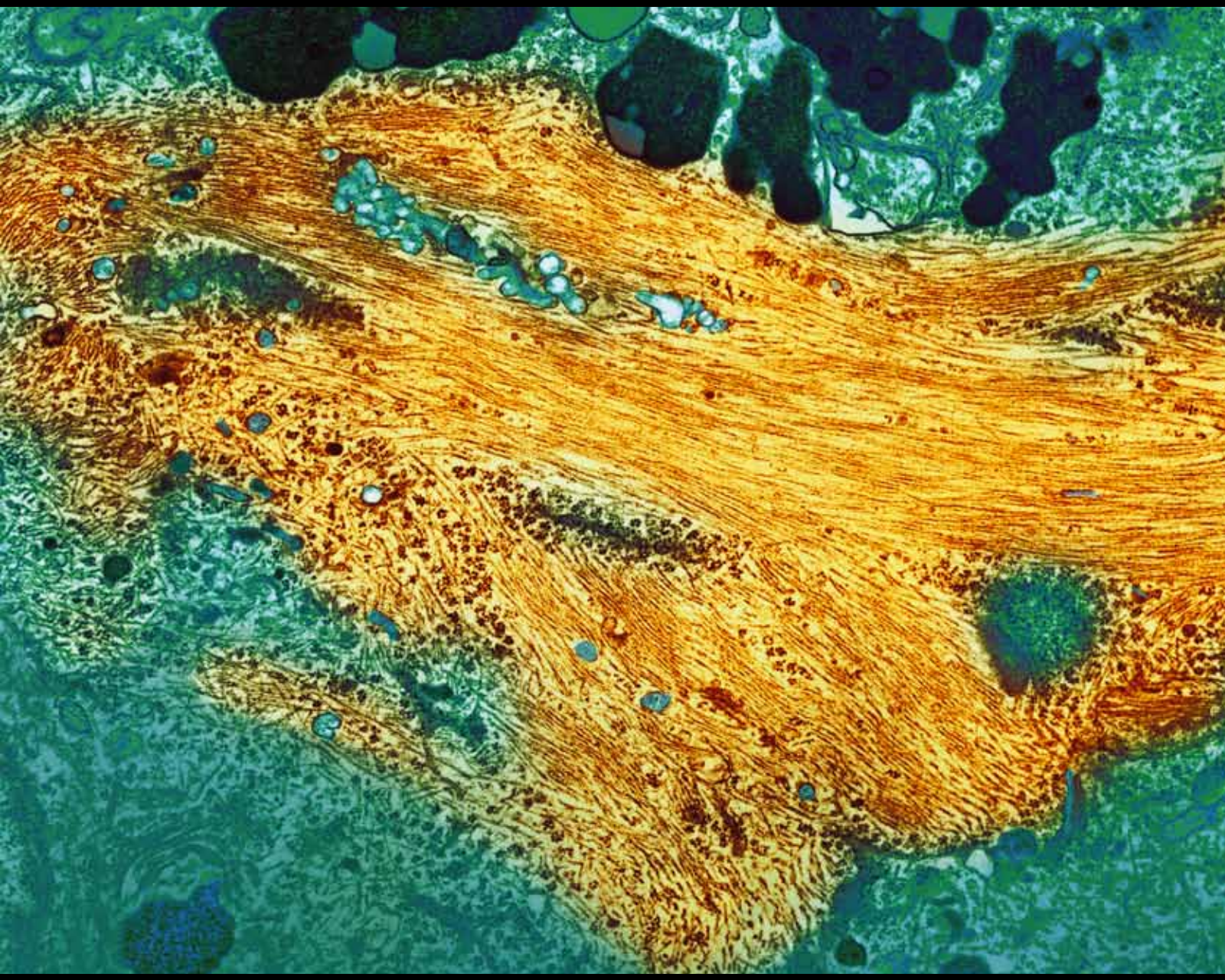
International Journal of Alzheimer's Disease

Copper Status in Alzheimer's Disease and Other Neurodegenerative Disorders 2013

Guest Editors: Rosanna Squitti, Tjaard Hoogenraad, George Brewer, Ashley I. Bush, and Renato Polimanti



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Editorial Board

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Editorial

Copper Status in Alzheimer's Disease and Other Neurodegenerative Disorders 2013

Rosanna Squitti,[1,2] Tjaard Hoogenraad,[3] George Brewer,[4] Ashley I. Bush,[5] and Renato Polimanti[6]

[1] *Department of Neuroscience, AFaR-Ospedale "San Giovanni Calibita" Fatebenefratelli, Isola Tiberina, 00186 Rome, Italy*
[2] *Neurodegeneration Laboratory, IRCCS San Raffaele Pisana, 00166 Rome, Italy*
[3] *Department of Neurology, University Hospital Utrecht, 3584 CX Utrecht, The Netherlands*
[4] *Departments of Human Genetics and Internal Medicine, University of Michigan Medical School, Ann Arbor, MI 48109, USA*
[5] *Florey Institute of Neuroscience & Mental Health, University of Melbourne, Melbourne, VIC 3053, Australia*
[6] *Department of Biology, University of Rome "Tor Vergata", 00133 Rome, Italy*

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

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In recent years, the number of studies about the role of copper metabolism in the pathophysiology of Alzheimer's disease (AD) has been rapidly increasing. A wide range of experimental approaches have been used to explore this important issue: in vitro analyses, investigations on animal models, bioinformatic surveys, and patients' studies. Most of these studies have indicated that systemic disarrangements of copper metabolism can be one of the pathologic pathways at the basis of AD, and moreover, preventive and therapeutic strategies based on copper may be developed in order to slow down or to block the disease progression. However, further studies are necessary to translate these findings into clinical practice. In 2011, the International Journal of Alzheimer's Disease (IJAD) published the annual issue "*Copper Status in Alzheimer's Disease and Other Neurodegenerative Disorders*", in order to collect some relevant studies about copper and AD. After two years, due to the growing literature about this topic, we and IJAD have decided to present the 2013 annual issue. In this issue, studies from different international authors are published about the recent advances in the field of copper metabolism in AD. We decided to include investigations based on different approaches in order to display the multiplicity of experimental methodologies, currently present to investigate this important subject.

One of the paper of this annual issue is a review article by B. G. Schreurs about the use of the AD rabbit model based on a 2% cholesterol diet to explore the role of copper in drinking water in determining the AD progression, especially highlighting the outstanding contribution of the late D. L. Sparks.

Another paper is provided by K. Singh and collaborators. They present novel data about the positive effects of a new copper chelator, L-2,6-pyridinedicarboxylic acid, 2,6-bis[2-[(4-carboxyphenyl) methylene] hydrazide], in the eye tissues of transgenic Drosophila expressing beta-amyloid (Aβ).

One of the paper is a review article by G. J. Brewer and S. Kaur about the importance of ingestion of inorganic copper (such as that of drinking water) as a possible factor of AD causation and of zinc therapy to reduce serum "free" copper levels and, consequently, to slow down cognition loss in AD patients. They also furnished novel data about a 6-month small double blind trial of a new zinc formulation on AD patients.

In another paper, A. Rembach and colleagues show their outcomes on the brain-specific alteration in copper levels of AD patients. Specifically, they observed that the decrease of copper in the human frontal cortex was confined in the soluble fractions.

Two of the papers are two research articles provided by N. Arnal and collaborators. In these papers, they display the interactions between copper and cholesterol in neurodegenerative processes of Wistar rats and the effects of copper and cholesterol on mitochondrial function in a rat model.

Another paper is a review article by X. Y. Choo and collaborators. In this review, the authors focused their attention on AD-related inflammation, highlighting recent advances on the role of copper as modulator of inflammatory processes in AD.

Finally, last but not least, there is the paper by C. Salustri and collaborators. In this research article, the authors provide novel information about the role of copper in the neurophysiology of AD. Specifically, they investigated whether copper dysfunction affects neurotransmission in AD patients, highlighting the association between copper and glutamatergic and Gabaergic transmission.

In conclusion, we provided an issue with a wide range of experimental evidence about the relevant role of copper in AD pathogenesis. Although we are still far to completely understand this important topic, the data present can help the reader to better understand the recent advance in this research field.

Rosanna Squitti
Tjaard Hoogenraad
George Brewer
Ashley I. Bush
Renato Polimanti

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Clinical Study

Sensorimotor Cortex Reorganization in Alzheimer's Disease and Metal Dysfunction: A MEG Study

C. Salustri,[1] F. Tecchio,[1,2] F. Zappasodi,[3] L. Tomasevic,[1] M. Ercolani,[4] F. Moffa,[4] E. Cassetta,[4] P. M. Rossini,[2,5] and R. Squitti[4,6]

[1] *Institute of Cognitive Sciences and Technologies (CNR), Unità MEG, Fatebenefratelli Hospital, Isola Tiberina, 00186 Rome, Italy*
[2] *Department of Imaging, IRCCS San Raffaele Pisana, 00163 Rome, Italy*
[3] *Department of Neuroscience and Imaging, Gabriele d'Annunzio University, 66100 Chieti, Italy*
[4] *AFaR, Department of Neuroscience, Fatebenefratelli Hospital, Isola Tiberina, 00186 Rome, Italy*
[5] *Institute of Neurology, Department of Neuroscience, Catholic University, A. Gemelli Polyclinic, 00168 Rome, Italy*
[6] *Laboratory of Neurodegeneration, IRCCS San Raffaele Pisana, 00163 Rome, Italy*

Correspondence should be addressed to C. Salustri; carlo.salustri@istc.cnr.it

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Objective. To verify whether systemic biometals dysfunctions affect neurotransmission in living Alzheimer's disease (AD) patients. *Methods*. We performed a case-control study using magnetoencephalography to detect sensorimotor fields of AD patients, at rest and during median nerve stimulation. We analyzed position and amount of neurons synchronously activated by the stimulation in both hemispheres to investigate the capability of the primary somatosensory cortex to reorganize its circuitry disrupted by the disease. We also assessed systemic levels of copper, ceruloplasmin, non-Cp copper (i.e., copper not bound to ceruloplasmin), peroxides, transferrin, and total antioxidant capacity. *Results*. Patients' sensorimotor generators appeared spatially shifted, despite no change of latency and strength, while spontaneous activity sources appeared unchanged. Neuronal reorganization was greater in moderately ill patients, while delta activity increased in severe patients. Non-Cp copper was the only biological variable appearing to be associated with patient sensorimotor transmission. *Conclusions*. Our data strengthen the notion that non-Cp copper, not copper in general, affects neuronal activity in AD. *Significance*. High plasticity in the disease early stages in regions controlling more commonly used body parts strengthens the notion that physical and cognitive activities are protective factors against progression of dementia.

1. Introduction

In the last decade, increasing evidence has revealed the role of biometals dysfunctions in Alzheimer's disease (AD) (see [1] for a review). AD is characterized by a progressive degradation of cognitive abilities, inexorably leading to dementia, due to a gradual loss of neurons and synapses. Also sensorimotor deficits are characteristic of the disease but they usually appear in the late severe stages of the disease.

Topographic and frequency changes of the sensorimotor cortex's electro- and magneto-encephalographic activities have been observed to follow the progression of the disease [2, 3]. These changes are not attributable to lesion densities, since the density of plaques and tangles in the sensorimotor cortex of AD brains has been shown to be equivalent to that of other brain areas more specific to AD [4]. Evidently, other phenomena play a role and one of them is likely to be the sensorimotor cortex's formidable capability to reorganize its circuitry damaged by AD degeneration into new and still healthy locations [3, 5, 6].

The aim of the present paper was to investigate reorganization within the primary sensorimotor cortex in AD and verify the role of systemic metal derangements in this reorganization. We studied both rest and stimulus-evoked magnetoencephalographic (MEG) activities in the sensorimotor cortex of a group of AD patients and a matched group of controls. The study of the spontaneous activity focused on the oscillatory rhythms, while we investigated the evoked

activity focusing on the first two components of the magnetic somatosensory Evoked Fields (SEFs), M20 and M30, recorded during stimulation of the median nerve. M20 is well known to represent the first synchronous activation of Brodmann area 3b, when the contralateral median nerve is stimulated [7]. It is stable, highly repeatable, and completely independent of subject's attention [8]. It is mediated by glutamate neurotransmission [9, 10]. M30, instead, appears to be generated by a joint contribution of the motor and somatosensory cortices and is mediated by a major contribution of both glutamate and Gaba neurotransmitters. Levels of copper, ceruloplasmin, non-Cp copper, peroxides, transferrin, and total antioxidant (TAS) capacity were correlated with M20 and M30 indices in both healthy and AD subjects.

2. Materials and Methods

The study was approved by the local ethics committee and each participant or caregiver signed an informed consent.

2.1. Subjects. Sixteen patients, age 55 to 86 years (mean 7 ± 7 years, 13 females), were enrolled in the study. "Probable AD" had been diagnosed in all patients according to NINCDS-ADRDA criteria [11]. Patients underwent general medical, neurological, and psychiatric assessments and were rated with standard neuropsychological instruments which included the mini mental state examination (MMSE) [12], the clinical dementia rating scale [13], the geriatric depression scale [14], the Hachinski ischemic scale [15], and the instrumental activities of daily living [16]. They also underwent neuroimaging diagnostics and standard laboratory analyses to rule out other causes of dementias. Exclusion criteria were (i) fronto-temporal dementia; (ii) vascular dementia; (iii) extra-pyramidal syndromes; (iv) Parkinson's disease; (v) reversible causes of dementias; (vi) Lewy body dementia. Detection of vascular burden was based on guidelines previously developed within our clinical network [17].

The control sample was made of 16 subjects (7 females) age-matched to the AD patients (mean age 72 ± 14 years, two-tailed t-tests $P = 0.801$). All controls underwent physical, neurological, and neuropsychological examinations.

2.2. MRI Investigation. Brain MRI was performed at 1.5 tesla. The imaging protocol consisted of axial T1W spin echo and T2W double Spin Echo sequences in axial, coronal, and sagittal planes, with 5 mm slice thickness and interslice gap = 0.5 mm. MR images were evaluated by two experienced neuroradiologists blind to the patients' diagnoses or laboratory results, who approached total agreement (95%). Atrophy was graded following standard visual rating scales on plain MRI. The degree of medial temporal lobe atrophy 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 the standard five-point rating scale of medial temporal lobe atrophy (MTA). Generalized brain atrophy (ventricular and sulcal atrophy) was rated as absent (=0) and present (=1). It must be noted that some degree of atrophy is normally present in the old age. Thus, atrophy was classified as absent when falling within the moderate levels shared by all elderly individuals.

2.3. MEG Investigation. A 28-channel MEG system [18], covering a total scalp area of about 180 cm^2, operating inside a magnetically shielded room (Vacuumschmelze GMBH), was used to record brain magnetic fields from left and right hemispheres. The sensors were positioned over the area of the contralateral sensorimotor cortex controlling the hand (C3/C4 site of the International 10–20 EEG system). Subjects lay on a nonmagnetic bed, with their eyes open to reduce disturbance by alpha activity. The recording procedure lasted about 30 min for all subjects.

Magnetic evoked fields were recorded under electrical stimulation of the contralateral median nerve at the wrist by 0.2 ms electric pulses (cathode proximal), with 631 ms interstimulus interval. Stimulus intensities were individually set just above the threshold inducing a painless twitch of the thumb.

Spontaneous brain activity with eyes open was recorded continuously for three minutes on each hemisphere.

Signals were bandpass filtered between 0.48–250 Hz, sampled at 1 kHz and stored for off-line process.

2.4. Biochemical Investigations. Sera from overnight fasting blood samples were drawn in the morning and rapidly stored at −80°C. Biochemical variables were determined according to the established methods reported in details elsewhere [19]. Briefly, serum copper concentration was measured following the method of Abe et al. [20] (Randox Laboratories, Crumlin, UK) and with an Analyst 600 Perkin Elmer atomic absorption spectrophotometer equipped with a graphite furnace with platform HGA 800. Transferrin [21] and ceruloplasmin [22] were measured by immunoturbidimetric assays (Horiba ABX, Montpellier, France). The iron level in serum was determined using Ferene colorimetric method (Horiba ABX, Montpellier, France). All biochemical measures were automated on a Cobas Mira Plus analyzer (Horiba ABX, Montpellier, France) and performed in duplicate. For each serum copper (total copper) and ceruloplasmin pair we computed the amount of copper bound to ceruloplasmin (CuB) and the amount of copper not bound to ceruloplasmin (free copper) following standard procedures [23]; briefly: CuB = ceruloplasmin (mg/dL) $*$ 10 $*$ n; $n = 0.0472$ (μmol/mg); free copper = total copper-CuB). This calculation expresses free copper in μmol/L and is based on the fact that ceruloplasmin contains 0.3% of copper [23]. Thus, for a subject with a serum copper concentration of 17.3 μmol/L and a serum ceruloplasmin concentration of 33 mg/dL, the bound copper concentration was $33 * 10 * 0.0472 = 15.6$ μmol/L, and the free copper concentration $17.3 - 15.6 = 1.7$ μmol/L.

2.5. Data Analysis. An *ad hoc* developed semiautomatic (i.e., partially based on visual inspection) artifact-rejection procedure [24] was first applied to all data to detect and discard the contribution of spurious sources, such as heart, eyes, and muscles, which normally generate signals in

the same frequency range of the sources under study. About 280 artifact-free trials were filtered between 3–150 Hz and averaged to obtain the time course of the magnetic evoked fields. The signal amplitude was measured as the deviation from a baseline defined as the mean value of the signal in the 5–15 ms poststimulus window.

M20 and M30 were identified by the maxima of the global field power within time windows centered respectively at 20 and 30 ms after stimulus delivery (Figure 1(a)). Latency, spatial coordinates and strength of the equivalent current dipoles (ECDs) generating M20 and M30 magnetic fields were computed by solving the inverse problem in the model of a dipole moving inside a homogeneously conducting sphere. ECD localizations were accepted only if they explained at least 90% of the variance. In our coordinate system (Figure 2), we defined as *displacement* the Euclidean distance between the patients' ECDs positions and the center of mass of the controls' ECDs.

Interhemispheric asymmetries were assessed by measuring the Euclidean distance between each component's left and right ECD positions, after relocating them in the same hemisphere (i.e., measuring in the left hemisphere after mirroring the right ECD position into the left hemisphere or vice versa).

Spectral characteristics of the background spontaneous activity were evaluated through the signal power spectral density (PSD), estimated for each MEG channel following the Welch procedure (2048 ms duration, Hanning window, 60% overlap, and about 180 artifact free trials used). The PSD mean of the 16 inner axial gradiometers, covering a circular area of about 12 cm diameter, was taken as the total PSD. Total signal power was computed by integrating the total PSD in the 2–44 Hz frequency interval. Spectral properties were investigated in the standard frequency bands: 2–3.5 Hz (delta), 4–7.5 Hz (theta), 8–12.5 Hz (alpha), 13–33 Hz (beta) and 33.5–44 Hz (gamma).

An MEG-MRI common reference system was used based on three head landmarks corresponding to nasion, left, and right preauricular points [25] (Figure 2). The MRI procedure lasted about 30 min and was well tolerated by all patients.

2.6. Statistical Analysis. M20 and M30 ECD coordinates and displacements displayed a Gaussian distribution (Kolmogorov-Smirnov test, $P > 0.2$). Absolute power values showed a Gaussian distribution after log transformation ($P > 0.2$).

In order to compare characteristics between patients and controls, ANOVA for repeated measures was applied to ECD latencies, positions and displacements, including *Hemisphere* (left, right) as within-subject factor and *Group* (patient, control) as between-subject factor. The 3-dimensional dipole coordinate vectors (x, y, and z) were used for the positions. The factor *Band* (delta, theta, alpha, beta, and gamma) was used when we analyzed the spontaneous activity spectral characteristics in left and right rolandic regions.

In order to pinpoint differences due to global atrophy patients were divided in two subgroups (atrophy, no-atrophy). ANOVA was then applied separately to ECD positions, displacements and delta and theta powers, with *Hemisphere* (left, right) as within-subjects and *Global Atrophy* (atrophy, no-atrophy) as between-subjects factors.

To pinpoint differences due to levels of illness progression, patients were divided in two subgroups, one including moderately ill patients (MMSE 25–20) and the other including severely ill patients (MMSE < 20). ANOVA was then applied separately to ECD displacements and delta and theta powers, with *Hemisphere* (left, right) as within-subjects and *Illness Severity* (severe, moderate, and controls) as between-subjects factors.

Inter-hemispheric asymmetries of the ECD positions were studied by ANOVA with *Component* as within-subjects and *Group* (patients, controls) as between-subjects factors.

3. Results

3.1. MEG Analysis of Plastic Phenomena

3.1.1. M20 and M30 ECDs Recruited by Median Nerve Stimulation. Table 1 shows the means of M20's and M30's latencies, position coordinates, and ECD strengths in patients and controls.

The M20 and M30 ECD positions of our patients resulted shifted with respect to the ones of our controls, as indicated by the ANOVA *Group* effect applied to the dipoles' coordinate vectors ($F(3, 18) = 4.146$, $P = 0.021$) (Figures 2 and 3 left). In particular, the patients' M20 and M30 appeared more posterior in both hemispheres, although the shift reached statistical significance only in the left hemisphere (post-hoc y-coordinate t-test: $P = 0.04$ and 0.02 for M20 and M30, resp.).

The sources' displacements showed evident differences between patients and controls in both hemispheres as indicated by the *Group* effect ($F(1, 20) = 6.587$, $P = 0.018$). Again, the difference resulted statistically significant only in the left hemisphere (Table 2). Inter-hemispheric asymmetries of ECD locations did not differ in patients and controls (*Group* effect $P > 0.5$, with no interaction with *Component*).

ANOVA applied to ECD's strengths and latencies showed no *Group* effect.

3.2. Spontaneous Activity. Since ANOVA applied to Rolandic rest activity showed a strong *Band* $*$ *Group* effect ($F(2.4, 59.6) = 7.052$; $P = 0.001$), we repeated the analysis separately for each frequency band. A *Group* effect was found in the delta ($F(2, 24) = 5.577$; $P = 0.010$) and theta ($F(2, 24) = 3.383$; $P = 0.051$) bands. Patients showed a higher power in low-frequency bands than controls in both hemispheres.

To test whether the spatial displacements observed in AD patients were due to structural displacements secondary to cortical atrophy, we investigated whether any rest activity was generated from cortical regions more frontal than the M20 generator. We considered the cortical patches 1.5 cm anterior and posterior to the central sulcus, and estimated the percentage of background activity dipoles falling in this spatial range both in patients and controls. A general linear model with *Hemisphere* (left, right) as within-subjects factor

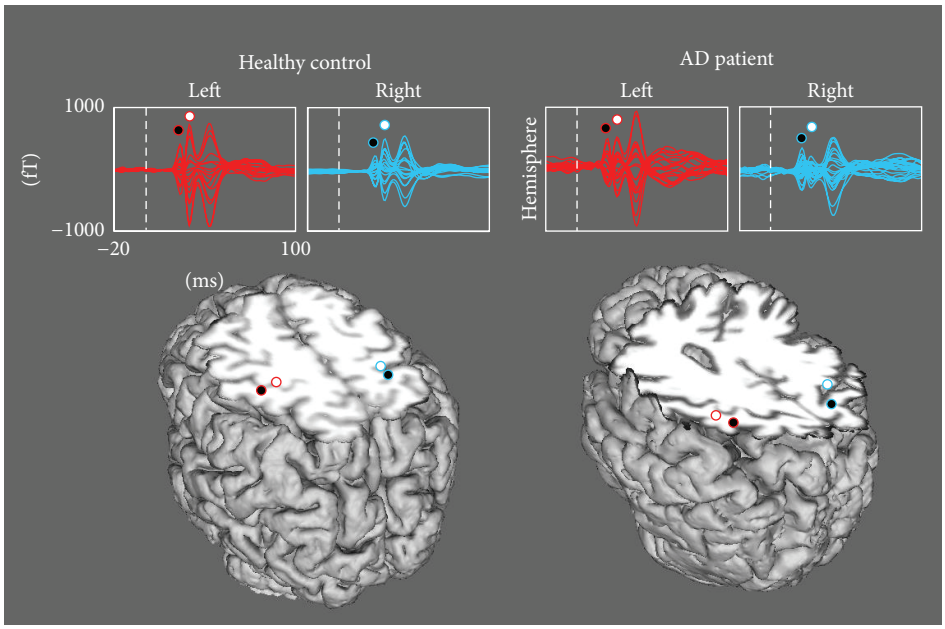

Figure 1: SEF and M20 and M30 ECD positions in a representative healthy control (*F*, 68 y.o., MMSE = 29) and an AD patient (*F*, 63 y.o., MMSE = 24). (Top) superimposition of all channels in the rolandic region, averaged in the [−20, 100] ms period, 0 representing the time of stimulus delivery. The amplitude of the magnetic field is represented in femtotesla (fT) on the *y*-axis; (Bottom) positions of the ECDs explaining the cerebral activation in correspondence to the M20 (black circle) and M30 (white circle) components, projected onto a suitable axial slice after volumetric head reconstruction from individual MR images.

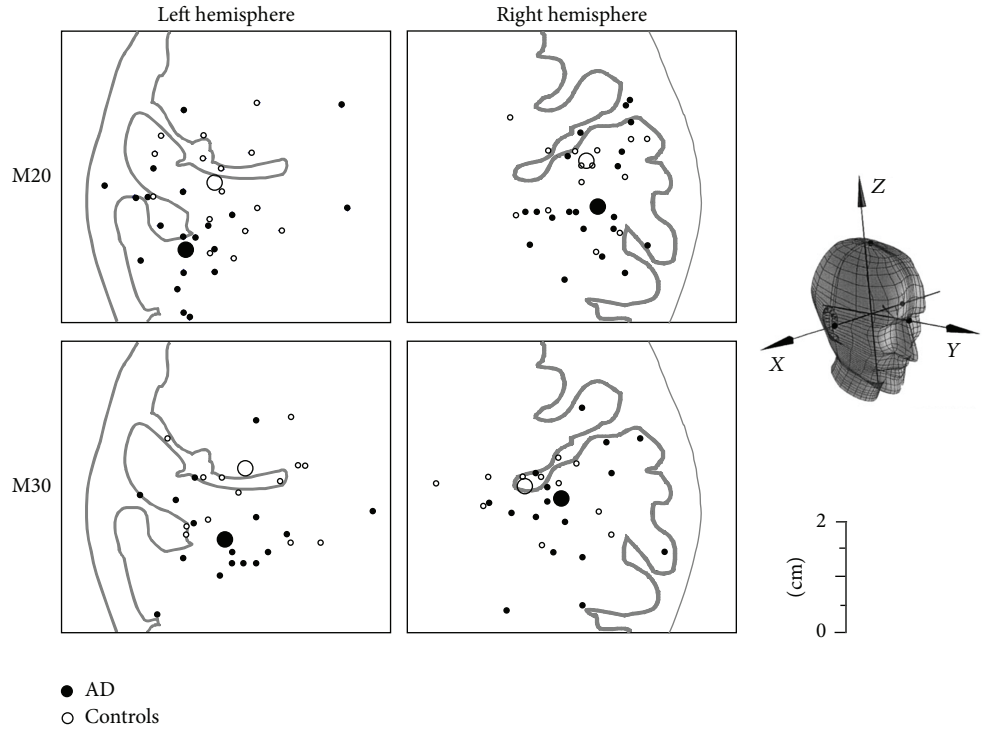

Figure 2: M20 and M30 ECD positions. Positions of individual M20 (top) and M30 (bottom) ECDs in healthy controls (empty dot) and AD patients (black dot) and of the average across the two groups (larger circles) projected on a representative axial graphic rendering in the region of the hand control. *Right side*: spatial coordinate system: the *y*-axis runs through the midpoint between the subject's preauricular points and his/her nasion; the *z*-axis is the line perpendicular to the *y*-axis that passes through the vertex; the *x*-axis is the line perpendicular to both *y* and *z*-axes that runs through their intersection. The intersection of the *x*-, *y*- and *z*-axes is the origin of the subject-based coordinate system, and *x* is positive on the head's right side, *y* towards the nasions and *z* towards the vertex.

Table 1: M20 and M30 ECD characteristics in patients and controls.

	Left hemisphere					Right hemisphere				
	t (ms)	x (mm)	y (mm)	z (mm)	s (nA m)	t (ms)	x (mm)	y (mm)	z (mm)	s (nA m)
M20										
Patients	21.3 ± 2.0	−48,9 ± 8,7	−20,9 ± 10.7	64,3 ± 15,6	17.9 ± 7.8	21.3 ± 1.9	48,5 ± 9,0	−14,5 ± 10,2	70,3 ± 13,9	15.7 ± 6.5
Controls	22.3 ± 1.7	−44,2 ± 7,0	−13,4 ± 9,3	67,7 ± 9,3	21.8 ± 11.6	22,3 ± 2.3	44,9 ± 7,5	−11,4 ± 9,8	71,7 ± 9,0	19.2 ± 8.0
M30										
Patients	28.9 ± 3.4	−37,6 ± 25,4	−21,5 ± 8,8	65,7 ± 15,3	29.2 ± 17.8	29.6 ± 4.6	41,9 ± 8,0	−19,7 ± 11,9	69,5 ± 15,1	26.3 ± 14.4
Controls	30.5 ± 3.4	−39,8 ± 9,0	−13,5 ± 7,3	69,1 ± 9,0	29.1 ± 18.4	30.3 ± 4.0	38,6 ± 9,4	−14,6 ± 7,9	74.8 ± 8,3	26.4 ± 16.4

Mean and standard deviation of latencies (t), positions (x, y, z) and strength (s) of equivalent current dipoles explaining M20 and M30 magnetic field distributions (see methods).

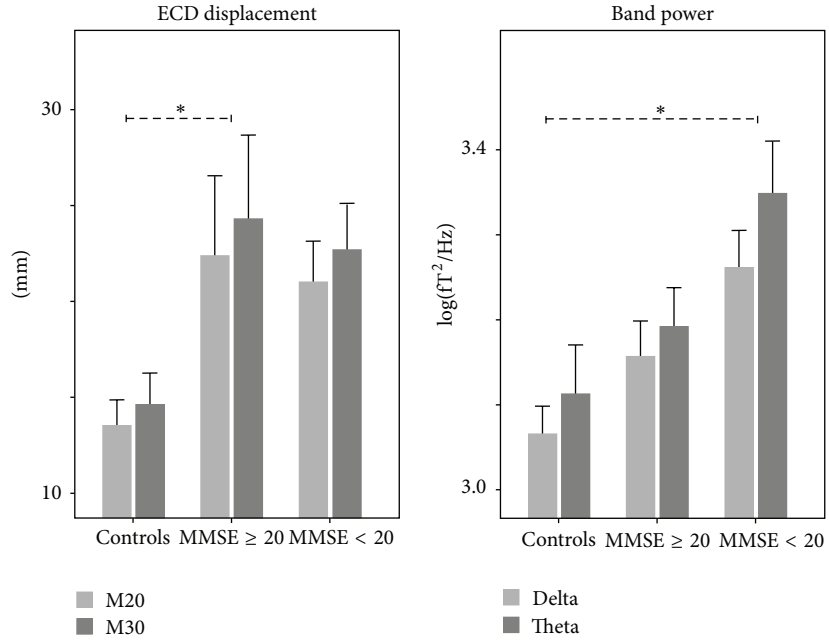

Figure 3: Patients' clinical severity in relationship with left displacements and rest low band powers. Mean and standard errors in controls and patients are subdivided in moderate (MMSE 25–21) and severe (MMSE ≤ 20) clinical severity. *Left*: M20 and M30 ECD in the left hemisphere. *Right*: delta and theta bend powers of left and right activity in resting state.

Table 2: M20 and M30 ECD displacements.

	M20		M30	
	Left H	Right H	Left H	Right H
Controls	13.3 ± 5.9	13.5 ± 6.4	13.1 ± 5.5	12.9 ± 6.3
Patients	21.3 ± 7.0	17.7 ± 8.5	19.1 ± 8.1	23.2 ± 21.2
P	**0.001**	0.132	**0.037**	0.121

Mean ± standard deviation of the displacements (mm) of M20 and M30 sources in the two hemispheres (H) for controls and patients. The last raw shows the P value of two-tailed independent t-test between the two groups.

and *Group* (patients, controls) as between-subjects factor revealed no significant difference ($P > 0.2$).

