proteins like calmodulin [69] or tubulin [70]. In general, the free radical scavenging action of melatonin does not involve receptors except for the induction of synthesis of some antioxidant enzymes like γ-glutamylcysteine synthase that involves RZR/ROR α receptors [71].

In view of the involvement of oxidative stress in AD, melatonin represents an interesting neuroprotective agent as it antagonizes oxidative stress both in a direct and in an indirect way [55, 56, 72, 73]. In the N2a murine neuroblastoma cell model Pappolla et al. [74] first demonstrated

that coincubation of Aβ with melatonin significantly reduced several features of apoptosis like cellular shrinkage or formation of membrane blubs. In a number of studies melatonin prevented the death of neuroblastoma cells exposed to Aβ [5, 75, 76].

Several animal models of AD have been used to study the possible antoxidative and antiapoptotic actions of melatonin in arresting neuronal lesions. Okadaic acid induces physiological and biochemical changes similar to those seen in AD. Increased levels of 4-hydroxynonenal in cultured neuronal cells have been found following administration of okadaic acid [77]. After the administration of antioxidants like melatonin or vitamin C, the effects of okadaic acid on NIE 115 neuronal cells were prevented effectively [78]. Melatonin was more effective than vitamin C, since it not only prevented the free radical-induced damage with greater efficiency but also increased the activity of the antioxidant enzymes glutathione-S transferase and glutathione reductase [78].

Several studies indicate that the apoptosis of astrocytes contribute to the pathogenesis of AD (sees [79]). Astrocytes exhibit tau phosphorylation and activation of stress kinases as seen in AD pathology. They also produce apolipoprotein E4 (apoE4) that aggravates Aβ neurodegenerative effects [80, 81]. During interaction with Aβ, astrocytes lose control over NO production leading to the neurotoxic peroxynitrate formation. By treating the C6 astroglioma cells with melatonin, the increase in NO production induced by Aβ was effectively prevented [82].

3. Molecular Mechanisms of Melatonin's Anti-Amyloid Actions

Melatonin not only reduced apoptosis but also exerts its antiamyloid actions through additional mechanisms. One of them is by preventing Aβ-induced mitochondrial damage and disruption of respiration. Melatonin administration prevented Aβ action on mitochondrial DNA proteins and level of lipid peroxidation [75]. In this aspect it is interesting to note that melatonin's metabolite AFMK also offered protection from Aβ-induced mitochondrial oxidative stress [83] although a higher concentration was needed.

Melatonin inhibits the formation of amyloid fibrils as demonstrated by different techniques [84, 85]. The structural analog of melatonin indole-3-propionic acid not only shares the radical scavenging activity of melatonin [86] but also exhibits similar or even higher antifibrillogenic activity [87].

Several lipoproteins can modulate fibrillogenesis [88]. Melatonin was shown to reverse the profibrillogenic activity of apoE4 and to antagonize the neurotoxic combinations of Aβ and apoE4 or apoE3 [83]. ApoE4 is also produced by astrocytes and aggravates Aβ effects showing thereby the mutual interaction of Aβ protein and apo-E4 in the astrocyte-neuron interactions [81]. The antifibrillogenic effects of melatonin and its metabolites were observed not only in vitro but also in vivo in transgenic mouse models [84, 89, 90]. Protection from Aβ toxicity was observed, especially at the mitochondrial level.

As mentioned above, chronic intermittent hypoxia has been shown to induce Aβ protein generation by upregulating the APP processing enzymes BACE and PSEN-1 [28–30]. The daily administration of melatonin (10 mg/kg) prior to a short-term hypoxia prevented the generation of Aβ protein but it did not reduce the increase of HIF-1 transcription factor induced by hypoxia [32]. Hence it was suggested that melatonin's neuroprotective effect against amyloid-β-peptide was due to its direct free radical scavenging properties actions [32].

Another manifestation of AD studied in experimental models is the expression of protein hyperphosphorylation and cytoskeletal disorganization. Calyculin A, an inhibitor of protein phosphatases (PP), was used in neuroblastoma N2 cells to examine this point. Calyculin A resulted in activation of glycogen synthase kinase 3 (GSK-3), a redox-controlled enzyme involved in various regulatory mechanisms of the cell, and the consequent hyperphosphorylation of tau [91]. Melatonin administration decreased oxidative stress and tau hyperphosphorylation and reversed GSK-3 activation showing thereby that it not only acts as an antioxidant but also interferes with the phosphorylation system, particularly stress kinases [91].

The inhibition of PP-2A and PP-1 brought about by calyculin A caused hyperphosphorylation of tau and of neurofilaments, synaptophysin loss, and spatial memory retention impairment, an effect counteracted by the administration of melatonin i.p. for 9 days before calyculin injection [92]. Melatonin also partially reversed the phosphorylation of the catalytic subunit of PP-2A at tyrosine 307 (Y307) crucial site regulating the activity of PP-2A, and reduced malondialdehyde levels induced by calyculin A [92]. Melatonin also attenuated tau hyperphosphorylation induced by wortmannin [93, 94] and isoproterenol [95].

Tyrosine kinase (trk) receptors, important elements of the phosphorylation system, as well as neurotrophins, are affected by Aβ and other oxidotoxins and melatonin normalized in neuroblastoma cells trk and neurotrophin expression [96]. Recent studies using organotypic hippocampal studies confirmed that the presence of melatonin (25–100 μM) prevented the cell damage induced by exposure to Aβ reducing the activation of GSK-3β, the phosphorylation of tau protein, and the Aβ-induced increases of TNF-α and IL-6 levels [97]. The chronobiological aspects of melatonin-Aβ interaction are underlined by a study describing the protective effect of melatonin against the circadian changes produced by $A\beta_{25–35}$ microinjection into the suprachiasmatic nuclei (SCN) of golden hamsters [98].

4. Potential Therapeutic Value of Melatonin in AD

A number of studies in AD patients have indicated that there is a profound disturbance in sleep/wake cycle associated with the progression of the disease. Cross-sectional studies reveal that sleep disturbances are associated with memory and cognitive impairment. [16–19]. A severe disruption of the circadian timing system occurs in AD as indicated by

alterations in numerous overt rhythms like body temperature, glucocorticoids, and/or plasma melatonin [22, 99, 100]. The internal desynchronization of rhythms is significant in AD patients [101, 102].

"Sundowning" is a chronobiological phenomenon observed in AD patients in conjunction with sleep-wake disturbances, including symptoms like disorganized thinking, reduced ability to maintain attention to external stimuli, agitation, wandering, and perceptual and emotional disturbances, all appearing in late afternoon or early evening [99, 103, 104]. Chronotherapeutic interventions such as exposure to bright light and/or timed administration of melatonin in selected circadian phases alleviated sundowning symptoms like wandering, agitation and delirium and also improved sleep-wake patterns of AD patients [105].

A number of studies have revealed that melatonin levels are lower in AD patients as compared to age-matched control subjects [20–22, 106]. The decreased CSF melatonin levels of AD patients were attributed to decreased melatonin production. CSF melatonin levels decreased even in preclinical stages (Braak stages-1) when patients did not manifest cognitive impairment [107] suggesting thereby that reduction in CSF melatonin may be an early marker (and cause) for incoming AD. The decrease of melatonin levels in AD was attributed to a defective retinohypothalamic tract or SCN-pineal connections [108]. The impaired melatonin production at night correlates significantly with the severity of mental impairment of demented patients [109]. As AD patients have profound deficiency of endogenous melatonin, replacement of levels of melatonin in the brain could be a therapeutic strategy for arresting the progress of the disease. Melatonin's neuroprotective and vasoprotective properties would help in enhancing cerebral blood flow and would help to improve the clinical condition of AD patients [23].

Sleep disturbances exacerbate memory and cognitive impairment [110]. Therefore, optimization in management of sleep disturbances is of paramount importance in treating AD patients. In an initial study on 14 AD patients with 6–9 mg of melatonin given for 2-3 year period it was noted that melatonin improved sleep quality [111]. Sundowning, diagnosed clinically, was no longer detectable in 12 out of 14 patients. Reduction in cognitive impairment and amnesia was also noted. This should be contrasted with the significant deterioration of the clinical conditions expected from patients after 1–3 year of evolution of AD [111, 112].

Several studies support the efficacy of melatonin in treating sleep and chronobiologic disorders in AD patients (Table 1). The administration of melatonin (6 mg/day) for 4 weeks to AD patients reduced nighttime activity as compared to placebo [113]. An improvement of sleep and alleviation of sundowning were reported in 11 AD patients treated with melatonin (3 mg/day at bedtime) and evaluated by using actigraphy [114]. Improvement in behavioral signs was reported with use of 6–9 mg/day of melatonin for 4 months in AD patients with sleep disturbances [115].

In a double blind study conducted on AD patients it was noted that 3 mg/day of melatonin significantly prolonged actigraphically evaluated sleep time, decreased activity in night, and improved cognitive functions [119]. In a multicenter, randomized, placebo-controlled clinical trial of a sample of 157 AD patients with sleep disturbances, melatonin or placebo was administered for a period of 2 months [120]. In actigraphic studies a trend to increased nocturnal total sleep time and decreased wake after sleep onset was noted in the melatonin-treated group. On subjective measures by caregiver ratings significant improvement in sleep quality was noted with 2.5 mg sustained release melatonin relative to placebo [120].

Negative results with the use of melatonin in fully developed AD were also published. For example, in a study in which melatonin (8.5 mg fast release and 1.5 mg sustained release) was administered at 10.00 PM for 10 consecutive nights to patients with AD, no significant difference was noticed with placebo on sleep, circadian rhythms and, agitation [124]. Although the lack of beneficial effect of melatonin in this study on sleep could be attributed to the short period of time examined, it must be noted that large interindividual differences between patients suffering from a neurodegenerative disease are not uncommon. It should be also taken into account that melatonin, though having some sedating and sleep latency-reducing properties, does not primarily act as a sleeping pill, but mainly as a chronobiotic.

Since the circadian oscillator system is obviously affected in AD patients showing severe sleep disturbances, the efficacy of melatonin should be expected to depend on disease progression. In a recent paper one of us summarized the published data concerning melatonin treatment of AD patients [125] (Table 1). Eight reports (5 open-label studies, 2 case reports) ($N = 89$ patients) supported a possible efficacy of melatonin: sleep quality improved and in patients with AD sundowning was reduced and cognitive decay showed less progression. In 6 double blind, randomized placebo-controlled trials ($N = 210$) sleep was objectively measured by wrist actigraphy and additionally neuropsychological assessment and sleep quality were subjectively evaluated. Sleep quality increased and sundowning decreased significantly and cognitive performance improved in 4 studies ($N = 143$) whereas there was absence of effects in 2 studies ($N = 67$) [125]. Therefore, the question whether melatonin has a causal value in preventing or treating AD, affecting disease progression of the neuropathology and the driving mechanisms, remains unanswered. Double-blind multicenter studies are needed to further explore and investigate the potential and usefulness of melatonin as an antidementia drug. Its apparent usefulness in symptomatic treatment, concerning sleep, sundowning, and so forth, even in a progressed state, further underlines the need for such decisive studies.

It has been shown that with degeneration of the SCN, the master body clock, there is a decrease in the expression of MT_1 receptors so that strength of melatonin as a synchronizing agent is reduced [126]. Moreover the input of neural pathways involved in entrainment (synchronization) of the central clock may become dysfunctional or less sensitive during aging and even more so in AD [127]. In a large multicentre trial only a nonsignificant trend to improvement in the circadian rhythm disturbance of AD is when treatment

Table 1: Clinical studies on melatonin efficacy in AD.

Design	Subjects (M, F)	Treatment	Study's duration	Measured	Results	Reference
Open-label study	10 (6, 4) demented patients	3 mg melatonin p.o./daily at bed time	3 weeks	Daily logs of sleep and wake quality completed by caretakers	Seven out of ten dementia patients having sleep disorders treated with melatonin showed a significant decrease in sundowning and reduced variability of sleep onset time	[116]
Open-label study	14 (8, 14) AD patients	9 mg melatonin p.o./daily at bed time	22 to 35 months	Daily logs of sleep and wake quality completed by caretakers. Neuropsychological assessment.	At the time of assessment, a significant improvement of sleep quality was found. Sundowning was not longer detectable in 12 patients and persisted, although attenuated in 2 patients. Clinically, the patients exhibited lack of progression of the cognitive and behavioral signs of the disease during the time they received melatonin.	[111]
Case report	Monozygotic twins with AD of 8 years duration	One of the patients was treated with melatonin 9 mg p.o./daily at bed time.	36 months	Neuropsychological assessment. Neuroimaging.	Sleep and cognitive function severely impaired in the twin not receiving melatonin as compared to the melatonin-treated twin.	[112]
Open-label, placebo-controlled trial	14 AD patients	6 mg melatonin p.o./daily at bed time or placebo	4 weeks	Daily logs of sleep and wake quality completed by caretakers. Actigraphy	The 7 AD patients receiving melatonin showed a significantly reduced percentage of nighttime activity compared to a placebo group	[113]
Open-label study	11 (3, 8) AD patients	3 mg melatonin p.o./daily at bed time	3 weeks	Daily logs of sleep and wake quality completed by the nurses.	Analysis revealed a significant decrease in agitated behaviors in all three shifts and a significant decrease in daytime sleepiness.	[117]
Open-label study	45 (19, 26) AD patients	6–9 mg melatonin p.o./daily at bed time	4 months	Daily logs of sleep and wake quality completed by caretakers. Neuropsychological assessment.	Melatonin improved sleep and suppressed sundowning, an effect seen regardless of the concomitant medication employed to treat cognitive or behavioral signs of AD.	[115]
Randomized double blind placebo controlled cross over study	25 AD patients	6 mg of slow release melatonin p.o. or placebo at bed time	7 weeks	Actigraphy	Melatonin had no effect on median total time asleep, number of awakenings, or sleep efficiency.	[118]
Double-blind, placebo-controlled study	20 (3, 17) AD patients	Placebo or 3 mg melatonin p.o./daily at bed time	4 weeks	Actigraphy. Neuropsychological assessment.	Melatonin significantly prolonged the sleep time and decreased activity in the night. Cognitive function was improved by melatonin.	[119]
Randomized, placebo-controlled clinical trial	157 (70, 87) AD patients	2.5 mg slow-release melatonin, or 10 mg melatonin or placebo at bed time	2 months	Actigraphy. Caregiver ratings of sleep quality	Nonsignificant trends for increased nocturnal total sleep time and decreased wake after sleep onset were observed in the melatonin groups relative to placebo. On subjective measures, caregiver ratings of sleep quality showed improvement in the 2.5 mg sustained-release melatonin group relative to placebo.	[120]

Table 1: Continued.

Design	Subjects (M, F)	Treatment	Study's duration	Measured	Results	Reference
Open-label study	7 (4, 3) AD patients	3 mg melatonin p.o./daily at bed time	3 weeks	Actigraphy. Neuropsychological assessment.	Complete remission of daynight rhythm disturbances or sundowning was seen in 4 patients, with partial remission in other 2.	[114]
Randomized, placebo-controlled study	17 AD patients	3 mg melatonin p.o./daily at bed time (7 patients). Placebo (10 patients)	2 weeks	Actigraphy. Neuropsychological assessment.	In melatonin-treated group, actigraphic nocturnal activity and agitation showed significant reductions compared to baseline.	[121]
Randomized, placebo-controlled study	50 AD patients	Morning light exposure (2,500 lux, 1 h) and 5 mg melatonin ($n = 16$) or placebo ($n = 17$) in the evening. Control subjects ($n = 17$) received usual indoor light (150–200 lux).	10 weeks	Night time sleep variables, day sleep time, day activity, day : night sleep ratio, and rest-activity parameters were determined using actigraphy.	Light treatment alone did not improve night time sleep, daytime wake, or rest-activity rhythm. Light treatment plus melatonin increased daytime wake time and activity levels and strengthened the rest-activity rhythm.	[122]
Case report	68-year-old man with AD who developed rapid eye movement (REM) sleep behavior disorder	5–10 mg melatonin p.o./daily at bed time.	20 months	Polysomnography	Melatonin was effective to suppress REM sleep behavior disorder	[123]
Randomized, placebo-controlled study	41 (13, 28) AD patients	Melatonin (8.5 mg immediate release and 1.5 mg sustained release) ($N = 24$) or placebo ($N = 17$) administered at 10 : 00 P.M.	10 days	Actigraphy.	There were no significant effects of melatonin, compared with placebo, on sleep, circadian rhythms, or agitation.	[124]

was done using melatonin [120]. Because MT_1 receptor expression in the SCN is decreased it is certainly possible that melatonin will be ineffective as a synchronizing agent although it is possible that a higher dose of melatonin or a more potent melatonin agonist such as ramelteon may be useful. Another strategy could be exposure to bright light [128] (see below).

5. Melatonin as a Therapeutic Agent for Mild Cognitive Impairment

As outlined, melatonin acts at different levels relevant to the development and manifestation of AD. The antioxidant, mitochondrial, and antiamyloidogenic effects may be seen as a possibility of interfering with the onset of the disease. Therefore, early beginning of treatment may be decisive [129].

Mild cognitive impairment (MCI) is an etiologically heterogeneous syndrome characterized by cognitive impairment shown by objective measures adjusted for age and education in advance of dementia [130]. Approximately 12% of MCI converts to AD or other dementia disorders every year. Since MCI may represent prodromal A,D it should be adequately diagnosed and treated. Indeed, the degenerative process in AD brain starts 20–30 years before the clinical onset of the disease [130]. During this phase, plaques and tangles loads increase and at a certain threshold the first symptom appears. As already mentioned, CSF melatonin levels decrease even in preclinical stages when the patients do not manifest any cognitive impairment (at Braak stages I-II), suggesting that the reduction in CSF melatonin may be an early trigger and marker for AD. Therefore, MCI could be an appropriate moment for initiating any melatonin treatment aiming to affect progression of the disease. Studies on melatonin effect on MCI are summarized in Table 2.

Table 2: Clinical studies on melatonin efficacy in MCI.

Design	Subjects (M, F)	Treatment	Study's duration	Measured	Results	Reference(s)
Double-blind, placebo-controlled, crossover study	10 (4, 6) patients with mild cognitive impairment (MCI)	6 mg melatonin p.o./daily at bed time	10 days	Actigraphy. Neuropsychological assessment.	Enhanced the rest-activity rhythm and improved sleep quality (reduced sleep onset latency and in the number of transitions from sleep to wakefulness Total sleep time unaffected. The ability to remember previously learned items improved along with a significant reduction in depressed mood.	[131]
Double-blind, placebo-controlled pilot study	26 individuals with age-related MCI	1 mg melatonin p.o. or placebo at bed time	4 weeks	Sleep questionnaire and a battery of cognitive tests at baseline and at 4 weeks	Melatonin administration improved reported morning "restedness" and sleep latency after nocturnal awakening and also improved scores on the California Verbal Learning Test-interference subtest.	[132]
Open-label, retrospective study	50 (13, 37) MCI outpatients	25 had received daily 3–9 mg of a fast-release melatonin preparation p.o. at bedtime. Melatonin was given in addition to the standard medication	9–18 months	Daily logs of sleep and wake quality. Initial and final neuropsychological assessment.	Patients treated with melatonin showed significantly better performance in neuropsychological assessment. Abnormally high. Beck Depression Inventory scores decreased in melatonin-treated patients, concomitantly with an improvement in wakefulness and sleep quality.	[133]
Randomized, double blind, placebo-controlled study	354 individuals with age-related cognitive decay	prolonged release melatonin (Circadin, 2 mg) or placebo, 2 h before bedtime	3 weeks	Leeds Sleep Evaluation and Pittsburgh Sleep Questionnaires, Clinical Global Improvement scale score and quality of life.	PR-melatonin resulted in significant and clinically meaningful improvements in sleep quality, morning alertness, sleep onset latency, and quality of life	[134]
Long-term, double-blind, placebo-controlled, 2×2 factorial randomized study	189 (19, 170) individuals with age-related cognitive decay	Long-term daily treatment with whole-day bright (1000 lux) or dim (300 lux) light. Evening melatonin (2.5 mg) or placebo administration	1 to 3.5 years	Standardized scales for cognitive and noncognitive symptoms, limitations of activities of daily living, and adverse effects assessed every 6 months.	Light attenuated cognitive deterioration and also ameliorated depressive symptoms. Melatonin shortened sleep onset latency and increased sleep duration but adversely affected scores for depression. The combined treatment of bright light plus melatonin showed the best effects.	[105]
Prospective, randomized, double-blind, placebo-controlled, study	22 (15, 7) individuals with age-related cognitive decay	Participants received 2 months of melatonin (5 mg o.o./day) and 2 months of placebo	2 months	Sleep disorders were evaluated with the Northside Hospital Sleep Medicine Institute (NHSMI) test. Behavioral disorders were evaluated with the Yesavage Geriatric Depression Scale and Goldberg Anxiety Scale.	Melatonin treatment significantly improved sleep quality scores. Depression also improved significantly after melatonin administration.	[135]

The first report on melatonin treatment of 10 MCI patients (6 mg/day for 10 days) indicated that besides enhancing the rest-activity rhythm and improved sleep quality the ability to remember previously learned items improved along with a significant reduction in depressed mood [131]. In another double-blind, placebo-controlled pilot study performed in 26 individuals with age-related MCI, the administration of 1 mg melatonin or placebo at bed time for 4 weeks resulted in improvement of sleep and of scores on the California Verbal Learning Test-interference subtest [132].

In a retrospective study of a group of 25 MCI patients who received melatonin (3–9 mg per day) for 9 to 18 months in comparison to a similar group of 25 MCI patients who did not receive it [133], patients treated with melatonin showed significantly better performance in a number of neuropsychological tests. Abnormally high Beck Depression Inventory scores decreased in melatonin treated patients, concomitantly with an improvement in wakefulness and sleep quality. The results suggested that melatonin could be a useful add-on drug for treating MCI in a clinic environment [133]. A follow up of that study has now been completed on a group of 35 MCI patients receiving melatonin for 9 to 24 months with essentially similar results [125].

A randomized controlled trial on the effect of bright light and melatonin on cognitive and noncognitive function in elderly residents of group care facilities was published [105]. The authors concluded that light has a benefit in improving some cognitive and noncognitive symptoms of MCI which was amplified by the conjoint administration of melatonin. In other two similar studies, one of them using the prolonged release preparation of melatonin (Circadin) recently approved by the European Medicines Agency, melatonin resulted in significant and clinically meaningful improvements of sleep quality, morning alertness, sleep onset latency and quality of life in old patients with mild cognitive impairment [134, 135]. In these studies melatonin treatment also improved mood. The evaluation of the published data concerning melatonin treatment of MCI that include 5 double blind, randomized placebo-controlled trials, and 1 open-label retrospective study ($N = 651$) all agreess in indicating that treatment with daily evening melatonin improves sleep quality and cognitive performance in MCI [125] (Table 2).

6. Use of Melatonin Agonist, Ramelteon in AD

As AD is associated with disturbed sleep/wake rhythms and circadian rhythm disturbances, a melatonin agonist with higher affinity to melatonin MT_1 and MT_2 receptors with a longer duration would theoretically be beneficial in tackling sleep-wake and circadian rhythm disturbances. In this aspect, ramelteon, which is the first melatonin receptor agonist approved by FDA with activity on MT_1 and MT_2 receptors, should be considered [136, 137].

The chemical structure of ramelteon is: (S)-*N*-[2-(1,6,7,8-tetrahydro-2Hindeno[5,4-b]furan-8-yl)ethyl] propionamide. This melatonin receptor agonist has a chemical formula $C_{16}H_{21}NO_2$ with a molecular weight 259.34. Receptor binding studies indicated that ramelteon has high selectivity for MT_1 and MT_2 receptors, with little affinity for quinone reductase 2 binding [138]. The selectivity of ramelteon for MT_1 has been found >1000-fold over that of MT_2 receptors. It is well known that melatonin exerts its hypnotic effects through the activation of the MT_1 and MT_2 melatonin receptors [139]. Although both MT_1 and MT_2 receptors are involved in the regulation of sleep, the selectivity of MT_1 receptors by ramelteon suggests that it targets sleep onset more specifically than melatonin [140]. Ramelteon has been found to have no affinity for benzodiazepine (BZP), dopamine, opiate, or serotonin receptor binding sites [138]. Hence ramelteon has advantages over other hypnotic drugs in not causing rebound insomnia, withdrawal symptoms, or dependence which is common with the activation of BZP, opiate, or dopamine receptors.

On oral administration, ramelteon is rapidly absorbed with a T_{max} of less than 1 hour [141]. The absolute bioavailability of the oral formulation of ramelteon is less than 2% (range 0.5% to 12%) [141]. It is metabolized mainly in the liver via oxidation to hydroxyl and carbonyl groups and then conjugated with glucuronide. CYP1A2 is the major hepatic enzyme involved in ramelteon metabolism. Four principal metabolites ramelteon, that is, M-I, M-II, M-III, and M-IV, have been identified [141]. Among these, M-II has been found to occur in much higher concentration with systemic concentration being 20- to 100- fold greater than ramelteon.

Ramelteon is rapidly excreted and its elimination is significantly higher in elderly than in younger adults [142]. The influence of age and gender on the pharmacokinetics and pharmacodynamics of ramelteon has been evaluated in healthy volunteers following the administration of a single dose of 16 mg of ramelteon. When compared to young volunteers, ramelteon clearance was significantly reduced in elderly volunteers and its half life significantly increased. No significant effect of gender was observed [142]. The contribution of ramelteon's metabolites on the net pharmacologic activity was also evaluated. Among the four metabolites produced, the activity of M-II was to be about 30-fold lower than that of ramelteon, but its exposure exceeds exposure to ramelteon by a factor 30. It was thus suggested that M-II may contribute to net clinical activity of ramelteon [142].

The subjective efficacy of ramelteon was evaluated in clinical trials consisting of 829 elderly outpatients with chronic insomnia; 701 patients (128 patients discontinued) were treated for a period of 5-weeks with 4 mg and 8 mg ramelteon [143]. Patients in both ramelteon groups reported significant reductions in sleep onset latency (SOL) and increases in total sleep time (TST). Continuation of this study on 100 elderly patients established the efficacy of ramelteon in improving TST and decreasing SOL [144]. A number of studies have now established the efficacy of ramelteon in treating patients with chronic insomnia [145–147].

Concerning the safety and adverse effects with ramelteon, in a double blind placebo controlled study of rebound insomnia (sleep latency after treatment discontinuation) Roth and co-workers [143] evaluated each of the 7 nights

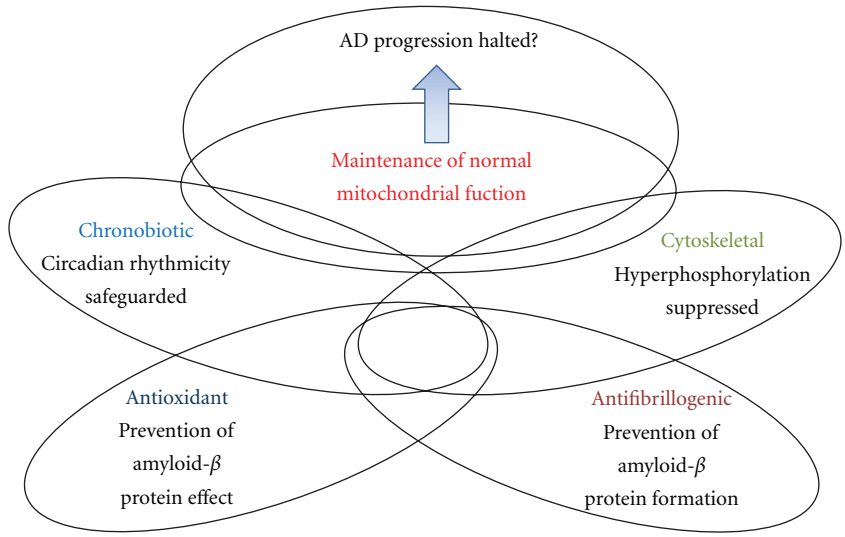


Figure 1: Melatonergic agonists in AD. The multiple effects of melatonin discussed in the text and the different degree of overlap (interrelations and mutual influences) are indicated by the respective intersections in the scheme.

of placebo run-out period. It was noted that during each of the 7 nights, patients in both ramelteon treatment groups (4 mg/day and 8 mg/day) maintained a similar or greater reduction in sleep latency from baseline as compared to those receiving placebo [143]. Withdrawal effects, as assessed by a BZP withdrawal symptom questionnaire, did not differ from the placebo group [143]. In another recent study it was noted that ramelteon did not affect alertness or the ability to concentrate, indicating no next-morning residual effects [148]. The incidence of adverse effects in ramelteon-treated patients in a 5 week study was found to be similar to that of placebo-treated patients. The adverse effects included mild gastrointestinal disturbances and nervous system effects such as dizziness, headache, somnolence, depression, fatigue, myalgia, and exacerbated eye pain [143].

Ramelteon not only has the potential in improving the sleep quality of AD and other neurodegenerative patients but can also offer neuroprotection as well in AD [149]. As ramelteon is a melatonin agonist with more potency and longer duration of action, it could act more efficiently than melatonin in its actions against neurotoxic effects involved in the pathogenesis of AD.

To what extent ramelteon reproduces the nonreceptor mediated effects of melatonin is not known. Ramelteon displays no relevant antioxidant capacity in the ABTS radical cation assay, as compared to luzindole or melatonin [150]. However, MT_1/MT_2 receptor-mediated effects on the upregulation of several antioxidant enzymes by physiological concentration of melatonin [151] such as glutathione peroxidase, glutathione reductase, γ-glutamylcysteine synthase, glucose-6-phosphate dehydrogenase, hemoperoxidase/catalase, Cu,Zn- and Mn-superoxide dismutases (reviewed in [152–155] can well give the basis for the use of ramelteon in AD. Since there are extensive data indicating a loss of melatonin receptors in AD patients, including the cerebral cortex and pineal gland (MT_1 and MT_2 receptors) [156], the hippocampus [157] and retina [158] (MT_2 receptors) and the cerebrovascular system [159], and SCN [126, 128] (MT_1 receptors), the chances of alleviating symptoms such as sundowning and disturbed sleep by giving the MT_1/MT_2 receptor agonist may vanish in late AD patients.

In addition, it has been suggested that melatonin and its receptors participate in neurodevelopment and regulation of neurotrophic factors [160]. In vitro studies have shown that melatonin promotes the viability and neuronal differentiation of neural stem cells and increases the production brain-derived neurotrophic factor (BDNF) by acting through MT_1 receptors [161]. In mouse cerebellar granule cells in culture ramelteon increased the neural content of BDNF [162]. Therefore, if ramelteon treatment is capable of regulating brain BDNF levels, it could be used as a possible therapeutic agent in neurodegenerative diseases like AD for treating symptoms other than sleep disturbances.

7. Concluding Remarks

As AD disease involves a complex physiopathology, it has been suggested that monotherapy targeting early single steps in this complex cascade process may not be of much help [149]. Pleiotrophic drugs that can act independently by different routes including antioxidant, antiinflammatory, and antiamyloid effects would be much beneficial in the treatment of AD and other neurodegenerative disorders. Available evidence indicates suppression of GSK-3β overactivity; neuroinflammation and mitochondrial impairment are some of the combined strategies required in AD.

Melatonin is a pleiotropic molecule with antioxidant, antiinflammatory and antinitridergic properties [56, 154, 163]. It has also a role in sleep induction, and this is important in view that sleep deprivation is one of the cardinal features seen in AD and other neurodegenerative diseases. Sleep deprivation is associated with GSK-3β activation [164], altered proteosomal processing [165],

oxidative damage [166], impaired mitochondrial integrity and function [167], and neurodegenerative inflammation [168]. Therefore, improvement of insomnia in neurodegenerative conditions and particularly in AD is a good practical approach for arresting the progression of the disease (Figure 1).