3.3. Relationship of Cortical Displacements and Background Activity with Brain Atrophy. No dependence on *Global Atrophy* was shown by the patients' M20 and M30 ECD positions ($F(3, 13) = 1.482$, $P = 0.266$), sources' displacements ($F(1, 15) = 0.058$, $P = 0.813$), and delta and theta band powers ($F(1, 12) = 0.062$, $P = 0.807$).

Middle temporal atrophy showed no nonparametric correlation with either any cortical displacements or background activity variable or clinical severity ($P > 0.200$ consistently).

Furthermore, clinical severity did not depend on *Global Atrophy* (independent t-test $t(15) = 0.828$, $P = 0.421$).

3.4. Relationship of Cortical Displacements and Background Activity with Clinical Status. ANOVA revealed a significant *Illness Severity* effect for displacements in the left hemisphere ($F(2, 20) = 5.005$, $P = 0.017$, Figure 3 right). Post-hoc comparison (Bonferroni corrected) revealed a significant difference between controls and moderate patients ($P = 0.032$). ANOVA on spectral background activity showed a significant *Illness Severity* effect for low band powers (delta: $F(2, 24) = 5.577$, $P = 0.010$; theta: $F(2, 24) = 3.383$, $P = 0.051$). Bonferroni post-hoc comparison revealed a difference

between controls and severe patients (delta: $P = 0.016$, theta: $P = 0.047$, Figure 3 right). Displacements differences between controls and severe patients ($P > 0.200$) were not significant. Also delta and theta powers differences between controls and moderate patients ($P > 0.200$) were not significant.

3.4.1. Cortical Excitability and Metal and Oxidative Stress Derangement. The biological variables under study did not correlate with age and sex. Table 3 shows the correlation among M20 and M30 ECD strengths as markers of glutamate S1 and glutamate-Gaba SM1 excitability in healthy controls and patients affected by AD. While in healthy controls M20 (s20II) was mainly associated with ceruloplasmin and peroxides [26], in AD these relationships were not evident. Instead, a significant association appeared between higher M30 ECD strength (s30II) and non-Cp copper, which is absent in healthy controls.

Table 3: Correlation between SM1 excitability and metal variables.

	Healthy control		AD patients	
	M20 ECD strength	M30 ECD strength	M20 ECD strength	M30 ECD strength
Copper	**−.760**	.486	−.010	.071
	.011	*.222*	*.973*	*.826*
Perox	**−.823**	.058	−.351	−.338
	.003	*.891*	*.239*	*.282*
Cerulopasmin	**−.882**	.136	−.463	−.539
	.002	*.771*	*.111*	*.070*
Transferrin	−.514	.017	−.461	−.559
	.193	*.975*	*.180*	*.118*
TAS	−.054	.290	−.043	.059
	.890	*.528*	*.888*	*.855*
Non-Cp copper	−.357	.561	.393	**.648**
	.345	*.190*	*.184*	***.023***

Pearson's coefficient (upper row) and two-tail significance (lower row) of the correlation between the metal variables and the ECD strengths (expressed as nA∗m) of glutamate-mediated M20 and glutamate- and gaba-mediated M30 for controls and patients. Bold font is used when $P < 0.05$.

4. Discussion

The main result of this study is that non-Cp copper is associated with the primary sensorimotor cortical excitability in AD patients and S1 area of the dominant hemisphere displays plastic changes in the early phase of the disease.

As we did previously with healthy subjects [26], we focused our study on a brain circuit which connects pyramidal neurons in the somatosensory cortex with a projection coming from neurons in the thalamus. We showed that a marker of glutamate-mediated cortical excitability, that is, the M20 ECD strength, is associated with the concentrations of a marker of copper status, that is, ceruloplasmin, in healthy controls, while the M30 ECD strength is associated with higher concentrations of non-Cp copper in AD patients.

We focused a previous study on systemic metal variations in healthy subjects in relation with indices of glutamatergic neurotransmission, observing that the higher the ceruloplasmin the lesser the cortical glutamatergic neurotransmission as revealed by MEG. Conversely, in AD patients, our results demonstrate that ceruloplasmin is not associated with either M20 or M30 ECD strength, differently from non-Cp copper. The shift in the correlation of this metal indices of copper status appears related to the diverse nature of the two copper components analysed. While ceruloplasmin is a protective factor in many diverse conditions, non-Cp copper still remains a toxic component of copper metabolism, specifically related to the AD condition. The fact that the AD brain is under the threat of oxidative stress, also mediated by metals via Haber Weiss and Fenton's chemistry, can be one of the mechanisms subtending the plastic phenomena presently detected. More specifically, we observed a spatial shift of the AD patients' sensorimotor generators with no change of latencies or strength and with neuronal reorganization more evident in moderate AD patients. Conversely, in severe patients delta activity increased, as previously observed [2, 3]. Plastic phenomena were more evident in the patients' dominant hemisphere, which is more used for daily activities. These plastic phenomena suggest that the cortex delays the appearance of evident deficits in the attempt of escaping the advancing neuronal degeneration, either by utilizing previously silent networks or by establishing brand new circuitries. Conceivably, symptoms appear when reorganization is overwhelmed by neurodegeneration. Non-Cp copper derangement, as we observed in AD, is among the biological processes underlying cortex activity reorganization.

We showed that the cortical sources of the earliest responses to contralateral median nerve stimulation are shifted posteriorly in AD patients with respect to controls and that these shifts are not due to brain atrophy. When the median nerve is stimulated at the wrist, the axonal signal traveling towards the brain reaches the ventroposterior lateral thalamic nucleus (VPL), which relays the majority of the information to areas 3a and 3b of the sensory cortex. Area 3a is invisible to MEG, due to the orientation of its pyramidal cells perpendicular to the scalp, but it quickly transfers information to the neighboring area 3b, whose pyramidal cells are instead parallel to the head surface [27, 28]. This cortico-cortical contribution adds to the activation generated by the signal arriving to area 3b directly from the VPL and the two produce a strong and reliable MEG signal, represented by M20. M30 is more puzzling. Some authors localized it in area 3b [29], while others in motor area 4 [30].

Our results showed no differences between AD patients and controls in M20's and M30's latencies and amplitudes ($P > 0.2$), an evidence that stresses the role of plasticity: since thalamic pathways to the cortex are relatively spared in AD [31], the amount of information relayed to the cortex remains approximately unaltered while neurodegeneration ravages the corticocortical interconnections, on which both M20 and M30 partially rely. This result appears in contrast with a recent work by Stephen et al. [32], who instead observed a significant diagnosis-dependent difference of the amplitude. However, they observed this difference only in their male participants,

although the wider part of their patient population consisted of females. The difference between the two studies possibly originates from the fact that we did not differentiate on the basis of sex and that we did not consider successive recruitments. We studied the single response separately, since we were interested in assessing the behavior of a specific portion of cortical activity.

The parietal direction of M20's drift was probably to be expected from the fact that the sulcus lies immediately ahead and that the VPL projections are mainly directed towards parietal regions. Nonetheless, our displacement results (Table 2), which show a much wider localization spread in patients than controls, indicate that occasional recruitments within the motor cortex cannot be ruled out. In the case of M30, we speculate that the shift of its ECDs could be interpreted as a sign of a gradual loss of the motor contribution to the component.

The fact that plasticity appeared stronger in our patients' dominant hemisphere suggests that it works to enhance preservation of the more common right-hand activities. This notion is in line with epidemiological data showing that individuals engaging in higher levels of mental and physical activity are at lower risk of developing AD and dementias (review in [33]). Regular activity increases the endurance of cells and tissues to oxidative stress and influences vascularisation, energy metabolism, and neurotrophin synthesis, which all play an important role in neurogenesis and plasticity.

The nature of our investigation does not allow definite statements on the actual mechanisms subtending the described plastic changes. The sensorimotor cortex may activate a number of mechanisms including changes in neuronal membrane excitability [34], strengthening [35], or weakening [36] of existing synapses, creation of new synapses [37], and even "unmasking" areas normally kept silent by tonic inhibition [38]. All these mechanisms are certainly not mutually exclusive. Since glutamate is the major excitatory neurotransmitter involved in sensory and motor thalamocortical projections, we can conceive that the glutamatergic system is mainly involved in the plasticity phenomena that we observed. This system suffers major disruptions in AD especially due to synaptic malfunctions and apoptosis, together with neurotransmitter demodulation. The correlation of non-Cp copper with M30 and not with M20 ECD strength suggests that complex neurotransmission is involved in the non-Cp copper dysfunction.

Finally, while plasticity phenomena appeared more evident in less impaired AD patients, their spontaneous activity showed a linear behavior with disease severity (Figure 3). The power of both delta and theta rhythms increased with increasing illness severity. A bulk of evidence from different approaches shows that the spectral characteristics of the electromagnetic background brain rhythms significantly reflect network functioning within various brain regions, especially in AD [39]. Discrimination between physiological and pathological brain aging clearly emerges at the group level, with low frequencies within the delta/theta range marking the neural degeneration. Delta dipole distributions used to estimate the neuronal sources in diverse regions of AD brains have shown increased densities in posterior temporal areas [40]. This topographic change in brain activity possibly reflects changes in the cholinergic system of AD brains, a view supported by the evidence that administration of scopolamine, an antagonist of muscarinic receptors, to healthy elderly subjects induces alterations of their spontaneous oscillatory activity similar to the ones seen in AD [41]. Functional deficits due to cholinergic dysfunctions are typical of the late stage of AD.

In summary, this study showed that the copper dysfunction typical of AD patients is associated with cortical excitability, both glutamate- and gaba-mediated, within the peripheral-central neural sensorimotor pathways.

Conflict of Interests

All authors declare no actual or potential conflicts of interests.

Authors' Contribution

These authors contributed equally to this work.

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Review Article

Neuroinflammation and Copper in Alzheimer's Disease

Xin Yi Choo, Lobna Alukaidey, Anthony R. White, and Alexandra Grubman

Department of Pathology, University of Melbourne, VIC 3010, Australia

Correspondence should be addressed to Alexandra Grubman; alexandra.grubman@unimelb.edu.au

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Inflammation is the innate immune response to infection or tissue damage. Initiation of proinflammatory cascades in the central nervous system (CNS) occurs through recognition of danger associated molecular patterns by cognate immune receptors expressed on inflammatory cells and leads to rapid responses to remove the danger stimulus. The presence of activated microglia and astrocytes in the vicinity of amyloid plaques in the brains of Alzheimer's disease (AD) patients and mouse models implicates inflammation as a contributor to AD pathogenesis. Activated microglia play a critical role in amyloid clearance, but chronic deregulation of CNS inflammatory pathways results in secretion of neurotoxic mediators that ultimately contribute to neurodegeneration in AD. Copper (Cu) homeostasis is profoundly affected in AD, and accumulated extracellular Cu drives Aβ aggregation, while intracellular Cu deficiency limits bioavailable Cu required for CNS functions. This review presents an overview of inflammatory events that occur in AD in response to Aβ and highlights recent advances on the role of Cu in modulation of beneficial and detrimental inflammatory responses in AD.

1. Inflammation

Inflammation is a protective response rapidly triggered by innate immune cells in the event of tissue injury, as well as endogenous or exogenous insults (reviewed in [1]). The process is highly complicated, involving the complex interplay of cells and mediators. In brief, acute inflammatory responses involve vasodilation to increase blood flow combined with alterations in microvascular structure to allow exit of circulating leukocytes and plasma proteins, followed by accumulation and activation of leukocytes at the site of injury, where leukocyte extravasation is largely facilitated by cytokines including tumour necrosis factor (TNF) and interleukin-1 (IL-1) [1]. In addition, activated innate immune cells at site of injury remove cellular debris and/or pathogens via phagocytosis with concomitant cytokine production to facilitate the initiation of adaptive responses [1].

Due to the variability in the nature, severity, and site of injuries, resolution of inflammatory processes, where all injury and insults become resolved with little tissue damage, is not always possible. For severe tissue damage where regeneration is insufficient, healing with fibrosis may occur instead. The third possible outcome is progression from acute to chronic inflammation. This occurs when danger signals persist and inflammation cannot be resolved. Notably, a wide range of diseases, including asthma [2], diabetes [3], coronary heart disease [4], cancer [5], and neurodegenerative diseases [6, 7], have been associated with chronic inflammation.

1.1. Inflammatory Signaling Cascades. The innate immune system functions as the first line of defense against cellular damage caused by danger stimuli including pathogenic organisms or damaging molecules. An array of innate immune cells including macrophages, mast cells, fibroblast, dendritic cells, monocytes, and neutrophils are involved in inflammatory responses. Innate immune cells sense danger signals by activation of membrane-bound pattern recognition receptors (PRRs), including Toll-like receptors (TLRs) and C-type lectin receptors (CLRs), and cytoplasmic PRRs, including nucleotide-binding oligomerization domain (NOD)-like receptors (NLRs) and RIG-I-like receptors (RLRs), to initiate immune responses [8]. Activation of PRRs by pathogen-associated molecular patterns (PAMPs), usually conserved molecular patterns expressed by pathogens, and/or danger-associated molecular patterns (DAMPs), endogenous molecules released by damaged cells,

triggers inflammatory signaling cascade(s) that drive a wide range of cellular responses [1].

The TLR family, with 10 identified members, is the most widely studied of all classes of PRRs [9]. TLRs are type 1 transmembrane proteins with an extracellular domain with leucine-rich repeats (LRRs) and a cytoplasmic Toll/IL-1 receptor domain [10]. TLRs play an important role in detecting microbial infection via recognition of ligands including lipids, nucleic acids, lipopolysaccharides, and other unique molecular microbial components [11, 12]. TLRs undergo conformational changes upon ligand binding to recruit adaptor molecules, which in different combinations contribute to the specificity in individual TLR responses [13]. The two major signaling pathways initiated by TLRs include the myeloid differentiation primary response gene 88 (MYD88)-dependent pathway and the Toll/interleukin-1 receptor (TIR)-domain-containing adapter-inducing interferon-β (TRIF)-dependent pathway [1]. MYD88-dependent signaling responses activate the c-Jun N-terminal kinases (JNK)/activator protein 1 (AP-1) and kappa-light-chain-enhancer of activated B cells (NF-κB) pathways [9]. AP-1 and NF-κB activation initiates a transcriptional response program of cytokine production specifically tailored to the insult. Classical NF-κB-regulated cytokines include IL-1, IL-6, IL-8, and TNF [14]. In the alternative TRIF-dependent pathway, activation of the transcription factors NF-κB and interferon regulatory factors (IRF3 and IRF7) can result in type 1 interferon (IFN) production, often in response to viral infection [1]. Type I IFNs are released by various peripheral cell types, including lymphocytes such as natural killer (NK) cells, B cells and T cells, macrophages, fibroblasts, and endothelial cells, and are likely to induce CNS inflammatory effects through infiltration of peripheral cells into the brain [15].

In contrast to the TLRs, NLRs are cytosolic PRRs that can be activated by both pathogens and endogenous components [16]. Making up the NLR family are 5 NOD receptors, 14 NALP receptors, NAIP, Ipaf, and CIITA type 1 and CIITA receptors [17]. In particular, NOD1 and NOD2, best known for their intercellular sensing of distinct peptidoglycan fragments released by bacteria, also signal to NF-κB [18] and mitogen-activated protein kinase (MAPK) [19] signaling pathways. On the other hand, NLRP1, NLRP3, and NLRC4 recognise specific PAMPS and DAMPS to form signaling complexes known as "inflammasomes." Inflammasome assembly may be triggered by diverse stimuli, including uric acid crystals, cholesterol, protein aggregates and, aluminium adjuvants [20–23]. Assembly of "inflammasome" complexes leads to caspase-1 activation resulting in caspase-1-mediated cleavage of NF-κB-dependent precursors of the proinflammatory cytokines IL-18 and IL-1β to produce their mature forms [1, 19]. Therefore inflammasome activation requires two signals, the first to induce transcription of pro-IL-1β and pro-IL-18 and the second to initiate inflammasome assembly.

Overall, inflammatory responses can be viewed as a system consisting of mediator-driven feedback loops. Subsets of inflammatory mediators can positively feedback into the system to intensify activation state of immune cells, leading to exacerbated inflammatory responses [24, 25]. Conversely, other inflammatory mediator subsets can function in a reverse manner. They can negatively feedback into the system leading to inhibited or downregulated inflammatory responses so to limit tissue injury for the resolution of inflammation [26]. Depending on the mediator secretion profile, inflammation can mediate different outcomes. From the brief outline of the inflammatory signaling cascades, it is evident that inflammation is a highly complex but tightly regulated process. More importantly, the regulation of inflammation can vary in different organs, due to tissue-specific expression of innate receptors and variations in inflammatory mediator secretion profiles.

2. Neuroinflammation

Being physically separated from the peripheral immune system, the CNS is conventionally recognized as being "immunologically privileged" [34]. With limited regenerating capacity, tight regulation of the immune responses in the CNS is necessary as chronic inflammatory responses in the CNS can lead to sustained neurodegeneration [35]. Immune regulation of the CNS is characterised by the absence of defined lymphatic channels, downregulated immune surveillance, absence of specialized antigen presenting cells, and the presence of the blood-brain barrier (BBB) [34, 36, 37]. Aside from its functions as a physical barrier to separate cerebrospinal fluid from circulating blood, the BBB also serves as a specialized physical barrier to limit immune responses.

Despite the immunologically privileged status, it is becoming increasingly recognized that the CNS is capable of independently shaping immune responses [37]. Lymphocytes, in particular T cells, can be trafficked into the CNS to survey the environment [37, 38]. Emerging evidence suggests that a lymph-like system is also present in the brain [36, 39]. But most importantly, specialized cells of the CNS express major histocompatibility complexes (MHC) classes I and II molecules and can be activated to participate in immune responses [37].

In an analogous manner to systemic organs, inflammatory reactions in the CNS play a critical role in maintaining tissue homeostasis [40]. Neuroinflammatory responses can also be classified as acute or chronic. Acute neuroinflammation usually occurs in reversible neuronal injury where glial cells in the CNS become activated [41]. The process is usually short-lived and responses by glial cells are generally subtle. On the other hand, chronic inflammation refers to prolonged inflammatory responses in the CNS due to a persistent presence of injurious stimuli. Notably, neuroinflammation described in diseases of the CNS, including neurodegenerative diseases, is generally chronic [41]. Despite differences in their clinical presentation and underlying mechanisms of disease, neuroinflammation has been identified as a process crucial to the progression of many neurodegenerative diseases including AD, motor neuron disease (MND), and Parkinson's disease (PD). This is supported by the observation in models of neurodegenerative disease, as well as patient tissues, of activation and/or proliferation of glia, the major cell types initiating the neuroinflammatory process [42–48].

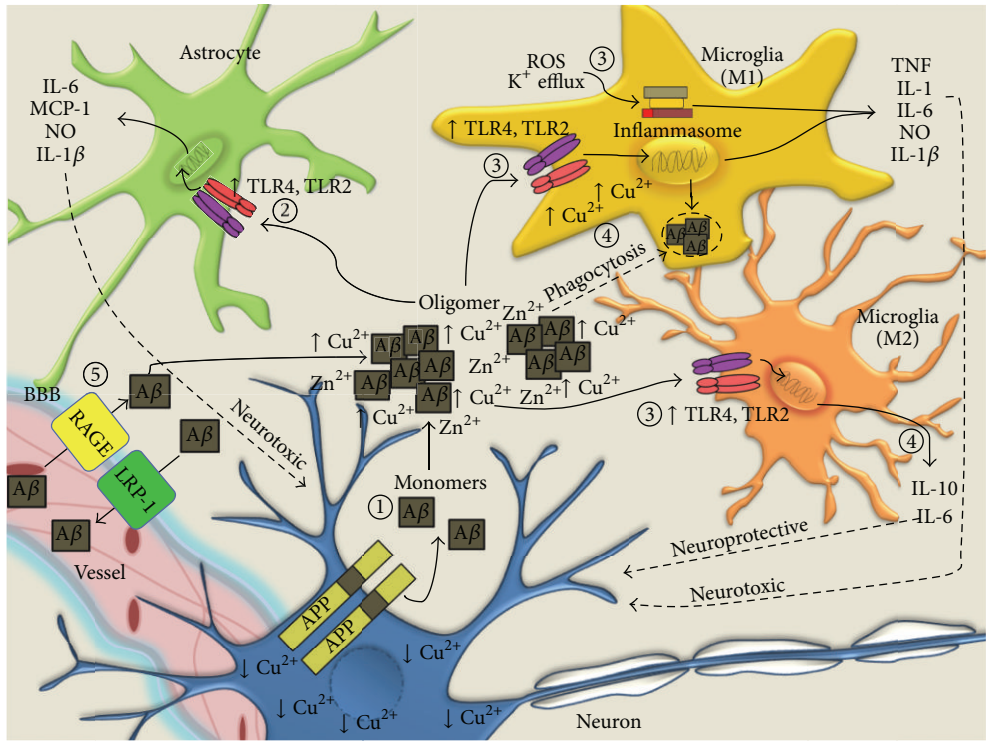

FIGURE 1: *Inflammatory processes in AD.* (1) Sequential cleavage of APP by secretase proteins generates extracellular Aβ monomers, which aggregate to form toxic oligomers and plaques, a process accelerated by Cu and Zn ions. (2) Aβ oligomers may directly interact with immune components on astrocytes, potentially through TLR2/4 recognition, resulting in astrocyte-derived secretion of toxic proinflammatory mediators that act on neurons. (3) Microglia surrounding Aβ plaques may be polarised to the neurotoxic M1 phenotype through Aβ- or ROS-dependent inflammasome and TLR activation. (4) Microglia may also exert protective functions through intracellular Cu sequestration, direct phagocytic activity on plaques, and secretion on neuroprotective M2 mediators including IL-10. (5) The brain levels of Aβ are also controlled by RAGE- and LRP-1 mediated transport between the plasma and brain. Increased vascular RAGE in AD contributes to impaired clearance of Aβ from the CNS.

In conclusion, the understanding of neuroinflammatory processes occurring in neurodegenerative diseases raises many as yet unanswered questions: does the inflammatory process occur before or after the occurrence of other pathological changes? Are the inflammatory processes beneficial or detrimental to disease progression? Nevertheless, it is clear that if neuroinflammation remains unresolved, as is the case for most neurodegenerative diseases, prolonged exposure to cytokine-induced injury is neurotoxic. As the clinical and pathological presentation of various neurodegenerative diseases can be very different, there are also disease-specific differences in the associated inflammatory profiles. Therefore, further understanding the triggers and consequences of inflammatory processes in a disease-specific context will have strong implications for potential therapeutic approaches.

2.1. Neuroinflammation in AD. There was little evidence to link neuroinflammation to disease progression of AD until immunohistochemistry techniques were used to study amyloid β (Aβ) plaques. Towards the end of the 1980s, several research groups detected clusters of activated microglia around Aβ plaques [49–51]. These observations support the hypothesis that Aβ plaques are key players in chronic neuroinflammation in AD. Later, it was suggested that the presence in AD brains of high levels of redox metals, including iron (Fe) and copper (Cu), promotes reactive oxygen species (ROS) production and these metals can also act as mediators to induce neuroinflammation in AD [52, 53]. Specifically, Cu concentrations are elevated in amyloid plaques and Cu binds with Aβ high affinity, thereby promoting Aβ oligomerization and neurotoxicity (Figure 1).

Further studies on the neuroinflammatory profile in AD also revealed abnormal upregulation of various cytokines and chemokines including TNF, IL-1, IL-6, monocyte chemoattractant protein-1 (MCP-1), nitric oxide (NO), and transforming growth factor β (TGFβ), which exert proinflammatory effects in AD brain [54–57]. Put together, these data suggest that neuroinflammation has a significant impact on the disease pathology of AD (for detailed reviews, see [58, 59]).

Ageing is still considered the greatest risk factor for AD although risk modifiers including environmental, genetic, and epigenetic factors have been described. Among the physiological and lifestyle changes during ageing, alterations to metal homeostasis and inflammatory processes have also been documented, which may have a causal connection with increased AD risk (reviewed in [60]). Plasma concentrations

of copper are elevated in ageing [61], which may contribute to metal imbalances that trigger ROS production and Aβ aggregation. Moreover, inadequate zinc absorption during ageing contributes to low-grade peripheral inflammation due to deregulated inflammatory transcription factors (including NF-κB) containing zinc-finger motifs [60]. Thus the compounding effects of metal and inflammatory dyshomeostasis in ageing may impede normal physiological responses to additional stressors such as increased Aβ deposition.

2.2. Cell Types Involved

2.2.1. Microglia. The key innate immune cell types mediating inflammatory process of AD brain include microglia and astrocytes. Microglia are the major immune surveillance cells accounting for 10–20% of the total CNS glial cell population. Also known as resident macrophages, microglia are of mesodermal origin and known to occupy all regions of the CNS, although microglial density can vary widely in different regions of the CNS [62, 63]. Microglial activity can vary between populations and regions of the CNS, based on differences in gene expression profiles [64]. Additionally, microglia also express different phenotypes depending on the surrounding environmental conditions. In healthy individuals, microglia have a "resting" phenotype, characterised by a ramified morphology, which are in constant motion, surveying their microenvironment [64]. In AD, microglia migrate to areas of Aβ plaque deposition [49–51] and were shown to participate in the clearance of Aβ plaques via phagocytosis or Aβ plaque degradation [65, 66]. While clearance of Aβ by microglia is suggestive of a beneficial response, the actual outcome of Aβ clearance by microglia remains unknown. Rather, phagocytosis of Aβ by microglia may drive further immune activation, as supported by increased microglial production of proinflammatory mediators, including IL-1 [57, 67, 68], IL-6 [57], TNF [57], macrophage inflammatory protein (MIP) [57], MCP-1 [57, 67], and ROS [69]. However, the precise role of microglia in the pathogenesis of AD has yet to be elucidated. This is due, in part, to the multiple activation states that can be expressed by microglia. Similarly to peripheral macrophages, microglia can be classically (M1) or alternatively (M2) activated. In brief, M1 microglia are primarily associated with proinflammatory responses and induce neurodegeneration, while M2 phenotypes are more closely associated with anti-inflammatory responses, which are neuroprotective [70]. In an *in vivo* study involving the APP/PS1 AD mouse model, an age-dependent switch of microglial activation state, from M2 to M1, was observed [71]. However, this does not rule out that both M1 and M2 populations can coexist and play varying roles in AD disease pathogenesis (Figure 1).

2.2.2. Astrocytes. Astrocytes, of ectodermal origin, are the most abundant glial cell type and the most abundant cell type in the CNS. They can be classified by morphological differences into protoplasmic and fibrous astrocytes, which are present in the gray and white matter regions, respectively [72]. It is well established that astrocytes play critical roles in supporting neuronal survival and maintaining homeostasis of the CNS by close contact with all CNS cell types through their extensive processes. Emerging evidence suggests that astrocytes also play a role in the innate immune system [73]. In the presence of injurious stimuli, astrocytes can be triggered to initiate immune responses. Common observation of astrocyte clusters around Aβ plaques in AD brains suggests that Aβ plaques are key endogenous stimuli driving reactive astrocytosis [74]. This is further supported by *in vitro* evidence of astrocyte activation in response to aggregated Aβ fragments [75]. In contrast to microglia, astrocytes do not play a significant role in the clearance of Aβ plaques [65, 66]. Instead, astrocytes downregulate microglial Aβ plaque clearance via secretion of glycosaminoglycans-sensitive molecules [65, 66]. This implies that astrocytes may act to hinder effective clearance of Aβ, indirectly promoting Aβ accumulation in AD brains. However, a recent study carried out in adult astrocytes suggests that astrocytes can contribute to Aβ degradation [76]. Additionally, astrocytes play significant role in AD neuroinflammation by producing a broad range of inflammatory mediators, including IL-1β [77, 78], IL-6 [79], MCP-1 [78], MIP [78], and NO [80], identified from various *in vivo* and *in vitro* studies. Long-term production of these mediators becomes a chronic "cytokine cycle" (Figure 1), as described by Griffin and colleagues, which plays detrimental role in influencing disease progression [24]. Therefore cytokine-induced feedback loops may present a target for therapeutic intervention using anti-inflammatory approaches.

2.3. Pathways and Mediators. Many studies report that Aβ oligomers and fibrils are key drivers of AD pathogenesis [81, 82]. Besides causing direct injury to neuronal cells [81, 83], Aβ oligomers and fibrils are endogenous stimuli that can be recognized by PRRs expressed on innate immune cells. Aβ species have been shown to induce inflammatory responses through activating various PRRs expressed by microglia and astrocytes including TLRs [84, 85], receptor for advanced glycation end products (RAGE) [86, 87], and the inflammasomes [23, 88].

2.3.1. TLR Signaling. Microglia and astrocytes can be differentiated based on the TLRs they express. Microglia express all TLRs 1–9, while astrocytes predominantly express TLR3, although low-level expression of TLR1, TLR4, TLR5, and TLR9 has also been detected [89, 90]. In particular, TLR2 [85] and TLR4 [84, 91] have been identified to be important for the recognition of Aβ species in AD. TLR activation by Aβ can function as a double-edged sword. Activation of TLR2 and TLR4 was shown to be beneficial through enhanced phagocytic microglial Aβ clearance, and TLR2 or TLR4 deficiency in AD mice has detrimental effects on Aβ deposition and cognitive function [92, 93]. However, TLR2 knockout and TLR4 loss-of-function mutant mouse models secreted less neurotoxic proinflammatory mediators IL-1β, IL-6, TNF, and inducible nitric oxide synthase (iNOS) with Aβ stimulation, suggesting that TLR-dependent signaling may contribute to neurotoxicity in AD [84, 85]. This is supported by further evidence showing that Aβ can initiate sterile inflammation

via heterodimeric TLR4/TLR6 when accompanied by regulatory signals from scavenger receptor CD36 [94]. Additionally, brains of human AD patients express high levels of TLR4, while APP mouse brains exhibit higher levels of *TLR4* mRNA [84]. Recently, upregulated TLR2 and TLR4 have been detected in peripheral mononuclear blood cells in 60 patients with late onset AD [95]. These studies suggest that inflammatory responses in AD brains can be further potentiated by Aβ-induced upregulation of TLR4 expression. Although TLRs can activate several transcription factors, including AP-1 and NF-κB [1, 9], the current understanding of the downstream signaling cascades in AD is limited.

2.3.2. RAGE Signaling. RAGE, a member of the immunoglobulin superfamily of cell surface proteins, is a multiligand receptor that functions as a PRR for Aβ oligomers [96]. RAGE exists in a membrane-bound full-length form and a soluble form (sRAGE) that competitively inhibits Aβ-mediated RAGE signaling. Emerging evidence suggests the involvement of RAGE in AD pathogenesis. RAGE promotes Aβ transport from plasma to the CNS (Figure 1). Conversely, low-density lipoprotein receptor related protein 1 (LRP-1) exerts the reverse function and increases plasma Aβ levels [97]. Thus the combined actions of RAGE and LRP-1 maintain the balance of plasma and CNS Aβ concentrations [97]. Significant elevation of the hippocampal microvascular ratio of RAGE to LRP-1 expression was reported in AD patients [98], resulting in impaired clearance of Aβ in AD with RAGE driving the influx of Aβ into the CNS [97]. Moreover, reduced sRAGE expression in AD further contributes to overactive RAGE-induced inflammation. Additionally, studies also revealed that Aβ binding to RAGE drives microglial activation, thereby initiating a positive feedback loop that further elevates RAGE expression and associated inflammation [87]. APP mutant AD model mice crossed with mice overexpressing RAGE demonstrated exacerbated disease outcomes, indicating that elevated RAGE expression is detrimental to cognitive function in AD [86, 99].

2.3.3. NLR Signaling. Another class of PRR involved in AD pathogenesis is the NLRs. In particular, IL-1β and IL-18, predominant cytokines released with inflammasome assembly, are significantly upregulated in both CNS and plasma components of human AD patients [100, 101]. In addition, IL-1β was also found to be significantly upregulated in the Tg2567 mouse model of AD [102]. These data support the involvement of inflammasome activation in AD neuroinflammation. NALP3 inflammasome activation can be induced by potassium (K^+) efflux [103]. In particular, Aβ species have been shown to induce reduced intracellular K^+ by disrupting K^+ channel function [104, 105]. Treatment of hippocampal neurons with Aβ induced upregulation of the KV3.4 channel subunit and increased K^+ efflux, and this is also evident in Tg2576 mouse model. In addition to the above, the NALP3 inflammasome can also be directly activated by Aβ upon phagocytosis of Aβ by microglia [23] due to consequential triggering of lysosomal damage [106].

Despite the rapidly increasing knowledge of the inflammatory cells, pathways, and mediators significantly altered in AD, several major questions remain unanswered. Critical to our understanding of the disease process as well as development of diagnostic and therapeutic tools is a clearer picture of the specific triggers of microglial activation in AD and the pathways that can be induced to shift microglial responses to protective M2 phenotypes. Moreover, a spatiotemporal analysis of the beneficial and detrimental consequences of microglial activation in AD is required to target pathways that selectively engage protective responses such as phagocytosis of amyloid deposits while limiting secretion of neurotoxic mediators.

3. A Role for Cu in Neuroinflammation in AD

3.1. Cu and Aβ in AD. Cu is essential for the development and maintenance of CNS functioning. It is becoming well established that deregulation of Cu homeostasis is a pathological feature associated with a number of neurodegenerative diseases including AD, PD, and MND [107]. Although the precise role(s) that Cu plays in the pathology of these diseases is not elucidated, Cu is a critical cofactor of numerous enzymes but in excess can mediate Fenton chemistry-dependent cytotoxicity and therefore must be tightly regulated [108]. Cu plays an important role in AD pathology by a twofold mechanism involving toxic Cu-induced Aβ deposition occurring concomitantly with reduced intracellular bioavailable Cu [32, 109–111] (Figure 2). Additionally, Zn is also reported to potently induce Aβ plaque deposition [112] and Fe can mediate ROS production [113]; thus it is not surprising that changes to homeostasis of both Zn and Fe in AD have been described [110]. As discussed above, although the mechanisms by which Aβ exerts its toxic effects on neurons are not fully elucidated, ROS and acute inflammatory mediators produced by glial cells enriched at amyloid plaques may contribute to Aβ-induced neuronal death.

Several *in vitro* studies have demonstrated that low levels of Cu ion can induce Aβ aggregation [114–117]. Cu can bind with high affinity to an amino terminal tyrosine residue in Aβ and induce oligomerization through oxidative modification [118]. APP knockout mice exhibit elevated brain Cu levels, whereas APP overexpressing transgenic mice have reduced brain Cu levels [32, 119]. APP or Aβ interactions with Cu^{2+} induce reduction to Cu^+ *in vitro*, promoting neurotoxic H_2O_2 production [120]. It is now well established that Cu binding induces Aβ deposition and promotes neurotoxicity [118]. Additionally, elevated free Cu can mediate ROS-dependent toxicity [53]. As chronic activation of inflammatory cells is commonly observed in the vicinity of Aβ plaques [49–51], it stands to reason that an agent that drives Aβ deposition would indirectly contribute to damaging chronic immune responses. However, as there is controversy regarding the role of activated microglia surrounding amyloid plaques, it is unclear whether the contribution of Cu to this process is beneficial.

3.2. Proinflammatory Role of Cu. A role for Cu in peripheral inflammatory responses is supported by *in vivo* and *in vitro* data. Cu was reported to induce IL-6 secretion in

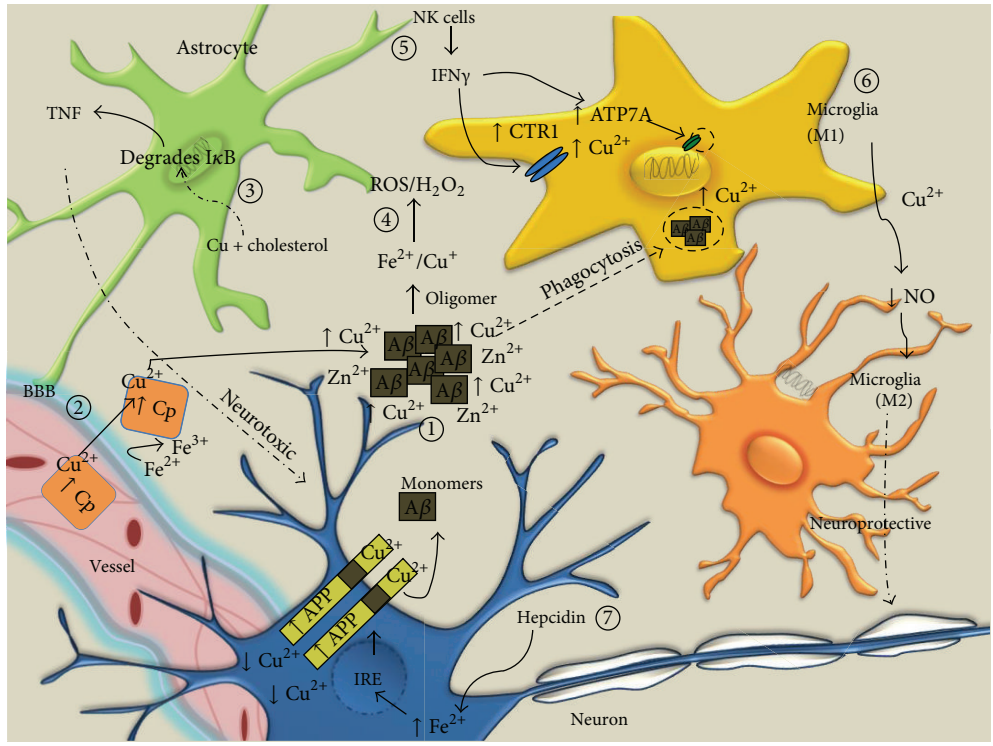

FIGURE 2: *Hypothesised roles of copper in the inflammatory process of AD.* (1) Clockwise from center. Cu and Zn induce the aggregation of Aβ in AD, leading to reduced neuronal intracellular bioavailable Cu. This may account for the reported reduced expression of the Cu-requiring proteins SOD1 and ATOX1 in AD. (2) Cp, the Cu-transport protein, which is elevated in AD, can promote Fe oxidation, inflammation [28], and increased extracellular Cu levels in the CNS. (3) Copper has been shown to potentiate the effects of cholesterol on inflammation-induced Aβ neurotoxicity through increased TNF production [30]. (4) APP or Aβ reduce Cu^{2+} to Cu^{+}—this redox cycling promotes production of ROS including H_2O_2. (5) NK cell-derived IFNγ can increase Cu uptake in microglia via enhanced CTR1 expression [27]. IFNγ also promotes ATP7A elevation and vesicular trafficking. These mechanisms of Cu sequestration by microglia may prevent further plaque formation. Phagocytosis of amyloid plaques also raises microglial Cu levels and promotes Aβ clearance. (6) Cu may polarize inflamed microglial populations from the neurotoxic (M1) phenotype to the neuroprotective (M2) phenotype via inhibition of NO production [31]. (7) The Fe master regulator, hepcidin, is induced by cytokines in AD and prevents Fe release from neurons. Excess Fe binds IREs in the APP promoter and upregulates APP production, promoting Cu export and mislocalisation.

a cell culture system composed of human keratinocytes and fibroblasts [121]. Implantation of female rats with Cu-coated discs caused NF-κB activation and IL-6 production and induced recruitment of IL-1α-secreting cells [122, 123]. Inhalation of Cu present as a particulate in air pollution also elicited inflammatory NF-κB activation [124]. Moreover, intratracheal instillation of Cu sulfate results in enhanced neutrophilia and MIP2 mRNA expression in rats [125] and Cu chloride can elicit IL-8 responses in human endothelial cells [126]. Together, these studies demonstrate that Cu can induce peripheral inflammation in numerous models, although there is limited direct evidence of the potential of Cu to initiate neuroinflammation. Synergistic effects of Cu and cholesterol in neuroinflammation have been described. A study demonstrated that trace Cu potentiated Aβ neurotoxicity in cholesterol-fed mice by Aβ-induced neurotoxic inflammatory responses [30] (Figure 2). No proinflammatory effects were observed upon treatment with Cu or cholesterol alone. However, cotreatment with Cu and cholesterol increased IκB degradation as well as TNF expression and production in the brains of the mice, implicating a TNF-dependent proinflammatory role for Cu and cholesterol in AD. Investigation of the role of cholesterol in Cu-induced inflammatory responses would provide critical insight into AD pathogenesis, as genetic variation in the cholesterol transport protein ApoE4 remains the greatest genetic risk factor for sporadic AD and cholesterol-rich lipid rafts are likely to be the site of Cu-Aβ interactions (reviewed in [127]). Moreover, AD-associated changes to cholesterol metabolism may impact membrane fluidics, which could affect associated copper transporter and PRR expression, as well as inflammatory signal transduction pathways.

3.3. Cu Trafficking and Inflammation. The major Cu-binding proteins in plasma are ceruloplasmin (Cp), albumin, and transcuprein, while subcellular Cu transport is controlled by membrane transporters and cytosolic chaperones that shuttle Cu between intracellular compartments and high-affinity cuproproteins, respectively. Loss of the Cu transporter ATP7B in Wilson's disease model mice presents as elevated

brain copper, neurodegeneration, and inflammation [128]. Interestingly, ATP7B gene polymorphisms have been associated with increased AD risk in certain populations [129, 130]. Moreover, an inflammatory milieu can affect Cu homeostasis via regulation of Cu transport proteins (Figure 2). IFNγ, which is secreted by NK cells in AD patients [131], stimulated ATP7A expression in cultured microglia and altered Cu homeostasis, including Cu-dependent trafficking of ATP7A from the Golgi to cytoplasmic vesicles [27]. IFNγ stimulation also increased Cu uptake and elevated expression of the CTR1 Cu importer [27]. The impact of this finding for AD is unclear, as IFNγ exerts multiple biological effects in AD. Aside from stimulating microglial activation, IFNγ was also reported to mediate neurogenesis and reduce Tau pathology in AD model mice [132, 133]. Zheng et al. [27] also reported elevated ATP7A expression in activated microglia surrounding Aβ plaques in the brains of TgCRND8 AD model mice [27], which may promote overall Cu uptake by upregulation of CTR1 expression. Cu sequestration by microglia may therefore provide a neuroprotective mechanism in AD by limiting the free extracellular Cu available to seed Aβ aggregation and plaque formation. Conversely, transfection of fibroblast cell lines with ATP7A resulted in loss of cellular copper and reduced APP expression [33], suggesting that it is the lack of intracellular copper induced by elevated ATP7A that may prevent Aβ production by downregulation of APP.

The major plasma Cu transport protein, Cp, which is elevated in the serum and brain in AD patients [134, 135], can also elicit proinflammatory responses in cultured primary and secondary microglia. These inflammatory responses include elevated NO release and induction of proinflammatory transcriptional programs involving TNF, IL-1β, COX-2, NADPH oxidase, iNOS, and prostaglandin E2 [28]. Cu-stimulated responses were significantly attenuated by a p38 inhibitor, SB203580, and the NF-κB inhibitor SN50 [28]. Moreover, as Cp is a Cu-containing ferroxidase [136], transport of Fe is inextricably linked to Cu mislocalisation and Cp levels.

The Fe regulating peptide, hepcidin, which is induced by cytokines including IL-6, inhibits Fe release from neurons by inducing lysosomal degradation of the Fe exporter, ferroportin [137]. A recent study proposed that the resultant intracellular excess Fe in ageing, which is further exacerbated by inflammation in AD, promotes APP production via an iron responsive element (IRE) in the promoter of APP [138, 139]. Enhanced APP expression thereby promotes neuronal Cu export, as APP binds Cu via its Cu-binding domain [139]. Cu mislocalisation by secreted APP could therefore act as a double-edged sword resulting in an excess extracellular Cu that may promote Aβ aggregation and deplete intracellular Cu stores available for physiological enzyme functions. However, another study reported that Cu but not Fe induced APP exocytosis *in vitro* [140], suggesting that complex regulatory mechanisms, which may be dependent on the surrounding inflammatory milieu, may be important for subtle control of APP trafficking.

3.4. Anti-Inflammatory Role of Cu in AD. Conversely, intracellular Cu deficiency, which was detected in brain of mice overexpressing APP [32, 109] and supported by studies of AD brain tissue [111], also promotes microglial activation. A loss of bioavailable brain Cu, in mice that had been perinatally weaned on Cu deficient diets, resulted in microglial and astrocytic activation in the cerebrum and thalamus and neurological signs [141]. This suggests that bioavailable physiological Cu concentrations are required to prevent CNS inflammation. For instance, expression of the Cu-requiring enzymes superoxide dismutase 1 (SOD1) and the ATX antioxidant protein homolog (ATOX1) was significantly reduced in AD brains as determined by several microarray studies, suggesting that neurons are Cu-deficient in AD [139, 142, 143]. As SOD1 itself may also exert anti-inflammatory functions through ROS detoxification, a loss of SOD1 activity would further exacerbate chronic inflammation.

Cu is also closely associated with regulation of cytokine signaling. Robust secretion of the anti-inflammatory cytokine, IL-4 was detected in the brains of mice coadministered Al and Cu in drinking water compared to mice administered Al alone [29]. Interestingly Cu administration alone had no effect on the inflammatory markers tested, although the study did not examine whether Cu was increased in the brain as a result of treatment. Stimulation of the microglial BV2 cell line with LPS in the presence of Cu(I) shifted the population from the neurotoxic M1 to the neurotrophic M2 phenotype and significantly reduced nitrite release [31]. Treatment with Cu alone (without LPS) had no effect on the microglial phenotype, nitrite release, or iNOS expression, thereby suggesting that Cu(I) can modulate inflamed microglia and may alter the cell signaling function of NO by altering its redox state. NO is an M1 mediator, and Cu-dependent inhibition of nitrite release may be the mechanism, which induces the M2 phenotype. Therefore, Cu sequestration by microglia may be a neuroprotective response in activated microglia surrounding Aβ plaques. These neuroprotective responses may be mediated, at least in part, by Cu-dependent induction of the metal-sequestering and antioxidant acute phase protein, metallothionein, as observed in rat microglia [144]. Administration of the therapeutic copper-bis(thiosemicarbazonato) complex, Cu(gtsm), delivered bioavailable Cu to the brain of APP/PS1 AD model mice and improved amyloid and tau pathology, as well as indicators of cognitive function [145]. As Cu(gtsm) is able to cross the BBB and release Cu inside brain cells, this study further supports the hypothesis that intracellular Cu pools are depleted in AD. It will be interesting to determine whether Cu delivery is anti-inflammatory or promotes M1 to M2 shift in the activated microglia surrounding amyloid deposits in these mice as part of the neuroprotective mechanism.

4. Conclusions

As discussed above, extensive evidence links neuroinflammation to AD. Whilst regulated neuroinflammation is an important neuroprotective mechanism in the CNS, unregulated, chronic neuroinflammation is toxic if not resolved as in AD. Cu appears to possess both pro- and anti-inflammatory

properties that may be mediated in part by its spatial proximity to amyloid plaques. Thus deregulation of Cu transport may be the precursor to initiation of toxic inflammatory reactions in the AD brain. Future investigation of the effects of both elevated and depleted discrete Cu pools on inflammatory pathways, as well as improved techniques for measurement of subcellular Cu trafficking in AD, will facilitate identification of novel therapeutic targets related to Cu homeostasis and inflammation.

Abbreviations

Aβ:	Amyloid β
AD:	Alzheimer's disease
AP-1:	Activator protein 1
BBB:	Blood-brain barrier
CLRs:	C-type lectin receptors
CNS:	Central nervous system
Cp:	Ceruloplasmin
Cu:	Copper
DAMPs:	Danger-associated molecular patterns
Fe:	Iron
IFN:	Interferon
IL-1:	Interleukin-1
iNOS:	Inducible nitric oxide synthase
IRF:	IFN regulatory factor
JNK:	c-Jun N-terminal kinases
LRP1:	Lipoprotein receptor related protein 1
LRRs:	Leucine-rich repeats
MAPK:	Mitogen-activated protein kinase
MCP-1:	Monocyte chemoattractant protein-1
MHC:	Major histocompatibility complexes
MIP:	Macrophage inflammatory protein
MND:	Motor neuron disease
MYD88:	Myeloid differentiation primary response gene 88
NK:	Natural killer
NF-κB:	Kappa-light-chain-enhancer of activated B cells
NLRs:	NOD-like receptors
NO:	Nitric oxide
NOD:	Nucleotide-binding oligomerization domain
PAMPs:	Pathogen-associated molecular patterns
PD:	Parkinson's disease
PRRs:	Pattern recognition receptors
RLRs:	RIG-I-like receptors
RAGE:	Receptor for advanced glycation end products
ROS:	Reactive oxygen species
sRAGE:	Soluble RAGE
TGFβ:	Transforming growth factor β
TIR:	Toll/interleukin-1 receptor
TLRs:	Toll-like receptors
TNF:	Tumour necrosis factor
TRIF:	TIR domain-containing adapter-inducing interferon-β
Zn:	Zinc.

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Research Article

Effects of Copper and/or Cholesterol Overload on Mitochondrial Function in a Rat Model of Incipient Neurodegeneration

Nathalie Arnal,[1] Omar Castillo,[2] María J. T. de Alaniz,[1] and Carlos A. Marra[1,3]

[1] *Instituto de Investigaciones Bioquímicas de La Plata (INIBIOLP), CCT La Plata, CONICET-UNLP, Cátedra de Bioquímica y Biología Molecular, Facultad de Ciencias Médicas, Universidad Nacional de La Plata, 60 y 120, 1900 La Plata, Argentina*
[2] *Centro de Investigaciones Cardiovasculares (CIC), CCT-CONICET, 1900 La Plata, Argentina*
[3] *INIBIOLP, Cátedra de Bioquímica, Facultad de Ciencias Médicas, Universidad Nacional de La Plata, Calles 60 y 120, 1900 La Plata, Argentina*

Correspondence should be addressed to Carlos A. Marra; contactocarlos@hotmail.com

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Copper (Cu) and cholesterol (Cho) are both associated with neurodegenerative illnesses in humans and animals models. We studied the effect in Wistar rats of oral supplementation with trace amounts of Cu (3 ppm) and/or Cho (2%) in drinking water for 2 months. Increased amounts of nonceruloplasmin-bound Cu were observed in plasma and brain hippocampus together with a higher concentration of ceruloplasmin in plasma, cortex, and hippocampus. Cu, Cho, and the combined treatment Cu + Cho were able to induce a higher Cho/phospholipid ratio in mitochondrial membranes with a simultaneous decrease in glutathione content. The concentration of cardiolipin decreased and that of peroxidation products, conjugated dienes and lipoperoxides, increased. Treatments including Cho produced rigidization in both the outer and inner mitochondrial membranes with a simultaneous increase in permeability. No significant increase in Cyt C leakage to the cytosol was observed except in the case of cortex from rats treated with Cu and Cho nor were there any significant changes in caspase-3 activity and the Bax/Bcl2 ratio. However, the Aβ(1–42)/(1–40) ratio was higher in cortex and hippocampus. These findings suggest an incipient neurodegenerative process induced by Cu or Cho that might be potentiated by the association of the two supplements.

1. Introduction

It is well known that copper (Cu) is an essential transition metal for all living organisms, functioning as cofactor for many enzymes [1–3]. However, we and other laboratories have demonstrated *in vivo* and *in vitro* that excess inorganic Cu produces increased levels of reactive oxygen species (ROS) and damage to biomolecules, ultimately promoting cell death [4–7]. Humans are continuously at risk from excess Cu due to involuntary exposure to pollution (contaminated water, food), professional activities [8–10], ingestion of dietary supplements [10–12], and prolonged use of intrauterine devices [13, 14]. Elevated Cu plasma levels, especially of free-Cu or the so-called nonceruloplasmin-bound Cu (NCBC), have been associated with neurodegenerative damage [10, 15, 16]. In recent years, there has been a considerable increase in the number of published papers relating Cu to the neurodegenerative process [15–18]. In line with this, Brewer [10] hypothesized that ingestion of inorganic Cu from different sources is at least a partial cause of Alzheimer disease (AD) in developed countries. Squitti et al. [17–19] reported that NCBC, which is loosely bound to molecules such as serum albumin and other proteins, is one of the main risk factors involved in AD development. They suggest that it is the ratio of CP to Cu that is the crucial biomarker for interpreting Cu-associated features in live AD patients.

AD has multiple conditioning and causative factors. However, it was reported that inorganic Cu in conjunction with a high fat diet sets the stage for the development of AD, particularly if other risk factors are present [19]. Pappolla et al. [20] proposed that hypercholesterolemia accelerates the biochemical neuropathologic damage observed in transgenic

mice. In addition, Notkola et al. [21] found that plasma cholesterol (Cho) levels were predictors of AD prevalence in a population-based sample of 444 men (aged 70–89 years). More recently, *in vivo* models of AD-like neurodegenerative diseases demonstrated that the association of Cu and Cho increases the risk of the development of neurodegenerative damage [22–24].

Cu overload has been extensively associated with ROS overproduction *in vitro* and *in vivo* [6, 7, 25]. Oxidative stress is considered a primary event in the development of AD [26]. Pope et al. [27] reported that ROS overproduction due to the dysfunction of the electron transport chain is causative of neurodegeneration associated with AD and Parkinson's disease. Moreover, some experimental evidence indicates that alterations in mitochondrial energetics occur along with the accumulation of oxidative damage before the cardinal signs of AD pathogenesis in the brains of transgenic animal models and human patients [28, 29].

As reviewed by Paradies et al. [30], the mitochondrion is considered the most important cellular organelle contributing to the neurodegenerative process, mainly through respiratory chain dysfunction and the formation of reactive oxygen species. Factors that increase the rate of mitochondrial ROS production lead to mitochondrial dysfunction because of the oxidative-induced damage caused to various crucial biomolecules such as mtDNA, lipids, and proteins, thus increasing mitochondrial deterioration in a self-perpetuating process [31]. Together with ROS accumulation, mitochondrial dysfunction is one of the earliest and most prominent features of AD [27, 28] and this condition is strongly associated with multiple negative consequences. Dysfunctional mitochondria generate high levels of ROS that are ultimately toxic to cells, particularly those with a long lifespan and low antioxidant defense system such as neurons [28, 29]. At the same time, mitochondria are also targets of the ROS produced by pro-oxidative factors such as Cu overload or increased levels of NCBC.

Among the mitochondrial lipids directly affected by ROS overproduction, cardiolipin (CL) is one of the most deeply involved in both synapses and neuronal loss [31]. CL is linked to the endogenous proapoptotic cascade that eventually leads to the integration of the apoptosome and the subsequent activation of the effector caspase system [28–31]. Moreover, CL is involved in the regulation of the bioenergetic process and in the integrity of mitochondrial membranes. Peroxidation of CL directly affects the biochemical functions that depend on the inner and outer mitochondrial membrane composition and structure. Experimental evidence clearly indicates that this lipid represents the most important target of ROS attack [30].

In summary, there is conclusive evidence linking ROS hyperproduction with mitochondrial dysfunction. Oxidative stress is also directly involved in neurodegeneration and Cu overload in association with cholesterol apparently acts as a synergistic risk factor in the etiology of neurological disorders. All the foregoing evidence suggests the need for more in-depth study of this phenomenon. In view of the absence of conclusive studies on the biochemical effects of the Cu + Cho (CuCho) association on mitochondrial-associated dysfunction, especially in relation to those aspects involved in programmed cell death, we aimed to investigate the effects of CuCho on mitochondria isolated from cortex and hippocampus of Wistar rats and explore (i) the Cho/Pi ratio and steady-state fluorescence anisotropy of the outer and inner mitochondrial membranes in order to assess their fluidities; (ii) mitochondrial membrane integrity by means of a probe that measures trans-membrane potential; (iii) the concentration of the main mitochondrial antioxidant glutathione; (iv) the concentration of the key mitochondrial phospholipid, cardiolipin, and the possible pro-oxidative damage it causes; (v) the activity of the apoptotic pathway depending on mitochondrial membrane integrity (caspase-3 and the Bax/Bcl-2 ratio); and (vi) the concentration of β-amyloid (Aβ) in the two brain zones and in peripheral plasma as a biomarker of a proneurodegenerative effect.

2. Materials and Methods

2.1. Chemicals. All chemicals used were of analytical grade and obtained from Sigma Chem. Co. (Buenos Aires, Argentina or USA), Merck (Darmstadt, Germany), and Carlo Erba (Milan, Italy).

2.2. Animals and Treatments. Certified pathogen-free male Wistar rats were used. The rats were maintained at a controlled temperature (25°C) and relative humidity of 60% with forced ventilation, under a normal photoperiod of 12 h darkness and 12 h light. The health of the animals was monitored in accordance with the internationally recommended practices of the *Institute of Laboratory Animal Resources, Commission of Life Sciences, National Research Council* (ILAR). Solid food and drinking water were provided *ad libitum*. The diets for the experiments were prepared in our laboratory according to the recommendations for Wistar rats [32]. All procedures for handling the animals followed the NIH regulations [33]. The experimental protocol was reviewed and approved by the Bioethics Committee of the Faculty of Medical Sciences, UNLP (number 00382/11).

2.3. Experimental Protocols. Rats (21 days old) were randomly assigned (ten animals per group) to the protocols detailed as follows and treated for eight weeks (2 months). Selecting very young rats lets us discard any neurodegenerative (unknown) event(s) associated to the age of the animal. By the way, this approach may be extrapolated to humans that are exposed to copper from the very beginning of their life. In addition, in previous experiments (not shown), we noted that—at lest during the first year of life—that we have no differences in the response of the model as a function of the starting age of treatment. The control group (C) was maintained on lab-prepared pellets as recommended for normal growth containing 7 ppm of Cu [34, 35]. The Cu-supplemented experimental group (Cu) was fed on control pellets and tap water supplemented with 3 mg/L (or ppm) of Cu in the form of ultrapure $CuSO_4$ (Merck, Darmstadt, Germany). The Cho-supplemented group (Cho) was fed on pellets containing

2% (W/W) of Cho (87% pure) (obtained from Saporiti SRL, Buenos Aires, AR), and the Cu + Cho-supplemented group (CuCho) was simultaneously treated with Cu in water + Cho in food. Rats were monitored during the experimental period to observe their behavior, quantify water and food consumption, and determine their body weight gain. Total Cu concentration in tap water supplemented with CuSO was determined by means of atomic absorption methodology and was 3.42 ± 0.21 ppm (means of all daily measurements taken along the experimental period). Considering that each animal imbibed between 4.9 ± 0.4 and 15.0 ± 1.1 mL water/day (at the beginning and the end of the protocol, resp.), a maximum of 0.01 to 0.05 mg Cu/day was acquired from water (a dose equivalent to 0.06 and 0.18 mg Cu/Kg live animal, resp.). Linear regression curves and ANOVA test for Cu content in food demonstrated that there were no significant variations between the 6 preparations used for the experiments (7.22 ± 0.31 ppm or mg Cu/Kg diet). Fe and Zn content (determined by atomic absorption spectrometry) was the same in all preparations (45.9 ± 0.8 and 66.6 ± 2.0 ppm, resp.). Ingestion of solid food along the experiments varied from 11.6 ± 0.8 to 29.7 ± 2.8 g/rat, implying that the oral ingestion of Cu was in the range from 0.08 to 0.21 mg Cu/day/rat (0.0032 to 0.0008 mg Cu/Kg live animal).

2.4. Sample Collection. At the end of the treatments, animals were deeply anesthetized with ketamine (70 mg/Kg) and xylazine (5 mg/Kg) applied intramuscularly and then sacrificed by decapitation. Brains were removed and dissected into two zones: cortex and hippocampus, using the atlas of Paxinos and Watson [36] as a guide for tissue dissection. Both brain regions were washed, weighed, and homogenized using a Tris/HCl buffer (10 mM pH 7.4) with sucrose (70 mM), mannitol (230 mM), ethylenediaminetetraacetic acid (EDTA) (1 Mm), and dithiothreitol (DTT) (1 mM) (Buffer I). Homogenates were centrifuged at 2°C 700 ×g for 10 min and the supernatants recentrifuged at 8,000 ×g (10 min/2°C) to obtain the cytoplasm fraction. Pure mitochondrial fractions were isolated from the pellets of the previous centrifugation by resuspending them in 3 mL of buffer I and centrifuging during 5 min at 1,000 ×g (2°C). The pellets were discarded and the supernatants re-centrifuged for 10 min at 11,000 ×g (2°C). Finally, the final pellet (mitochondrial fraction) was resuspended in buffer II (buffer I with 0.015% mg pure albumin (Sigma Chem. Co.; Buenos Aires, AR)). Cytosol fractions were prepared by ultracentrifugation of the supernatants at 110,000 ×g for 1 h at 2°C. During the sacrifice of the animals, blood was also collected using heparin as anticoagulant (1 IU/5 mL) in ice-cold polypropylene tubes. The plasma samples were immediately prepared by centrifugation in the cold (4,000 ×g, 10 min) and stored at −70°C until analyzed.

2.5. Atomic Absorption Measurements. Aliquots of sample were digested with a mixture of 4 mL of HNO_3 (c) and 1 mL $HClO_4$ (Aldrich or Sigma Chem. Co., Buenos Aires, Argentina) by heating at 120°C for 60 min in a mineralization block [37]. The digests were cooled, diluted with ultrapure water (18 mΩ cm, Carlo Erba, Milan, Italy), and ultrafiltered with a 0.22 μm Millipore membrane (Milli-Q Purification System, from Millipore, CA, USA). Ultrafiltered dissolutions were directly aspirated into the flame of a PerkinElmer 1100 B Spectrophotometer equipped with a Perkin-Elmer cathode lamp (Perkin-Elmer Corp., Norwalk, CT, USA) at a spectral width of 1 nm. Standard solutions of 100 ppm Zn and Fe from HCR Inc. (QuimiNet, Buenos Aires, AR) were used. Cu determinations were calibrated with a standard solution (200 ppm) of $Cu(NO_3)_2$ in HNO_3 0.5 N (Titrisol from Merck Co., Darmstadt, Germany). All measurements were carried out in peak height mode (324.7 nm line). The intra-$[(SD/x) \cdot 100]$ and inter-$[(\Delta SD/\Delta x) \cdot 100]$ assay coefficients of variation were 15.5 and 6.0%, respectively. We routinely obtained a similar equation for the calibration curve (IR = $55 \cdot 10^{-5}$ + 0.048 [Cu, mg/L]), and the statistical analyses demonstrated a correlation coefficient always between 0.95 and 0.99. In addition, we explored the so-called matrix effects that might have modified the slopes of the standard regressions. In spiked samples, the obtained values varying from 48 to $60 \cdot 10^{-5}$ were very similar to those of Cu standard solutions, indicating that the matrix effect was considered nonsignificant or was negligible. The mean for recovery and RSD for spiked samples was 99.7% and 3.3%, respectively, and the detection limit was 0.09 mg/L. In order to verify the accuracy of the method, we explored the influence of time after dilution, temperature of acid digestion, and concentration of $HNO_3/HClO_4$ following the suggestions of Terrés-Martos et al. [38]. We also checked our results with biological samples (plasma and homogenates) against a Seronorm Trace Elements Serum (from Sero Labs, Billingstad, Norway) and found no significant differences between the obtained and the declared (certified) concentrations.

2.6. Ceruloplasmin (CP) Levels and Nonceruloplasmin-Bound Copper (NCBC). Samples were analyzed by the enzyme conversion of p-phenylenediamine into a blue-colored product [39] which was then measured at 550 nm. Reaction proceeded at 37°C in buffer glacial acetic/sodium acetate (50 mM, pH 5.5) directly into flat-bottomed plates, using a Microplate Reader SpectraMax M2/m2^e model from Molecular Devices Analytical Technologies (Sunnyvale, CA, USA) for 3 min. Intra- and interassay coefficients of variation were 8.3 and 4.4%, respectively. CP concentrations were calculated by comparison with the reaction rate of pure CP standard (Sigma Chem. Co., Buenos Aires, Argentina). Using the Cu and CP data we calculated the non-CP-bound Cu (NCBC, or so-called free Cu) as described by Brewer [19] by subtracting the amount of Cu bound to each mg of CP from data of total Cu. This parameter can be easily expressed in percentages using the formula $(([Cu] - 47.2 \times [CP]) \times 100/[Cu])$ where Cu is in μmol/L and CP in g/L [40].