Melatonin and particularly ramelteon can be greatly beneficial in preventing the insomnia-induced damage of neuronal cells and can be of therapeutic value in treating AD. Owing to its potent effect on MT_1 and MT_2 receptors, ramelteon activates sleep onset by influencing the hypothalamic "sleep switch" downstream from the SCN more efficiently than melatonin itself [35]. Multicenter, placebo-controlled clinical trials using ramelteon are needed to prove the efficacy of this drug in arresting the progression or prevention of AD or remission in the early stages of AD such as MCI.

Acknowledgment

D. P. Cadinale is a Research Career Awardee from the Argentine National Research Council (CONICET), Argentina.

Conflict of Interest Statement and Disclosure Statement

S. R. Pandi-Perumal is a stockholder and the President and Chief Executive Office of Somnogen Inc., a New York Corporation. He declared no competing interests that might be perceived to influence the content of this article. All remaining authors declare that they have no proprietary, financial, professional, nor any other personal interest of any kind in any product or services and/or company that could be construed or considered to be a potential conflict of interest that might have influenced the views expressed in this manuscript.

References

[1] J. J. Palop and L. Mucke, "Amyloid-β-induced neuronal dysfunction in Alzheimer's disease: from synapses toward neural networks," *Nature Neuroscience*, vol. 13, no. 7, pp. 812–818, 2010.

[2] M. Mancuso, D. Orsucci, A. LoGerfo, V. Calsolaro, and G. Siciliano, "Clinical features and pathogenesis of Alzheimer's disease: involvement of mitochondria and mitochondrial DNA," *Advances in Experimental Medicine and Biology*, vol. 685, pp. 34–44, 2010.

[3] C. Behl, J. B. Davis, R. Lesley, and D. Schubert, "Hydrogen peroxide mediates amyloid β protein toxicity," *Cell*, vol. 77, no. 6, pp. 817–827, 1994.

[4] K. Hensley, J. M. Carney, M. P. Mattson et al., "A model for β-amyloid aggregation and neurotoxicity based on free radical generation by the peptide: relevance to Alzheimer disease," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 91, no. 8, pp. 3270–3274, 1994.

[5] M. A. Pappolla, Y. J. Chyan, B. Poeggeler et al., "An assessment of the antioxidant and the antiamyloidogenic properties of melatonin: implications for Alzheimer's disease," *Journal of Neural Transmission*, vol. 107, no. 2, pp. 203–231, 2000.

[6] R. H. Swerdlow, J. M. Burns, and S. M. Khan, "The Alzheimer's disease mitochondrial cascade hypothesis," *Journal of Alzheimer's Disease*, vol. 20, supplement 2, pp. S265–S279, 2010.

[7] W. E. Müller, A. Eckert, C. Kurz, G. P. Eckert, and K. Leuner, "Mitochondrial dysfunction: common final pathway in brain aging and Alzheimer's disease-therapeutic aspects," *Molecular Neurobiology*, vol. 41, no. 2-3, pp. 159–171, 2010.

[8] J. Viña and A. Lloret, "Why women have more Alzheimer's disease than men: gender and mitochondrial toxicity of amyloid-β peptide," *Journal of Alzheimer's Disease*, vol. 20, supplement 2, pp. S527–S533, 2010.

[9] B. F. Oliveira, J. A. Nogueira-Machado, and M. M. Chaves, "The role of oxidative stress in the aging process," *Scientific World Journal*, vol. 10, pp. 1121–1128, 2010.

[10] A. R. Hipkiss, "Mitochondrial dysfunction, proteotoxicity, and aging: causes or effects, and the possible impact of NAD+-controlled protein glycation," *Advances in Clinical Chemistry*, vol. 50, pp. 123–150, 2010.

[11] V. Calabrese, C. Cornelius, C. Mancuso, R. Lentile, A. M. Stella, and D. A. Butterfield, "Redox homeostasis and cellular stress response in aging and neurodegeneration," *Methods in Molecular Biology*, vol. 610, pp. 285–308, 2010.

[12] G. Candore, M. Bulati, C. Caruso et al., "Inflammation, cytokines, immune response, apolipoprotein E, cholesterol, and oxidative stress in alzheimer disease: therapeutic implications," *Rejuvenation Research*, vol. 13, no. 2-3, pp. 301–313, 2010.

[13] S. Mandrekar-Colucci and G. E. Landreth, "Microglia and inflammation in Alzheimer's disease," *CNS and Neurological Disorders—Drug Targets*, vol. 9, no. 2, pp. 156–167, 2010.

[14] M. Fiala, "Re-balancing of inflammation and Aβ immunity as a therapeutic for Alzheimer's disease-view from the bedside," *CNS and Neurological Disorders—Drug Targets*, vol. 9, no. 2, pp. 192–196, 2010.

[15] T. Nakagawa, H. Zhu, N. Morishima et al., "Caspase-12 mediates endoplasmic-reticulum-specific apoptosis and cytotoxicity by amyloid-β," *Nature*, vol. 403, no. 6765, pp. 98–103, 2000.

[16] M. Fotuhi, V. Hachinski, and P. J. Whitehouse, "Changing perspectives regarding late-life dementia," *Nature Reviews Neurology*, vol. 5, no. 12, pp. 649–658, 2009.

[17] S. Beaulieu-Bonneau and C. Hudon, "Sleep disturbances in older adults with mild cognitive impairment," *International Psychogeriatrics*, vol. 21, no. 4, pp. 654–666, 2009.

[18] V. Cochen, C. Arbus, M. E. Soto et al., "Sleep disorders and their impacts on healthy, dependent, and frail older adults," *Journal of Nutrition, Health and Aging*, vol. 13, no. 4, pp. 322–329, 2009.

[19] M. F. Vecchierini, "Sleep disturbances in Alzheimer's disease and other dementias," *Psychologie et NeuroPsychiatrie du Vieillissement*, vol. 8, no. 1, pp. 15–23, 2010.

[20] D. J. Skene, B. Vivient-Roels, D. L. Sparks et al., "Daily variation in the concentration of melatonin and 5-methoxytryptophol in the human pineal gland: effect of age and Alzheimer's disease," *Brain Research*, vol. 528, no. 1, pp. 170–174, 1990.

[21] R. Y. Liu, J. N. Zhou, J. van Heerikhuize, M. A. Hofman, and D. F. Swaab, "Decreased melatonin levels in postmortem cerebrospinal fluid in relation to aging, Alzheimer's disease, and apolipoprotein E-ε4/4 genotype," *Journal of Clinical*

Endocrinology and Metabolism, vol. 84, no. 1, pp. 323–327, 1999.

[22] K. Mishima, T. Tozawa, K. Satoh, Y. Matsumoto, Y. Hishikawa, and M. Okawa, "Melatonin secretion rhythm disorders in patients with senile dementia of Alzheimer's type with disturbed sleep-waking," *Biological Psychiatry*, vol. 45, no. 4, pp. 417–421, 1999.

[23] V. Srinivasan, S. R. Pandi-Perumal, D. P. Cardinali, B. Poeggeler, and R. Hardeland, "Melatonin in Alzheimer's disease and other neurodegenerative disorders," *Behavioral and Brain Functions*, vol. 2, article 15, 2006.

[24] C. Kaur, V. Sivakumar, J. Lu, and E. A. Ling, "Increased vascular permeability and nitric oxide production in response to hypoxia in the pineal gland," *Journal of Pineal Research*, vol. 42, no. 4, pp. 338–349, 2007.

[25] A. Sakotnik, P. M. Liebmann, K. Stoschitzky et al., "Decreased melatonin synthesis in patients with coronary artery disease," *European Heart Journal*, vol. 20, no. 18, pp. 1314–1317, 1999.

[26] L. Girotti, M. Lago, O. Ianovsky et al., "Low urinary 6-sulphatoxymelatonin levels in patients with coronary artery disease," *Journal of Pineal Research*, vol. 29, no. 3, pp. 138–142, 2000.

[27] L. Girotti, M. Lago, O. Ianovsky et al., "Low urinary 6-sulfatoxymelatonin levels in patients with severe congestive heart failure," *Endocrine*, vol. 22, no. 3, pp. 245–248, 2003.

[28] C. Peers, H. A. Pearson, and J. P. Boyle, "Hypoxia and Alzheimer's disease," *Essays in Biochemistry*, vol. 43, pp. 153–164, 2007.

[29] M. Guglielmotto, M. Aragno, R. Autelli et al., "The up-regulation of BACE1 mediated by hypoxia and ischemic injury: role of oxidative stress and HIF1α," *Journal of Neurochemistry*, vol. 108, no. 4, pp. 1045–1056, 2009.

[30] X. Zhang, K. Zhou, R. Wang et al., "Hypoxia-inducible factor 1α (HIF-1α)-mediated hypoxia increases BACE1 expression and β-amyloid generation," *Journal of Biological Chemistry*, vol. 282, no. 15, pp. 10873–10880, 2007.

[31] X. Zhang and W. Le, "Pathological role of hypoxia in Alzheimer's disease," *Experimental Neurology*, vol. 223, no. 2, pp. 299–303, 2010.

[32] K.-M. Ng, C.-F. Lau, and M.-L. Fung, "Melatonin reduces hippocampal β-amyloid generation in rats exposed to chronic intermittent hypoxia," *Brain Research*, vol. 1354, no. C, pp. 163–171, 2010.

[33] M. Aldhous, C. Franey, J. Wright, and J. Arendt, "Plasma concentrations of melatonin in man following oral absorption of different preparations," *British Journal of Clinical Pharmacology*, vol. 19, no. 4, pp. 517–521, 1985.

[34] S. A. Ferguson, S. M. W. Rajaratnam, and D. Dawson, "Melatonin agonists and insomnia," *Expert Review of Neurotherapeutics*, vol. 10, no. 2, pp. 305–318, 2010.

[35] S. R. Pandi-Perumal, V. Srinivasan, D. W. Spence et al., "Ramelteon: a review of its therapeutic potential in sleep disorders," *Advances in Therapy*, vol. 26, no. 6, pp. 613–626, 2009.

[36] G. M. Brown, D. P. Cardinali, and S. R. Pandi-Perumal, "Melatonin and mental illness," in *Sleep and Mental Illness*, S. R. Pandi-Perumal and M. Kramer, Eds., Cambridge University Press, Cambridge, UK, 2010.

[37] D. C. Klein, "Arylalkylamine N-acetyltransferase: "the timezyme"," *Journal of Biological Chemistry*, vol. 282, no. 7, pp. 4233–4237, 2007.

[38] D. C. Klein, M. J. Bailey, D. A. Carter et al., "Pineal function: impact of microarray analysis," *Molecular and Cellular Endocrinology*, vol. 314, no. 2, pp. 170–183, 2010.

[39] H. Tricoire, A. Locatelli, P. Chemineau, and B. Malpaux, "Melatonin enters the cerebrospinal fluid through the pineal recess," *Endocrinology*, vol. 143, no. 1, pp. 84–90, 2002.

[40] J. Leston, C. Harthé, J. Brun et al., "Melatonin is released in the third ventricle in humans. A study in movement disorders," *Neuroscience Letters*, vol. 469, no. 3, pp. 294–297, 2010.

[41] R. J. Reiter and D. X. Tan, "Role of CSF in the transport of melatonin," *Journal of Pineal Research*, vol. 33, no. 1, article 61, 2002.

[42] S. F. Pang and G. M. Brown, "Regional concentrations of melatonin in the rat brain in the light and dark period," *Life Sciences*, vol. 33, no. 12, pp. 1199–1204, 1983.

[43] M. D. Catalá, W. B. Quay, and P. S. Timiras, "Lower tryptophan:phenylalanine ratios in culture media increase medium: pineal melatonin ratios in early dark but not late light phase," *Journal of Pineal Research*, vol. 4, no. 3, pp. 267–275, 1987.

[44] D. P. Cardinali, R. E. Rosenstein, D. A. Golombek et al., "Melatonin binding sites in brain: single or multiple?" *Advances in Pineal Research*, vol. 5, pp. 159–165, 1991.

[45] G. Facciolá, M. Hidestrand, C. von Bahr, and G. Tybring, "Cytochrome P isoforms involved in melatonin metabolism in human liver microsomes," *European Journal of Clinical Pharmacology*, vol. 56, no. 12, pp. 881–888, 2001.

[46] F. Hirata, O. Hayaishi, T. Tokuyama, and S. Senoh, "In vitro and in vitro formation of two new metabolites of melatonin," *Journal of Biological Chemistry*, vol. 249, no. 4, pp. 1311–1313, 1974.

[47] R. Hardeland, D. X. Tan, and R. J. Reiter, "Kynuramines, metabolites of melatonin and other indoles: the resurrection of an almost forgotten class of biogenic amines," *Journal of Pineal Research*, vol. 47, no. 2, pp. 109–126, 2009.

[48] D. X. Tan, L. C. Manchester, R. J. Reiter et al., "A novel melatonin metabolite, cyclic 3-hydroxymelatonin: a biomarker of in vivo hydroxyl radical generation," *Biochemical and Biophysical Research Communications*, vol. 253, no. 3, pp. 614–620, 1998.

[49] R. J. Reiter, "The melatonin rhythm: both a clock and a calendar," *Experientia*, vol. 49, no. 8, pp. 654–664, 1993.

[50] D. Dawson and S. M. Armstrong, "Chronobiotics—drugs that shift rhythms," *Pharmacology and Therapeutics*, vol. 69, no. 1, pp. 15–36, 1996.

[51] R. J. Wurtman and I. Zhdanova, "Improvement of sleep quality by melatonin," *Lancet*, vol. 346, no. 8988, p. 1491, 1995.

[52] J. M. Monti, F. Alvariño, D. Cardinali, I. Savio, and A. Pintos, "Polysomnographic study of the effect of melatonin on sleep in elderly patients with chronic primary insomnia," *Archives of Gerontology and Geriatrics*, vol. 28, no. 2, pp. 85–98, 1999.

[53] A. Carrillo-Vico, R. J. Reiter, P. J. Lardone et al., "The modulatory role of melatonin on immune responsiveness," *Current Opinion in Investigational Drugs*, vol. 7, no. 5, pp. 423–431, 2006.

[54] V. Srinivasan, G. J. M. Maestroni, D. P. Cardinali, A. I. Esquifino, S. R. Pandi Perumal, and S. C. Miller, "Melatonin, immune function and aging," *Immunity and Ageing*, vol. 2, article 17, 2005.

[55] R. J. Reiter, J. J. Garcia, and J. Pie, "Oxidative toxicity in models of neurodegeneration: responses to melatonin,"

Restorative Neurology and Neuroscience, vol. 12, no. 2-3, pp. 135–142, 1998.

[56] R. J. Reiter, D. X. Tan, and L. Fuentes-Broto, "Melatonin: a multitasking molecule," *Progress in Brain Research*, vol. 181, pp. 127–151, 2010.

[57] R. J. Reiter, "The pineal and its hormones in the control of reproduction in mammals," *Endocrine Reviews*, vol. 1, no. 2, pp. 109–131, 1980.

[58] R. J. Reiter, D. X. Tan, L. C. Manchester, S. D. Paredes, J. C. Mayo, and R. M. Sainz, "Melatonin and reproduction revisited," *Biology of Reproduction*, vol. 81, no. 3, pp. 445–456, 2009.

[59] V. Srinivasan, W. D. Spence, S. R. Pandi-Perumal, R. Zakharia, K. P. Bhatnagar, and A. Brzezinski, "Melatonin and human reproduction: shedding light on the darkness hormone," *Gynecological Endocrinology*, vol. 25, no. 12, pp. 779–785, 2009.

[60] D. E. Blask, L. A. Sauer, and R. T. Dauchy, "Melatonin as a chronobiotic/anticancer agent: cellular, biochemical, and molecular mechanisms of action and their implications for circadian-based cancer therapy," *Current Topics in Medicinal Chemistry*, vol. 2, no. 2, pp. 113–132, 2002.

[61] B. Jung-Hynes, R. J. Reiter, and N. Ahmad, "Sirtuins, melatonin and circadian rhythms: building a bridge between aging and cancer," *Journal of Pineal Research*, vol. 48, no. 1, pp. 9–19, 2010.

[62] A. J. Lewy, "Circadian misalignment in mood disturbances," *Current Psychiatry Reports*, vol. 11, no. 6, pp. 459–465, 2009.