2.7. Lipid Analysis. Total lipids were extracted by the method of Folch et al. [41]. An aliquot of each Folch extract was evaporated and the residue dissolved in 50 mM sodium phosphate buffer (pH 7.4) containing digitonin 1%. Aliquots of this solution were taken to enzymatically measure cholesterol (Cho) and phospholipids (PL) using commercial kits from Wienner Lab (Rosario, Argentina).

To determine the mitochondrial cardiolipin (CL) content, samples were separated by high-performance thin-layer chromatography (HPTLC) on precoated silica gel plates (10 × 20 cm) with concentration zone from Whatman Schleicher and Schuell (Maidstone, England). The mobile phase was chloroform : methanol : ammonium hydroxide (65 : 25 : 4; by volume). Spots were localized using iodine vapor on a lateral lane spotted with authentic standards of CL, mono- and dilyso-CL, and other reference phospholipids (Avanti Polar Lipids, Ontario, Canada). The rest of the plate was covered with a glass to avoid possible spontaneous oxidation. The CL and lyso-CL zones were scraped off the plates and eluted from the silica with "inverse" Folch extraction (chloroform : methanol; 1 : 2). After evaporation under nitrogen at room temperature, the selected zones were dissolved in 200 μL of Folch solvent mixture. The total amount of CL and lyso-CL was quantified by means of phosphorous measurement using the method of Chen et al. [42].

2.8. Mitochondrial Membrane Integrity. The mitochondrial membrane potential ($\Delta\psi$) was measured by estimating the integrity of the mitochondrial membrane using the isolated mitochondrial staining kit from Sigma-Aldrich, Inc. (Buenos Aires, AR). This kit is based on the uptake of the lipophilic cationic probe 5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethyl-benzimidazolcarbocyanine iodide dye (JC-1) into the mitochondrial matrix which directly depends on the $\Delta\psi$. In healthy cells, the dye concentrates in the matrix, where it forms bright red fluorescent agglomerates. Uptake by the mitochondria of JC-1 can be utilized as an effective distinction between apoptotic and healthy cells. Any event that dissipates the $\Delta\psi$ prevents the accumulation of the JC-1 dye in the mitochondria, and the dye is thus dispersed in the cytoplasm, leading to a shift from red (JC-1 agglomerated) to green fluorescence (JC-1 monomers). To measure the samples, we used a PerkinElmer LS 55 Fluorescence Spectrometer, set at 525 nm excitation wavelength and 590 nm emission.

2.9. Steady-State Fluorescence Anisotropy of DPH and TMA-DPH. Submitochondrial fractions (inner and outer membranes) were obtained following the method described by Fraser and Zammit [43] with the modifications of Pellon-Maison et al. [44]. Prior to membrane fluidity assays, total protein concentration was determined in each suspension and adjusted to approximately 300 μg protein/mL using 5 mM TRIS/HCL buffer pH 7.40. Membrane fluidity was determined by the fluorescence anisotropy (or polarization) technique using two fluorescent probes: 1,6-diphenyl-1,3,5-hexatriene (DPH; Sigma-Aldrich, St. Louis, MO) and 1-[4-(trimethylamino)phenyl]-6-phenyl-1,3,5-hexatriene (TMA-DPH; Molecular Probes, Eugene, OR). TMA-DPH was used to monitor fluidity near the surface of the cell membrane. The polar region of this probe is anchored at the lipid-water interface and the hydrocarbon moiety enters the lipid part of the membrane. The length of the hydrophobic part of the TMA-DPH molecule is approximately equivalent to that of a plasma membrane [45]. DPH is incorporated into the hydrophobic regions of the lipid bilayer [46]. DPH or TMA-DPH anisotropy is inversely correlated with membrane fluidity [47]. Anisotropy measurements were performed as previously described [48], with slight modifications. Briefly, membranes were vigorously homogenized using a vortex vibrator for 1 min and subjected to mild sonication in a Branson sonifier 450 (Branson Ultrasonic SA, CA, USA) (set at 40% output) following a 3 min incubation period on ice. The sonication cycle was controlled by the turbidity of the suspension as evaluated at 600 nm and was interrupted when an absorbance value of 0.2 or less was reached. This procedure does not interfere with the transition of lipid bilayers but disperses aggregates, facilitating fluorescence readings and decreasing light scattering [49]. The samples for the DPH and TMA-DPH assays (3 mL) contained 5 mM Tris–HCl buffer pH 7.4, 60 μg of protein from diluted suspensions, and 50 μM MDPH in tetrahydrofuran or 25 μM TMA-DPH in dimethylformamide and were incubated for 45 min at 37°C. The steady-state anisotropy was measured in a Heλ10Sβ spectrofluorophotometer (Thermoelectron Corp., Sydney, Australia) equipped with a thermostated cell holder. The sample's temperature was checked to an accuracy of ±0.1°C using a thermistor thermometer. Excitation and emission were set at 357/435 nm and 358/428 nm for DPH and TMA-DPH (resp.) using 5 nm excitation and emission slits. Samples were illuminated with linear (vertically, *v*, or horizontally, *h*) polarized monochromatic light and the fluorescence intensities emitted (*I*, in arbitrary units) parallel or perpendicular to the direction of the excitation beam were recorded. Blank samples without the addition of the probe(s) were also measured to estimate unspecific light that might reach the detector. The fluorescence anisotropy of the probes (X) was calculated as $r(X) = [Ivv - Ivh \times G] = [Ivv + 2Ivh \times G]$, where Ivv and Ivh are the intensities of the fluorescence emitted, respectively, parallel and perpendicular to the direction of the vertically polarized excitation light; G is the correction factor ($G = Ihv/Ihh$) for the optical system given by the ratio of the vertically to the horizontally polarized emission components when the excitation light is polarized in the horizontal direction; and X represents TMA-DPH or DPH. According to Shinitzky and Barenholz [50], fluorescence anisotropy values are inversely proportional to cell membrane fluidity. Thus, a high degree of fluorescence anisotropy represents a high structural order or low cell membrane fluidity.

2.10. Mitochondrial Reduced (GSH) and Oxidized (GSSG) Glutathione Content. Total glutathione was determined by the glutathione reductase/dithionitrobenzoic (DTNB) method that can measure both GSH and GSSG [51]. Mitochondrial GSH (mGSH) were calculated by subtracting GSSG from the total glutathione content (GSH + GSSG). For this reason samples were run in the presence and absence of 2 mM divinylpyridine.

2.11. Programmed Cell Death Biomarkers

2.11.1. Caspase-3 Activity. Caspase-3 activity was measured by a colorimetric assay kit (CASP-3-C), based on the hydrolysis of the synthetic peptide substrate acetyl-Asp-Glu-Val-Asp-p-nitroaniline (Ac-DEVD-pNA) by caspase-3 (Sigma Chem.

Co., Buenos Aires, Argentina). The resulting p-nitroaniline (p-NA) released was monitored at 405 nm. Each assay was run in parallel with inhibitor-treated cell lysate (to measure the nonspecific hydrolysis of the substrate) and caspase-3 positive control (using commercial caspase-3, 5 mg/mL provided by the kit manufacturer). A calibration curve using a standard solution of p-nitroaniline (p-NA) was also run for each assay to calculate the activity of the protease expressed as μmol p-NA released/min · mL of sample.

2.11.2. Bax/Bcl-2 Ratio. Western blot assays of Bax and Bcl-2 were performed to calculate the Bax/Bcl-2 ratio. Equal quantities (30 μg protein per lane) of total protein were separated by SDS-PAGE (15% gels) under reducing conditions and a Miniprotean VI unit (Bio-Rad, CA, USA). The proteins were then electrophoretically transferred to PVDF membranes (IPVH00010 Immobilon, Millipore, USA). The membranes were blocked with 5% skimmed milk and incubated with anti-Bcl-2 and anti-Bax antibodies, respectively (1 : 1000; sc-492 rabbit polyclonal and sc-493 rabbit polyclonal from Santa Cruz CA, USA, resp.), at 4°C overnight. This was followed by incubation with goat anti-rabbit secondary antibody conjugated with horseradish peroxidase (1 : 20.000; Santa Cruz, CA, USA). Photographs were taken using the Molecular Imager Gel Doc XR and ChemiDoc XRS systems (Bio-Rad) and the optical densities of the bands were quantified.

2.12. Beta-Amyloid Peptides (1–40) and (1–42). Beta-amyloid peptides (Aβ) (1–40) and (1–42) were measured using the Human/Rat β Amyloid-40 ELISA kit Wako II and the Human Amyloid-42 ELISA kit Wako High-sensitive, respectively. Before the assay the samples were centrifuged (2°C) at 5000 ×g for 15 min and the supernatants diluted 1 : 1 with the buffer provided by the manufacturer. Results for plasma were expressed in pmoles/L and for brain tissues in pmol/mg protein. The Aβ(1–42)/(1–40) ratios were calculated from each individual pair of data.

2.13. Biomarkers of Cardiolipin Peroxidation

2.13.1. Authentic Lipoperoxides. Lipoperoxides (LPOO) in cardiolipin extracts (CL) were determined according to the method of Nourooz-Zadeh et al. [52]. Results were expressed as μmoles LPOO/μmoles CL (for calculation we used an extinction coefficient of 4.60 (10^{-4}) · mol^{-1} · cm^{-1} at 560 nm).

2.13.2. Conjugated Dienes. The spectrophotometric detection of conjugated dienes was performed according to the methodology described by Recknagel and Glende [53]. The spectral register between 300 and 220 nm was obtained and subtracted from a blank spectrum of pure cyclohexane. Conjugated dienes show a typical absorption band in the range of 230–240 nm. The results were expressed as relative units of optical density (UDO)/mg mitochondrial CL.

2.14. Statistical Analysis. All values represent the mean of 10 rats assayed in triplicate expressed as mean ± standard deviation (SD). Data were analyzed by ANOVA plus the Tukey test with the aid of SPSS 11.0.1 software (SPSS Inc., Chicago, IL). To analyze the bands obtained by western blot, we used the Image J software for image processing (National Institute of Health, USA). Results were also plotted and analyzed using Sigma Scientific Graphing Software (version 11.0) from Sigma Chem. Co. (St. Louis, MO). The statistical significance ($P \leq 0.01$) of differences is indicated by superscript letters (values with distinct letters are statistically different between them).

3. Results

Growth parameters (final weight gain, rate of body weight, food efficiency ratio, etc.) showed no significant differences between the experimental groups (data not shown). Also, water intake among the experimental groups was essentially identical, which is a reason to assume that the rats did not note any difference in the palatability of the supplemented water compared with the nonsupplemented one. The same happened with solid food.

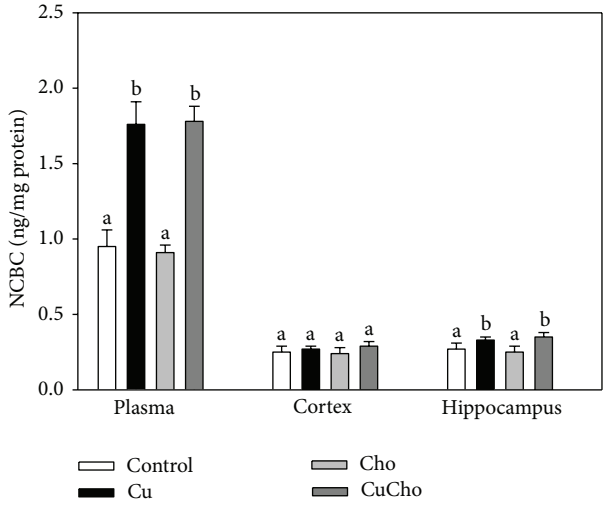
3.1. Copper Content in Mitochondrial Suspensions. Atomic absorption measurements demonstrated that the total Cu content in digested mitochondrial suspensions did not differ significantly among the experimental groups. However, mean values for cortex were higher than those for hippocampus (5.9 ± 0.4 versus 4.0 ± 0.2 g/mg mitochondrial proteins, resp.).

3.2. Nonceruloplasmin-Bound Copper (NCBC) and Ceruloplasmin (CP) Concentrations. Figure 1(a) shows the concentration of free Cu (expressed as NCBC) in plasma and in both brain zones analyzed. Plasma and hippocampus showed significant increases after Cu and CuCho treatments compared to the control group. In cortex, we observed no significant differences.

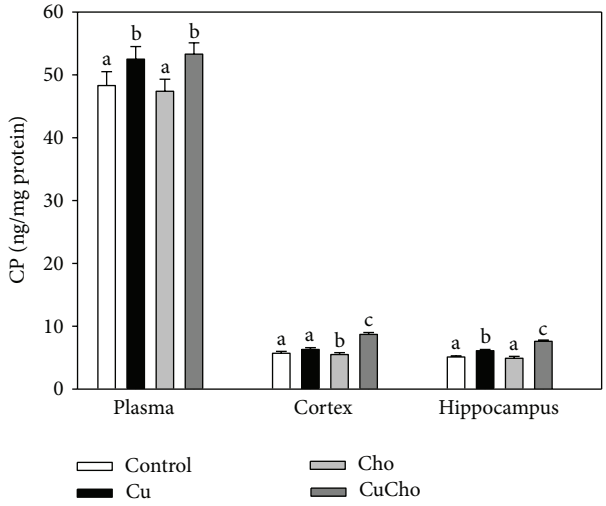
We also analyzed CP concentration in plasma, cortex, and hippocampus after the experimental diets (Figure 1(b)). CP increased significantly after Cu and CuCho supplementation in plasma and in both brain zones. Cortex and hippocampus from rats fed on the CuCho diet also showed significant increases with respect to the Cu treatment alone.

3.3. Effect of Diets on Mitochondrial Membrane Physicochemical Properties. Figure 2 shows the ratio between Cho and total phospholipids (measured as total inorganic phosphate or Pi) in cortex and hippocampus. In both brain zones, Cu treatment produced a significant increase in this ratio. Supplementation with Cho and with Cu + Cho (CuCho) also produced significant increases in the Cho/Pi ratio compared to the control or Cu treatment alone.

The mitochondrial membrane potential was estimated by fluorimetric determination of the degree of aggregation of the JC-1 dye in mitochondrial suspensions from brain cortex and hippocampus (Figure 3). Supplementation with Cu alone produced no significant changes in hippocampus and a discrete although significant increase in the membrane permeability in cortex. In both brain zones cholesterol supplementation also produced a clear loss of integrity which was



(a)



(b)

Figure 1: Free Cu (NCBC) (a) and ceruloplasmin (CP) (b) levels in brain cortex and hippocampus homogenates (ng/mg total protein). Treatments are indicated with different colors, control (white bars), Cu (black bars), Cho (gray bars), and CuCho (dark gray bars). Samples were analyzed as indicated in Sections 2.5 and 2.6. Results are expressed as mean of 10 rats assayed in triplicate ± standard deviation (SD). Significant differences among the experimental groups are indicated with superscript letters (values with distinct letters are significantly different between them, $P < 0.01$).

more pronounced when the lipid was associated with Cu in drinking water (especially in hippocampus).

3.4. Glutathione Content and Biomarkers of Lipid Peroxidation. Cho and CuCho treatments produced a significant decrease in total mitochondrial glutathione (GSH + GSSG) in both brain zones compared to the control data and to supplementation with Cu alone (Figure 4). Reductions were more evident in hippocampus than in cortex. Addition of inorganic Cu to drinking water did not produce significant changes with respect to the control group.

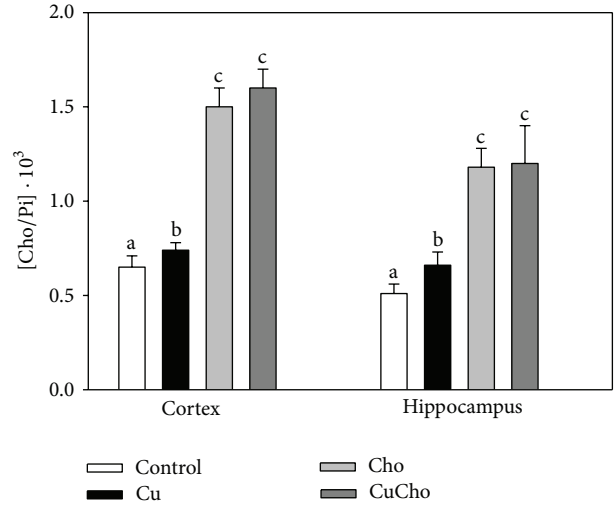


Figure 2: Cholesterol (Cho) and total phospholipids (measured as total inorganic phosphate or Pi) ratio (Cho/Pi) in brain cortex and hippocampus homogenates (ng/mg total protein). Samples were analyzed as indicated in Section 2.7. Treatments are indicated with different colors, control (white bars), Cu (black bars), Cho (gray bars), and CuCho (dark gray bars). Results are expressed as mean of 10 rats assayed in triplicate ± standard deviation (SD). Significant differences among the experimental groups are indicated with superscript letters (values with distinct letters are significantly different between them, $P < 0.01$).

Figure 5 shows the concentration of two products of CL fatty acids oxidation, lipoperoxides (LPOO), and conjugated dienes (Figures 5(a) and 5(b), resp.). Significant increases in LPOO were observed after Cu addition in hippocampus and in cortex with respect to control data. Cho treatments produced a significant decrease in this biomarker in hippocampus. A very significant increase was observed for the CuCho treatment with respect to supplementation with Cu or Cho alone for both brain zones. After Cu and CuCho treatments, the amount of conjugated dienes was also higher compared to control data in hippocampus and in cortex.

3.5. Cardiolipin (CL) and Lyso-Cardiolipin (LCL) Content in Mitochondrial Suspensions. Figure 6(a) shows the total concentration of mitochondrial CL in μmol of inorganic phosphate (Pi)/mg protein. After Cu treatment, CL content decreased in both brain zones analyzed. The Cho-supplemented diet increased CL in both cortex and hippocampus, whereas cosupplementation with Cu and Cho produced no significant changes with respect to the control diet. Figure 6(b) shows the levels of lyso-CL (LCL) after dietary treatments. All diets induced significant increases in LCL concentrations in hippocampus and cortex compared to control data; however, the higher increases were observed for the groups treated with Cu (alone or in combination with Cho).

3.6. Mitochondrial Membrane Apparent Microviscosities. Isolated outer and inner mitochondrial membranes were examined for their apparent microviscocities using two fluorescent

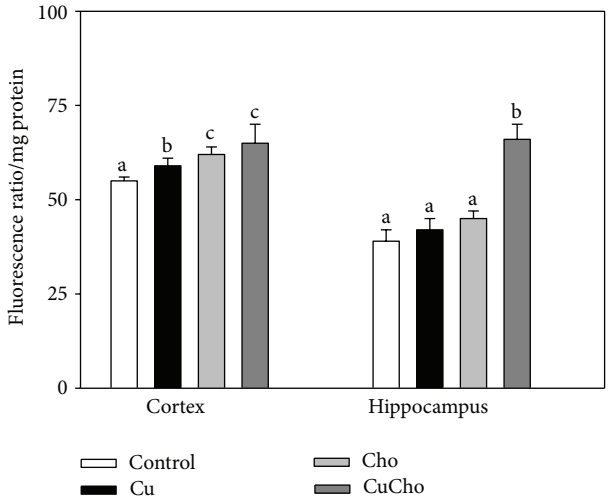


Figure 3: Mitochondrial membrane potential in brain cortex and hippocampus. Treatments are indicated with different colors, control (white bars), Cu (black bars), Cho (gray bars), and CuCho (dark gray bars). Samples were analyzed as indicated in Section 2.8. Results are expressed as mean of 10 rats assayed in triplicate ± standard deviation (SD). Significant differences among the experimental groups are indicated with superscript letters (values with distinct letters are significantly different between them, $P < 0.01$).

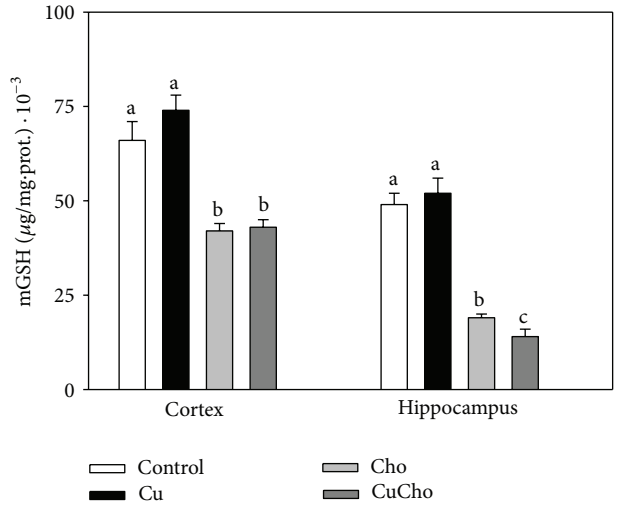


Figure 4: Mitochondrial glutathione (mGSH) content in brain cortex and hippocampus (ng/mg total protein). Treatments are indicated with different colors, control (white bars), Cu (black bars), Cho (gray bars), and CuCho (dark gray bars). Samples were analyzed as indicated in Section 2.10. Results are expressed as mean of 10 rats assayed in triplicate ± standard deviation (SD). Significant differences among the experimental groups are indicated with superscript letters (values with distinct letters are significantly different between them, $P < 0.01$).

Table 1: Caspase-3 activity in brain cortex and hippocampus from the experimental diets.

Diets	Cortex	Hippocampus
C	2.1 ± 0.1	1.4 ± 0.1
Cu	2.3 ± 0.2	1.5 ± 0.1
Cho	2.0 ± 0.1	1.4 ± 0.3
CuCho	2.4 ± 0.1	1.7 ± 0.1

Results were obtained as described in Section 2.11.1. They were expressed as the mean of 10 independent determinations ± SD. There are no statistical differences ascribed to the experimental diet.

Table 2: Ratio Aβ(1–42)/(1–40) in plasma and brain cortex and hippocampus after the experimental diets.

Diets	Plasma	Brain	
		Cortex	Hippocampus
C	6.5 ± 1.0^{a}	7.3 ± 0.9^{a}	6.3 ± 0.7^{a}
Cu	6.8 ± 0.8^{a}	9.2 ± 0.5^{b}	6.6 ± 0.4^{a}
Cho	7.1 ± 1.1^{a}	9.2 ± 0.6^{b}	9.6 ± 0.5^{b}
CuCho	8.5 ± 0.8^{a}	10.9 ± 0.5^{c}	9.3 ± 0.6^{b}

Data were obtained as described in Section 2.12. They correspond to the mean of 10 individual determinations assayed in triplicate ± SD. Differences statistically significant among the different diets are indicated with distinct superscript letters.

probes (DPH or TMA-DPH) that explore different zones of the lipid bilayer. Figures 7 (cortex) and 8 (hippocampus) show the values of the anisotropies which are inversely correlated with (outer and inner) membrane fluidities. In both brain regions and with both probes tested, we observed decreased fluidities and increased fluorescence anisotropies produced by cholesterol supplementation either alone or in combination with Cu.

3.7. Programmed Cell Death Biomarkers. Despite the damage observed in the integrity of the inner mitochondrial membrane (JC-1 measurements), we did not detect any significant increase in Cyt C in cytosol fractions with the sole exception of a slight—although significant—increase in brain cortex from rats simultaneously treated with Cu and Cho (data not shown). In agreement with this finding, caspase-3 activity was not significantly modified in any experimental group (Table 1) nor was there any significant alteration in the Bax/Bcl2 ratio (Figure 9).

3.8. Biomarker of Neurodegeneration. Table 2 shows the Aβ(1–42)/(1–40) ratio in plasma and in cortex and hippocampus homogenates as a biomarker of neurodegeneration. Cu or Cho treatments produced a significant increase in this biomarker in brain cortex and the CuCho association produced an even more significant increase. Cho supplementation increased the ratio in hippocampus with a value indistinguishable from that observed for the simultaneous treatment with Cu and Cho. These changes were not reflected in peripheral plasma.

4. Discussion

Inappropriate dietary copper and cholesterol intakes are implicated in the development of Alzheimer's disease. This study follows previous experimental evidence reporting

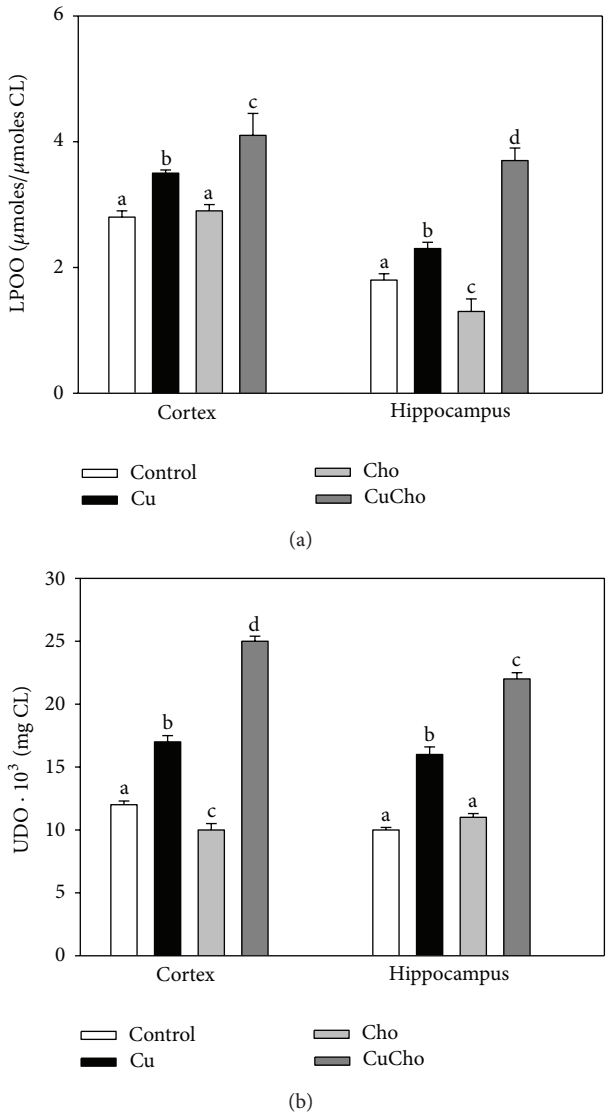


FIGURE 5: Levels of lipoperoxides (LPOO) (a) and conjugated dienes (b) in mitochondrial fractions from brain cortex and hippocampus. Results are expressed as μmoles of LPOO/μMoles of Pi in CL, or UDO/mg protein, respectively. Treatments are indicated with different colors, control (white bars), Cu (black bars), Cho (gray bars), and CuCho (dark gray bars). Samples were analyzed as indicated in Sections 2.13.1 and 2.13.2. Results are the mean of 10 rats assayed in triplicate ± standard deviation (SD). Significant differences among data are indicated with superscript letters (values with distinct letters are significantly different between them, $P < 0.01$).

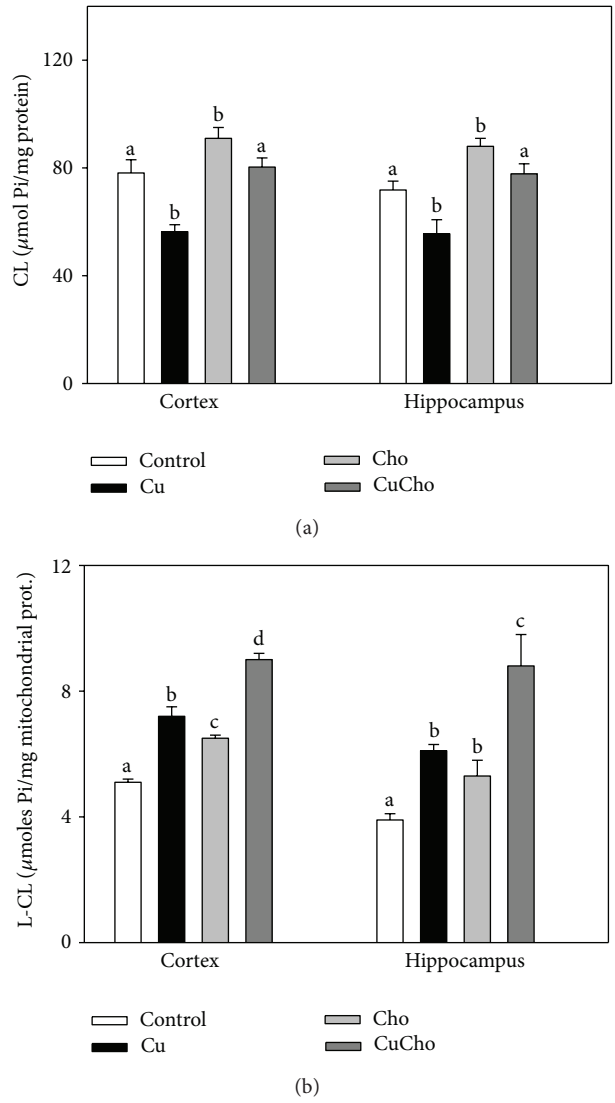
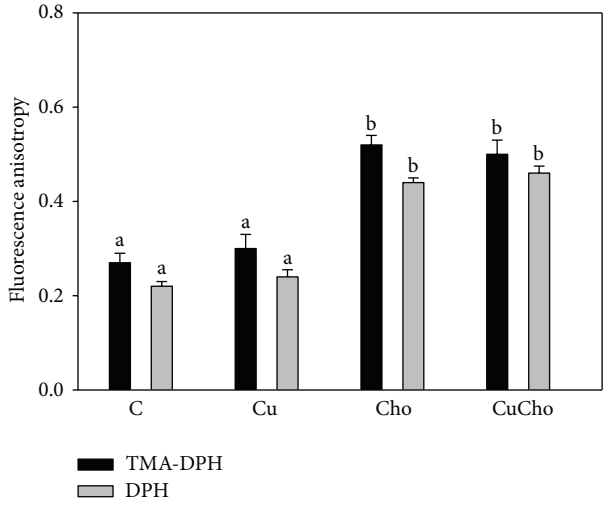


FIGURE 6: Cardiolipin (CL) (a) and lyso-CL (LCL) (b) levels in mitochondrial fractions from brain cortex and hippocampus (μmoles of Pi/mg total protein). Treatments are indicated with different colors, control (white bars), Cu (black bars), Cho (gray bars), and CuCho (dark gray bars). Samples were analyzed as indicated in Section 2.7. Results are the mean of 10 rats assayed in triplicate ± standard deviation (SD). Significant differences among the experimental groups are indicated with superscript letters (values with distinct letters are significantly different between them, $P < 0.01$).

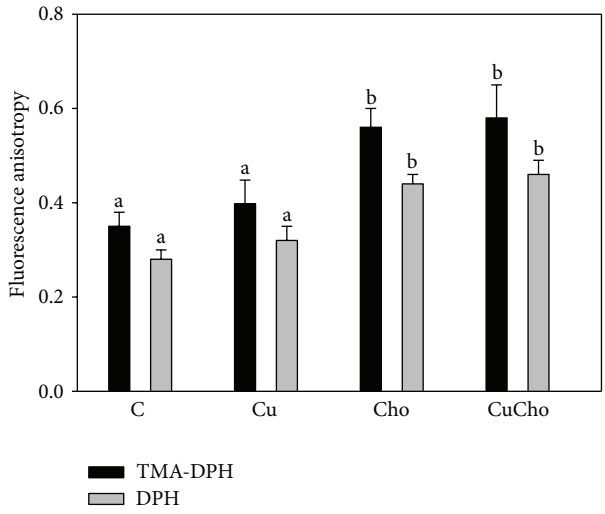
the impact of dietary Cu and Cho manipulations as pro-neurodegenerative cause in animal models.

The impact of elevated inorganic Cu, Cho, or a combination of both in the diet (for 2 months) significantly modifies the mitochondrial function. Results suggest that these dietary supplements are able to increase oxidative damage in brain cortex and hippocampus (with a substantial increment of the lipoperoxides and conjugated dienes in the cardiolipin subfraction), increase the proportion Cho/phospholipids, and consequently decrease the concentration of mGSH, decrease the membrane potential, modify the proportion of cardiolipin and lyso-derivatives, alter inner and outer mitochondrial membrane fluidities, and increase Aβ(1–42)/(1–40) ratio.

It is well known that the metabolism of Cu in humans is under strictly regulated control. The usual concentration of total Cu in human plasma (as determined by us and other groups) is in the range of 0.3 to 2.1 mg/L for intakes of 1.4 to 2.0 mg Cu/day [16]. However, the amount of Cu not bound to ceruloplasmin (NCBC) is very low and has been associated with some of the features of neurodegenerative

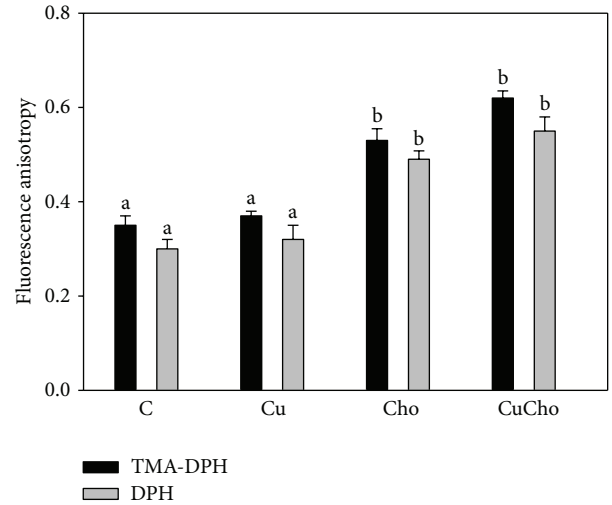


(a) Cortex (OMM)

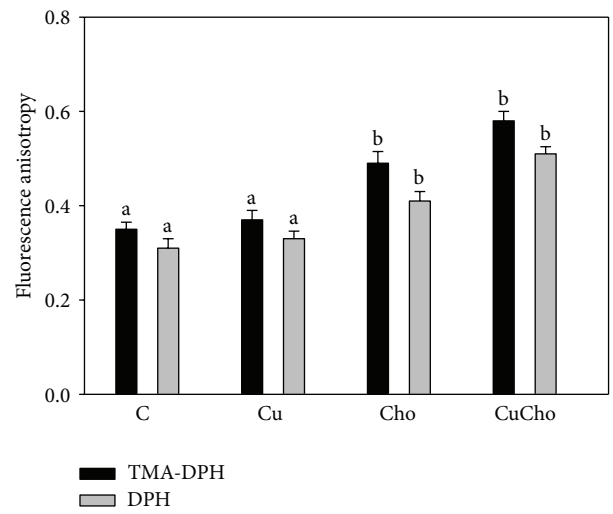


(b) Cortex (IMM)

Figure 7: Mitochondrial outer (a) and inner (b) membrane fluidities (fluorescence anisotropy) in suspension isolated from brain cortex. Treatments are indicated with different colors, control (white bars), Cu (black bars), Cho (gray bars), and CuCho (dark gray bars). Samples were analyzed as indicated in Section 2.9. Results are expressed as the mean ± standard deviation (SD) of 10 rats. Significant differences among the experimental groups are indicated with superscript letters (values with distinct letters are significantly different between them, $P < 0.01$).



(a) Hippocampus (OMM)



(b) Hippocampus (IMM)

Figure 8: Mitochondrial outer (a) and inner (b) membrane fluidities (fluorescence anisotropy) in suspension isolated from brain hippocampus. Treatments are indicated with different colors, control (white bars), Cu (black bars), Cho (gray bars), and CuCho (dark gray bars). Samples were analyzed as indicated in Section 2.9. Results are expressed as the mean ± standard deviation (SD) of 10 rats. Significant differences among the experimental groups are indicated with superscript letters (values with distinct letters are significantly different between them, $P < 0.01$).

disorders such as AD [16, 17]. Cholesterol was also associated with the etiology or increased risk of developing AD [54–57]. Thus, our experimental system was designed to investigate the possible concurrence of these two factors in promoting neurodegeneration, with a focus on their role in mitochondrial function. Mitochondrial dysfunction due to ROS overproduction and/or the alteration of the physicochemical properties of their membranes (mainly lipid composition) was reported to be the main factor in the advance of AD, featuring early on in the progression of the disease [25–27].

In discussing the validity and/or limitations of our experimental system, it is necessary to consider the level of the supplementation with Cu and Cho and the characteristics of the metabolism of these two dietary supplements. With respect to Cu overload using oral administration, our experimental conditions were based on previous work [23, 24] and resemble the Cu levels commonly found as a consequence of involuntary exposure through air, food, and water pollution [8, 58–60], ingestion of dietary mineral supplements, and in professionals engaged in agrochemical activities [9–12] or female users of Cu-based intrauterine devices [13, 14]. Studies

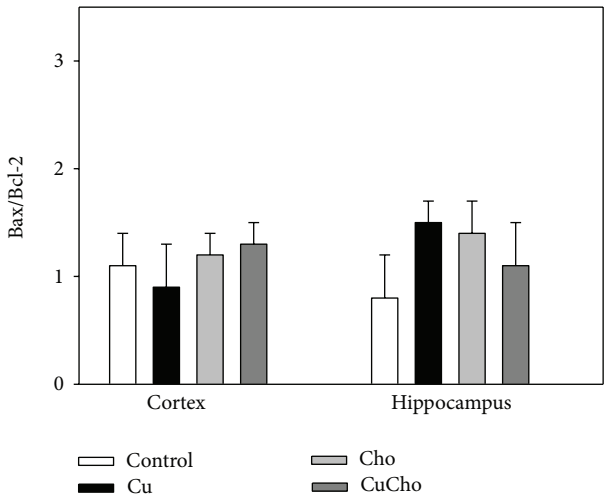


Figure 9: Bax/BcL-2 ratio in brain cortex and hippocampus. Treatments are indicated with different colors, control (white bars), Cu (black bars), Cho (gray bars), and CuCho (dark gray bars). Samples were analyzed as indicated in Section 2.11.1. Results are expressed as the mean ± standard deviation (SD) of 10 rats. There were no significant differences between data.

performed in rats demonstrated that Cu metabolism and homeostasis are essentially identical to those in humans [61]. Similarly, oral Cho supplementation was selected on the basis of previous experiments by other researchers [22–24], and the plasma Cho concentration represents an approximately 30% increase over the medium values obtained for the control group. This increase is frequently observed in humans with dyslipemia [62]. We chose a treatment duration equivalent to approximately 6 years in human terms and therefore assume that the experimental exposure conditions can reasonably be extrapolated to human populations. The known differences between the metabolism of lipoproteins in rats as opposed to humans could be considered a limitation to this assumption, but this can be taken into account as in the case of other experimental models (rabbits or hamsters, e.g.). It is important to recall that rats are quite unique in terms of their sterol metabolism, since in other species studied previously (rabbit, guinea pig, hamster, and squirrel monkey) the liver displays a secondary function, whereas extrahepatic tissues play a quantitatively major role in whole animal sterol biosynthesis and metabolism [63].

Cu supplementation in the trace amounts used here did not substantially modify the concentration of the metal inside the mitochondria isolated from cortex or hippocampus. However, after two months of treatment, we observed an increase in CP concentration in both brain zones in those experimental groups that received Cu. We attribute this increase mainly to a response to the proinflammatory condition induced by the Cu-induced oxidative stress as described in our previous work [64]. We were unable to measure the NCBC *inside* the purified mitochondrial fraction mainly due to the lack of CP or to the presence of this protein in amounts beyond detection by our method of measurement. However, in plasma and in whole homogenates we found that NCBC was increased by Cu administration even in the small amounts used here. Concomitantly, the CP concentration was also higher in all samples except cortex, which probably has a strong homeostatic system to control Cu levels. In agreement with previous findings, Das et al. [65] reported that increases of free Cu (NCBC) stimulate the expression of CP through the activation of AP-1.

Experimental diets also produced significant changes in the lipid composition of mitochondrial fractions. Cu supplementation increased the Cho/phospholipid ratio in the cortex and hippocampus of Wistar rats. This increase was even more pronounced after Cho or CuCho addition. These changes must obviously be associated with the modification of the metabolism (uptake, biosynthesis, and/or degradation) of both subtypes of lipids. The elucidation of these effects remains to be investigated in detail, opening a new avenue of research of potential importance in the search for therapeutic targets in neurodegenerative processes. As a consequence of the aforementioned changes, we observed significant modifications in the physicochemical properties of mitochondrial membranes. The transmembrane potential was altered in Cu- and Cu + Cho-treated rats. Furthermore, the apparent microviscocity of both mitochondrial membranes (outer and inner) was profoundly altered by Cho and CuCho treatments. Generalized and extensive damage in the form of increased permeability (JC-1) and rigidization was mainly demonstrated by the use of two probes with substantially different capacities of lipid bilayer exploration (DPH and TMA-DPH). These findings are in agreement with previously reported data associating the loss of membrane fluidity with an increase in the Cho/Pi ratio [66]. These changes are of relevance from the physiological point of view. It is known that many membrane protein functions including carriers, enzymes, and receptors, among others, can be modulated by the microenvironment of the membrane where they are embedded [66]. Since ROS overproduction has a key role in AD and other neurodegenerative illnesses [67, 68], we will focus on the possible consequences of changes in membrane properties on mitochondrial redox homeostasis and the possible activation of programmed cell death signals.

Mitochondrial glutathione (mGSH) is considered the key antioxidant for mitochondrial functioning and survival. It is the main line of defense for the maintenance of the appropriate mitochondrial redox environment by preventing or repairing oxidative modifications that could lead to mitochondrial dysfunction and cell death. The importance of mGSH lies not only in its abundance but also in its extreme versatility to counteract hydrogen peroxide, lipid hydroperoxides, or xenobiotics, mainly as a cofactor of enzymes such as glutathione peroxidase or glutathione-S-transferase. Many cell-inducing stimuli, such as excess NCBC or other transition metals that induce oxidative stress, reduce the levels of mGSH and sensitize the mitochondria to additional insults (e.g., excess cholesterol in the composition of their biomembranes) [66]. Interestingly, the transport of GSH into the mitochondria is critically dependent on the maintenance of physiologically regulated membrane parameters, especially the Cho/Pi ratio and the apparent microviscocity of the inner mitochondrial membrane [66–70]. The dicarboxylate carrier

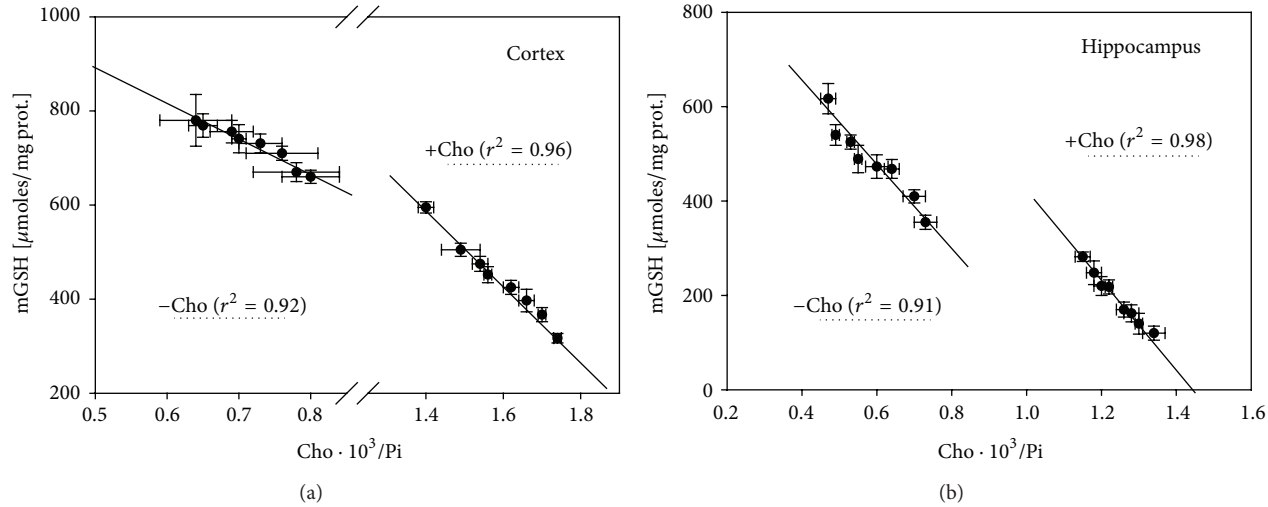


Figure 10: Lineal correlation coefficients for the relationship between mGSH and Cho/Pi ratio in cortex (a) and hippocampus (b) grouped according to two groups of data independently of Cu supplementation, one for rats that did not receive cholesterol (−Cho) and the other for animals fed on diets supplemented with Cho (+Cho). The values of the r^2 for each curve adjustment appear in parenthesis.

and the 2-oxoglutarate carrier have been shown to function as GSH transporters highly sensitive to the physiochemical state of mitochondrial membranes [69]. The activities of both GSH carriers were inhibited for a decrease in the membrane fluidity. In agreement with these facts, we not only observed a decrease in mGSH in the Cho-supplemented rats but also a strong negative correlation between mGSH and the Cho/Pi ratio in mitochondrial membranes (Figure 10). Cu supplementation also produced an increase in the Cho/Pi ratio, though not sufficiently to significantly modify the membrane fluidity, leaving it unable to modify the mGSH levels.

Depletion of the main antioxidant defense system in mitochondria is directly linked to increased lipid peroxidation. A pro-oxidative environment clearly promotes the oxidation of CL and also stimulates its regeneration or remodeling activity by increasing the intermediary metabolites, lyso-CL (mono- and dilyso-CL in cortex and hippocampus). In line with this, we found higher LPOO and conjugated diene production in isolated CL subfractions after Cu addition that was even more pronounced after cosupplementation with Cho (CuCho). Peroxidized CL decreases the pool of CL available in mitochondria, thus preventing the formation of the transition pore and the subsequent leakage of Cyt C to the cytosol. Moreover, oxidized CL was able to produce mitochondrial dysfunction, preventing electron transport through the protein complexes [28, 68]. Our finding of lower mitochondrial CL content after Cu treatment in both brains zones is in agreement with previous observations of Yurkova et al. [71]. They reported that Cu ions possess the ability to fragment CL with the subsequent formation of phosphatidic acid and phosphatidyl hydroxyacetone in mitochondria from a mouse model of Wilson's disease. Interestingly, Cho supplementation produced an increase in CL levels probably through an ROS-dependent overstimulation of acyltransferase-1 activity [72]. The fact that no significant changes with respect to control rats were observed under CuCho treatment could be explained in terms of a compensatory mechanism to counteract the opposite effects produced by Cu and Cho supplementations.

Cho enrichment of mitochondrial membranes has other important consequences. Alterations in the physical properties of mitochondrial membranes, such as those revealed by the response to DPH and TMA-DPH probes, could modify mitochondrial dynamics, which are critical for the maintenance of mitochondrial integrity and functions including energy production, ROS generation and apoptosis regulation [26–31]. Modifications in mitochondrial dynamics lead to structural changes in cristae formation and the assembly of electron transport complexes, compromising bioenergetics and causing calcium dyshomeostasis, increased oxidative stress (consumption of mGSH), mitochondrial DNA damage, and synaptic dysfunction [28–30].

We have mentioned that the pro-oxidative condition induces CL peroxidation and in consequence diminishes CL's ability join to proteins like Cyt C. Montero et al. [73] and Eckmann et al. [74] emphasized that even though only 15% of the Cyt C is strongly bound to CL, the oxidation of minor amounts of this phospholipid could trigger the mobilization of the Cyt C from the mitochondrial inner membrane to the intermembrane space and then to the cytosol. However, we found no significant changes in Cyt C release to the cytoplasm after any treatments, except in the case of CuCho addition to the cortex. There were also no significant changes in the Bax/BcL-2 ratio and in caspase-3 activity, indicating that the intrinsic apoptotic pathway was still not significantly activated. The pro-oxidative conditions likely induced by Cu, together with the enrichment of cholesterol that reduces mGSH levels, produce CL peroxidation; however, it appears that the level of damage is insufficient to produce a significant stimulation of apoptosome formation and subsequent activation of the caspase-3 pathway [73]. Our lab is now studying the effect of a more prolonged time of exposure in order to investigate the chronic effects of these experimental treatments.

Lu et al. [24] reported that Cu is associated with Aβ in senile plaques and that this complex can recruit Cho molecules, oxidizing them and producing even more ROS. Despite the clear activation of the proapoptotic cascade, we found an increase in Aβ formation in the brain, a condition which is a recognized biomarker of neurodegeneration. These changes in cortex and hippocampus were not reflected in peripheral plasma, most likely because the latter is less sensitive or the duration of the treatment was not sufficient to affect the proportion between Aβ(1–42)/(1–40) in peripheral plasma, or both. Oxidative stress induces Aβ accumulation in mitochondrial membranes, causing structural and functional damage. Amyloid-β interacts with the mitochondrial protein Aβ-binding alcohol dehydrogenase (ABAD) which is upregulated in the temporal lobe of AD patients as well as in AβPP transgenic mice [28]. This complex prevents the binding of nicotinamide adenine dinucleotide to ABAD, thereby changing mitochondrial membrane permeability (as we observed in our model) and reducing the activities of respiratory enzymes triggering elevated ROS production [28–30]. Increased Aβ production and Aβ-ROS-dependent formation are linked to other important issues such as the inactivation of the presequence protease (PreP), one of the most important proteins involved in Aβ degradation; alteration of mitochondrial dynamics (previously discussed); increased nitrosative stress; activation of cyclophilin D (an integral part of the permeability transition pore or mPTP); potentiation of synaptic failure; and cytoskeletal aberrations, among others [26–31]. All these facts indicate the importance of monitoring the level of Aβ production not only at the local (SNC) but also at the systemic (peripheral plasma) level.

5. Conclusions

Our work demonstrates that dietary supplementation with trace amounts of inorganic Cu in drinking water + Cho supplementation in solid food causes oxidative stress in brain cortex and hippocampus by depletion of mGSH in an inverse Cho/Pi-dependent fashion; CL peroxidation and stimulation of the remodeling route; major alterations in mitochondrial membrane integrity and fluidity; and a higher Aβ(1–42)/(1–40) ratio as a biomarker of neurodegeneration. These changes may be additive and could lead—under more prolonged exposure—to activation of the proapoptotic cascade. Ongoing research will shed further light on the real significance of the present results and establish the intimate mechanisms underlying the damage induced by these two very common factors (Cu and Cho overload) with a view to defining potential preventive strategies in human populations at risk.

Abbreviations

Cu: Copper
CL: Cardiolipin
CP: Ceruloplasmin
Cho: Cholesterol
LPOO: Lipoperoxides
mGSH: Mitochondrial glutathione
ROS: Reactive oxygen species.

Conflict of Interests

The authors declare that there is no conflict of interests.

Disclosure

All authors disclose any financial and personal relationships with other people or organisations that could inappropriately influence this work.

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Research Article

Role of Copper and Cholesterol Association in the Neurodegenerative Process

Nathalie Arnal,[1] Gustavo R. Morel,[1] María J. T. de Alaniz,[1] Omar Castillo,[2] and Carlos A. Marra[1]

[1] *INIBIOLP (Instituto de Investigaciones Bioquímicas de La Plata), CCT La Plata, CONICET-UNLP, Cátedra de Bioquímica y Biología Molecular, Facultad de Ciencias Médicas, Universidad Nacional de La Plata, 60 y 120 (1900) La Plata, Argentina*
[2] *CIC (Centro de Investigaciones Cardiovasculares), CCT La Plata, CONICET-UNLP, Facultad de Ciencias Médicas, Universidad Nacional de La Plata, 60 y 120 (1900) La Plata, Argentina*

Correspondence should be addressed to Carlos A. Marra; contactocarlos@hotmail.com

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Age is one of the main factors involved in the development of neurological illnesses, in particular, Alzheimer, and it is widely held that the rapid aging of the world population is accompanied by a rise in the prevalence and incidence of Alzheimer disease. However, evidence from recent decades indicates that Cu and Cho overload are emerging causative factors in neurodegeneration, a hypothesis that has been partially investigated in experimental models. The link between these two variables and the onset of Alzheimer disease has opened up interesting new possibilities requiring more in-depth analysis. The aim of the present study was therefore to investigate the effect of the association of Cu + Cho (CuCho) as a possible synergistic factor in the development of an Alzheimer-like pathology in Wistar rats. We measured total- and nonceruloplasmin-bound Cu and Cho (free and sterified) contents in plasma and brain zones (cortex and hippocampus), markers of oxidative stress damage, inflammation, and programmed cell death (caspase-3 and calpain isoforms). The ratio beta-amyloid (1-42)/(1-40) was determined in plasma and brain as neurodegenerative biomarker. An evaluation of visuospatial memory (Barnes maze test) was also performed. The results demonstrate the establishment of a prooxidative and proinflammatory environment after CuCho treatment, hallmarked by increased TBARS, protein carbonyls, and nitrite plus nitrate levels in plasma and brain zones (cortex and hippocampus) with a consequent increase in the activity of calpains and no significant changes in caspase-3. A simultaneous increase in the plasma Aβ1-42/Aβ1-40 ratio was found. Furthermore, a slight but noticeable change in visuospatial memory was observed in rats treated with CuCho. We conclude that our model could reflect an initial stage of neurodegeneration in which Cu and Cho interact with one another to exacerbate neurological damage.

1. Introduction

The aging of the world population is being accompanied by a rise in the prevalence and incidence of Alzheimer disease (AD) and other neurodegenerative illnesses. In the USA, the number of patients with AD is expected to increase to 13 million by 2050 and in the European Union (EU) to over 4 million [1]. The mortality rate of AD is second only to cancer and stroke. Epidemiologic data indicates that the world population will have grown considerably by 2025 and the percentage of elderly people will be significantly higher [2]. Since age is one of the major risk factors in the development of AD, it is expected that the incidence of this disease will also increase. However, experimental evidence detailed in a review by Brewer indicates that other factors in addition to age could play a critical role [3] in the development of diseases such as Alzheimer and that the contribution of inorganic copper (Cu) has been underestimated. It is widely known that more than 95% of AD cases are sporadic and only 2–7% are genetically determined [4]. Thus, any environmental factors likely to have an etiopathogenic role in AD should be investigated.

Though Cu is essential to human health, Cu overload has been associated with mental decline [5] and particularly with AD development [3, 6, 7]. Data from Squitti's group specifically demonstrated that free Cu (also known as NCBC

or nonceruloplasmin-bound Cu) is elevated in the blood of AD patients, negatively correlates with cognition, and predicts the rate of loss of cognition [8–10]. More recently, our group corroborated these findings in an independent human cohort and demonstrated that increased NCBC has a direct impact on the disease duration [11]. We also proposed the NCBC/ceruloplasmin ratio as a predictive marker of risk for the first-degree relatives of AD patients. Sparks and Schreurs [12] first demonstrated that Cu supplementation in drinking water given to rabbits under a diet with excess cholesterol (Cho) produced an induction of β-amyloid plaques and a learning deficit. In addition, Lu et al. reported that trace amounts of Cu activate the apoptotic cascade and exacerbate beta amyloid-induced neurotoxicity in Cho-fed mice through a TNF-mediated inflammatory pathway [13, 14]. Very recently, Brewer has reviewed the theory of inorganic Cu toxicity in Alzheimer disease as a causative factor in cognitive loss [6]. However, the question of how inorganic Cu might trigger a neurodegenerative process is still a matter of debate [15].

The degree of exposure of human populations to Cu is also a controversial issue. There is little data on Cu overload in humans since most of the available evidence (experimental or epidemiological) was obtained from animal models. However, the regulatory framework for chronic Cu exposure in large human populations indicates that pollution, drinking water, and dietary Cu-containing supplements are the main sources of exposure [6, 16]. The dietary reference intake for people in the USA, United Kingdom, Europe, and Australia varies from 0.16 to 0.98 (Estimated Average Requirements) EAR or (Recommended Dietary Allowance) RDA expressed in mg Cu/kg body weight, with considerable variations as a function of age. The (Population Reference Intake) PRI was reported between 0.3 and 1.5 mg Cu/kg body weight [16]; however, these limits were largely surpassed in many circumstances such as ingestion of fish, bivalves, or contaminated drinking water [3, 17]. In accumulated data on 280 samples of household drinking water all across North America, 72% of the samples have Cu levels above those enhancing AD in experimental models [3]. The content of inorganic Cu in dietary supplements can be as high as 3 mg/pill (approx. 2 mg above the EAR or RDA). Furthermore, we and others have demonstrated significantly higher Cu levels in plasma of women using Cu-IUDs and in blood samples from farmers working with Cu-based pesticides [17–19]. Most importantly, we found alterations in Cu homeostatic biomarkers in neurodegenerative patients and their first-degrees relatives [11].

Fat ingestion has also been linked to AD prevalence [20]. Specifically, Cho has been associated with oxidative stress and AD development [21, 22], with most of the experimental evidence emerging from exploration of the role of Cho in β-amyloid formation [23]. Despite the paucity of epidemiological data from human studies, it is reasonable to assume that Cho plays at least some role in learning and memory and is associated with AD pathogenesis [24]. Of particular interest is evidence showing that Cu greatly exacerbates cognitive decline in those people included in the highest quintile of fat ingestion [25]. Experimental evidence obtained from rabbit and mouse models suggests that the association of Cu and Cho can be risk factor for AD development [12–14]. Moreover, it was hypothesized that Cu could oxidize Cho, generating substances toxic to the brain [3].

However, the mechanism of action of Cu and Cho in AD incidence and development is poorly understood and requires further investigation. Thus, the aim of this work was to study the effects of Cu and Cho association on the two main brain areas affected in AD, cortex and hippocampus, using a model of Wistar rats. Specifically, we aimed to determine for each nutritional supplement alone or in combination (i) the capacity to install oxidative/nitrative damage; (ii) changes in the levels of the main antioxidant molecules (glutathione and α-tocopherol); (iii) the possible development of a pro-inflammatory condition by analyzing the concentration of prostaglandins PGE2 and PGF2α; (iv) the activities of the two main protease systems associated with programmed cell death, caspase-3 and calpains (μ- and m-); and (v) possible changes in visuospatial memory as assessed by means of the Barnes maze test.

Our findings could be useful in further investigating the mechanisms underlying the neurodegenerative process and also in localizing putative targets for preventive interventions associated with endogenous and/or exogenous causative factors such as Cu and Cho, alone or in combination.

2. Material and Methods

2.1. Chemicals. All chemicals used were of analytical grade and obtained from Sigma Chem. Co. (Buenos Aires, Argentina or USA), Merck (Darmstadt, Germany), and Carlo Erba (Milan, Italy).

2.2. Animals and Treatments. Certified pathogen-free male Wistar rats were used. The rats were maintained at a controlled temperature (25°C) and relative humidity of 60% with forced ventilation, under a normal photoperiod of 12 h darkness and 12 h light. The health of the animals was monitored in accordance with the internationally recommended practices of the (*Institute of Laboratory Animal Resources, Commission of Life Sciences, National Research Council*) ILAR. Solid food and drinking water were provided *ad libitum*. The diets for the experiments were prepared in our laboratory according to the recommendations for Wistar rats [26]. All procedures for handling the animals followed the NIH regulations [27]. The experimental protocol was reviewed and approved by (Bioethics Committee of the Faculty of Medical Sciences, UNLP) COBIMED under the code # 00382/11.

2.3. Experimental Protocols. Rats (21 days old) were randomly assigned (ten animals per group) to the protocols detailed as follows and treated during eight weeks. The control group (C) was maintained on lab-prepared pellets as recommended for normal growth, containing 7 ppm of Cu [28, 29]. The Cu-supplemented experimental group (Cu) was fed on control pellets and tap water supplemented with 3 mg/L (or ppm) of Cu in the form of ultrapure $CuSO_4$ (Merck, Darmstadt, Germany), the Cho-supplemented group (Cho) was fed on pellets containing 2% (W/W) of Cho (87% pure) (obtained from

Saporiti SRL, Buenos Aires, Argentina), and the Cu + Cho-supplemented group (CuCho) was simultaneously treated with Cu in water + Cho in food. Rats were monitored during the experimental period to observe their behavior, quantify water, and food consumption and determine their body weight gain. Total Cu concentration in tap water supplemented with $CuSO_4$ was determined by means of atomic absorption methodology and was 3.42 ± 0.21 ppm (means of all daily measurements along the experimental period). Considering that each animal imbibed between 4.9 ± 0.4 and 15.0 ± 1.1 mL water/day (at the beginning and the end of the protocol, resp.), a maximum of 0.01 to 0.05 mg Cu/day was acquired from water (a dose equivalent to 0.06 and 0.18 mg Cu/Kg live animal, resp.). Linear regression curves and ANOVA test for Cu content in food demonstrated that there were no significant variations between the 6 preparations used for the experiments (7.22 ± 0.31 ppm or mg Cu/Kg diet). Fe and Zn content (determined by atomic absorption spectrometry) were the same in all preparations (45.9 ± 1.8 and 66.6 ± 2.0 ppm, resp.). Ingestion of solid food along the experiments varied from 11.6±0.8 to 29.7±2.8 g/rat, implying that the oral ingestion of Cu was in the range of 0.08 to 0.21 mg Cu/day/rat (0.90 to 1.21 mg Cu/Kg live animal, a mean of 1.06 ± 0.11 mg Cu/Kg).

2.4. Sample Collection. At the end of the treatments, animals were deeply anesthetized with ketamine (70 mg/Kg) and xylazine (5 mg/Kg) applied intramuscularly and then sacrificed by decapitation. Brains were rapidly taken out and dissected in two zones, cortex and hippocampus, using the atlas of Paxinos and Watson [30] as a guide for tissue dissection and a Binocular Stereoscopic Arcano Ztx-1065 Microscope (Instrumental Pasteur, Buenos Aires, Argentina). Both brain regions were washed, weighed, and homogenized using a buffer Tris/HCl (10 mM pH 7.4) with sucrose (70 mM), mannitol (230 mM), ethylenediaminetetraacetic acid (EDTA) (1 mM), and dithiothreitol (DTT) (1 mM).

2.5. Atomic Absorption Measurements. Aliquots of sample were digested with a mixture of 4 mL of HNO_3 (c) and 1 mL $HClO_4$ (Aldrich or Sigma Chem. Co., Buenos Aires, Argentina) by heating at 120°C for 80 min in a mineralization block [31]. The digests were cooled, diluted with ultrapure water (18 mΩ cm, Carlo Erba, Milan, Italy), and ultrafiltered by a 0.22 μm Millipore membrane (Milli-Q Purification System, from Millipore, CA, USA). Ultrafiltered dissolutions were directly aspirated into the flame of a Perkin-Elmer 1100 B Spectrophotometer equipped with a Perkin-Elmer cathode lamp (Perkin-Elmer Corp., Norwalk, CT, USA) at a spectral width of 1 nm. Standard solutions of 100 ppm from HCR Inc. (QuimiNet, Buenos Aires, Argentina) were used for Zn and Fe. Cu determinations were calibrated with a standard solution (200 ppm) of Cu $(NO_3)_2$ in HNO_3 0.5 N (Tritrisol from Merck Co., Darmstadt, Germany). All measurements were carried out in peak height mode (324.7 nm line). The intra-[(SD/x)·100] and inter-[(ΔSD/Δx)·100] assay coefficients of variation were 15.5 and 6.0%, respectively. We routinely obtained a similar equation for the calibration curve ($IR = 55.10^{-5} + 0.048 \cdot$[Cu, mg/L]), and the statistical analyses demonstrated a correlation coefficient always between 0.95 and 0.99. In addition, we explored the so-called matrix effects that could have modified the slopes of the standard regressions. In spiked samples the obtained values varying from 48 to 60.10^{-5} were very similar to those of Cu standard solutions, indicating that the matrix effect was not significant or was negligible. The mean for recovery and RSD for spiked samples was 99.7% and 3.3%, respectively, and the detection limit was 0.09 mg/L. In order to verify the accuracy of the method, we explored the influence of time after dilution, temperature of acid digestion, and concentration of $HNO_3/HClO_4$ following the suggestions of Terrés-Martos et al. [32]. We also checked our results with biological samples (plasma and homogenates) against a Seronorm Trace Elements Serum (from Sero Labs, Billingstad, Norway) and found no significant differences between the obtained and the declared (certified) concentrations.