[63] S. R. Pandi-Perumal, I. Trakht, G. M. Brown, and D. P. Cardinali, "Melatonin, circadian dysregulation, and sleep in mental disorders," *Primary Psychiatry*, vol. 15, no. 5, pp. 77–82, 2008.

[64] S. M. Reppert, D. R. Weaver, and T. Ebisawa, "Cloning and characterization of a mammalian melatonin receptor that mediates reproductive and circadian responses," *Neuron*, vol. 13, no. 5, pp. 1177–1185, 1994.

[65] S. M. Reppert, C. Godson, C. D. Mahle, D. R. Weaver, S. A. Slaugenhaupt, and J. F. Gusella, "Molecular characterization of a second melatonin receptor expressed in human retina and brain: the Mel(1b) melatonin receptor," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 92, no. 19, pp. 8734–8738, 1995.

[66] M. L. Dubocovich, P. Delagrange, D. N. Krause, D. Sugden, D. P. Cardinali, and J. Olcese, "International union of basic and clinical pharmacology. LXXV. Nomenclature, classification, and pharmacology of G protein-coupled melatonin receptors," *Pharmacological Reviews*, vol. 62, no. 3, pp. 343–380, 2010.

[67] C. Carlberg and I. Wiesenberg, "The orphan receptor family RZR/ROR, melatonin and 5-lipoxygenase: an unexpected relationship," *Journal of Pineal Research*, vol. 18, no. 4, pp. 171–178, 1995.

[68] A. M. Jetten, S. Kurebayashi, and E. Ueda, "The ROR nuclear orphan receptor subfamily: critical regulators of multiple biological processes," *Progress in Nucleic Acid Research and Molecular Biology*, vol. 69, pp. 205–247, 2001.

[69] G. Benítez-King, "Melatonin as a cytoskeletal modulator: implications for cell physiology and disease," *Journal of Pineal Research*, vol. 40, no. 1, pp. 1–9, 2006.

[70] D. P. Cardinali and F. Freire, "Melatonin effects on brain. Interaction with microtubule protein, inhibition of fast axoplasmic flow and induction of crystaloid and tubular formations in the hypothalamus," *Molecular and Cellular Endocrinology*, vol. 2, no. 5, pp. 317–330, 1975.

[71] Y. Urata, S. Honma, S. Goto et al., "Melatonin induces γ-glutamylcysteine synthetase mediated by activator protein-1 in human vascular endothelial cells," *Free Radical Biology and Medicine*, vol. 27, no. 7-8, pp. 838–847, 1999.

[72] V. Srinivasan, S. R. Pandi-Perumal, G. J. M. Maestroni, A. I. Esquifino, R. Hardeland, and D. P. Cardinali, "Role of melatonin in neurodegenerative diseases," *Neurotoxicity Research*, vol. 7, no. 4, pp. 293–318, 2005.

[73] C. Kaur and E. A. Ling, "Antioxidants and neuroprotection in the adult and developing central nervous system," *Current Medicinal Chemistry*, vol. 15, no. 29, pp. 3068–3080, 2008.

[74] M. A. Pappolla, M. Sos, R. A. Omar et al., "Melatonin prevents death of neuroblastoma cells exposed to the Alzheimer amyloid peptide," *Journal of Neuroscience*, vol. 17, no. 5, pp. 1683–1690, 1997.

[75] M. A. Pappolla, Y. J. Chyan, B. Poeggeler et al., "Alzheimer β protein mediated oxidative damage of mitochondrial DNA: prevention by melatonin," *Journal of Pineal Research*, vol. 27, no. 4, pp. 226–229, 1999.

[76] M. A. Pappolla, M. J. Simovich, T. Bryant-Thomas et al., "The neuroprotective activities of melatonin against the Alzheimer β-protein are not mediated by melatonin membrane receptors," *Journal of Pineal Research*, vol. 32, no. 3, pp. 135–142, 2002.

[77] M. Pérez, F. Hernández, A. Gómez-Ramos, M. Smith, G. Perry, and J. Avila, "Formation of aberrant phosphotau fibrillar polymers in neural cultured cells," *European Journal of Biochemistry*, vol. 269, no. 5, pp. 1484–1489, 2002.

[78] G. Benitez-King, I. Túnez, A. Bellon, G. G. Ortíz, and F. Antón-Tay, "Melatonin prevents cytoskeletal alterations and oxidative stress induced by okadaic acid in N1E-115 cells," *Experimental Neurology*, vol. 182, no. 1, pp. 151–159, 2003.

[79] K. K. Ting, B. Brew, and G. Guillemin, "The involvement of astrocytes and kynurenine pathway in Alzheimer's disease," *Neurotoxicity Research*, vol. 12, no. 4, pp. 247–262, 2007.

[80] F. M. Harris, I. Tesseur, W. J. Brecht et al., "Astroglial regulation of apolipoprotein E expression in neuronal cells: implications for Alzheimer's disease," *Journal of Biological Chemistry*, vol. 279, no. 5, pp. 3862–3868, 2004.

[81] F. Malchiodi-Albedi, M. R. Domenici, S. Paradisi, A. Bernardo, M. A. Ajmone-Cat, and L. Minghetti, "Astrocytes contribute to neuronal impairment in βA toxicity increasing apoptosis in rat hippocampal neurons," *GLIA*, vol. 34, no. 1, pp. 68–72, 2001.

[82] Z. Feng and J. T. Zhang, "Protective effect of melatonin on β-amyloid-induced apoptosis in rat astroglioma c6 cells and its mechanism," *Free Radical Biology and Medicine*, vol. 37, no. 11, pp. 1790–1801, 2004.

[83] B. Poeggeler, L. Miravalle, M. G. Zagorski et al., "Melatonin reverses the profibrillogenic activity of apolipoprotein E4 on the alzheimer amyloid Aβ peptidet," *Biochemistry*, vol. 40, no. 49, pp. 14995–15001, 2001.

[84] E. Matsubara, T. Bryant-Thomas, J. P. Quinto et al., "Melatonin increases survival and inhibits oxidative and amyloid pathology in a transgenic model of Alzheimer's disease," *Journal of Neurochemistry*, vol. 85, no. 5, pp. 1101–1108, 2003.

[85] X. Cheng and R. B. van Breemen, "Mass spectrometry-based screening for inhibitors of β-amyloid protein aggregation," *Analytical Chemistry*, vol. 77, no. 21, pp. 7012–7015, 2005.

[86] B. Poeggeler, M. A. Pappolla, R. Hardeland et al., "Indole-3-propionate: a potent hydroxyl radical scavenger in rat brain," *Brain Research*, vol. 815, no. 2, pp. 382–388, 1999.

[87] Y. J. Chyan, B. Poeggeler, R. A. Omar et al., "Potent neuroprotective properties against the Alzheimer β-amyloid by an endogenous melatonin-related indole structure, indole-3-propionic acid," *Journal of Biological Chemistry*, vol. 274, no. 31, pp. 21937–21942, 1999.

[88] J. R. Harris and N. G. Milton, "Cholesterol in Alzheimer's disease and other amyloidogenic disorders," *Sub-Cellular Biochemistry*, vol. 51, pp. 47–75, 2010.

[89] Z. Feng, Y. Chang, Y. Cheng et al., "Melatonin alleviates behavioral deficits associated with apoptosis and cholinergic system dysfunction in the APP 695 transgenic mouse model of Alzheimer's disease," *Journal of Pineal Research*, vol. 37, no. 2, pp. 129–136, 2004.

[90] J. M. Olcese, C. Cao, T. Mori et al., "Protection against cognitive deficits and markers of neurodegeneration by long-term oral administration of melatonin in a transgenic model of Alzheimer disease," *Journal of Pineal Research*, vol. 47, no. 1, pp. 82–96, 2009.

[91] X. C. Li, ZE. F. Wang, J. X. Zhang, Q. Wang, and J. Z. Wang, "Effect of melatonin on calyculin A-induced tau hyperphosphorylation," *European Journal of Pharmacology*, vol. 510, no. 1-2, pp. 25–30, 2005.

[92] X. Yang, Y. Yang, Z. Fu Jr et al., "Melatonin ameliorates alzheimer-like pathological changes and spatial memory retention impairment induced by calyculin A," *Journal of Psychopharmacology*. In press.

[93] Y. Q. Deng, G. G. Xu, P. Duan, Q. Zhang, and J. Z. Wang, "Effects of melatonin on wortmannin-induced tau hyperphosphorylation," *Acta Pharmacologica Sinica*, vol. 26, no. 5, pp. 519–526, 2005.

[94] S. J. Liu and J. Z. Wang, "Alzheimer-like tau phosphorylation induced by wortmannin in vivo and its attenuation by melatonin," *Acta Pharmacologica Sinica*, vol. 23, no. 2, pp. 183–187, 2002.

[95] X. C. Wang, J. Zhang, X. Yu et al., "Prevention of isoproterenol-induced tau hyperphosphorylation by melatonin in the rat," *Acta Physiologica Sinica*, vol. 57, no. 1, pp. 7–12, 2005.

[96] G. Olivieri, U. Otten, F. Meier et al., "β-amyloid modulates tyrosine kinase B receptor expression in SHSY5Y neuroblastoma cells: influence of the antioxidant melatonin," *Neuroscience*, vol. 120, no. 3, pp. 659–665, 2003.

[97] J. B. Hoppe, R. L. Frozza, A. P. Horn et al., "Amyloid-β neurotoxicity in organotypic culture is attenuated by melatonin: involvement of GSK-3β, tau and neuroinflammation," *Journal of Pineal Research*, vol. 48, no. 3, pp. 230–238, 2010.

[98] A. M. Furio, R. A. Cutrera, V. C. Thea et al., "Effect of melatonin on changes in locomotor activity rhythm of Syrian hamsters injected with beta amyloid peptide 25-35 in the suprachiasmatic nuclei," *Cellular and Molecular Neurobiology*, vol. 22, no. 5-6, pp. 699–709, 2002.

[99] D. A. Weldemichael and G. T. Grossberg, "Circadian rhythm disturbances in patients with Alzheimer's disease: a review," *International Journal of Alzheimer's Disease*, vol. 2010, Article ID 716453, 2010.

[100] D. G. Harper, E. G. Stopa, A. C. McKee et al., "Differential circadian rhythm disturbances in men with Alzheimer disease and frontotemporal degeneration," *Archives of General Psychiatry*, vol. 58, no. 4, pp. 353–360, 2001.

[101] E. J. W. van Someren, "Circadian and sleep disturbances in the elderly," *Experimental Gerontology*, vol. 35, no. 9-10, pp. 1229–1237, 2000.

[102] E. J. W. van Someren, "More than a marker: interaction between the circadian regulation of temperature and sleep, age-related changes, and treatment possibilities," *Chronobiology International*, vol. 17, no. 3, pp. 313–354, 2000.

[103] S. Klaffke and J. Staedt, "Sundowing and circadian rhythm disorders in dementia," *Acta Neurologica Belgica*, vol. 106, no. 4, pp. 168–175, 2006.

[104] S. R. Pandi-Perumal, L. K. Seils, L. Kayumov et al., "Senescence, sleep, and circadian rhythms," *Ageing Research Reviews*, vol. 1, no. 3, pp. 559–604, 2002.

[105] R. F. Riemersma-van der Lek, D. F. Swaab, J. Twisk, E. M. Hol, W. J. G. Hoogendijk, and E. J. W. van Someren, "Effect of bright light and melatonin on cognitive and noncognitive function in elderly residents of group care facilities: a randomized controlled trial," *Journal of the American Medical Association*, vol. 299, no. 22, pp. 2642–2655, 2008.

[106] Y. Ohashi, N. Okamoto, K. Uchida, M. Iyo, N. Mori, and Y. Morita, "Daily rhythm of serum melatonin levels and effect of light exposure in patients with dementia of the Alzheimer's type," *Biological Psychiatry*, vol. 45, no. 12, pp. 1646–1652, 1999.

[107] J. N. Zhou, R. Y. Liu, W. Kamphorst, M. A. Hofman, and D. F. Swaab, "Early neuropathological Alzheimer's changes in aged individuals are accompanied by decreased cerebrospinal fluid melatonin levels," *Journal of Pineal Research*, vol. 35, no. 2, pp. 125–130, 2003.

[108] D. J. Skene and D. F. Swaab, "Melatonin rhythmicity: effect of age and Alzheimer's disease," *Experimental Gerontology*, vol. 38, no. 1-2, pp. 199–206, 2003.

[109] F. Magri, M. Locatelli, G. Balza et al., "Changes in endocrine circadian rhythms as markers of physiological and pathological brain aging," *Chronobiology International*, vol. 14, no. 4, pp. 385–396, 1997.

[110] S. M. McCurry, C. F. Reynolds, S. Ancoli-Israel, L. Teri, and M. V. Vitiello, "Treatment of sleep disturbance in Alzheimer's disease," *Sleep Medicine Reviews*, vol. 4, no. 6, pp. 603–628, 2000.

[111] L. I. Brusco, M. Marquez, and D. P. Cardinali, "Melatonin treatment stabilizes chronobiologic and cognitive symptoms in Alzheimer's disease," *Neuroendocrinology Letters*, vol. 19, no. 3, pp. 111–115, 1998.

[112] L. I. Brusco, M. Márquez, and D. P. Cardinali, "Monozygotic twins with alzheimer's disease treated with melatonin: case report," *Journal of Pineal Research*, vol. 25, no. 4, pp. 260–263, 1998.

[113] K. Mishima, M. Okawa, S. Hozumi, and Y. Hishikawa, "Supplementary administration of artificial bright light and melatonin as potent treatment for disorganized circadian rest-activity and dysfunctional autonomic and neuroendocrine systems in institutionalized demented elderly persons," *Chronobiology International*, vol. 17, no. 3, pp. 419–432, 2000.

[114] R. Mahlberg, D. Kunz, I. Sutej, K. P. Kühl, and R. Hellweg, "Melatonin treatment of day-night rhythm disturbances and sundowning in Alzheimer disease: an open-label pilot study using actigraphy," *Journal of Clinical Psychopharmacology*, vol. 24, no. 4, pp. 456–459, 2004.

[115] D. P. Cardinali, L. I. Brusco, C. Liberczuk, and A. M. Furio, "The use of melatonin in Alzheimer's disease," *Neuroendocrinology Letters*, vol. 23, no. 1, pp. 20–23, 2002.

[116] I. Fainstein, A. J. Bonetto, L. I. Brusco, and D. P. Cardinali, "Effects of melatonin in elderly patients with sleep disturbance: a pilot study," *Current Therapeutic Research—Clinical and Experimental*, vol. 58, no. 12, pp. 990–1000, 1997.

[117] J. Cohen-Mansfield, D. Garfinkel, and S. Lipson, "Melatonin for treatment of sundowning in elderly persons with

dementia—a preliminary study," *Archives of Gerontology and Geriatrics*, vol. 31, no. 1, pp. 65–76, 2000.

[118] M. Serfaty, S. Kennell-Webb, J. Warner, R. Blizard, and P. Raven, "Double blind randodmised placebo controlled trial of low dose melatonin for sleep disorders in dementia," *International Journal of Geriatric Psychiatry*, vol. 17, no. 12, pp. 1120–1127, 2002.

[119] K. Asayama, H. Yamadera, T. Ito, H. Suzuki, Y. Kudo, and S. Endo, "Double blind study of melatonin effects on the sleep-wake rhythm, cognitive and non-cognitive functions in Alzheimer type dementia," *Journal of Nippon Medical School*, vol. 70, no. 4, pp. 334–341, 2003.

[120] C. Singer, R. E. Tractenberg, J. Kaye et al., "A multicenter, placebo-controlled trial of melatonin for sleep disturbance in Alzheimer's disease," *Sleep*, vol. 26, no. 7, pp. 893–901, 2003.

[121] R. Mahlberg and S. Walther, "Actigraphy in agitated patients with dementia: monitoring treatment outcomes," *Zeitschrift fur Gerontologie und Geriatrie*, vol. 40, no. 3, pp. 178–184, 2007.

[122] G. A. Dowling, R. L. Burr, E. J. W. van Someren et al., "Melatonin and bright-light treatment for rest-activity disruption in institutionalized patients with Alzheimer's disease," *Journal of the American Geriatrics Society*, vol. 56, no. 2, pp. 239–246, 2008.