2.6. Ceruloplasmin (CRP) Levels and Nonceruloplasmin-Bound Copper (NCBC). Samples were analyzed by the enzyme conversion of p-phenylenediamine into a blue-colored product [33] which was then measured at 550 nm. Reaction proceeded at 37°C in buffer glacial acetic/sodium acetate (50 mM, pH 5.5) directly into flat-bottomed plates, using a Microplate Reader SpectraMax M2/m2^e model from Molecular Devices Analytical Technologies (Sunnyvale, CA, USA) for 3 min. Intra- and interassay coefficients of variation were 8.3 and 4.4%, respectively. CRP concentrations were calculated by comparison with the reaction rate of pure CRP standard (Sigma Chem. Co., Buenos Aires, Argentina). Using the Cu and CRP data, we calculated the non-CRP-bound Cu (NCBC, or so-called free Cu) as described by Brewer [3] by subtracting the amount of Cu bound to each mg of CRP from data of total Cu. This parameter can be easily expressed in percentages using the formula (([Cu]-47.2 × [CRP]) × 100/[Cu]), where Cu is in μmol/L and CRP is in g/L [34].

2.7. Lipid Analysis. Total lipids were extracted by the method of Folch et al. [35]. Aliquots of this solution were taken to measure total Cho by an enzymatic method using a commercial kit from Wienner Lab (Rosario, Argentina). To estimate the amount of esterified Cho (ECho), aliquots of the lipid extracts were seeded in high-resolution pre-coated silica gel plates (10 × 20 cm) with a concentration zone for thin layer chromatography (Whatman Adsorption 60 Å Silica Gel HP-TLC Plates, CA, USA) and developed with diethyl ether : hexane : acetic acid (90 : 4 : 1, by vol) as described elsewhere [36]. Authentic standards of Cho and Cho-esthers (from Avanti Polar Lipids, Ontario, Canada) were run in parallel and revealed by iodine vapors. Identified zones were scraped off the plates, eluted with Folch reactive, evaporated, dissolved in 50 mM phosphate buffer (pH 7.4) with 1% sodium deoxycholate, and enzymatically analyzed using the commercial kits from Wienner Lab (Rosario, Argentina).

2.8. Biomarkers of ROS Production. Thiobarbituric acid-reactive substances (TBARS) were measured in brain homogenates as previously described [37]. TBARS (mainly malondialdehyde, MDA) reacted with 2-thiobarbituric acid (TBA)

to yield TBA-MDA adducts which were quantified at 532 nm. The concentration of the chromophore was calculated from a calibration curve prepared with fresh tetramethoxypropane (TMP) solutions (TMP was purchased from Sigma Chem. Co., Buenos Aires, Argentina). Nitrate and nitrite [NOx] concentration were measured using the method of Griess on samples previously reduced with vanadium chlorohydrate according to Miranda et al. [38]. Quantification was performed after calibration with standard solutions of sodium nitrate from Merck Co. (Darmstadt, Germany). Protein carbonyls (PCs) were determined by the method of Reznick and Packer [39]. The concentrations of PCs were calculated from a calibration curve prepared with a stock solution of sodium pyruvate (Sigma Chem. Co.).

2.9. Antioxidant Defense System

2.9.1. Reduced (GSH) and Oxidized (GSSG) Glutathione Content. Total glutathione was determined by the glutathione reductase/dithionitrobenzoic (DTNB) method that can measure both GSH and GSSG [40]. To calculate the GSH/GSSG ratio, samples were reanalyzed after derivatization with divinylpyridine (3 mM final concentration).

2.9.2. Vitamin E (α-tocopherol). Vitamin E (α-tocopherol) was measured after extraction with the Buttriss and Diplock method [41] using the HPLC technique of Bagnati et al. [42] in a reverse phase/C-18 silica column from ALLTECH Associates, Inc. (Deerfield, IL, USA). The ECONOSIL C_{18} column with a Direct-connect Cartridge Guard Column System was operated at a maximum pressure of 500 psi in a Hitachi HPLC System (Tokyo, Japan). The amount of vitamin was electronically calculated using internal calibration with pure α-tocopherol (Sigma, Bs. As.), and the results were expressed in μM concentration of α-tocopherol.

2.9.3. Glutathione Reductase (GR). GR activity was determined by the method of Carlberg and Mannervik [43]. The specific activity of the enzyme was calculated for each sample in terms of U/min·mg protein ($\varepsilon = 6.22\ \text{nM}^{-1}\cdot\text{cm}^{-1}$ for absorbance at 340 nm).

2.10. Biomarkers of Inflammation. Prostaglandin F2α (PG F2α) and prostaglandin E2 (PGE2) were measured using the PGF2α EIA Kit and PGE2 Express EIA Kit, respectively (Cayman, Migliore Laclaustra S.R). The results were expressed as ng of each prostaglandin/mg total protein.

2.11. Programmed Cell Death Biomarkers

2.11.1. Caspase-3 Activity. Caspase-3 activity was measured by a colorimetric assay kit (CASP-3-C) based on the hydrolysis of the synthetic peptide substrate acetyl-Asp-Glu-Val-Asp-p-nitroaniline (Ac-DEVD-pNA) by caspase-3 (Sigma Chem. Co., Buenos Aires, Argentina). The resulting p-nitroaniline (p-NA) was monitored at 405 nm. Each assay was run in parallel with inhibitor-treated homogenates (to measure the nonspecific hydrolysis of the substrate) and caspase-3 positive control (using commercial caspase-3, 5 mg/mL provided by the kit manufacturer). A calibration curve using a standard solution of p-nitroaniline (p-NA) was also run for each assay to calculate the activity of the protease expressed as μmol p-NA released/min·mg protein.

2.11.2. Milli- (m-) and Micro- (μ-) Calpains. The assay involves the hydrolysis of whole ultrapure casein (Sigma, Chem. Co., CA, USA) by calpain activity and the subsequent detection of trichloroacetic acid (TCA) soluble peptidic fragments at 280 nm [44]. To select the activity of the calpain isoforms, the level of calcium in the medium was regulated (5 mM or 500 μM of $CaCl_2$ for m- or μ-calpain, resp.). The activity of calpains was calculated considering that a unit of calpain is the amount of enzyme that produces a change of absorbance of 0.01 at 280 nm. Results were expressed as units/min·mg of protein.

2.12. Markers of Neurodegeneration

2.12.1. Beta Amyloid Peptides (1-40) and (1-42). Beta amyloid peptides (Aβ) (1-40) and (1-42) were measured using Human/Rat β Amyloid-40 ELISA kit Wako II and the Human Amyloid-42 ELISA kit Wako High-Sensitivity, respectively. The Aβ(1-42)/(1-40) ratio was then calculated from the individual data expressed as picomole of the respective Aβ peptide/mg total protein.

2.12.2. Barnes Maze Test. The Barnes maze is a black acrylic circular platform, 122 cm in diameter and elevated 108 cm off the floor, containing twenty holes around the periphery. The 10 cm diameter holes are uniform in appearance but, one hole is connected to an escape box, consisting of a 38.7 cm long × 12.1 cm wide × 14.2 depth cm removable box. Four proximal visual cues (30 cm high, opaque black geometric figures: a cross, a circle, a square, and a triangle) were located in the room 50 cm from the circular platform. The escape box remained in a fixed position relative to the cues, to ensure randomization of the hole associated with the escape tunnel. In the center of the platform is a starting chamber (an opaque, 26 cm diameter, 20 cm high, and white plastic open-ended cylinder). A 90 dB white-noise generator and a white-light 500 W bulb provided motivation for escaping from the platform. The acquisition session and the probe trial session were performed on the same day. In brief, the experiment consisted of eight acquisition trials (t1–t8) followed by a single evaluation trial (t9). Acquisition trials began with the animal inside the starting chamber for 30 seconds in the presence of a constant buzz. The chamber was then raised, the aversive stimulus (intense bright light) was switched on, and the rat was allowed to freely explore the maze. The rats were each given 120 s to locate the correct hole. If by the end of this period they had not entered the escape box of their own accord, they were gently picked up and placed over the hole above the escape box. The evaluation trial proceeded in the same manner as described above but without the start box. At the end of each trial, the aversive stimulus was switched off, the rat remained on the escape box during 60 s, and the white light was switched off. In order to eliminate any possible olfactory clues from the maze, it was cleaned with

Table 1: Copper (Cu) levels in animals fed with the experimental diets.

Diets	Cu (ng/mg prot)					
	Plasma		Brain			
			Cortex		Hippocampus	
	Total	NCBC	Total	NCBC	Total	NCBC
Control	18.5 ± 0.8^{a}	0.95 ± 0.11^{a}	5.5 ± 0.2^{a}	0.25 ± 0.04^{a}	4.8 ± 0.1^{a}	0.27 ± 0.04^{a}
Cu	23.2 ± 0.7^{b}	1.76 ± 0.15^{b}	7.1 ± 0.1^{b}	0.27 ± 0.02^{a}	6.1 ± 0.1^{b}	0.33 ± 0.02^{b}
Cho	18.1 ± 1.0^{a}	0.91 ± 0.05^{a}	5.4 ± 0.2^{a}	0.24 ± 0.04^{a}	5.1 ± 0.2^{a}	0.25 ± 0.04^{a}
CuCho	22.9 ± 0.6^{b}	1.78 ± 0.10^{b}	7.0 ± 0.1^{b}	0.29 ± 0.03^{a}	6.2 ± 0.04^{b}	0.35 ± 0.03^{b}

Cu content was determined after mineral digestion of the samples by atomic absorption spectrometry as described in Section 2.5. Results are the mean of 10 independent measurements analyzed in triplicate ± SD. Comparisons between data were performed by ANOVA + Tukey test at $P < 0.01$. Statistical differences among the experimental diets were indicated with distinct superscript letters (values within the same column with different superscript letters are statistically significant between them).

Table 2: Esterfied (ECho) and free (FCho) cholesterol levels in animals fed with the experimental diets.

Diets	Cho (nmoles/mg·prot)								
	Plasma			Brain zones					
				Cortex			Hippocampus		
	Esterified	Free	FCho/Echo	Esterified	Free	FCho/Echo	Esterified	Free	FCho/Echo
C	16.4 ± 0.3^{a}	10.6 ± 0.2^{a}	0.64 ± 0.05^{a}	28.2 ± 1.9^{a}	257.8 ± 10.6^{a}	9.14 ± 0.71^{a}	28.7 ± 2.2^{a}	247.1 ± 9.7^{a}	8.61 ± 0.60^{a}
Cu	16.8 ± 0.4^{a}	11.7 ± 0.3^{a}	0.70 ± 0.05^{a}	35.8 ± 2.2^{b}	260.2 ± 7.3^{a}	7.26 ± 0.42^{b}	39.2 ± 1.8^{b}	252.1 ± 11.4^{a}	6.43 ± 0.33^{b}
Cho	22.2 ± 0.4^{b}	11.5 ± 0.4^{a}	0.51 ± 0.03^{c}	30.6 ± 2.1^{a}	281.3 ± 12.2^{b}	9.19 ± 0.55^{a}	28.0 ± 1.7^{a}	269.4 ± 11.4^{b}	9.62 ± 0.21^{c}
CuCho	18.3 ± 0.5^{a}	15.5 ± 0.5^{c}	0.85 ± 0.05^{d}	70.0 ± 3.1^{c}	225.3 ± 15.0^{c}	3.21 ± 0.13^{c}	77.5 ± 2.7^{c}	233.6 ± 11.5^{a}	3.01 ± 0.11^{d}

Cho and Cho-esters contents were determined enzymatically after HP-TLC as described in Section 2.7. Results are the mean of 10 independent measurements analyzed in triplicate ± SD. Comparisons between data were performed by ANOVA + Tukey test at $P < 0.01$. Statistical differences among the experimental diets were indicated with distinct superscript letters (values within the same column with different superscript letters are statistically significant between them).

10% ethyl alcohol solution at the beginning of the 15 min intertrial period. An individual hole exploration was defined as being a single downward head deflection toward the inside of the hole. The following parameters were determined: (i) first-hole latency time (in s) spent by the animal between being released from the start box and exploring a hole in the maze for the first time; (ii) escape-box latency time (defined in the acquisition and retention test trials as the time (in s) spent by the animal between being released from the start box and entering the escape box and, in the case of the preference test and extinction trials, the time elapsed before the first exploration of the escape hole); and (iii) nongoal hole exploration (defined as the number of explorations of holes other than the escape hole, the explorations being considered as errors during the acquisition and probe trials). In the case of the evaluation trials, we evaluated the hole exploration frequency (the number of explorations of each hole during the trial in which the escape hole was numbered as hole 0 for normalized graphical representation purposes, 1 to 10 clockwise, and −1 to −9 counterclockwise). The behavioral measurements were recorded using a video camera mounted 110 cm above the platform, linked to a computer. The video performances of each rat were analyzed using the video analysis software Kinovea-Creative Commons Attribution (v 0.7.6).

2.13. Statistical Analysis. All values represent the mean of 6 rats assayed in triplicate expressed as mean ± standard deviation (SD). Data were analyzed by ANOVA plus Tukey test with the aid of SPSS 11.0.1 software (SPSS Inc., Chicago, IL). To analyze the data from the Barnes maze test, multiple comparisons were drawn with the control group using two-way ANOVA plus the Holm-Sidak *post hoc* test at two levels of significance ($P < 0.05$ and 0.01). Data were also analyzed using MANOVA with identical final conclusions. Results were also plotted and analyzed using Sigma Scientific Graphing Software (version 11.0) from Sigma Chem. Co. (St. Louis, MO). The statistical significance of differences is indicated by distinct superscript letters (data with distinct superscript letters are statistically different ($P < 0.01$) between them).

3. Results

3.1. Cholesterol and Copper Levels. Table 1 shows the levels of total and free Cu (NCBC or nonceruloplasmin-bound Cu) in plasma and in brain cortex and hippocampus after the experimental treatments. Plasma total Cu and NCBC exhibited discrete (but statistically significant) increases in those groups receiving Cu (Cu and CuCho). Total Cu also increased in both cortex and hippocampus after Cu supplementation (alone or in combination with Cho). Cortex shows a nonsignificant tendency towards higher NCBC after Cu or CuCho treatments, whereas hippocampus shows a significant increase in this parameter with respect to the control group.

The results of the Cho analysis (free and esterified) are shown in Table 2, from which it is evident that tissue from

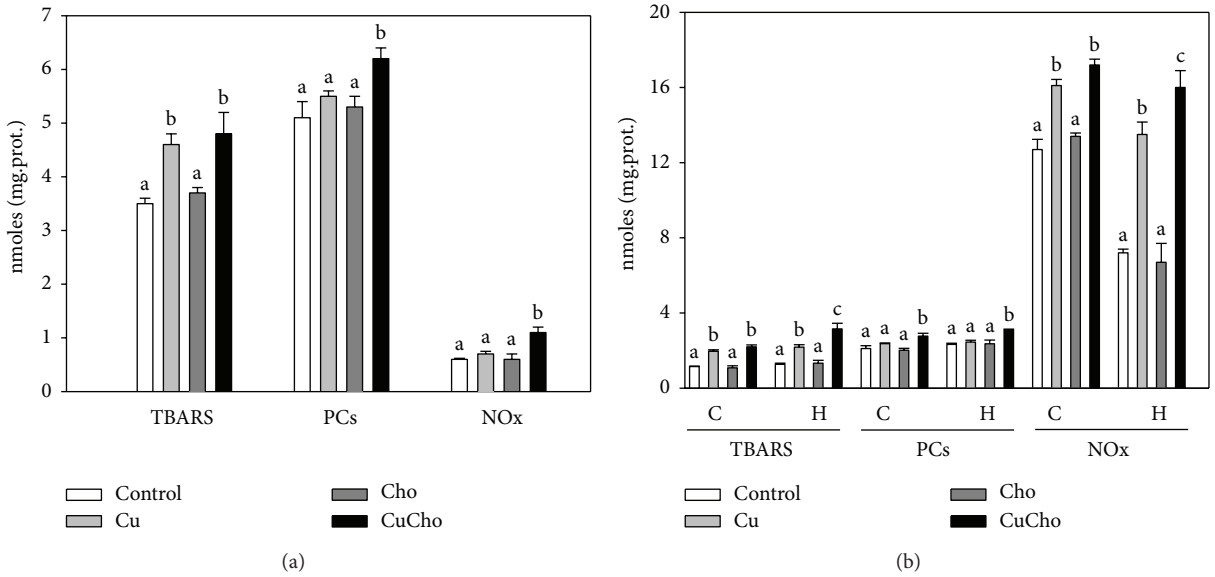

Figure 1: Oxidative/nitrative stress biomarkers in plasma (a) and brain cortex and hippocampus (b). Lipid peroxidation (TBARS) (nmol MDA/mg protein), protein oxidation (PCs) (nmol MDA/mg protein), and concentration of nitrites and nitrates ([NOx]) (μmol/mg protein) were determined in plasma and brain cortex and hippocampus following the procedures described in described in Material and Methods Section. Treatments are indicated with different colors. Control (white bars), Cu (gray bars), Cho (dark gray bars), and CuCho (black bars). Results are expressed as mean of 10 rats assayed in triplicate ± standard deviation (SD). Significant differences were indicated by letters (data with distinct letters are statistically different between them at $P < 0.01$).

the central nervous system has a particular Cho metabolism characterized by higher levels of free Cho (25 times higher than in the case of plasma). Samples from brain and plasma behave differently from one another. Cu addition to drinking water produced no significant changes in plasma total Cho and a slight increase in cortex and hippocampus at the expense of the esterified form (ECho). Furthermore, Cu did not change the ratio between free and ECho in plasma samples but did lower this parameter in the brain homogenates. Supplementing food with Cho caused a higher level of total Cho in peripheral plasma (from 27.0 ± 0.5 in the control group to 33.7 ± 0.6 nmoles/mg protein in Cho-supplemented rats) with a slight but significant decrease in the proportion of free/Echo. In brain, all the treatments led to an increase in total (free + esterified) Cho with respect to control data, though the increase was substantially higher and differentially orchestrated in those groups receiving Cho supplementation. In the case of Cho-treated rats, this occurred at the expense of free Cho; however, in rats under CuCho treatment, it was at the expense of the ECho form. Under CuCho treatment, hippocampus reached even higher values of total Cho than cortex (approx. 311 versus 295 nmoles/mg protein). Addition of Cu alone significantly elevated the ECho in both brain tissues, more noticeably in hippocampus than in cortex. Another differential result for the CuCho group was a decrease in free Cho with respect to control rats in cortex, a phenomenon not observed in hippocampus. The results obtained for the ratios between free and ECho strongly suggest that Cu addition was able to modify the balance of these two parameters, triggering the accumulation of ECho in cortex and hippocampus, perhaps facilitating its esterification or impeding its degradation, or both. This effect is especially noticeable under conditions of simultaneous Cu and Cho overload and, even more interestingly, was contrary to the effect observed in peripheral plasma samples.

3.2. Biomarkers of Oxidative Stress. In order to detect whether inorganic Cu in drinking water and Cho in food produced oxidative/nitrative stress, we measured the oxidation end products of lipids (TBARS) and proteins (PCs) and also the levels of NOx (nitrates and nitrites derived from the spontaneous dismutation of nitric oxide) as biomarkers of damage. Levels of the three markers analyzed were higher after CuCho treatment both in plasma and in brain cortex and hippocampus (Figures 1(a) and 1(b)). In plasma, treatment with Cu alone increased TBARS levels (Figure 1(a)), whereas all three biomarkers were significantly increased after CuCho cosupplementation. Brain homogenates exhibited similar results, with increased levels of all three biomarkers after Cu or CuCho supplementation (with the solo exception of PCs in both brain regions, which increased only with simultaneous exposure to Cu and Cho).

We also measured the levels of the two main antioxidant molecules for water- and lipid-soluble cellular compartments (total glutathione-GSH+GSSG- and α-tocopherol, resp.) (Table 3). Plasma levels of total glutathione were higher in rats fed on Cu plus Cho supplements. In the same experimental group (CuCho), the α-tocopherol concentration was 27% lower than in the control data. In brain cortex, we also observed an increase in total glutathione after CuCho treatments and a simultaneous increment in the GSSG/GSH ratio as a consequence of a higher level of GSSG. Concomitantly, the activity of glutathione reductase (GR) in cortex was

Table 3: Total glutathione content, ratio oxidized (GSSG)/reduced (GSH) glutathione, glutathione reductase activity (GR), and concentration of α-tocopherol (α-Toc) in plasma and brain cortex and hippocampus from the different experimental groups.

Diets	Plasma			Brain zones							
				Cortex				Hippocampus			
	GSH + GSSG	GSSG/GSH	α-Toc	GSH + GSSG	GSSG/GSH	GR	α-Toc	GSH + GSSG	GSSG/GSH	GR	α-Toc
C	13.0 ± 0.05^{a}	0.06 ± 0.01^{a}	20.9 ± 0.06^{a}	796.5 ± 33.3^{a}	0.11 ± 0.01^{a}	184.6 ± 7.2^{a}	0.35 ± 0.02^{a}	701.0 ± 30.5^{a}	0.08 ± 0.01^{a}	236.7 ± 5.6^{a}	0.27 ± 0.01^{a}
Cu	13.5 ± 0.11^{a}	0.08 ± 0.02^{a}	18.8 ± 0.12^{a}	808.8 ± 41.0^{a}	0.12 ± 0.02^{a}	200.4 ± 7.9^{b}	0.36 ± 0.02^{a}	715.8 ± 41.1^{a}	0.10 ± 0.02^{a}	250.2 ± 4.6^{b}	0.25 ± 0.02^{a}
Cho	12.7 ± 0.20^{a}	0.06 ± 0.02^{a}	19.5 ± 0.10^{a}	835.2 ± 41.2^{a}	0.10 ± 0.02^{a}	185.67 ± 7.8^{a}	0.35 ± 0.03^{a}	728.4 ± 38.5^{a}	0.07 ± 0.01^{a}	240.8 ± 3.2^{a}	0.26 ± 0.03^{a}
CuCho	15.8 ± 0.14^{b}	0.12 ± 0.03^{b}	15.2 ± 0.08^{b}	889.2 ± 41.3^{b}	0.16 ± 0.01^{b}	222.33 ± 6.5^{c}	0.29 ± 0.01^{b}	735.8 ± 31.7^{a}	0.19 ± 0.01^{b}	284.5 ± 10.3^{c}	0.18 ± 0.02^{b}

Results (mean of 10 animals assayed in triplicate ± SD) were obtained as described in Sections 2.9.1, 2.9.2, and 2.9.3. Total glutathione (GSH + GSSG) and GSH are expressed as μmoles/mg protein. GR activity was expressed as U/mg·prot·min. α-Toc was calculated in nmoles/mg·protein. Results statistically significantly different ($P < 0.01$) are indicated with distinct superscript letters (values within the same column with different superscript letters are statistically significant between them).

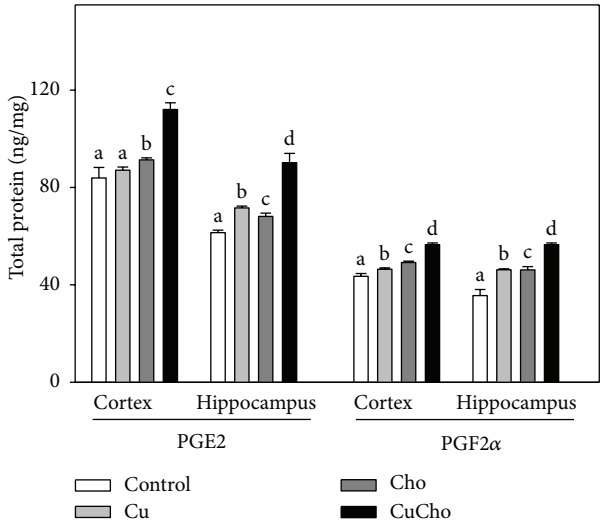

Figure 2: Prostaglandin PGF2α and PGE2 levels in brain cortex and hippocampus homogenates (ng/mg total protein). Treatments are indicated with different colors. Samples were analyzed as indicated in Section 2.10. Control (white bars), Cu (gray bars), Cho (dark gray bars), and CuCho (black bars). Results are expressed as mean of 10 rats assayed in triplicate ± standard deviation (SD). Significant differences were indicated by letters (data with distinct letters are statistically different between them at $P < 0.01$).

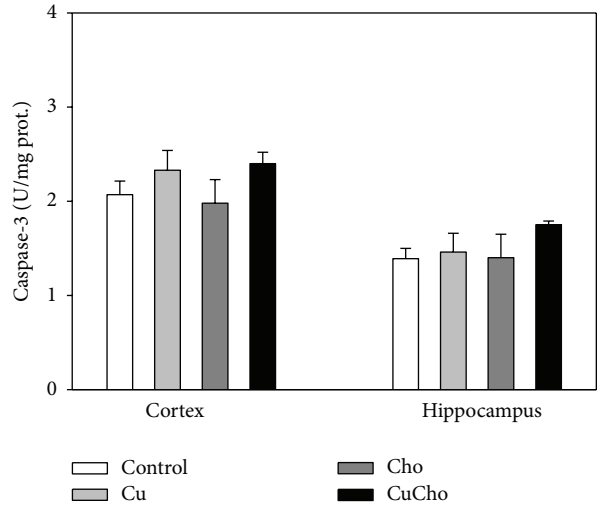

Figure 3: Caspase-3 (U/mg total protein) activity in brain cortex and hippocampus homogenates. Samples were analyzed according to the procedure described in Section 2.11.1. Treatments are indicated with different colors. Control (white bars), Cu (gray bars), Cho (dark gray bars), and CuCho (black bars). Results are expressed as mean of 10 rats assayed in triplicate ± standard deviation (SD). There are no statistically significant differences ($P < 0.01$) between treatments.

enhanced by Cu supplementation and even more so by simultaneous treatment with Cu + Cho. The level of α-tocopherol (α-Toc) decreased significantly only after CuCho treatment (approx. 17% compared to control data). In hippocampus, the behavior was very similar, with the exception of the increment in total glutathion content. Thus, hippocampus homogenates showed a significant increase in the GSSG/GSH ratio, activation of GR in Cu- and CuCho-treated rats, and a ca. 34% decrease in α-Toc content compared to control data, with this latter being observed only in the CuCho experimental group.

3.3. Markers of Inflammation. In order to evaluate whether simultaneous supplementation with Cu and Cho also produced an inflammatory condition, we analyzed the levels of two proinflammatory prostaglandins, PGE2 and PGF2α (Figure 2). In both brain regions, cosupplementation significantly increased prostaglandin levels. Interestingly, Cu and Cho alone also increased prostaglandins levels with respect to control data, and association of the two supplements produced an additive effect.

3.4. Caspase-3 and Calpains Activities. We also explored whether the prooxidative and proinflammatory environment developed after CuCho treatment was able to trigger apoptotic signals, to which end we determined the activities of the two main protease systems involved in programmed cell death, caspase-3, and calpains (μ- and m-). Caspase-3 activity tends to increase at least in cortex homogenates, but not to a statistically significant degree (Figure 3). Both calpain (milli- and microisoforms) activities increased after Cu treatment and even more so after CuCho supplementation in both brain zones (Figure 4).

3.5. Biomarkers of Neurodegeneration. The concentration of the β-amyloid peptides (1-42 and 1-40) and the Aβ 1-42/1-40 ratio in cortex and hippocampus are shown in Table 4. The ratio (which is the main indicator of neurodegenerative process) was different, depending on the brain zone examined. In cortex, it was found to increase after Cu and Cho treatments and to further increase after CuCho supplementation. In hippocampus, the Aβ 42/40 ratio increased only in the CuCho experimental group. There were no statistically significant changes of the ratio (1-42)/(1-40) in peripheral plasma.

We also investigated possible alterations in the visuospatial learning capabilities through the Barnes maze test. We observed minor (not statistically different) changes in latency to the first hole and more spatial preference for the escape region (holes −1, 0, and 1) regardless of treatment (data not shown). Taken together, these changes demonstrate minor alterations in visuospatial memory suggesting that simultaneous supplementation with Cu and Cho produces an increment in exploratory activity—or a sort of overexcited behavior—but with similar final results to those observed in the other experimental groups.

4. Discussion

Supplementation of drinking water with low amounts of inorganic Cu such as those used in our experiments was able to modify the basal status of redox biomarkers not only in peripheral plasma but also in the two zones of the central nervous system explored, cortex and hippocampus.

Table 4: Aβ (1–40 and 1–42) peptide concentrations and the ratio Aβ (1–42)/(1–40) in plasma and in brain regions (cortex and hippocampus) in rats fed with the experimental diets.

Treatments	Plasma			Brain zones					
				Cortex			Hippocampus		
	Aβ (1–40)	Aβ (1–42)	Aβ (1–42)/(1–40)·10^2	Aβ (1–40)	Aβ (1–42)	Aβ (1–42)/(1–40)·10^2	Aβ (1–40)	Aβ (1–42)	Aβ (1–42)/(1–40)·10^2
C	82.65 ± 1.91^a	7.06 ± 0.15^a	8.55 ± 0.15^a	59.49 ± 1.91^a	8.15 ± 0.33^a	7.30 ± 0.16^a	31.56 ± 1.85^a	5.01 ± 0.26^a	6.30 ± 0.12^a
Cu	94.62 ± 1.55^b	8.44 ± 0.31^b	8.92 ± 0.23^a	88.87 ± 1.44^b	9.66 ± 0.45^a	9.20 ± 0.22^b	41.18 ± 1.81^b	6.24 ± 0.20^a	6.60 ± 0.24^a
Cho	112.05 ± 2.33^c	9.95 ± 0.33^c	8.88 ± 0.33^a	115.58 ± 1.29^c	12.04 ± 0.28^b	9.60 ± 0.18^b	72.65 ± 2.22^c	7.49 ± 0.18^b	9.70 ± 0.15^b
CuCho	132.76 ± 2.40^d	12.56 ± 0.22^d	9.46 ± 0.28^a	176.40 ± 2.03^d	15.74 ± 0.25^c	11.20 ± 0.26^c	87.76 ± 1.44^d	8.82 ± 0.21^c	9.97 ± 0.22^b

Results (mean of 10 animals assayed in triplicate ± SD) were obtained as described in Section 2.12.1. Individual peptides are expressed as picomoles/L (plasma) or picomoles/mg protein (brain homogenates). Results statistically significantly different ($P < 0.01$) are indicated with distinct superscript letters (values within the same column with different superscript letters are statistically significant between them).

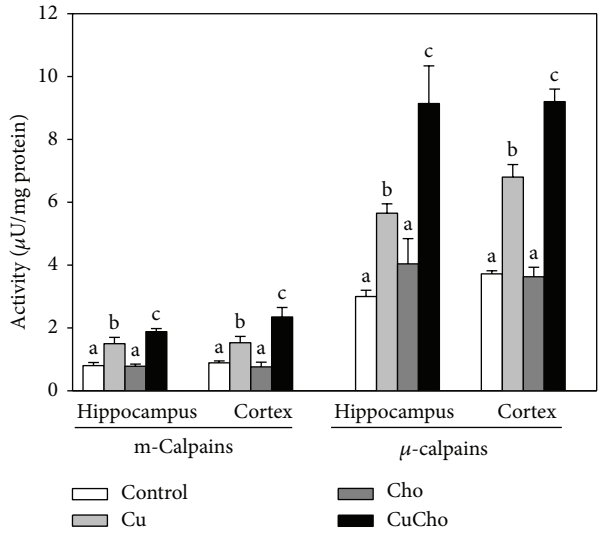

Figure 4: Calpains (μ- and m-) (U/min·mg total protein) activities in brain cortex and hippocampus homogenates. Samples were analyzed according to the procedures described in Section 2.11.2. Treatments are indicated with different colors. Control (white bars), Cu (gray bars), Cho (dark gray bars), and CuCho (black bars). Results are expressed as mean of 6 rats assayed in triplicate ± standard deviation (SD). Significant differences were indicated by letters (data with distinct letters are statistically different between them at $P < 0.01$).

Because of their short life span, free radicals cannot be measured directly, for which reason it is usual to analyze the products arising from reactions caused by reactive species with biomolecules. In line with this, we measured the end-oxidation products of lipids (TBARS) and proteins (PCs) and the production of NOx as biomarkers of prooxidative tissue damage. PCs increased only after CuCho treatment; however, TBARS and NOx increased after a sole exposure to trace amounts of inorganic Cu in drinking water. The biomarkers assayed were reproducible and sensitive to the experimental stimuli. Many authors have associated neurodegenerative illnesses with both a local (brain) and a systemic (plasma) increase in oxidative stress [45–52]. Irrespective of whether biomarkers are causative factors or whether they merely constitute phenomenologically associated changes, they are useful tools for evaluating the extent of damage and provide a simple methodology for monitoring large populations subjected to environmental Cu pollution or exposed to other risks associated with the development of AD. There is abundant evidence that transition metals in general, and specially Cu, are causative factors for oxidative stress and, as we mentioned previously, are strongly associated with the neurodegenerative process [53–56].

The decrease of α-tocopherol in plasma and brain zones homogenates and the increased GSSG/GSH ratio are both markers of accumulation of reactive species in lipid- and water-soluble cell compartments. The activation of GR can be interpreted as a compensatory mechanism of the enzymatic antioxidant defense system in order to normalize the altered GSSG/GSH ratio induced by cotreatment with Cu and Cho. In agreement with our results, Kojima et al. [57] have demonstrated the induction of mRNA coding for GR in the brain of mice irradiated with a low dose of γ-rays. Also in line with this, we can speculate that the observed increase in plasma total glutathione levels may be due to a compensatory mechanism under the oxidative insult evoked by the treatment studied here. This explanation is in agreement with the suggestions of other authors [58] and may be the consequence of an induction (overexpression) of cysteinyl-synthetase, the enzyme that controls the biosynthesis of glutathione [59, 60].

The oxidative stress induced by NCBC could have multiple effects on the signals cascade that depends on the redox state and can also modify the activity of ionic channels, transporters, and enzymes. Pallottini et al. [61] observed, in liver of Wistar rats treated with thioacetamide, that HMGCoA-reductase overactivation was strictly related to the magnitude of the reactive species accumulated. Our results demonstrate that inorganic Cu supplementation—even at the low levels assayed—produced an increase in ECho levels in cortex and hippocampus. Attributing the increase in Cho in brain to oxidative stress-induced HMGCoA-reductase hyperactivity could be oversimplistic, and this intriguing question remains to be resolved on the basis of new experimental evidence. Cho in the nervous system (10-fold higher than in any other organ) is mainly unesterified [62, 63]. Furthermore, most of the Cho content in brain depends on the *in situ* biosynthesis that appears to be regulated by similar mechanisms both outside and inside the brain, with HMGCoA-reductase being the most important regulatory effector [63]. However, the exact extent of Cho biosynthesis in neurons and astrocytes *in vivo* remains unknown [62], making it difficult to estimate the real effect of the accumulation of reactive species (NCBC-induced) on brain Cho biosynthesis. Interestingly, Cho turnover in individual neurons and astrocytes may in fact be very high and reach an estimated 20% per day, depending on the brain zone [62]. Unfortunately, the direct effect of higher Cho levels in blood on Cho concentration in brain is difficult to assess. Cho trafficking between brain and peripheral blood implies the participation of the blood-brain barrier (BBB) that hinders the direct passage into or out of the central nervous system. However, for reasons not yet understood, this restriction appears to be reduced in AD and other neurodegenerative disorders [64]. Also, in certain circumstances, for example, in cases of vascular injury due to oxidative stress, the BBB could be permeable to interaction with the peripheral pool of Cho [64]. Moreover, recent experimental evidence in a mouse model of AD demonstrated that inflammation is one of the key factors in determining increased BBB vulnerability [65]. Thus, from the above, we can assume that peripheral Cho might cross the BBB into the brain, in particular when the tissue is subjected to oxidative stress and redox-induced inflammation as observed in our experimental model.

Despite extensive research in recent years, the role of Cho as a risk factor for AD remains controversial [64], likely due to the still unresolved questions relating to the exact role of peripheral Cho in its level in brain. We can speculate that NCBC induces nitrative/oxidative and pro-inflammatory conditions that probably facilitate endothelial

damage and indirectly modify BBB properties [64, 65], allowing peripheral Cho to enter the central nervous system. Obviously, a great deal of experimental work is still required to either confirm or refute this working hypothesis. Several unresolved issues raise doubts concerning the beneficial effects of statins in neurodegenerative patients and the notion that high blood Cho is associated with brain dysfunction. However, one possibility is that the real risk is the association of hypercholesterolemia with a prooxidative and proinflammatory environment induced, for example, by NCBC.

Brain Cho is involved in synaptogenesis, the turnover, maintenance, stabilization, and restoration of synapses [63]. The functionality of synapses requires specific lipid domains in neuronal and axon membranes whose composition is critical for the correct targeting of the major membrane proteins, myelin biogenesis, cellular differentiation, signal transduction, and many other functions that depend on microdomains and specific lipid rafts. The proportion of free Cho to ECho in these membrane domains is a crucial factor in their biological activities [63]. For example, the enzymes responsible for the processing of the amyloid precursor protein (APP) to Aβ (β- and γ-secretases) reside in Cho-rich lipid rafts of plasma membrane [66]. It was suggested that a higher total Cho/phospholipid ratio in cellular membranes could affect secretase activities and determine preferential APP processing pathways [66], though *in vitro* studies suggest that Cho might impair the transcription of APP and consequently decrease the availability for its conversion to βA. However, it appears that this effect has no significant impact on the amount of protein (that still exceeds the capacity of the secretase system to process it) and only a slight impact on the levels of the mRNA encoding APP protein [67]. To date, there are no findings elucidating the exact role of ECho and how the ratio of free to esterified forms is able to modify secretase activity and other process associated with the amyloidogenic cascade [63]. Zana et al. [45] suggest that different sources of oxidative stress, such as NCBC, could trigger the amyloidogenic pathway, which may explain the higher Aβ 1-42/1-40 ratio we observed in the brain cortex of Cu-treated rats and in the group receiving both treatments (CuCho).

The question as to how Cho and Cu interact to lower the production of βA and enhance oxidative stress and inflammation is difficult to address. Experimental evidence obtained in culture cells demonstrates that exposure of macrophages to $CuSO_4$, at a level equivalent to NCBC in humans, induces SREBP-2 and consequently the expression of cholesterogenic enzymes [68], thus tentatively providing a new explanation for the apparently additive effect we observed between Cu and Cho. However, we were unable to find similar evidence in neuron or astrocyte cultures, or even in experiments conducted on live animals to explore these findings. There is yet another possibility: that the association of the two supplements (Cu and Cho) may affect the clearance of βA and facilitate its accumulation. Working with rabbits fed on a diet with excess Cho and inorganic Cu in their drinking water, Sparks et al. [69] proposed that Cho caused alterations in the BBB associated with an inflammatory condition and a concomitant overproduction of βA in the brain. In this model, Cu decreased the clearance of βA to the blood via inhibition of LRP at the vascular interface [69]. Impaired Cho metabolism, oxidative stress, and inflammation were all factors associated with the decreased clearance of the βA peptide [63, 69]. In addition, Lu et al. [14] also demonstrated that Cu exacerbates βA amyloid-induced neurotoxicity through a TNF-mediated inflammatory pathway.

Though the exact mechanism(s) underlying all these effects is still unknown, it is nevertheless widely accepted that the AD pathological cascade is likely to be a 2-stage event where deposition of βA and neuronal pathology (tauopathy, neuronal injury, and programmed cell death, or subsequent neurodegeneration with synapse and cognitive loss) are sequential rather than simultaneous processes [70, 71]. Our model likely represents a very early step in these successive events since the screening of the damages observed in visuospatial memory revealed only slight modifications. The Barnes maze test analysis demonstrated that all groups display almost normal locomotor and exploratory activities and spatial memory retention. The behavioral modifications indicate that the animals of the control and Cho groups were fully able to acquire the necessary knowledge for the spatial task through training, whereas the Cu and CuCho groups were only partially able to do so or presented slight learning difficulties. Interestingly, these learning difficulties were more evident during the evaluation trials of Cho animals, which explored holes very distant from the escape-hole region. Nevertheless, Cho and CuCho animals both showed a minor degree (not statistically significant) of deficit in learning and spatial memory capabilities.

Plasma levels of βA, particularly the βA 1-40/βA 1-42 ratio, are well-recognized biomarkers of sporadic AD [72, 73] and even indicators of early stages of the pathology [74]. However, apart from its role as a biomarker, accumulation of βA peptide in brain is a complex phenomenon with multiple and consecutive (sometimes unexpected) consequences [75]. In agreement with this, Tamagno et al. [76] demonstrated that oxidative stress induced by Aβ25–35 resulted in an early, significant, and time-dependent generation of free HNE (hydroxyl-nonenal) and H_2O_2. Also, other authors reported increased levels of oxidative stress biomarkers after Aβ exposure both *in vivo* and *in vitro* [77–79]. Our results are therefore in agreement with those of other groups reporting that oxidative stress and Aβ are linked to one another. Apparently, Aβ can induce oxidative stress [80, 81], and prooxidants such as NCBC can increase Aβ production [82–84] in the manner of an autostimulated process.

The increased levels of the two main proinflammatory prostaglandins (E2 and F2α) are consistent with the inflammatory condition characteristic of AD [85]. This proinflammatory and prooxidative environment triggers the activation of calpains, whereas caspase-3 activity was not significantly stimulated under our experimental conditions. In previous papers, we also found such dissociation between the effects of inorganic Cu overload on the relative activities of the two protease systems, which—at least *in vitro* experiments—appear to depend on the extent and intensity of exposure to Cu overload [86]. However, in view of previous experimental evidence, we cannot conclude that neuronal death is actually

occurring. So far, Saito et al. [87] have reported that the activation of μ-calpains in AD brain is not necessarily a consequence of the endstage of neuronal degeneration and may reflect a more widespread metabolic alteration that precedes and contributes to neuronal death. In fact, they observed increased activity of μ-calpains in the cerebellum of AD without any increase in the rate of death neurons [87]. In line with this, Nixon [88] established that different factors could lead to calpain activation triggering neurodegeneration in the early stages of AD development. Moreover, Trinchese et al. [89] also reported that calpains have many substrates that could be affected in AD patients but do not necessarily lead to immediate cell death. They also stated that μ-calpains are present predominantly in synapses, which is in agreement both with the fact that Cu concentration is particularly high (micromolar) in the synaptic cleft [90] and with the well-established synaptic pathology in AD [90, 91]. Nixon [88] suggested that increased activity of calpains during normal aging may also promote the development of neurological disorders and impaired calcium homeostasis, both of which could impact on the role of this cation in the function of cellular membrane receptors and metallosignaling in brain [91].

Finally, in discussing the validity and/or limitations of our experimental system, it is necessary to consider the level of the supplementation with Cu using oral administration. Our experimental conditions were based on previous work [13, 14] and resemble the Cu levels commonly found as a consequence of involuntary exposure through air, food and water pollution [16, 92–94], ingestion of dietary mineral supplements, exposure of professionals engaged in agrochemical activities [6, 11, 95], neurodegenerative patients and their first-degree relatives, or female users of Cu-based intrauterine devices [17, 19]. Studies performed in rats demonstrated that Cu metabolism and homeostasis are essentially identical to those in humans [96]. In terms of dosage, the rats from the groups supplemented with Cu received 1.06 ± 0.11 mg Cu/Kg/day (including Cu acquire from food and water ingestion). From the available data in humans, we can assume that general population are receiving 0.16 to 0.98 EAR or RDA which is equivalent to 0.3 to 1.5 mg Cu/kg body weight [97]. Very probably, humans are exposed to several types of Cu-based compounds of different chemical structures with differences in their physical stabilities, solubility, absorption capacities, life's times into the organism, and many other particularities related to their excretion or bioaccumulation rates. However, only inorganic Cu should be dangerous for its probable role as causative factor for neurodegeneration [3, 6, 8–10]. Thus, we think that there are a lot of questions to be answered before drawing a realistic conclusion about the comparisons between our experimental conditions and the actual human expose to Cu.

5. Conclusions

In conclusion, this *in vivo* study reveals that the association of inorganic Cu and Cho gives rise to a prooxidative and proinflammatory environment more pronounced than that produced by Cu and Cho administered alone. As described before, the combination of these two factors is common in many human populations. We suggest that the biochemical changes observed, in particular, oxidative stress, inflammation, and the higher Aβ 1-42/1-40 ratio in the cortex of rats fed on Cu + Cho (CuCho), could constitute the initial stages of the development of neurodegenerative disease. In view of the abundant evidence of disturbed Cu homeostasis in AD [7, 56, 98, 99], we strongly recommend more in-depth studies on the mechanism(s) responsible for the pro-neurodegenerative effect(s) of the association between NCBC and Cho. Furthermore, it is recommended that the present experimental evidence be used to promote the investigation of the emerging biomarkers—such as those examined in this work—to be applied in peripheral plasma as predictive tool(s) in high-risk populations.

Conflict of Interests

All authors disclose any financial and personal relationships with other people or organizations that could inappropriately influence this work. The authors declare that there is no conflict of interests.

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Research Article

Decreased Copper in Alzheimer's Disease Brain Is Predominantly in the Soluble Extractable Fraction

Alan Rembach,[1] Dominic J. Hare,[1,2] Monica Lind,[1] Christopher J. Fowler,[1] Robert A. Cherny,[1] Catriona McLean,[3] Ashley I. Bush,[1] Colin L. Masters,[1] and Blaine R. Roberts[1]

[1] *The Florey Institute of Neuroscience and Mental Health, The University of Melbourne, 30 Royal Parade Parkville, Parkville, VIC 3010, Australia*
[2] *Elemental Bio-Imaging Facility, University of Technology, Broadway, Sydney, NSW 2007, Australia*
[3] *Anatomical Pathology, The Alfred Hospital, Melbourne, VIC 3004, Australia*

Correspondence should be addressed to Blaine R. Roberts; blaine.roberts@florey.edu.au

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Alzheimer's disease (AD) is the leading cause of dementia and represents a significant burden on the global economy and society. The role of transition metals, in particular copper (Cu), in AD has become of significant interest due to the dyshomeostasis of these essential elements, which can impart profound effects on cell viability and neuronal function. We tested the hypothesis that there is a systemic perturbation in Cu compartmentalization in AD, within the brain as well as in the periphery, specifically within erythrocytes. Our results showed that the previously reported decrease in Cu within the human frontal cortex was confined to the soluble ($P < 0.05$) and total homogenate ($P < 0.05$) fractions. No differences were observed in Cu concentration in erythrocytes. Our data indicate that there is a brain specific alteration in Cu levels in AD localized to the soluble extracted material, which is not reflected in erythrocytes. Further studies using metalloproteomics approaches will be able to elucidate the metabolic mechanism(s) that results in the decreased brain Cu levels during the progression of AD.

1. Introduction

Alzheimer's disease (AD) is the predominant cause of dementia in the aging population and represents a mounting health epidemic [1]. Despite advances in understanding the events leading to the onset of cognitive decline, the principal cause of AD is still undetermined. The role of copper (Cu), iron (Fe), and zinc (Zn) in AD has become of significant interest because the dyshomeostasis of essential trace elements has been observed to have profound effects on cell viability and neuronal function [2, 3], which have been previously reviewed [4].

Cu, an essential element in the central nervous system (CNS), is crucial for life, but its unique redox propensity renders it toxic in circumstances of an increase pool of labile species [5–8]. Specific lesions in the Cu pathway can lead to a severe but treatable neurological impairment, including Menkes and Wilson's disease [9–11]. Cu displays a distinctly compartmentalized distribution throughout the brain, reflecting its diverse function in various neurological processes [12, 13].

Within the CNS, Cu is known to decrease in the frontal, occipital, and parietal lobes [14] amygdala and hippocampus in AD [15]. The process for this decline is not well understood, though extracellular plaques of aggregated amyloid-β (Aβ) are reported to be enriched with trace elements including Fe, Zn, and Cu [16]. Recently, it was also reported that frontal cortex from AD subjects had an increased propensity to bind exchangeable Cu, which correlated with oxidative damage observed in the tissue [17].

In cerebral spinal fluid (CSF), Cu levels are not observed at significantly different concentrations between AD and healthy controls (HC) [18–20]. However, within peripheral fluids, Cu dyshomeostasis has been more intensely studied.

Reports of increased [19, 21], decreased [22, 23], or unchanged [24–26] serum or plasma Cu in AD have rendered total Cu levels too variable to be of diagnostic utility, for review see [27]. Yet, many studies have concluded that there is a subtle but consistent excess of nonbound or diffusible Cu in serum [21, 22, 28–34]. Despite this, a consensus has been thwarted by a lack of standardization and limitations driven by covariate influences on peripheral "high throughout" screening of Cu concentrations [35]. In other peripheral tissues, such as erythrocytes, superoxide dismutase 1 (SOD1) activity has been found to be diminished in AD [23]. This is thought to be due to a Cu deficiency in the enzyme, as reported previously [36].

In this study, we tested the hypothesis that there is a systemic perturbation in Cu compartmentalization in AD, within the frontal cortex as well as in the periphery, within erythrocytes.

2. Methods

2.1. Subjects. The AIBL study incorporates longitudinal neuroimaging, biomarker, neuropsychometric, and lifestyle data, see [37] for a detailed description of methods. Briefly, participants over the age of 65 years and fluent in English were divided into three groups; cognitively healthy individuals (HC), participants with mild cognitive impairment (MCI) based on the established criteria [38, 39], and participants diagnosed with *possible* or *probable* AD as defined by NINCDS-ADRDA criteria [40]. Written informed consent was obtained from all participants, and the study was approved by the appropriate institutional ethics committees.

2.2. Erythrocyte Preparation. Whole blood was collected from overnight fasted participants with a 27 g needle, into Sarstedt S-Monovette Lithium-Heparin 7.5 mL tubes (01.1608.100). The tubes were spun at 3,200 ×g for 30 min at room temperature, and the plasma was carefully taken off the hematocrit. The buffy coat was prepared by ficoll gradient centrifugation to extract the white blood cells. The erythrocytefraction was washed 3 times by adding 0.9% normal saline to an end volume of approximately 14 mL. Erythrocytes were dispersed by gently inverting the tubes 10 times and then centrifuged at 650 ×g for 10 minutes at 20°C with braking on. The final centrifugation was 1,500 ×g for 10 minutes at 20°C with braking on. The final saline wash was discarded, and the erythrocytes resuspended to an end volume of 6 mL in phosphate buffered saline (PBS) (pH 7.4), then aliquoted into polypropylene (Nunc cryobank, Denmark) tubes and snap-frozen in liquid nitrogen.

2.3. Fractionation of Brain Tissue for Biochemical Analysis. Brain tissues were obtained from the Victorian brain bank network, and all experiments were approved by the University of Melbourne health sciences, human ethics subcommittee (ID1136882). Hemisected frozen brains at −80°C were thawed to −20°C and sectioned in 1 cm slices. The meninges were removed from approximately 5 g of frontal cortex (Brodmann area 9), and the grey matter was dissected in to 0.2–0.5 g aliquots and stored at −80°C. The grey matter was allowed to thaw on ice and then homogenized using a BioMasher (Omni International). Tissue was placed in the BioMasher, the plunger was inserted, and then the apparatus was centrifuged at 100,000 ×g with a desktop centrifuge. After centrifugation, Tris buffered saline (TBS, 50 mM Tris pH 8.0, 150 mM NaCl) containing EDTA free protease inhibitors (Roche, 05056489001) was added at a ratio of 1 : 4 (tissue : buffer, w/v). The sample was then transferred to ultracentrifuge tubes and centrifuged at 100,000 ×g for 30 minutes at 4°C. The TBS supernatant, or "soluble" material, was collected and stored at −80°C before Western blot analysis. The pellet was resuspended in 100 mM $NaCO_3$ pH 11.0 (1 : 4, tissue:buffer) and further centrifuged at 100,000 ×g for 30 minutes at 4°C. The supernatant, "peripheral membrane/vesicular" material was recovered, and the pellet was resuspended with 7 M urea, 2 M thiourea, 4% 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS), 30 mM Bicine pH 8.5, and centrifuged at 100,000 ×g for 30 minutes at 4°C. The supernatant, "membrane" material, was recovered, and the resulting pellet was then incubated at room temperature with 70% formic acid for 16–18 hours before being centrifuged at 100,000 ×g for 30 minutes. After the sequential extraction, little to no observable material remained.

2.4. Induction Coupled Plasma-Mass Spectrometry (ICP-MS). Frozen aliquots of erythrocytes or brain tissue homogenate fractions were thawed at room temperature. For brain homogenates, 50 μL was diluted (1 : 20) with 950 μL of 1% HNO_3 (v/v). 50 μL of washed erythrocytes were digested in equivalent volumes of concentrated (65%) HNO_3 and H_2O_2 (Merck Millipore) at 80°C for 5 minutes, then diluted 1 : 20 with 1% HNO_3. Cu concentration was determined using an Agilent Technologies 7700x ICP-MS system. The sample introduction system used a Teflon MiraMist parallel path nebulizer (Burgener Research Inc.) and standard Scott-type double-pass spray chamber (Glass Expansion). Helium was used as a collision gas. ICP-MS conditions were replicated from previously reported studies from our laboratory [35]. The instrument was calibrated using multielement standards (Accustandard, ICP-MS-2-1, ICP-MS-3, and ICP-MS-4; total of 44 elements) containing copper at 0, 5, 10, 50, and 100 ppb with ^{89}Y as the internal standard for all isotopes of Cu. Interday relative standard deviations were determined using a quality control serum (Seronorm) with a certified copper level (84.55 μg/L 95% CI 80.35–88.75 μg/L) and were consistently between 2.0–5.0%.

2.5. Statistical Analysis. Statistical analyses were performed with Prism version 6.0 (Graphpad Inc). To compare differences between the groups, a one-way ANOVA Bonferroni's multiple comparison test was used. Significant P values were <0.05.

Table 1: Distribution of Cu in different cellular fractions and total levels from human brain.

Brain fraction	Cu (μg/g of wet weight)		P value
	HC	AD	
Soluble	1.93 ± 0.6 (1.0–3.2)	1.46 ± 0.6 (0.6–3.3)	<**0.05**
Peripheral/vesicular	0.57 ± 0.2 (0.3–0.9)	0.38 ± 0.3 (0.7–1.1)	>0.05
Membrane	0.52 ± 0.2 (0.2–1.2)	0.36 ± 0.2 (0.05–0.8)	>0.05
Formic acid[¶]	0.52 ± 0.2 (0.3–1.0)	0.38 ± 0.2 (0.05–0.7)	>0.05
Total homogenate[§]	3.33 ± 2.2 (1.5–13)	2.29 ± 1.0 (0.9–4.7)	<**0.0001**

Concentration based on wet weight of tissue, mean ± standard deviation (range).
[¶](HC) $N = 20$, (AD) $N = 22$, [§](HC) $N = 24$, and (AD) $N = 23$. Numbers in brackets are the 95% confidence intervals. P values were calculated using one-way ANOVA Bonferroni multiple comparison post hoc test. (NS: nonsignificant).

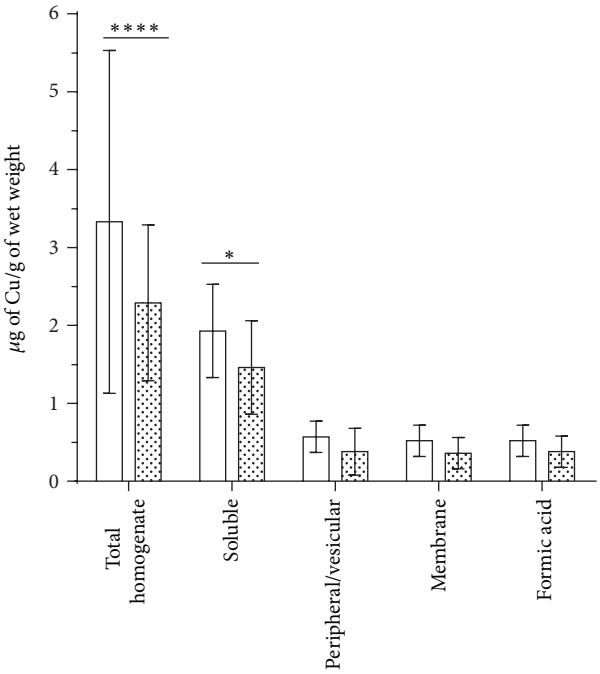

Figure 1: Cu content in human brain. Illustrates a significant decrease in total Cu in the soluble fraction of the extracted brain tissue. $^{*}P < 0.05$, $^{****}P < 0.0001$; one-way ANOVA with Bonferroni's multiple comparison post hoc test of log transformed data. Cu is decreased in AD frontal cortex. Copper is significantly decreased in the total homogenate and soluble extracted material ($P < 0.05$). The only fraction that had a significant decrease was the soluble fraction indicating that the decrease in Cu observed in the total homogenate is localized to changes in the soluble fraction. HC *Healthy Control* (clear boxes), AD *Alzheimer's disease* (filled boxes).

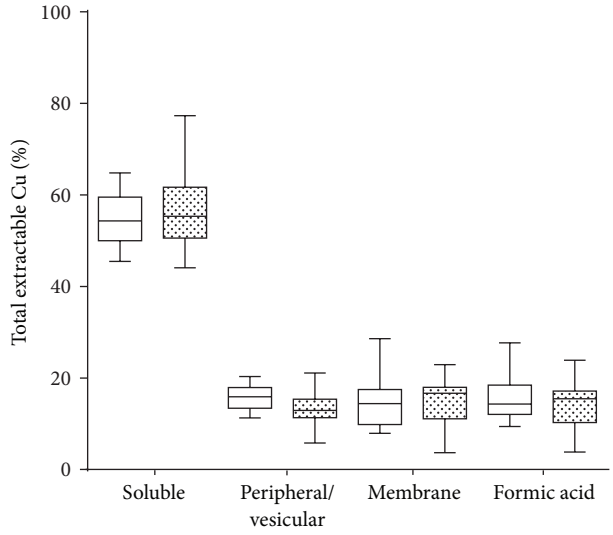

Figure 2: Cu content in human brain expressed as percentage distribution. The percent distribution of Cu extracted from human frontal cortex in the brain is conserved in AD and HC (total pooled). Box and whisker plots show the range, interquartile range, and median values. No significant difference was observed in the percentage of Cu in each of the corresponding fractions. Between 50–60% of the total Cu in human brain tissue is extractable in the soluble phase. No significant difference was observed between HC (clear boxes) and AD (filled boxes).

3. Results

Table 1 and Figure 1 show that changes in Cu within the human frontal cortex were localized to the soluble fraction. We observed a significant decrease in total Cu levels consistent with previous studies [14, 15]. To investigate if the change in Cu was global or localized to a specific cellular compartment, we fractionated the brain tissue into four groups: soluble, peripheral membrane, and vesicular material, integral membrane and formic acid extractable material that contains predominantly insoluble plaques [41]. We only observed a significant decrease in Cu in the soluble fraction between AD and HC (Figure 1, $P < 0.05$, one-way ANOVA Bonferroni's multiple comparison test). Table 2 shows the demography for the postmortem brain samples. Although we did observe a decreasing trend for Cu in each, we found that when Cu is expressed as a percentage of total Cu for each individual (Figure 2), no significant differences were observed, suggesting a conservation of Cu equilibrium that may be homoeostatically regulated. We observed that 50–60% of total tissue Cu content was localized to the soluble extractable material (Figure 2).

Previous studies have associated AD specific changes in erythrocytes [42, 43], including changes in the Cu dependent enzyme Cu, Zn-superoxide dismutase 1 (SOD1) [23]. We used well-characterized samples from the AIBL study to investigate the level of Cu in erythrocytes. Table 3 shows

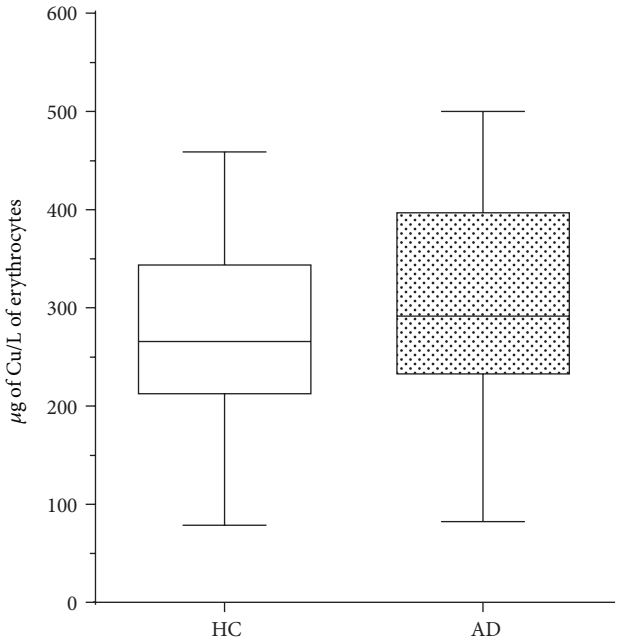

Figure 3: Cu concentration in human erythrocytes. The total Cu content of erythrocytes was determined using ICP-MS. No significant change ($P = 0.53$) in the level of Cu in red blood cells was observed between HC ($N = 40$) (clear boxes) and AD ($N = 40$) (filled boxes).

Table 2: Post-mortem subject demography.

	AD ($N = 30$)	HC ($N = 27$)	P value
Age (years)	78.0 (9.2)	77.0 (7.6)	>0.05
Gender females (%)	27	33	>0.05
ApoEε4 carriers (%)	76	15	<**0.0001**
PMI (hours)	33.9 (22)	38.4 (14.3)	>0.05

Values are means (SD) unless noted above as otherwise. P values were calculated using t-test (two tailed). HC: healthy control, AD: Alzheimer's disease, *ApoEε4*: Apolipoprotein E epsilon 4, PMI: postmortem interval.

the AIBL cohort demographics for individuals analysed for erythrocyte Cu concentrations. Figure 3 shows that there was no significant difference in erythrocytic Cu concentration observed between AD and control samples.

4. Discussion

The aim of this study was to investigate the distribution and concentration of Cu in the frontal cortex and periphery of AD subjects when compared to age-matched healthy control samples. A number of studies have indicated that there is a significant perturbation in Cu coordination in AD within the frontal cortex [14, 17, 44] and periphery [3, 27]. Using well-characterized subjects, we were unable to demonstrate a significant difference in Cu levels within erythrocytes, a finding which is consistent with our investigations of serum Cu levels [35]. However, we did observe a significant difference within the frontal cortex, where AD tissue had

Table 3: AIBL cohort demographics for individuals analysed for erythrocyte Cu levels.

	HC ($N = 40$)	AD ($N = 40$)	P value
Age (years)	76.8 (8.0)	77.3 (8.0)	>0.05
Gender females (%)	47.5	55	>0.05
ApoEε4 carriers (%)	37.5	60	<**0.05**
MMSE	28.4 (1.4)	18.1 (6.0)	<**0.0001**
CDR	0.075 (0.2)	6.175 (3.2)	<**0.0001**

Values are means (SD) unless noted above as otherwise. P values were calculated using one-way ANOVA Bonferroni multiple comparison post hoc test. HC: healthy control, AD: Alzheimer's disease, and MMSE: Mini-Mental State Examination. CDR: clinical dementia rating scale, *ApoEε4*: Apolipoprotein E epsilon 4.

significantly less Cu than controls ($P = <0.0001$). This observation is consistent with the frontal cortex having a unique susceptibility for Cu deficiency, compared to the periphery. Previous studies have used fractionation to investigate trace elements in AD brain [45], but data on changes to Cu concentrations is lacking. By fractionating brain tissue into several biochemical distinct subunits we observed that the decrease in Cu is mainly confined to the soluble fraction. The change in the soluble fraction is consistent with the reported deficiency of metallothioneins in the AD brain [46], though further metalloproteomic investigations are required to determine the extent that the Cu proteome is altered in AD neuropathology [47]. Surprisingly, no significant difference was observed in the formic acid fraction (Figure 1), contrary to our expectation that the AD plaques would demonstrate increased Cu in line with previous reports [48, 49]. The absence of an increase in Cu in the formic acid fraction highlights the importance of using spatially resolved techniques to measure tissue distribution of trace elements, including X-ray microfluorescence microscopy [50] and laser ablation ICP-MS [51, 52].