[123] K. N. Anderson, S. Jamieson, A. J. Graham, and J. M. Shneerson, "REM sleep behaviour disorder treated with melatonin in a patient with Alzheimer's disease," *Clinical Neurology and Neurosurgery*, vol. 110, no. 5, pp. 492–495, 2008.

[124] P. R. Gehrman, D. J. Connor, J. L. Martin, T. Shochat, J. Corey-Bloom, and S. Ancoli-Israel, "Melatonin fails to improve sleep or agitation in double-blind randomized placebo-controlled trial of institutionalized patients with alzheimer disease," *American Journal of Geriatric Psychiatry*, vol. 17, no. 2, pp. 166–169, 2009.

[125] D. P. Cardinali, A. M. Furio, and L. I. Brusco, "Clinical aspects of melatonin intervention in Alzheimer's disease progression," *Current Neuropharmacology*, vol. 8, no. 3, pp. 218–227, 2010.

[126] Y. H. Wu, J. N. Zhou, J. van Heerikhuize, R. Jockers, and D. F. Swaab, "Decreased MT1 melatonin receptor expression in the suprachiasmatic nucleus in aging and Alzheimer's disease," *Neurobiology of Aging*, vol. 28, no. 8, pp. 1239–1247, 2007.

[127] Y. H. Wu, D. F. Fischer, A. Kalsbeek et al., "Pineal clock gene oscillation is disturbed in Alzheimer's disease, due to functional disconnection from the "master clock"," *The FASEB Journal*, vol. 20, no. 11, pp. 1874–1876, 2006.

[128] Y. H. Wu and D. F. Swaab, "Disturbance and strategies for reactivation of the circadian rhythm system in aging and Alzheimer's disease," *Sleep Medicine*, vol. 8, no. 6, pp. 623–636, 2007.

[129] J. Quinn, D. Kulhanek, J. Nowlin et al., "Chronic melatonin therapy fails to alter amyloid burden or oxidative damage in old Tg2576 mice: implications for clinical trials," *Brain Research*, vol. 1037, no. 1-2, pp. 209–213, 2005.

[130] S. Gauthier, B. Reisberg, M. Zaudig et al., "Mild cognitive impairment," *Lancet*, vol. 367, no. 9518, pp. 1262–1270, 2006.

[131] G. Jean-Louis, H. von Gizycki, and F. Zizi, "Melatonin effects on sleep, mood, and cognition in elderly with mild cognitive impairment," *Journal of Pineal Research*, vol. 25, no. 3, pp. 177–183, 1998.

[132] J. S. Peck, D. B. LeGoff, I. Ahmed, and D. Goebert, "Cognitive effects of exogenous melatonin administration in elderly persons: a pilot study," *American Journal of Geriatric Psychiatry*, vol. 12, no. 4, pp. 432–436, 2004.

[133] A. M. Furio, L. I. Brusco, and D. P. Cardinali, "Possible therapeutic value of melatonin in mild cognitive impairment: a retrospective study," *Journal of Pineal Research*, vol. 43, no. 4, pp. 404–409, 2007.

[134] A. G. Wade, I. Ford, G. Crawford et al., "Efficacy of prolonged release melatonin in insomnia patients aged 55-80 years: quality of sleep and next-day alertness outcomes," *Current Medical Research and Opinion*, vol. 23, no. 10, pp. 2597–2605, 2007.

[135] C. Garzón, J. M. Guerrero, O. Aramburu, and T. Guzmán, "Effecf of melatonin administration on sleep, behavioral disorders and hypnotic drug discontinuation in the elderly: a randomized, double-blind, placebo-controlled study," *Aging—Clinical and Experimental Research*, vol. 21, no. 1, pp. 38–42, 2009.

[136] M. Miyamoto, "Pharmacology of ramelteon, a selective MT/MT receptor agonist: a novel therapeutic drug for sleep disorders," *CNS Neuroscience and Therapeutics*, vol. 15, no. 1, pp. 32–51, 2009.

[137] E. J. Sanchez-Barcelo, C. M. Martinez-Campa, M. D. Mediavilla et al., "Melatonin and melatoninergic drugs as therapeutic agents: ramelteon and agomelatine, the two most promising melatonin receptor agonists," *Recent Patents on Endocrine, Metabolic & Immune Drug Discovery*, vol. 1, pp. 142–151, 2007.

[138] K. Kato, K. Hirai, K. Nishiyama et al., "Neurochemical properties of ramelteon (TAK-375), a selective MT_1/MT_2 receptor agonist," *Neuropharmacology*, vol. 48, no. 2, pp. 301–310, 2005.

[139] N. Zisapel, "Sleep and sleep disturbances: biological basis and clinical implications," *Cellular and Molecular Life Sciences*, vol. 64, no. 10, pp. 1174–1186, 2007.

[140] C. Cajochen, "TAK-375 Takeda," *Current Opinion in Investigational Drugs*, vol. 6, no. 1, pp. 114–121, 2005.

[141] S. Stevenson, S. Bryson, D. Amayke et al., "Study to investigate the absolute bioavailability of a single oral dose of ramelteon (TAK-375) in healthy male subjects," *Clinical Pharmacology and Therapeutics*, vol. 75, p. P22, 2004.

[142] D. J. Greenblatt, J. S. Harmatz, and A. Karim, "Age and gender effects on the pharmacokinetics and pharmacodynamics of ramelteon, a hypnotic agent acting via melatonin receptors MT_1 and MT_2," *Journal of Clinical Pharmacology*, vol. 47, no. 4, pp. 485–496, 2007.

[143] T. Roth, D. Seiden, S. Sainati, S. Wang-Weigand, J. Zhang, and P. Zee, "Effects of ramelteon on patient-reported sleep latency in older adults with chronic insomnia," *Sleep Medicine*, vol. 7, no. 4, pp. 312–318, 2006.

[144] T. Roth, D. Seiden, S. Wang-Weigand, and J. Zhang, "A 2-night, 3-period, crossover study of ramelteon's efficacy and safety in older adults with chronic insomnia," *Current Medical Research and Opinion*, vol. 23, no. 5, pp. 1005–1014, 2007.

[145] G. Zammit, M. Erman, S. Wang-Weigand, S. Sainati, J. Zhang, and T. Roth, "Evaluation of the efficacy and safety of ramelteon in subjects with chronic insomnia," *Journal of Clinical Sleep Medicine*, vol. 3, no. 5, pp. 495–504, 2007.

[146] G. Zammit, H. Schwartz, T. Roth, S. Wang-Weigand, S. Sainati, and J. Zhang, "The effects of ramelteon in a first-night model of transient insomnia," *Sleep Medicine*, vol. 10, no. 1, pp. 55–59, 2009.

[147] G. Mayer, S. Wang-Weigand, B. Roth-Schechter, R. Lehmann, C. Staner, and M. Partinen, "Efficacy and safety of 6-month

nightly ramelteon administration in adults with chronic primary insomnia," *Sleep*, vol. 32, no. 3, pp. 351–360, 2009.

[148] M. Kryger, S. Wang-Weigand, and T. Roth, "Safety of ramelteon in individuals with mild to moderate obstructive sleep apnea," *Sleep and Breathing*, vol. 11, no. 3, pp. 159–164, 2007.

[149] E. C. Lauterbach, J. Victoroff, K. L. Coburn, S. D. Shillcutt, S. M. Doonan, and M. F. Mendez, "Psychopharmacological neuroprotection in neurodegenerative disease: assessing the preclinical data," *Journal of Neuropsychiatry and Clinical Neurosciences*, vol. 22, no. 1, pp. 8–18, 2010.

[150] A. M. Mathes, D. Kubulus, L. Waibel et al., "Selective activation of melatonin receptors with ramelteon improves liver function and hepatic perfusion after hemorrhagic shock in rat," *Critical Care Medicine*, vol. 36, no. 10, pp. 2863–2870, 2008.

[151] D. Tan, R. J. Reiter, L. Chen, B. Poeggeler, L. C. Manchester, and L. R. Barlow-Walden, "Both physiological and pharmacological levels of melatonin reduce DNA adduct formation induced by the carcinogen safrole," *Carcinogenesis*, vol. 15, no. 2, pp. 215–218, 1994.

[152] R. J. Reiter, D. X. Tan, J. C. Mayo, R. M. Sainz, J. Leon, and Z. Czarnocki, "Melatonin as an antioxidant: biochemical mechanisms and pathophysiological implications in humans," *Acta Biochimica Polonica*, vol. 50, no. 4, pp. 1129–1146, 2003.

[153] R. Hardeland and S. R. Pandi-Perumal, "Melatonin, a potent agent in antioxidative defense: actions as a natural food constituent, gastrointestinal factor, drug and prodrug," *Nutrition and Metabolism*, vol. 2, article 22, 2005.

[154] S. R. Pandi-Perumal, V. Srinivasan, G. J. M. Maestroni, D. P. Cardinali, B. Poeggeler, and R. Hardeland, "Melatonin: nature's most versatile biological signal?" *FEBS Journal*, vol. 273, no. 13, pp. 2813–2838, 2006.

[155] R. Hardeland and B. Poeggeler, "Melatonin beyond its classical functions," *Open Physiology Jounal*, vol. 1, pp. 1–23, 2008.

[156] P. Brunner, N. Sözer-Topcular, R. Jockers et al., "Pineal and cortical melatonin receptors MT_1 and MT_1 are decreased in Alzheimer's disease," *European Journal of Histochemistry*, vol. 50, no. 4, pp. 311–316, 2006.

[157] E. Savaskan, M. A. Ayoub, R. Ravid et al., "Reduced hippocampal MT_2 melatonin receptor expression in Alzheimer's disease," *Journal of Pineal Research*, vol. 38, no. 1, pp. 10–16, 2005.

[158] E. Savaskan, R. Jockers, M. Ayoub et al., "The MT_2 melatonin receptor subtype is present in human retina and decreases in Alzheimer's disease," *Current Alzheimer Research*, vol. 4, no. 1, pp. 47–51, 2007.

[159] E. Savaskan, G. Olivieri, L. Brydon et al., "Cerebrovascular melatonin MT_1-receptor alterations in patients with Alzheimer's disease," *Neuroscience Letters*, vol. 308, no. 1, pp. 9–12, 2001.

[160] L. P. Niles, K. J. Armstrong, L. M. Rincón Castro et al., "Neural stem cells express melatonin receptors and neurotrophic factors: colocalization of the MT_1 receptor with neuronal and glial markers," *BMC Neuroscience*, vol. 5, article 41, 2004.

[161] X. Kong, X. Li, Z. Cai et al., "Melatonin regulates the viability and differentiation of rat midbrain neural stem cells," *Cellular and Molecular Neurobiology*, vol. 28, no. 4, pp. 569–579, 2008.

[162] M. Imbesi, T. Uz, S. Dzitoyeva, and H. Manev, "Stimulatory effects of a melatonin receptor agonist, ramelteon, on BDNF in mouse cerebellar granule cells," *Neuroscience Letters*, vol. 439, no. 1, pp. 34–36, 2008.

[163] R. E. Rosenstein, S. R. Pandi-Perumal, V. Srinivasan, D. W. Spence, G. M. Brown, and D. P. Cardinali, "Melatonin as a therapeutic tool in ophthalmology: implications for glaucoma and uveitis," *Journal of Pineal Research*, vol. 49, no. 1, pp. 1–13, 2010.

[164] F. Benedetti, A. Serretti, C. Colombo, C. Lorenzi, V. Tubazio, and E. Smeraldi, "A glycogen synthase kinase 3-β promoter gene single nucleotide polymorphism is associated with age at onset and response to total sleep deprivation in bipolar depression," *Neuroscience Letters*, vol. 368, no. 2, pp. 123–126, 2004.

[165] A. Sehgal, W. Joiner, A. Crocker et al., "Molecular analysis of sleep: wake cycles in Drosophila," *Cold Spring Harbor Symposia on Quantitative Biology*, vol. 72, pp. 557–564, 2007.

[166] L. Lavie and P. Lavie, "Molecular mechanisms of cardiovascular disease in OSAHS: the oxidative stress link," *European Respiratory Journal*, vol. 33, no. 6, pp. 1467–1484, 2009.

[167] C. Cirelli and G. Tononi, "Uncoupling proteins and sleep deprivation," *Archives Italiennes de Biologie*, vol. 142, no. 4, pp. 541–549, 2004.

[168] C. Cirelli, U. Faraguna, and G. Tononi, "Changes in brain gene expression after long-term sleep deprivation," *Journal of Neurochemistry*, vol. 98, no. 5, pp. 1632–1645, 2006.

SAGE-Hindawi Access to Research
International Journal of Alzheimer's Disease
Volume 2011, Article ID 623051, 9 pages
doi:10.4061/2011/623051

Review Article

Recent Development of Bifunctional Small Molecules to Study Metal-Amyloid-β Species in Alzheimer's Disease

Joseph J. Braymer,[1] Alaina S. DeToma,[1] Jung-Suk Choi,[2] Kristin S. Ko,[1] and Mi Hee Lim[1,2]

[1] *Department of Chemistry, University of Michigan, Ann Arbor, MI 48109-1055, USA*
[2] *The Life Sciences Institute, University of Michigan, Ann Arbor, MI 48109-2216, USA*

Correspondence should be addressed to Mi Hee Lim, mhlim@umich.edu

Received 18 September 2010; Accepted 25 October 2010

Academic Editor: Peter Faller

Copyright © 2011 Joseph J. Braymer et al. This is an open access article distributed under the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original work is properly cited.

Alzheimer's disease (AD) is a multifactorial neurodegenerative disease related to the deposition of aggregated amyloid-β (Aβ) peptides in the brain. It has been proposed that metal ion dyshomeostasis and miscompartmentalization contribute to AD progression, especially as metal ions (e.g., Cu(II) and Zn(II)) found in Aβ plaques of the diseased brain can bind to Aβ and be linked to aggregation and neurotoxicity. The role of metal ions in AD pathogenesis, however, is uncertain. To accelerate understanding in this area and contribute to therapeutic development, recent efforts to devise suitable chemical reagents that can target metal ions associated with Aβ have been made using rational structure-based design that combines two functions (metal chelation and Aβ interaction) in the same molecule. This paper presents bifunctional compounds developed by two different design strategies (linkage or incorporation) and discusses progress in their applications as chemical tools and/or potential therapeutics.

1. Introduction

Alzheimer's disease (AD) is a progressive neurodegenerative disorder that is a growing worldwide health concern among aging populations [1, 2]. Attempts to unravel the underlying causes of AD have been made across a variety of disciplines, but despite the recognition of the physical and mental symptoms of the disease, little is understood about its etiology and pathological development [1–4]. It has been established, however, that accumulated amyloid-β (Aβ) plaques and neurofibrillary tangles are two of the defining pathological characteristics of AD [1–4].

One of the currently accepted hypotheses of AD, the amyloid cascade hypothesis, states that Aβ and its aggregated forms may cause the neurodegeneration observed in diseased brains [1–7]. Aβ is a peptide (38–43 amino acids in length) cleaved from the transmembrane amyloid precursor protein (APP) by β- and γ-secretases [1, 3–7]. Once generated, Aβ peptides can aggregate into oligomers, protofibrils, and fibrils that adopt the well-organized β-sheet structure [1, 3, 4]. To date, the aggregation pathways and neurotoxicity of Aβ have been extensively studied, but their involvement in the pathology of AD remains elusive [1, 3, 4, 7–12]. Current evidence suggests that monomers and fibrils are relatively benign, while soluble oligomers, including dimers, are responsible for the neurotoxicity leading to dementia [8, 9]. It also has been suggested that the formation of amyloid plaques may be an effect rather than a cause of AD development, and further insight into the biological role of Aβ may help elucidate this aspect [10–12]. Thus far, Aβ has been identified as a causative agent in AD and ongoing studies regarding the role of the Aβ peptide may contribute to the establishment of a fundamental understanding of AD neuropathogenesis.

Metal ion dyshomeostasis and miscompartmentalization also are thought to play a significant part in the progression of AD [1, 3, 4, 13–23]. The role of metal ions, including their effects on Aβ production/aggregation and neurotoxicity, is a relevant but contentious topic in the research field of AD. Particularly, Al, Fe, Cu, and Zn have been found to be associated with Aβ plaques in the brains of AD patients; however, like Aβ, how these metals

participate in AD pathogenesis is still unclear [1, 3, 4, 13–25]. It has been suggested that Al(III) can cause a change in the Aβ conformation that stabilizes the oligomeric state and increases surface hydrophobicity, which may cause an increase in toxicity and membrane permeability [16, 19, 26]. The implication of Al(III) in AD has been debatable, as studies have been conducted using nonphysiological conditions [16].

Recent interest in this field has focused on the association of Fe(II/III), Cu(I/II), and Zn(II) with AD development [1, 3, 4, 13–25]. Redox active Fe(II/III) and Cu(I/II) bound to Aβ species can undergo Fenton chemistry leading to the generation of reactive oxygen species (ROS) such as hydrogen peroxide and hydroxyl radical. ROS can cause oxidative damage to biological molecules, which may trigger neurodegeneration. An additional effect of these ions, especially Cu(II) and Zn(II), in the AD brain is the facilitation of Aβ aggregation upon binding to the peptide. Elevated concentrations of Cu(II) and Zn(II) have been observed in senile plaques [1, 4, 13–15, 24, 25]; in particular, high concentrations of labile zinc are found in the regions of the brain most affected by AD, the cortex and hippocampus [4, 13–17, 19]. Although these metal ions have been shown to be involved in Aβ aggregation events, their direct interactions with the peptide are not completely established. To understand metal coordination properties of Aβ, structural investigations of metal-bound Aβ species have been performed employing a wide range of techniques such as nuclear magnetic resonance (NMR) spectroscopy, electron paramagnetic resonance (EPR) spectroscopy, circular dichroism (CD) spectroscopy, and mass spectrometry (MS) [3, 4, 18, 21, 22]. These studies have suggested that the coordination of Cu(II) and Zn(II) in Aβ species could occur *via* three histidine residues (H6, H13, and H14) and possibly another *N*-terminal residue or the peptide backbone. Structural characterization of metal binding to Aβ is of great interest and has been reviewed elsewhere [4, 18, 21, 22].

The involvement of metal ions in AD has been integrated into the established amyloid cascade hypothesis to form the "metal hypothesis of Alzheimer's disease," which states that the interactions between metal ions and Aβ, as well as abnormal metal ion homeostasis, are connected with AD neuropathogenesis [3, 4, 13–23]. Based on this hypothesis, disruption of metal-Aβ interactions *via* metal chelation therapy has been proposed in order to reduce neurotoxicity of metal-Aβ species and restore metal ion homeostasis in the brain [1, 3, 4, 13, 15–17, 19, 27–35]. To date, several chelators such as EDTA (*N*,*N*′-1,2-ethanediylbis[*N*-(carboxymethyl)]glycine), clioquinol (CQ, 5-chloro-7-iodo-8-hydroxyquinoline), and an 8-hydroxyquinoline derivative (PBT2) have been utilized as agents for metal ion chelation therapy in AD (Figure 1). Among these compounds, CQ and PBT2 improved cognition in phase II clinical trials [30–33]; however, long-term use of CQ is limited by a side effect, subacute myelo-optic neuropathy [36]. Overall, the studies using these and other traditional metal chelating agents have exhibited modulation of metal-induced Aβ events including aggregation and neurotoxicity *in vitro* and *in vivo*, which suggests that the continued development of metal ion chelation therapy is a valuable pursuit for AD.

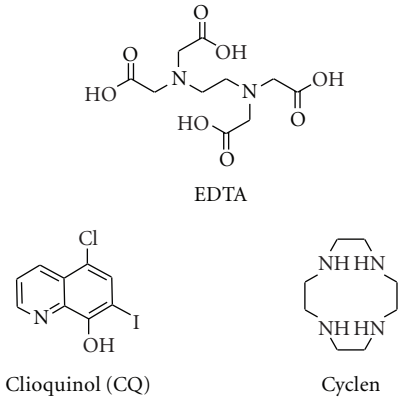


Figure 1: Chemical structures of EDTA (*N*,*N*′-1,2-ethaned- iylbis[*N*-(carboxymethyl)]glycine), clioquinol (CQ, 5-chloro-7-iodo-8-hydroxyquinoline), and cyclen (cyc, 1,4,7,10-tetraazacyclododecane).

To obtain a greater understanding of the role of metal ions associated with Aβ and fashion potential therapeutic agents for AD, improvement upon the metal ion chelation therapy approach would be desirable. Current efforts have been to apply rational structure-based design toward construction of small molecules that are capable of synergistically recognizing both metal ions and Aβ [4, 37–42]. This approach could improve the limitations of the traditional metal chelating compounds (e.g., nonspecific metal chelation and low blood-brain barrier (BBB) permeability). The reported small molecules that have bifunctionality (metal chelation and Aβ interaction) are hybrids of a metal chelation moiety like EDTA, CQ, or cyclen (cyc, 1,4,7,10-tetraazacyclododecane) (Figure 1) and a known Aβ interaction scaffold, including ThT, the KLVFF peptide, curcumin, IMPY, and *p*-I-stilbene (ThT = thioflavin-T, 2-[4-(dimethylamino)phenyl]-3,6-dimethylbenzothiazolium; curcumin = (1*E*,6*E*)-1,7-bis(4-hydroxy-3-methoxyphenyl)-1,6-heptadiene-3,5-dione; IMPY = 4-(7-iodoimidazo[1,2-*a*]pyridin-2-yl)-*N*,*N*-dimethylaniline; *p*-I-stilbene = *N*,*N*-dimethyl-4-[(1*E*)-2-(4-iodophenyl)ethenyl]benzenamine) (Figure 2) [4, 37–42]. Bifunctional small molecules targeted to metal ions surrounded by Aβ species have been developed following two different design principles (Figure 3, Approach I: linkage of metal chelation and Aβ interaction structural components; Approach II: incorporation of a metal-binding site into an Aβ interacting framework). The capability of these bifunctional compounds to regulate metal-Aβ-involved events such as aggregation and neurotoxicity *in vitro* and *in vivo* is promising for further applications. The progress to date in the design and utilization of these compounds is discussed herein.

2. Approach I: Linked Compounds

The "linked" compounds are those in which a structural moiety for metal binding is connected to an Aβ interacting

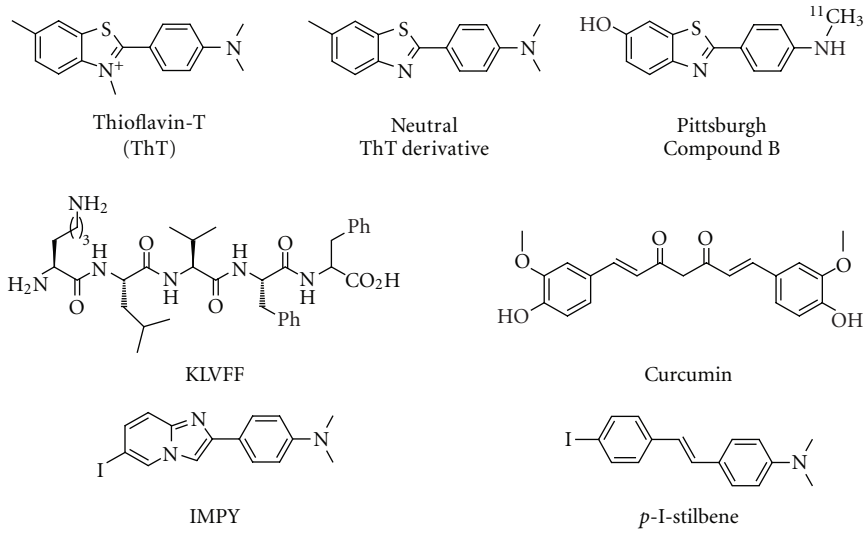


Figure 2: Chemical structures of ThT (thioflavin-T), a neutral ThT derivative, Pittsburgh Compound B, the KLVFF peptide, curcumin, IMPY, and *p*-I-stilbene (ThT = 2-[4-(dimethylamino)phenyl]-3,6-dimethylbenzothiazolium; neutral ThT derivative = 2-(4-(dimethylamino)phenyl)-6-methylbenzothiazole; Pittsburgh Compound B = 2-[4-(methyl-^{11}C-amino)phenyl]-6-benzothiazolol; curcumin = ((1*E*,6*E*)-1,7-bis(4-hydroxy-3-methoxyphenyl)-1,6-heptadiene-3,5-dione; IMPY = 4-(7-iodoimidazo[1,2-*a*]pyridin-2-yl)-*N*,*N*-dimethylaniline; *p*-I-stilbene = *N*,*N*-dimethyl-4-[(1*E*)-2-(4-iodophenyl)ethenyl]benzenamine).

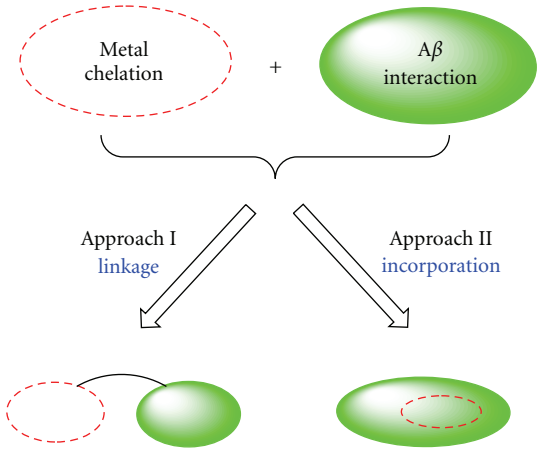


Figure 3: Overview of the two rational structure-based design strategies currently used to develop small molecules having bifunctionality (metal chelation and Aβ interaction). The first approach is based on the connection of metal chelation and Aβ recognition structural moieties (Approach I: linkage). The second approach is direct incorporation of a metal chelation site into an Aβ interacting framework (Approach II: incorporation).

molecule (Approach I, Figure 3). The linked bifunctional molecules XH1 (*N*,*N*-bis[2-[[2-[[4-(2-benzothiazolyl) phenyl]amino]-2-oxoethyl](carboxymethyl)amino]ethyl]glycine), cyc-KLVFF, and cyc-Curcumin are discussed in this paper (Figure 4) [4, 37–40].

2.1. XH1. One of the small molecules most frequently used to understand Aβ aggregation pathways is ThT (Figure 2) [43–46]. Due to the ability of ThT to selectively bind to aggregated amyloid forms and thereby generate a fluorescence response, it can be employed to monitor the degree of Aβ aggregation. It has been proposed that ThT could be a useful framework to pursue in the development of Aβ imaging agents [47]. The application of ThT as a chemical probe *in vivo*, however, is limited by its positive charge, which impairs its ability to diffuse through the BBB [47–49]. Neutral ThT derivatives (Figure 2) prepared to alleviate this limitation of ThT exhibited more favorable properties, such as 600-fold higher lipophilicity and greater binding affinity to Aβ [47]. These compounds also adhere to the restrictive terms of Lipinski's rules (low molecular weight (MW $\leq$ 450), relatively lipophilic ($c\log P$, calculated logarithm of the octanol/water partition coefficient, $\leq$5), hydrogen-bond donor atoms (HBD $\leq$ 5), hydrogen-bond acceptor atoms (HBA $\leq$ 10), and small polar surface area (PSA $\leq$ 90 Å^2)), which, along with the calculated logBB, can be used to predict BBB permeability [48, 49]. The modifications of the neutral ThT derivatives have been conducted to devise applicable *in vivo* imaging agents. One of the most successful examples has been the preparation and utilization of a carbon-11-labeled compound, Pittsburgh Compound B (Figure 2), as an agent for positron emission tomography (PET) [47, 50, 51]. This compound has shown high affinity for Aβ, specificity for staining plaques, and good brain entry and clearance. Overall, the ThT scaffold is an ideal candidate for chemical modification in order to produce new bifunctional molecules capable of targeting metal-Aβ species (*vide infra*).

Dedeoglu et al. were the first to report a neutral ThT-conjugated EDTA derivative, XH1 (Figure 4), for metal-associated Aβ species [39]. Computational ligand/receptor docking studies using XH1 indicated its specific interactions with Aβ_{1-40}. XH1 was able to decrease Zn(II)-induced

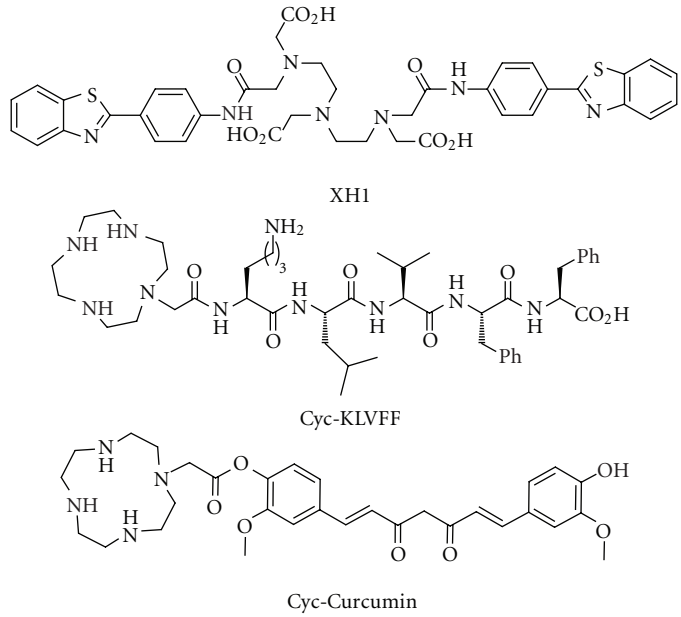


Figure 4: Chemical structures of small molecules XH1 (*N*,*N*-bis[2-[[2-[[4-(2-benzothiazolyl)phenyl]amino]-2-oxoethyl](carboxymethyl) amino]ethyl]glycine), cyc-KLVFF, and cyc-Curcumin that were designed by Approach I (Figure 3).

$A\beta_{1-40}$ aggregation as well as APP expression in human SH-SY5Y neuroblastoma cells, indicating the possible modification of metal-Aβ interactions by XH1. Given these positive results *in vitro* and no significant neurotoxicity at low micromolar concentrations in living cells, XH1 was tested further in the presenilin 1 (PS1)/APP transgenic mice model. While no behavior differences were observed in mice treated with XH1 for four weeks compared to those untreated, the deposition of Aβ plaques and the concentration of cortical Aβ were reduced in the treated mice. Therefore, these observations suggest that XH1 may be BBB permeable. Overall, the Aβ interaction and nontoxic nature of XH1 are a promising beginning for designing a new class of bifunctional small molecules.

2.2. Cyc-KLVFF. "β-sheet blockers" that interfere with amyloidogenesis by interacting with the portions of the peptide responsible for facilitating self-aggregation have been investigated [52–56]. The peptide sequence containing residues 16–20 (KLVFF) from the full-length peptide has been found to be the most effective at hindering Aβ fibrillogenesis. A bifunctional small molecule, cyc-KLVFF (Figure 4), was fashioned by the coupling of the KLVFF peptide as the amyloid recognition moiety with the metal chelator cyclen (Figures 1 and 2) [40]. Cyclen is a macrocyclic metal chelator that can modulate Aβ aggregation and neurotoxicity *in vitro* and in cortical neuronal cells of C57BL6/J mice, and it has been shown that cyclen-Co(III) complexes have proteolytic activity [57, 58]. Cyc-KLVFF (Figure 4) was effective toward reducing metal-triggered Aβ aggregation and neurotoxicity including Cu(II)-induced ROS production *in vitro* and in neuronal cell culture [40]. Aggregated forms of the peptide also were cleaved upon incubation with cyc(Cu(II))-KLVFF complex. Apo-cyc-KLVFF was nontoxic in primary mouse N2a neuroblastoma cells and could recover the toxicity of $A\beta_{1-42}$ in cells pretreated with Cu(II). These results indicate the potential of apo-cyc-KLVFF to sequester Cu(II) from heterogeneous environments to protect against metal-Aβ events. Cyc-KLVFF potentially is limited by nonspecific cleavage of other Cu-containing proteins while BBB permeability may present an additional challenge due to the high MW of the compound [40, 48, 49]. Taken together, the design and studies of cyc-KLVFF suggest that the linkage of two structural frameworks for Aβ interaction and metal chelation (Approach I, Figure 3) could enhance reactivity toward metal-Aβ species.

2.3. Cyc-Curcumin. Curcumin (Figure 2) has shown anti-amyloidogenic, antioxidative, and anti-inflammatory properties [59–61]. The structure-activity relationships of Aβ aggregation inhibitors based on curcumin have been studied, and three necessary structural features were identified: the presence of two terminal aromatic groups, the substitution of these aromatics, and the length and flexibility of the linker between the aromatic groups [62]. The reported properties of curcumin could afford a strong prototype recognition framework to incorporate into new bifunctional small molecules. Another bifunctional small molecule containing cyclen, cyc-Curcumin (Figure 4), was prepared to evaluate the ability of other recognition frameworks to function in this platform [40]. The corresponding metal complex, cyc(Cu(II))-Curcumin, presented Aβ aggregation inhibition and Aβ cleavage activity similar to cyc(Cu(II))-KLVFF. The cyc(Cu(II))-Curcumin complex showed modest rescue of Aβ-induced neurotoxicity. This compound demonstrates that linking a nonpeptide Aβ recognition/interaction

molecule to a metal chelation framework may exert a similar effect on Aβ aggregation pathways, introducing another avenue to develop bifunctional compounds targeted to metal-Aβ species following Approach I.

3. Approach II: Incorporated Compounds

The second class of bifunctional small compounds reviewed herein was designed based on direct incorporation of a metal-binding site into the structural framework of an Aβ recognition/interaction molecule (Approach II, Figure 3). Utilizing Approach II, three categories of compounds have been reported: (a) the ThT-based molecules, HBX, HBT, BM, and their iodinated derivatives (HBX = 2-(2-hydroxyphenyl) benzoxazole; HBT = 2-(2-hydroxyphenyl)benzothiazole; BM = 2-(2-aminophenyl)benzimidazole), (b) the IMPY derivative (**1**) (**1** = 2-[4-(dimethylamino)phenyl]imidazo[1,2-*a*] pyridine-8-ol), and (c) the stilbene derivative (**2**) (**2** = N^1,N^1-dimethyl-N^4-(pyridin-2-ylmethylene)benzene-1,4-diamine) (Figure 5) [37, 38, 41, 42].

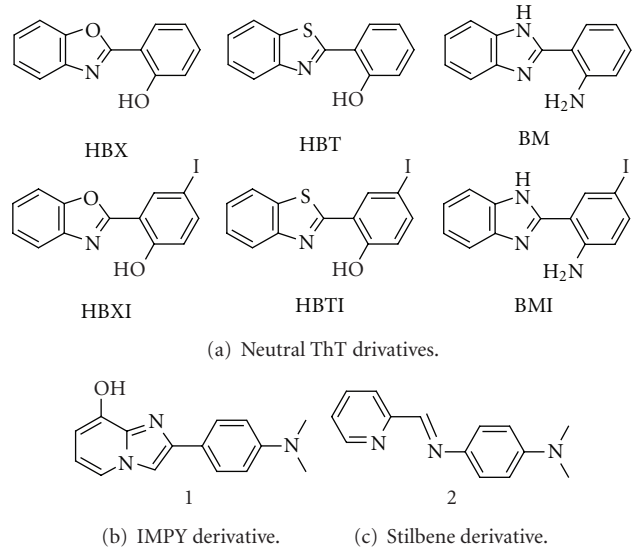


Figure 5: Chemical structures of small molecules (a) HBX/HBXI, HBT/HBTI, and BM/BMI, (b) **1**, and (c) **2** that were constructed employing Approach II (Figure 3) (HBX = 2-(2-hydroxyphenyl) benzoxazole; HBT = 2-(2-hydroxyphenyl)benzothiazole; BM = 2-(2-aminophenyl)benzimidazole; **1** = 2-[4-(dimethylamino)phenyl]imidazo[1,2-*a*]pyridine-8-ol; **2** = N^1,N^1-dimethyl-N^4-(pyridin-2-ylmethylene)benzene-1,4-diamine).

3.1. ThT Derivatives. The neutral ThT derivative (Figure 2) was used as a framework for designing bifunctional small molecules studied by Rodríguez-Rodríguez et al. [41]. By virtual screening of commercially available neutral ThT derivatives containing a bidentate metal chelation site, small molecules that could target metal ions surrounded by Aβ were identified. The criteria applied to select desired compounds required that their structures could be iodinated and satisfy the restrictive terms of Lipinski's rules, along with logBB [41, 48, 49]. Their virtual screen identified 36 candidates, which were narrowed to three small molecules, HBX, HBT, and BM (Figure 5(a)). The iodinated versions of these three compounds, HBXI, HBTI, and BMI (Figure 5(a)), also were prepared and investigated due to their potential contribution to the development of new SPECT imaging agents (SPECT = single photon emission computed tomography).

HBXI and HBTI were found to be primarily neutral at physiological pH (e.g., 7.4), which indicates potential BBB permeability *in vivo* while BMI existed in both the protonated and neutral forms at this pH [41]. The solution speciation studies regarding the binding affinities of Cu(II) and Zn(II) to these compounds suggest their capability to sequester metal ions associated with soluble forms of Aβ. Inhibition of both Cu(II)- and Zn(II)-induced Aβ aggregation was most significant for HBTI, while HBXI and BMI showed a similar effect only for Cu(II)-triggered Aβ aggregation. Also, fluorescence measurements were conducted to assess the possible use of HBX, HBT, and their iodinated analogues as potential *in vivo* biomarkers for amyloid aggregates. Promisingly, HBX and HBT exhibited stronger fluorescence responses with mature amyloid fibrils than Pittsburgh Compound B, used in clinical trials. HBXI and HBTI demonstrate the potential for improving radioisotopic detection of Aβ aggregates in the human brain [41]. Taken together, the studies using small, neutral ThT derivatives suggest that bifunctional small molecules could have multiple applications in AD diagnosis and therapy.

3.2. IMPY Derivative. The need for *in vivo* Aβ plaque imaging molecules also has led to the development of a ThT analogue, IMPY (Figure 2) [63–68]. This radiolabeled iodinated ligand can be a useful SPECT reagent over some current PET probes for a number of reasons including practicality of administering the compound as well as cost and availability of SPECT equipment in clinical settings [64–66]. IMPY binds to Aβ_{1-40} aggregates with nanomolar affinity and likely occupies a similar binding site as the thioflavin dyes due to its planarity and hydrophobicity. IMPY also has shown favorable pharmacokinetic properties in the Tg2576 and PS1/APP transgenic mouse models as well as safety in human patients [64–66]. The studies performed thus far illustrate the promise of radiolabeled IMPY as an *in vivo* SPECT imaging agent for potential diagnosis of AD in human patients.

The positive outcomes from studies with IMPY suggest that the modification of this framework to include a metal chelation site could be effective for developing bifunctional small molecules toward metal ions associated with Aβ. One bifunctional IMPY derivative, **1**, has been reported by Lim and coworkers (Figure 5(b)) [42]. An OH functionality was incorporated into the core framework of IMPY to generate a metal chelation site. Additionally, the structure fulfills the criteria of the restrictive terms of Lipinski's rules and the logBB values, indicating that it may be drug-like and BBB permeable [48, 49]. Along with metal-binding studies of **1** by UV-visible spectroscopy, two-dimensional (2D)

^{1}H-^{15}N TROSY-HSQC NMR spectroscopy was employed in order to understand the interaction of **1** with Aβ_{1-40} monomer (TROSY = transverse relaxation optimized spectroscopy; HSQC = heteronuclear single quantum correlation). Changes in the shifts of the NMR spectrum indicated that **1** interacts with Aβ residues including E11 and H13. Based on the close proximity of these residues to the metal chelation site (H6, H13, and H14) [3, 4, 18, 21, 22], **1** could possibly interact with metal ions in that portion of the peptide. This implies that **1** may have the potential to block Aβ fibrillogenesis from the monomeric state *via* disruption of metal-Aβ interactions. Compared with the traditional metal chelators CQ, phen (1,10-phenanthroline), and EDTA, **1** more effectively inhibited Cu(II)-triggered Aβ aggregation and disassembled Cu(II)-associated Aβ aggregates [42]. Furthermore, **1** was able to modulate ROS generated by Cu-Aβ species. Although this compound showed cytotoxicity in human neuroblastoma cells at low micromolar concentrations, the favorable reactivity toward Cu(II)-associated Aβ aggregation pathways warrants further chemical modifications of the IMPY scaffold for serving in future investigations.

3.3. Stilbene Derivative. Stilbene derivatives (Figure 2) have been widely devised and used as imaging agents for amyloid plaques [69–71]. Overall, stilbene derivatives are small, simple structures that could be labeled easily for *in vivo* imaging applications. While *trans*-stilbene has low affinity for Aβ aggregates, derivatives that contain electron-donating functionalities such as *p*-N$(CH_3)_2$, *p*-OCH_3, or *p*-OH groups bind to Aβ with higher affinity. These characteristics have made it an exemplar framework to modify for construction of bifunctional molecules. Thus, Hindo et al. have recently reported a stilbene derivative (**2**, Figure 5(c)) containing two N donor atoms as a bidentate metal chelation site [42]. Based on the restrictive terms of Lipinski's rules and the calculated logBB value, **2** satisfies the requirements for potential BBB permeability [48, 49].

The stilbene derivative, **2**, presented encouraging reactivity toward metal-Aβ species [42]. This molecule was shown to interact with Cu(II) studied by UV-visible spectroscopy and the interaction of the compound with Aβ_{1-40} monomer was investigated using 2D ^{1}H-^{15}N TROSY-HSQC NMR spectroscopy. Similar to **1**, upon addition of **2** to the peptide solution, the residues E11 and H13 were most significantly shifted, which suggests close contact of **2** with the metal-binding site in Aβ. The bifunctional compound **2** not only controlled Cu(II)-induced formation of Aβ aggregation but also disaggregated preformed Cu(II)-treated Aβ aggregates. In addition, this compound attenuated ROS formation by Cu-Aβ species, indicated no cytotoxicity up to high micromolar concentrations, and, more importantly, was capable of diminishing neurotoxicity of Cu-Aβ species in human neuroblastoma cells. Though **2** has shown promising results thus far, the instability of this compound in aqueous media due to the susceptibility of the imine functionality to hydrolysis would hinder its *in vivo* applications. Therefore, structural modifications of **2** will lead to the next generation of biologically compatible small molecules as chemical tools and/or potential therapeutic agents for AD [72].

4. Conclusion

Metal ions associated with Aβ have been suggested to be related to AD neuropathogenesis; however, the detailed mechanisms are not fully understood. To elucidate the role of metal ions surrounded by Aβ and eventually to diagnose, treat, and prevent AD, small molecules that have bifunctionality (metal chelation and Aβ interaction) have been designed as chemical tools and/or potential therapeutic agents based on the two rational structure-based design strategies. The first approach involves linking two structural components for metal chelation and Aβ interaction. According to this approach, XH1, cyc-KLVFF, and cyc-Curcumin were constructed by combining the Aβ recognition/interaction molecules (ThT, the KLVFF peptide, or curcumin) with metal chelation moieties (EDTA or cyclen). The second approach represents direct incorporation of N and/or O donor atoms for metal chelation into Aβ interacting frameworks (ThT, IMPY, or *p*-I-stilbene) producing HBX/HBXI, HBT/HBTI, BM/BMI, **1**, and **2**. The bifunctional compounds described in this paper have shown promising preliminary results toward metal-induced Aβ aggregation and neurotoxicity *in vitro* and/or *in vivo*; however, comprehensive *in vivo* studies of these molecules would be valuable. Common challenges of developing effective small molecules in biological systems (in particular, in the brain) include optimization of BBB permeability, stability, and toxicity in order to accomplish their future applications. Overall, the bifunctional small molecules discussed herein have laid a foundation for the emerging field of rational structure-based design of small molecules for targeting metal ions surrounded by Aβ species and regulating metal-involved Aβ events. Future endeavors in this area will provide inspiration for uncovering details of the roles of metal-Aβ species in AD neuropathogenesis and may offer insight into the applicability to and understanding of other neurodegenerative diseases.

Acknowledgments

This work was supported by generous startup funding and a Rackham Faculty Grant from the Horace H. Rackham School of Graduate Studies from the University of Michigan, the Alzheimer's Art Quilt Initiative (AAQI), as well as the Alzheimer's Association (NIRG-10-172326) (to M. H. Lim). A. S. DeToma thanks the Graduate Assistance in Areas of National Need Fellowship from the University of Michigan. J.-S. Choi is grateful to the National Research Foundation of Korea funded by the Korean Government for a Postdoctoral Fellowship (NRF-2009-352-F00042). The authors thank Nichole Schmidt for helpful assistance. J. J. Braymer and A. S. DeToma contributed equally to this paper.

References

[1] R. Jakob-Roetne and H. Jacobsen, "Alzheimer's disease: from pathology to therapeutic approaches," *Angewandte Chemie International Edition*, vol. 48, no. 17, pp. 3030–3059, 2009.

[2] M. Citron, "Alzheimer's disease: strategies for disease modification," *Nature Reviews Drug Discovery*, vol. 9, no. 5, pp. 387–398, 2010.

[3] A. Rauk, "The chemistry of Alzheimer's disease," *Chemical Society Reviews*, vol. 38, no. 9, pp. 2698–2715, 2009.

[4] L. E. Scott and C. Orvig, "Medicinal inorganic chemistry approaches to passivation and removal of aberrant metal ions in disease," *Chemical Reviews*, vol. 109, no. 10, pp. 4885–4910, 2009.

[5] J. A. Hardy and G. A. Higgins, "Alzheimer's disease: the amyloid cascade hypothesis," *Science*, vol. 256, no. 5054, pp. 184–185, 1992.

[6] J. Hardy and D. J. Selkoe, "The amyloid hypothesis of Alzheimer's disease: progress and problems on the road to therapeutics," *Science*, vol. 297, no. 5580, pp. 353–356, 2002.

[7] G. Verdile, S. Fuller, C. S. Atwood, S. M. Laws, S. E. Gandy, and R. N. Martins, "The role of beta amyloid in Alzheimer's disease: still a cause of everything or the only one who got caught?" *Pharmacological Research*, vol. 50, no. 4, pp. 397–409, 2004.

[8] C. Haass and D. J. Selkoe, "Soluble protein oligomers in neurodegeneration: lessons from the Alzheimer's amyloid β-peptide," *Nature Reviews Molecular Cell Biology*, vol. 8, no. 2, pp. 101–112, 2007.

[9] G. M. Shankar, S. Li, T. H. Mehta et al., "Amyloid-β protein dimers isolated directly from Alzheimer's brains impair synaptic plasticity and memory," *Nature Medicine*, vol. 14, no. 8, pp. 837–842, 2008.

[10] H.-G. Lee, G. Casadesus, X. Zhu, A. Takeda, G. Perry, and M. A. Smith, "Challenging the amyloid cascade hypothesis: senile plaques and amyloid-β as protective adaptations to Alzheimer disease," *Annals of the New York Academy of Sciences*, vol. 1019, pp. 1–4, 2004.

[11] S. J. Soscia, J. E. Kirby, K. J. Washicosky et al., "The Alzheimer's disease-associated amyloid β-protein is an antimicrobial peptide," *PLoS ONE*, vol. 5, no. 3, article no. e9505, 2010.

[12] J. J. Palop and L. Mucke, "Amyloid-β-induced neuronal dysfunction in Alzheimer's disease: from synapses toward neural networks," *Nature Neuroscience*, vol. 13, no. 7, pp. 812–818, 2010.

[13] C. J. Maynard, A. I. Bush, C. L. Masters, R. Cappai, and Q.-X. Li, "Metals and amyloid-β in Alzheimer's disease," *International Journal of Experimental Pathology*, vol. 86, no. 3, pp. 147–159, 2005.

[14] K. J. Barnham and A. I. Bush, "Metals in Alzheimer's and Parkinson's diseases," *Current Opinion in Chemical Biology*, vol. 12, no. 2, pp. 222–228, 2008.

[15] A. I. Bush and R. E. Tanzi, "Therapeutics for Alzheimer's disease based on the metal hypothesis," *Neurotherapeutics*, vol. 5, no. 3, pp. 421–432, 2008.

[16] J. A. Duce and A. I. Bush, "Biological metals and Alzheimer's disease: implications for therapeutics and diagnostics," *Progress in Neurobiology*, vol. 92, no. 1, pp. 1–18, 2010.

[17] F. Molina-Holgado, R. C. Hider, A. Gaeta, R. Williams, and P. Francis, "Metals ions and neurodegeneration," *Biometals*, vol. 20, no. 3-4, pp. 639–654, 2007.

[18] E. Gaggelli, H. Kozlowski, D. Valensin, and G. Valensin, "Copper homeostasis and neurodegenerative disorders (Alzheimer's, prion, and Parkinson's diseases and amyotrophic lateral sclerosis)," *Chemical Reviews*, vol. 106, no. 6, pp. 1995–2044, 2006.

[19] P. Zatta, D. Drago, S. Bolognin, and S. L. Sensi, "Alzheimer's disease, metal ions and metal homeostatic therapy," *Trends in Pharmacological Sciences*, vol. 30, no. 7, pp. 346–355, 2009.

[20] X. Zhu, B. Su, X. Wang, M. A. Smith, and G. Perry, "Causes of oxidative stress in Alzheimer disease," *Cellular and Molecular Life Sciences*, vol. 64, no. 17, pp. 2202–2210, 2007.

[21] P. Faller, "Copper and zinc binding to amyloid-β: coordination, dynamics, aggregation, reactivity and metal-ion transfer," *ChemBioChem*, vol. 10, no. 18, pp. 2837–2845, 2009.

[22] P. Faller and C. Hureau, "Bioinorganic chemistry of copper and zinc ions coordinated to amyloid-β peptide," *Dalton Transactions*, no. 7, pp. 1080–1094, 2009.

[23] C. Hureau and P. Faller, "Aβ-mediated ROS production by Cu ions: structural insights, mechanisms and relevance to Alzheimer's disease," *Biochimie*, vol. 91, no. 10, pp. 1212–1217, 2009.

[24] M. A. Lovell, J. D. Robertson, W. J. Teesdale, J. L. Campbell, and W. R. Markesbery, "Copper, iron and zinc in Alzheimer's disease senile plaques," *Journal of the Neurological Sciences*, vol. 158, no. 1, pp. 47–52, 1998.

[25] L. M. Miller, Q. Wang, T. P. Telivala, R. J. Smith, A. Lanzirotti, and J. Miklossy, "Synchrotron-based infrared and X-ray imaging shows focalized accumulation of Cu and Zn co-localized with β-amyloid deposits in Alzheimer's disease," *Journal of Structural Biology*, vol. 155, no. 1, pp. 30–37, 2006.

[26] F. Ricchelli, D. Drago, B. Filippi, G. Tognon, and P. Zatta, "Aluminum-triggered structural modifications and aggregation of β-amyloids," *Cellular and Molecular Life Sciences*, vol. 62, no. 15, pp. 1724–1733, 2005.

[27] M. P. Cuajungco, K. Y. Fagét, X. Huang, R. E. Tanzi, and A. I. Bush, "Metal chelation as a potential therapy for Alzheimer's disease," *Annals of the New York Academy of Sciences*, vol. 920, pp. 292–304, 2000.

[28] P. J. Crouch, A. R. White, and A. I. Bush, "The modulation of metal bio-availability as a therapeutic strategy for the treatment of Alzheimer's disease," *The FEBS Journal*, vol. 274, no. 15, pp. 3775–3783, 2007.

[29] S. Bolognin, D. Drago, L. Messori, and P. Zatta, "Chelation therapy for neurodegenerative diseases," *Medicinal Research Reviews*, vol. 29, no. 4, pp. 547–570, 2009.

[30] R. A. Cherny, C. S. Atwood, M. E. Xilinas et al., "Treatment with a copper-zinc chelator markedly and rapidly inhibits β-amyloid accumulation in Alzheimer's disease transgenic mice," *Neuron*, vol. 30, no. 3, pp. 665–676, 2001.

[31] C. W. Ritchie, A. I. Bush, A. Mackinnon et al., "Metal-protein attenuation with iodochlorhydroxyquin (clioquinol) targeting Aβ amyloid deposition and toxicity in Alzheimer disease: a pilot phase 2 clinical trial," *Archives of Neurology*, vol. 60, no. 12, pp. 1685–1691, 2003.

[32] P. A. Adlard, R. A. Cherny, D. I. Finkelstein et al., "Rapid restoration of cognition in Alzheimer's transgenic mice with 8-hydroxy quinoline analogs is associated with decreased interstitial Aβ," *Neuron*, vol. 59, no. 1, pp. 43–55, 2008.

[33] L. Lannfelt, K. Blennow, H. Zetterberg et al., "Safety, efficacy, and biomarker findings of PBT2 in targeting Aβ as a modifying therapy for Alzheimer's disease: a phase IIa, double-blind, randomised, placebo-controlled trial," *The Lancet Neurology*, vol. 7, no. 9, pp. 779–786, 2008.

[34] M. B. Cardelli, M. Russell, C. A. Bagne, and N. Pomara, "Chelation therapy. Unproved modality in the treatment of

Alzheimer-type dementia," *Journal of the American Geriatrics Society*, vol. 33, no. 8, pp. 548–551, 1985.

[35] J.-Y. Lee, J. E. Friedman, I. Angel, A. Kozak, and J.-Y. Koh, "The lipophilic metal chelator DP-109 reduces amyloid pathology in brains of human β-amyloid precursor protein transgenic mice," *Neurobiology of Aging*, vol. 25, no. 10, pp. 1315–1321, 2004.

[36] J. L. Arbiser, S.-K. Kraeft, R. van Leeuwen et al., "Clioquinol-zinc chelate: a candidate causative agent of subacute myelo-optic neuropathy," *Molecular Medicine*, vol. 4, no. 10, pp. 665–670, 1998.

[37] C. Hureau, I. Sasaki, E. Gras, and P. Faller, "Two functions, one molecule: a metal-binding and a targeting moiety to combat Alzheimer's disease," *ChemBioChem*, vol. 11, no. 7, pp. 950–953, 2010.

[38] L. R. Perez and K. J. Franz, "Minding metals: tailoring multifunctional chelating agents for neurodegenerative disease," *Dalton Transactions*, vol. 39, no. 9, pp. 2177–2187, 2010.

[39] A. Dedeoglu, K. Cormier, S. Payton et al., "Preliminary studies of a novel bifunctional metal chelator targeting Alzheimer's amyloidogenesis," *Experimental Gerontology*, vol. 39, no. 11-12, pp. 1641–1649, 2004.

[40] W.-h. Wu, P. Lei, Q. Liu et al., "Sequestration of copper from β-amyloid promotes selective lysis by cyclen-hybrid cleavage agents," *Journal of Biological Chemistry*, vol. 283, no. 46, pp. 31657–31664, 2008.

[41] C. Rodríguez-Rodríguez, N. Sánchez de Groot, A. Rimola et al., "Design, selection, and characterization of thioflavin-based intercalation compounds with metal chelating properties for application in Alzheimer's disease," *Journal of the American Chemical Society*, vol. 131, no. 4, pp. 1436–1451, 2009.

[42] S. S. Hindo, A. M. Mancino, J. J. Braymer et al., "Small molecule modulators of copper-induced Aβ aggregation," *Journal of the American Chemical Society*, vol. 131, no. 46, pp. 16663–16665, 2009.

[43] P. S. Vassar and C. F. A. Culling, "Fluorescent stains, with special reference to amyloid and connective tissues," *Archives of Pathology*, vol. 68, no. 5, pp. 487–498, 1959.

[44] H. LeVine III, "Thioflavine T interaction with synthetic Alzheimer's disease β-amyloid peptides: detection of amyloid aggregation in solution," *Protein Science*, vol. 2, no. 3, pp. 404–410, 1993.

[45] R. Leuma Yona, S. Mazères, P. Faller, and E. Gras, "Thioflavin derivatives as markers for amyloid-β fibrils: insights into structural features important for high-affinity binding," *ChemMedChem*, vol. 3, no. 1, pp. 63–66, 2008.

[46] C. Rodríguez-Rodríguez, A. Rimola, L. Rodríguez-Santiago et al., "Crystal structure of thioflavin-T and its binding to amyloid fibrils: insights at the molecular level," *Chemical Communications*, vol. 46, no. 7, pp. 1156–1158, 2010.

[47] W. E. Klunk, Y. Wang, G.-f. Huang, M. L. Debnath, D. P. Holt, and C. A. Mathis, "Uncharged thioflavin-T derivatives bind to amyloid-beta protein with high affinity and readily enter the brain," *Life Sciences*, vol. 69, no. 13, pp. 1471–1484, 2001.

[48] C. A. Lipinski, F. Lombardo, B. W. Dominy, and P. J. Feeney, "Experimental and computational approaches to estimate solubility and permeability in drug discovery and development settings," *Advanced Drug Delivery Reviews*, vol. 46, no. 1–3, pp. 3–26, 2001.

[49] D. E. Clark and S. D. Pickett, "Computational methods for the prediction of 'drug-likeness'," *Drug Discovery Today*, vol. 5, no. 2, pp. 49–58, 2000.

[50] W. E. Klunk, H. Engler, A. Nordberg et al., "Imaging brain amyloid in Alzheimer's disease with Pittsburgh compound-B," *Annals of Neurology*, vol. 55, no. 3, pp. 306–319, 2004.

[51] D. A. Wolk and W. E. Klunk, "Update on amyloid imaging: from healthy aging to Alzheimer's disease," *Current Neurology and Neuroscience Reports*, vol. 9, no. 5, pp. 345–352, 2009.

[52] C. Soto, E. M. Sigurdsson, L. Morelli, R. A. Kumar, E. M. Castaño, and B. Frangione, "β-sheet breaker peptides inhibit fibrillogenesis in a rat brain model of amyloidosis: implications for Alzheimer's therapy," *Nature Medicine*, vol. 4, no. 7, pp. 822–826, 1998.

[53] J. F. Poduslo, G. L. Curran, A. Kumar, B. Frangione, and C. Soto, "β-Sheet breaker peptide inhibitor of Alzheimer's amyloidogenesis with increased blood-brain barrier permeability and resistance to proteolytic degradation in plasma," *Journal of Neurobiology*, vol. 39, no. 3, pp. 371–382, 1999.

[54] L. O. Tjernberg, J. Näslund, F. Lindqvist et al., "Arrest of β-amyloid fibril formation by a pentapeptide ligand," *Journal of Biological Chemistry*, vol. 271, no. 15, pp. 8545–8548, 1996.

[55] J. Ghanta, C.-L. Shen, L. L. Kiessling, and R. M. Murphy, "A strategy for designing inhibitors of β-amyloid toxicity," *Journal of Biological Chemistry*, vol. 271, no. 47, pp. 29525–29528, 1996.

[56] M. M. Pallitto, J. Ghanta, P. Heinzelman, L. L. Kiessling, and R. M. Murphy, "Recognition sequence design for peptidyl modulators of β-amyloid aggregation and toxicity," *Biochemistry*, vol. 38, no. 12, pp. 3570–3578, 1999.

[57] T. Chen, X. Wang, Y. He et al., "Effects of cyclen and cyclam on zinc(II)- and copper(II)-induced amyloid β-peptide aggregation and neurotoxicity," *Inorganic Chemistry*, vol. 48, no. 13, pp. 5801–5809, 2009.

[58] J. Suh and W. S. Chei, "Metal complexes as artificial proteases: toward catalytic drugs," *Current Opinion in Chemical Biology*, vol. 12, no. 2, pp. 207–213, 2008.

[59] K. Ono, K. Hasegawa, H. Naiki, and M. Yamada, "Curcumin has potent anti-amyloidogenic effects for Alzheimer's β-amyloid fibrils in vitro," *Journal of Neuroscience Research*, vol. 75, no. 6, pp. 742–750, 2004.

[60] E. K. Ryu, Y. S. Choe, K.-H. Lee, Y. Choi, and B.-T. Kim, "Curcumin and dehydrozingerone derivatives: synthesis, radiolabeling, and evaluation for β-amyloid plaque imaging," *Journal of Medicinal Chemistry*, vol. 49, no. 20, pp. 6111–6119, 2006.

[61] J. Kim, H. J. Lee, and K. W. Lee, "Naturally occurring phytochemicals for the prevention of Alzheimer's disease," *Journal of Neurochemistry*, vol. 112, no. 6, pp. 1415–1430, 2010.

[62] A. A. Reinke and J. E. Gestwicki, "Structure-activity relationships of amyloid beta-aggregation inhibitors based on curcumin: influence of linker length and flexibility," *Chemical Biology and Drug Design*, vol. 70, no. 3, pp. 206–215, 2007.

[63] M.-P. Kung, C. Hou, Z.-P. Zhuang et al., "IMPY: an improved thioflavin-T derivative for in vivo labeling of β-amyloid plaques," *Brain Research*, vol. 956, no. 2, pp. 202–210, 2002.

[64] M.-P. Kung, C. Hou, Z.-P. Zhuang, D. Skovronsky, and H. F. Kung, "Binding of two potential imaging agents targeting amyloid plaques in postmortem brain tissues of patients with Alzheimer's disease," *Brain Research*, vol. 1025, no. 1-2, pp. 98–105, 2004.

[65] M.-P. Kung, C. Hou, Z.-P. Zhuang, A. J. Cross, D. L. Maier, and H. F. Kung, "Characterization of IMPY as a potential imaging agent for β-amyloid plaques in double transgenic PSAPP mice," *European Journal of Nuclear Medicine and Molecular Imaging*, vol. 31, no. 8, pp. 1136–1145, 2004.

[66] A. B. Newberg, N. A. Wintering, K. Plössl et al., "Safety,

biodistribution, and dosimetry of ^{123}I-IMPY: a novel amyloid plaque-imaging agent for the diagnosis of Alzheimer's disease," *Journal of Nuclear Medicine*, vol. 47, no. 5, pp. 748–754, 2006.

[67] M.-P. Kung, Z.-P. Zhuang, C. Hou, L.-W. Jin, and H. F. Kung, "Characterization of radioiodinated ligand binding to amyloid β plaques," *Journal of Molecular Neuroscience*, vol. 20, no. 3, pp. 249–253, 2003.

[68] L. Cai, J. Cuevas, S. Temme et al., "Synthesis and structure-affinity relationships of new 4-(6-iodo-*H*-imidazo[1,2-*a*]pyridin-2-yl)-*N*-dimethylbenzeneamine derivatives as ligands for human β-amyloid plaques," *Journal of Medicinal Chemistry*, vol. 50, no. 19, pp. 4746–4758, 2007.

[69] H. F. Kung, C.-W. Lee, Z.-P. Zhuang, M.-P. Kung, C. Hou, and K. Plössl, "Novel stilbenes as probes for amyloid plaques," *Journal of the American Chemical Society*, vol. 123, no. 50, pp. 12740–12741, 2001.

[70] W. Zhang, S. Oya, M.-P. Kung, C. Hou, D. L. Maier, and H. F. Kung, "F-18 stilbenes as PET imaging agents for detecting β-amyloid plaques in the brain," *Journal of Medicinal Chemistry*, vol. 48, no. 19, pp. 5980–5988, 2005.

[71] H. F. Kung, S. R. Choi, W. Qu, W. Zhang, and D. Skovronsky, "^{18}F stilbenes and styrylpyridines for PET imaging of Aβ plaques in Alzheimer's disease: a miniperspective," *Journal of Medicinal Chemistry*, vol. 53, no. 3, pp. 933–941, 2010.

[72] J.-S. Choi, J. J. Braymer, R. P. R. Nanga, A. Ramamoorthy, and M. H. Lim, "Design of small molecules that target metal-Aβ species and regulate metal-induced Aβ aggregation and neurotoxicity," 2010, *Proceedings of the National Academy of Sciences of the United States of America*. In press.