We have shown that over 50% of the Cu in human brain tissue can be extracted in the soluble or cytosolic portion of the homogenate. Further, the changes observed in the AD brain are due to specific changes in this soluble phase. As Cu has a strong propensity to participate in free radical chemistry, the distribution and delivery of Cu are carefully controlled by a set of Cu specific protein machinery [13, 53, 54]. This Cu handling system maintains less than one free Cu ion per cell [55]. Therefore, essentially all of the Cu is bound to biological ligands or is chaperoned by Cu regulatory proteins. Future investigations using a metalloproteomic approach [56–58] will be able to determine if the changes in the soluble Cu levels are specific to changes in binding partners, such as the reported decrease in metallothionein observed in AD [46]. As Cu is tightly regulated and exists as a ligated entity in the cell, it will be interesting to investigate if the change in soluble Cu are global, suggesting all Cu-proteins are decreased in AD, or are the changes restricted to selective proteins. In particular, detailed investigations of the stoichiometry of Cu proteins, like ceruloplasmin and the three reported metallothionein isoforms, will also be

informative concerning Cu perturbations that may lead to deficiency in the CNS.

In conclusion, this study has examined two pools of Cu in AD compared to age matched HCs. We showed that there is a specific change in the frontal cortex, indicating there is a perturbation in Cu homeostasis leading to a local diminution in concentration. We did not detect a significant difference in erythrocytes, suggesting that Cu disturbance may be confined to the brain in AD, precluding peripheral Cu levels as a useful biomarker for AD. The timing, systemic covariates, and mechanism(s) of this alteration still need to be systemically investigated, and advanced analytical metalloproteomic techniques will go a long way to answer these questions in the future.

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Review Article

Zinc Deficiency and Zinc Therapy Efficacy with Reduction of Serum Free Copper in Alzheimer's Disease

George J. Brewer[1] and Sukhvir Kaur[2]

[1] *Department of Human Genetics, University of Michigan Medical School, Ann Arbor, MI 48109, USA*
[2] *Monmouth University, West Long Branch, NJ 07764, USA*

Correspondence should be addressed to George J. Brewer; brewergj@umich.edu

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We are in the midst of an epidemic of Alzheimer's disease (AD) in developed countries. We have postulated that ingestion of inorganic copper from drinking water and taking supplement pills and a high fat diet are major causative factors. Ingestion of inorganic copper can directly raise the blood free copper level. Blood free copper has been shown by the Squitti group to be elevated in AD, to correlate with cognition, and to predict cognition loss. Secondly, we have shown that AD patients are zinc deficient compared to age matched controls. Zinc is important in neuronal protection. We carried out a 6-month small double blind trial of a new zinc formulation on AD patients. We found that in patients 70 years and older, zinc therapy protected against cognition decline compared to placebo controls. We also found that zinc therapy significantly lowered blood free copper levels. So zinc efficacy could be due to restoring neuronal zinc levels, to lowering blood free copper levels, or to both.

1. Introduction

We are in the midst of an epidemic of Alzheimer's disease (AD), particularly in developed countries [1]. We have previously hypothesized that ingestion of inorganic copper from drinking water and supplement pills and a high fat diet are main causal factors in AD [2–5]. These two factors interact synergistically because copper oxidizes fats to molecules that are toxic, particularly to neurons. The epidemic correlates temporally with the use of copper plumbing in developed countries, and there is a great amount of additional data that draws the net tighter around a causative role for inorganic copper. Inorganic copper must not be confused with organic copper, the copper in food that is safely bound to protein. Inorganic copper is a simple salt of copper, not bound to anything, and is in part handled differently by the intestinal absorption mechanism, such that some of it contributes immediately to the serum free copper pool [6]. This pool has been shown by Squitti and her group to be expanded in AD [7], to correlate negatively with cognition [8], and to predict deterioration in cognition [9]. So, our inorganic copper hypothesis fits well with the work of the Squitti et al. group.

While we believe ingestion of inorganic copper and a high fat diet are major causal factors, there are a number of other known risk factors in AD. These include having an apolipoprotein E4 allele [10], having elevated homocysteine levels [11], or having certain alleles of the iron management genes, hemochromatosis [12] and transferrin [13]. These latter fit with the copper hypothesis since iron and copper are toxic through the same mechanism, generation of oxidant damage. In addition, the Squitti group has shown that possession of certain variants of the ATP7B gene is a risk factor for AD [14]. Since ATP7B gene products control copper levels in the body, this risk factor also fits beautifully with the copper hypothesis.

A risk factor for AD that we have recently observed has largely been ignored by the scientific community concerned with AD. This is zinc deficiency in AD patients. We have found [15], as has another group [16], that AD patients are zinc deficient. Zinc has many protective roles in neurons, and zinc deficiency may play a causal role in AD. We have found and will report here that zinc therapy appears to prevent at

least some cognition decline. Our findings on zinc therapy may also lend further support to the copper hypothesis, because we have found that zinc therapy, in addition to restoring normal zinc levels, reduces serum free copper levels in AD.

2. Demonstration of Zinc Deficiency in AD

The following are the highlights of our study [15]. A total of 29 patients with AD, diagnosed by standard criteria, were compared to 29 age and sex matched controls. Since elderly people tend to take a variety of supplements, many of which contain copper or zinc, we halted all supplement use beginning 30 days prior to the study, the first time that has been done in studying AD patients, to our knowledge. Serum zinc was assayed by atomic absorption.

Serum zinc declines with age in people for unknown reasons. Thus, in young adults, the mean serum zinc is around 100 μg/dL. In our group of 29 normal but aged controls, the mean was well below that at 82.7 μg/dL. But the mean of the 29 AD patients, 76.2 μg/dL, was significantly ($P = 0.027$) below the mean of the normal aged group. Thus, the AD patients are relatively zinc deficient compared to age matched controls. It should be kept in mind that serum zinc is a relatively late indication of zinc deficiency, but when it is low, the patient is zinc deficient.

3. Is Adequate Available Zinc Important in Neuronal Health?

The neurons of many parts of the brain have high zinc levels, and it is clear that zinc plays many critical roles in neurons. In some neurons, high concentrations of zinc are secreted along with glutamate into the synapse. Glutamate initiates firing, and zinc quenches, or shuts down, the firing [17]. With inadequate zinc, glutamate-induced firing persists and can damage the neuron. Excess glutamatergic excitotoxicity is believed to be a common occurrence in many neurodegenerative disorders, including AD.

Another possible mechanism by which low availability of zinc in the brain can have harmful effects is through failure of adequate inhibition of calcineurin [18]. Calcineurin activity is known to be high in AD brain, and this can have many negative downstream effects. Calcineurin is normally inhibited by zinc but is stimulated by beta amyloid.

Additional information about the key role of zinc in the brain is provided by the studies of Adlard et al. [19] in which they did a knockout of mice of the zinc transporter-3 (ZnT3) gene. ZnT3 is the pump that puts zinc into the vesicle to be discharged into the synapse. These mice, with a deficiency of zinc in the synapse, are a phenocopy of deficits in AD.

To add to the problem created by systemic zinc deficiency, there is another mechanism in the AD brain that depletes neurons of much-needed zinc. The beta amyloid plaques, that build up in the AD brain, are avid binders of zinc [19]. Thus, it seems very likely that the neurons of the AD brain are seriously lacking in available zinc and probably many are injured and die as a result.

4. Random Controlled Trial of Zinc Therapy

Given the above discussion of the critical roles of zinc in maintaining healthy neurons and the demonstration that AD patients are zinc deficient, it is logical to consider a trial of zinc therapy in AD. Interestingly, an uncontrolled trial of zinc therapy, both oral and parenteral, was carried out in 1992, with reportedly dramatic positive effects [20]. The uncontrolled nature of this study detracts from its significances, but it is puzzling that this very positive study has not been followed up. The probable reasons are that controlled drug trials are expensive and that the lack of a patent on zinc makes it unattractive to drug companies. In addition to the former human work, there has been a positive animal study, in which zinc supplementation caused improvement in an AD animal model [21].

We decided to pursue a controlled zinc trial in AD. By way of background, one of us (G. J. Brewer) had developed zinc therapy for Wilson's disease, an inherited disease of copper accumulation and copper toxicity [22]. Zinc acts in this disease by blocking intestinal copper absorption. We developed a zinc acetate salt, with the trademark name, Galzin, that was approved by the FDA for Wilson's disease in 1997. While effective, Galzin had to be given three times a day in 50 mg doses and each dose had to be separated from food in order to have the desired effect in the intestine. It turned out that up to 50% of patients had some gastric discomfort from Galzin and up to 10% could not take it at all. The problem was that the zinc capsules dissolved in one spot in the empty stomach, releasing all the zinc salt in that location, causing irritation in many patients.

At Adeona Pharmaceuticals, we developed a new zinc formulation with a zinc-binding agent that allowed the slow release of zinc. The kinetics of this formulation are such that in dissolution studies, peak zinc release is at 6–8 hours, and it is still releasing zinc at 24 hours. Thus, this pill caused no gastric intolerance, and because of the slow release, could be given one time/day, with around-the-clock elevation of serum zinc. We used this new zinc formulation in the controlled AD trial reported here.

4.1. Methods. The study was carried out in Clearwater, Florida, USA with Dr. Diana Pollock, of the Ptak Alzheimer's Center, Morton Plant Neuroscience Institutes, Morton Plant Hospital, Clearwater, FL, USA as the principal investigator. It was designed to recruit 60 AD patients. Patients were diagnosed using Alzheimer's Disease and Related Disorders Association criteria. They had mild to moderate AD with a Clinical Dementia Rating (CDR) of 0.5–1.5 and a mean Mini Mental State Examination (MMSE) of 25.6. Patients were randomly assigned to receive either 150 mg of the new zinc formulation in a once-a-day dose or matching placebo. The end points were improvement in cognition relative to controls, improvement in serum zinc, and reduction in serum free copper. Cognition was measured by three tests, the Alzheimer's Disease Assessment Scale (ADAS-Cog), the Mini Mental State Examination (MMSE), and CDR Sum of Boxes (CDR-SOB). Treatment was for 6 months. Zinc and copper

Table 1: Post hoc analysis of cognition change in patients 70 years and older during the six-month controlled trial.

Cognition test	Direction of deterioration	Zinc-treated group ($N = 14$)	Placebo group ($N = 15$)	P value
ADAS-Cog	Positive change	−0.76	+1.27	0.037
CDR-SOB	Positive change	+0.25	+0.87	0.032
MMSE	Negative change	+0.58	−1.0	0.067

were assayed by atomic absorption spectroscopy. Ceruloplasmin was measured by a nephelometric immunologic technique [23]. Student's t-test was used to test for statistical significance which was set at P at or below 0.05.

4.2. Results. Since this is a high dose of zinc, patients were monitored for copper deficiency by following serum ceruloplasmin. Either serum ceruloplasmin or serum copper can be followed for this purpose since almost all serum copper is in ceruloplasmin. We choose ceruloplasmin because the assay is more generally available in clinical laboratories. Only one patient had a sufficient decrease in ceruloplasmin to merit a reduction in zinc dose to 75 mg/day. This patient's data were included in the results of the study. The data on cognition were tested for statistical significance by Student's t-test. As reported by Dr. Pollock at the 2011 meeting of the American Academy of Neurology in Hawaii, there was a trend in the data from all three cognition screening tests favoring the zinc-treated group versus the placebo group. The P values for ADAS-Cog ($P = 0.36$), MMSE ($P = 0.42$), and CDR-SOB ($P = 0.1$) were not statistically significant although CDR-SOB was close at $P = 0.1$.

In examining the data, we noted that zinc was essentially stabilizing the cognition in patients, both young and old. However, in the placebo group, younger patients did not show much cognition change, but at about age 70 and older, they showed considerable deterioration. So, we analyzed the data comparing the two groups looking at patients aged 70 and over, the concept being that the greater variability and inconsistency in deterioration in the younger placebo patients were preventing the differences from being significant.

The very exciting results are shown in Table 1. With ADAS-Cog, a more positive change over the 6-month period indicates deterioration, and the placebo group deteriorated +1.27 points while the treatment group actually improved −0.76 points with a statistically significant difference at $P = 0.037$. With CDR-SOB, a more positive change again indicates deterioration, and the placebo group deteriorated +0.87 points, while the treatment group deteriorated significantly less ($P = 0.032$). With MMSE, a negative change indicates deterioration and the placebo group deteriorated −1.0 points while the treatment group actually improved to +0.58 points, but results were not quite statistically significant at $P = 0.067$.

We also reached our end points with serum zinc and serum free copper. In the placebo group, baseline serum zinc was 70.8 μg/dL, and 6 months later, it was 75.3 ($P = 0.153$). In the zinc treatment group, baseline serum zinc was 76.4, and 6 months later, it was 151.8 ($P = 0.002$). Regarding serum free copper in the placebo group, baseline serum free copper was 34.8 μg/dL, and 6 months later, it was 34.9 ($P = 0.486$). In the zinc-treated group, baseline serum free copper was 37.0, and 6 months later, it was 30.8 ($P = 0.004$). So, zinc deficiency was very effectively eliminated and serum free copper was very significantly lowered by zinc therapy.

5. Discussion

The first question to discuss is whether we now know for sure that zinc therapy will slow or halt cognitive decline in AD. At this point, we cannot give an absolute "yes"; the answer has to be qualified. We think the data are quite strong that this is the case. First, there is the randomized controlled trial (RCT) just reviewed in the previous sections. Second, there are the 1992 uncontrolled studies of Constantinidis [20], which were strongly positive. Third, there are the animal model studies of Corona et al. [21], in which zinc supplementation significantly improved cognition. Fourth, there are the ZnT3 mouse knockout studies of Adlard et al. [19], in which mice deprived of much of their neuronal zinc were a phenocopy of AD. Fifth, there are clear data that AD patients are more zinc deficient than age matched controls [15, 16]. Sixth, there are all the known protective roles of adequate zinc levels in the neuron, which were reviewed earlier.

So, the evidence is quite strong, with the RCT being the potential linchpin for a "yes" answer to the question. But the weakness of the RCT is that the statistically significant benefit to cognition was a post hoc analysis of the data on those aged 70 and over. Why are post hoc data weaker than pre hoc hypothesis testing? The major factor is that if you manipulate data in multiple ways, it becomes increasingly likely that one of the analyses will become significant by chance. Our post hoc analysis was limited to two evaluations (the other analysis asked whether there was a difference in results between the two sites of the study). So the P value is not weakened very much.

The other factor to consider about our RCT is that the end sample of patients is rather small, 14 AD patients of age 70 or over who were treated with zinc, and 15 AD patients of age 70 or over who were controls.

A factor which strengthens the conclusion of efficacy of zinc is the consistency of the three cognitive scoring tests. All three indicated benefit to the zinc-treated group. Two were strongly statistically significant, and one was close. If chance were the main operative, one would suspect heterogeneity among the tests. The consistency strongly supports that a biologic signal was operating, that is, a positive effect of zinc therapy on protecting cognition.

So, at this point we draw the conclusion, admittedly not quite final yet, that zinc therapy significantly slows cognition loss in AD.

The second question to discuss, assuming zinc efficacy, is what is the mechanism? In the first part of this paper, we laid out the background for two likely mechanisms. First, we laid out our hypothesis that ingestion of inorganic copper, such as from drinking water or taking supplement pills, increases the serum free copper level. Squitti and her group have shown in several elegant studies that serum free copper is high in AD [7], that it is correlated with cognition measures [8], and it is predictive of cognition loss over time [9]. These data indicate that elevated serum free copper is causative of cognition decline in AD. We significantly lowered serum free copper in the zinc-treated group; thus, a mechanism of benefit from zinc is very likely reduction in serum free copper.

However, earlier in the paper, we also pointed out that AD patients are more zinc deficient than age matched controls, and that zinc deficiency by amyloid plaques further depletes the neurons of zinc, and we pointed out how important adequate zinc is for neuronal health. Then there is the ZnT3 knockout mice that produce a phenocopy of AD [19]. The neurons in AD patients are very likely zinc deficient, so a case can be made that restoring zinc to the neurons of AD patients was a mechanism for improved cognition in the zinc group in our trial.

Or perhaps it is both the beneficial lowering of serum free copper and the beneficial restoration of neuronal zinc that played a role in the zinc therapy benefit we saw. This can be studied by a trial in which we have three groups of patients, a control group, a group receiving zinc as in the trial described, and a group getting zinc as in group two plus a copper supplement to maintain baseline serum free copper.

Such a trial would be expensive. Perhaps of higher priority is a trial to simply confirm zinc benefit in a larger number of patients over a longer period of time. We are not aware of any plans for such a trial at present. In the meantime, our study is there for all to see.

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Research Article

Neuroprotective Role of a Novel Copper Chelator against Aβ_{42} Induced Neurotoxicity

Sandeep Kumar Singh,[1] Priti Sinha,[2] L. Mishra,[2] and S. Srikrishna[1]

[1] *Cell and Neurobiology Laboratory, Department of Biochemistry, Faculty of Science, Banaras Hindu University, Varanas 221005, India*
[2] *Department of Chemistry, Faculty of Science, Banaras Hindu University, Varanasi 221 005, India*

Correspondence should be addressed to S. Srikrishna; sskrishna2000@yahoo.com

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Alzheimer's disease (AD) is a progressive neurodegenerative disease and associated with the extracellular deposits of amyloid-β peptide in hippocampus region. Metal ions like Cu, Fe and Zn are known to associate with the amyloid beta (Aβ) at high concentration and interaction of these ions with soluble and aggregated forms of Aβ peptide help in development of AD. Here we showed Cu mediated neurotoxicity in the eye tissues of transgenic *Drosophila* expressing human amyloid β and its rescue through a novel Cu chelator. In this context, we have synthesised and characterized the compound L 2,6-Pyridinedicarboxylic acid, 2,6-bis[2-[(4-carboxyphenyl) methylene] hydrazide] by Mass spectra (MS) and Elemental analysis (EA). The Cu chelation potential of the compound L is tested *in vivo* in *Drosophila*. Oral administration of Copper to the transgenic larvae resulted in severe degeneration in eye tissues, which was rescued by the supplementation of compound L. The levels of anti-oxidant markers like SOD and MDA were measured in compound L treated flies and found a significant rescue ($P < 0.001$). Further rescue of the eye degeneration phenotypes as revealed by SEM affirm the role of copper in Aβ toxicity. Hence, use of compound L, an amidoamine derivative, could be a possible therapeutic measure for Aβ induced neurotoxicity.

1. Introduction

Alzheimer's disease (AD) is the most common cause of dementia in the aging population. It is the major neurodegenerative disease of aging brain, mainly associated with the extracellular deposits of amyloid-β plaques and intracellular neurofibrillary tangles (NFT) in hippocampus region of the brain. Several studies across the globe show a strong association between loss of metal homeostasis and AD. Consequently, the research community is seriously considering the role of various bimetals and environmental metal toxins in progression and clinical outcomes of Alzheimer's disease and other forms of neurodegenerative disorders. Metals play an important role in Alzheimer's pathology; heavy metals such as lead, cadmium, and mercury especially are highly neurotoxic and have no other biological functions. However, nowadays, people are mainly focusing on biologically important metals such as iron (Fe), zinc (Zn), and copper (Cu) because their imbalance is related to AD. Earlier studies demonstrated that metals like copper and zinc play a critical role in amyloid beta (Aβ) aggregation and neurotoxicity [1–4]. Metal ions, mainly Cu, Fe, and Zn, have been found to colocalize with the amyloid beta (Aβ) in high concentrations, and interaction of these metal ions with soluble/aggregated forms of Aβ peptides has been associated to the development of AD [5]. Among these, copper has attracted the most attention because both the Amyloid beta protein (APP) and amyloid-β (Aβ) peptides have significant interaction with the copper.

Location of copper binding domain (CuBD) is found in the N-terminal region of the APP, a Type I transmembrane protein [6]. The CuBD is found in cysteine rich region (between residues 124–189) [7, 8]. His-147, His-151, Tyr-168, and Met-170 are the main amino acid residues of the CuBD, which is involved in the mechanism of Cu coordination and reduction of Cu (II) into Cu (I) [6]. In addition to binding Cu to CuBD of APP, copper is also found to interact with aggregated Aβ and lead to the production of ROS via Fenton's

chemistry mechanism [9, 10]. In Aβ, amino acid residues His-6, His-13, His-14, and Tyr-10 are mainly involved in binding with the copper. The copper binding domain present in APP reduces Cu (II) to Cu (I) causes the production of reactive oxygen species, and results in oxidative damage [10, 11].

There are many defence mechanisms that protect the cells from oxidative injuries caused by reactive oxygen species (ROS) like hydroxyl radicals, hydrogen peroxide, superoxide radicals, and singlet oxygen. Superoxide dismutase (SOD) is one of the major antioxidative enzymes which catalyze the conversion of superoxide radical to hydrogen peroxide in the presence of molecular hydrogen [12, 13]. Mainly 3 forms of SOD are present in mammals; copper/zinc SOD (CuZn-SOD, SOD1), which is localized in the cytosol; manganese SOD (Mn-SOD, SOD2), which occurs in the mitochondrial matrix, and SOD3 that is located extracellularly is also a complex of Cu and Zn. Oxidative injury in case of Alzheimer's is well established [14], but the exact role of Aβ peptide and copper ions during this process is controversial [15, 16]. Some people adduced that Aβ toxicity is due to ROS generation in the presence of the Aβ-Cu (II) complex, while others argued that Aβ has antioxidant role. However, Cu plays an important role in the generation of reactive oxygen species [17, 18]. Ongoing research in this area focuses on the prevention of Cu mediated Aβ neurotoxicity and ROS production by Cu chelating therapy, which is an emerging trend in current research. Hence, there is immense need to develop such a suitable copper chelator that could prevent amyloid-β aggregation by effectively sequestering extra Cu^{2+} ions. Recently, several groups are focusing on developing such types of new molecules [19–25]. More particularly, in a pioneering work, Storr et al. have developed two carbohydrate-containing compounds, N,N′-bis[(5-β-D-glucopyranosyloxy-2-hydroxy)benzyl]-N,N′-dimethyl-ethane-1,2-diamine (H2GL1) and N,N′-bis[(5-β-D-glucopyranosyloxy-3-tert-butyl-2-hydroxy)benzyl]-N,N′-dimethyl-ethane-1,2-diamine (H2GL2), that have shown to be promising *in vitro* properties as therapeutic tools against AD [21]. Herein, we designed and synthesized novel compound L, 2, 6-Pyridinedicarboxylic acid, 2,6-bis[2-[(4-carboxyphenyl) methylene] hydrazide], to test the *in vivo* neuroprotective efficacy in a well-established *Drosophila* transgenic model system.

2. Materials and Methods

2.1. Chemical Requirements and List of Instruments Used. 2,6-Pyridinedicarboxylic acid, hydrazine hydrate, and 4-carboxybenzaldehyde were purchased from Sigma-Aldrich Chem Co., whereas the solvents were purchased from E. Merck and freshly distilled prior to their use. MALDI-TOF Autoflex Speed (Bruker, Germany) was used for MS study.

2.2. Synthesis and Characterization of Compound L. The compound L was synthesized in three steps as reported by us earlier [26] starting from 2,6-pyridine dicarboxylic acid (1 mmol, 0.167 g). Its methyl ester was prepared by stirring it in excess methanol in the presence of catalytic amount of concentrated thionyl chloride ($SOCl_2$) at room temperature for one day. The ester (1 mmol, 0.171 g) thus isolated was then reacted with aqueous hydrazine hydrate (2.1 mmol, 0.12 mL) in methanol under reflux for 3 h which resulted the production of solid 2, 6-Pyridinedicarboxylic acid, 2,6-bis[2-[(4-carboxyphenyl) methylene] hydrazide]. It (1 mmol, 0.195 g) was finally reacted with 4-formyl-benzoic acid (2 mmol, 0.300 g) in methanol at room temperature. Reaction was monitored using TLC. The product thus obtained was filtered and then purified by repeated recrystallization from hot ethanol. Yield: 85%, elemental analysis calculation for $C_{23}H_{17}N_5O_6$ (%): C, 60.13; H, 3.70 and N, 15.25. Found (%): C, 60.11; H, 3.67; and N, 15.24. MALDI-TOF/MS, $[M + H^+]$ = 460.18, $[M + Na^+]$ = 482.17. See Supplementary Figure (S1) available online at http://dx.doi.org/10.1155/2013/567128. ^{1}H NMR (DMSO - d_6, 300 MHz): δ (ppm) 13.21 (b, 2H, –COOH), 12.43 (s, 2H, –NH), 8.80 (s, 2H, CH=N), 8.36 (m, 2H, CHpy), 8.34 (m, 1H, CHpy), 8.03 (d, 4H, ArH), and 7.91 (d, 4H, ArH). ^{13}C NMR (DMSO - d_6, 300 MHz): δ(ppm) 166.98 (C_1, –COOH), 138.18 (C_2, ArH), 127.41 (C_4, ArH), 132.12 (C_5, ArH), 140.11 (C_6, –CH=N), 159.70 (C_7, –C(O)NH), 148.89–148.15 (C_8, C_9, Py), and 125.79 (C_{10}, Py). IR (KBr pellet, cm^{-1}): 3463 (–CONH), 1671 (–COOH), and 1609 (–C=N).

2.2.1. Crystal Data. C27H35N5O11S2, M = 669.74, monoclinic, a = 26.7068(15), b = 10.1394(4), c = 12.4951(6), α = 90, β = 110.310(6), and γ = 90; space group C 2/c, Z = 4, V/Å^3 = 3173.2(3), reflections collected/unique = 6463/3552 [R (int) = 0.0196], and final R indices [$I > 2$sigma(I)] = R1 = 0.0360, $wR2$ = 0.0871.

2.3. Synthesis and Characterization of Complex [Cu(L)]·2NO$_3$. A solution of $Cu(NO_3)_2$·3H2O (0.241 g, 1 mmol) in water (5 mL) was added dropwise to a solution of L (0.459 g, 1 mmol) in DMSO (10 mL). The reaction mixture after stirring for one day at room temperature was left for slow evaporation to get green precipitate. The precipitate was washed with MeOH followed by diethyl ether and then dried in air. Yield: 60%, M.P. >250°C, elemental analysis calc for $C_{23}H_{17}N_7O_{12}Cu$ (%): C, 39.42; H, 2.42 and N, 14.00. Found (%): C, 39.40; H, 2.43, and N, 14.02. MALDI-TOF/MS [M + H] = 699.17 (S2). IR (KBr pellet, cm^{-1}): 3417 (–CONH), 1686 (–COOH), and 1651 (–C=N). UV-vis absorbance: λ_{max} (DMSO-water mixture, 10^{-5} M), nm ($\varepsilon/10^5$ M^{-1} cm^{-1}) 315 (0.66), 352 (0.45), 385 (0.26), and 568 (0.150).

2.4. Photophysical Properties of Compound L. UV-vis absorption spectra were recorded on "Jasco V-630" spectrophotometer at 25°C. The absorption titrations of L with copper salt are performed by monitoring the changes in the absorption spectrum of L (10^{-5} M) in DMSO-water mixture (1 : 9, v/v). The concentration of L is kept constant at 10^{-5} M, while the concentrations of copper salt are varied within (1–10) $\times$ 10^{-6} M. The absorption of guest molecule is eliminated initially by keeping their equal quantities separately in host L and reference solution. From the absorption data, the intrinsic association constant K_a was determined from a plot of [guest]/($\varepsilon_a - \varepsilon_f$) versus [guest] using [27] equation

$[\text{guest}]/(\varepsilon_a - \varepsilon_f) = [\text{guest}]/(\varepsilon_b - \varepsilon_f) + [K_a\ (\varepsilon_b - \varepsilon_f)]^{-1}$ where [guest] is the concentration of copper salt. The apparent absorption coefficients ε_a, ε_f, and ε_b correspond to *A*obsd/[L], the extinction coefficient of the free L and extinction coefficient of L in fully bound form, respectively. K_a is given by the ratio of slope to the intercept.

2.5. Fly Stocks and Genetics. The *Drosophila* transgenic strain expressing Aβ_{42} under the control of UAS (UAS-A$\beta^{H32.12}$/CyO) was a generous gift from Dr. M. Konsolaki (Department of Genetics, Rutgers, The State University of New Jersey, USA) and eye specific GAL4 line (ey-GAL4 (w[∗]; P{w[+mC]=UAS-Dab.W}2, P{w[+mC]=GAL4-ninaE.GMR}12/CyO), which directs the expression specifically in eye tissue, used in this study was obtained from Bloomington Stock Center (Bloomington Stock no. 9511), Indiana University, USA. Flies and larvae were reared at 24 ± 1°C on standard *Drosophila* medium containing agar-agar, maize powder, sugar, yeast, nepagin (methyl-p-hydroxybenzoate), and propionic acid. Over expression of the Aβ_{42} transgene under UAS control was achieved by crossing it with ey-GAL4 fly.

2.6. Cu/Cu Chelator (Compound L) Treatment. The *UAS-A*β_{42}/ey-GAL4 larvae were cultured in normal food (NF) to achieve eye neurodegeneration phenotypes. The effect of copper on eye degeneration phenotypes was tested by feeding the Aβ_{42} expressing larvae in 500 μM copper nitrate [$Cu(NO_3)_2$] supplemented food. Further, copper (500 μM) along with compound L (250 μM) was tested to see if copper chelation has any effect on eye degeneration in Aβ expressing flies. Wild type Oregon R and undriven *UAS*-Aβ_{42}/*UAS*-Aβ_{42} flies were taken as controls in every case (data not shown). The *UAS*-Aβ_{42}/ey-GAL4 flies from F1 generation with noncurly wings ($n = 100$ in each case) were observed under stereo zoom binocular microscope for scoring eye phenotypes. Data of eye phenotypes was collected in each case of *UAS*-Aβ_{42}/ey-GAL4 flies cultured on normal food (NF), Cu treated food (Cu food), Compound L treated food, and Cu + compound L supplemented food media, and statistical analysis was done by using one-way ANOVA analysis (PRISM 3 Software).

2.7. Superoxide Dismutase (SOD) Assay. Adult flies were homogenized in homogenizing buffer following a method described previously [28]. The homogenate was centrifuged, and SOD activity was estimated as described by Nishikimi et al. [29] with minor modifications as per Singh et al. [28]. One unit of enzyme activity is defined as enzyme concentration required for inhibiting chromogen production (optical density 560 nm) by 50% in 1 min under assay conditions, and the data were expressed as the specific activity in units/min/mg protein.

2.8. Assay for Lipid Peroxidation (LPO). Adult flies were homogenized in homogenizing buffer following a method described previously [28].Malondialdehyde (MDA) content as a measure of LPO was assayed using tetraethoxypropane as an external standard [30]. Lipid peroxide levels were expressed in terms of nmoles MDA formed/h/mg protein.

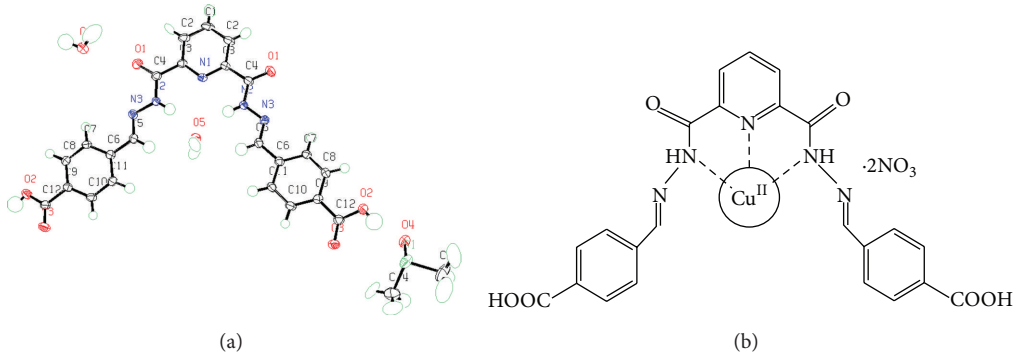
2.9. Scanning Electron Microscopy (SEM) of Drosophila Compound Eye. We followed the method of Wolff 2011 [31] with minor modifications for the Scanning Electron Microscopy of compound eyes. About 4–6 representative flies from each group with different treatments were etherized, and heads were detached carefully under the binocular microscope to leave the eyes intact. The decapitated heads with intact eyes were put into 1.5 mL eppendorf tube and fixed overnight in 1.5 mL fixative (0.1 M PBS, 25% glutaraldehyde and dH_2O), dehydrated in ethanol (once in 25%, 50%, 75%, and 100% ethanol each with 3 hrs and then thrice in absolute ethanol, 15 min each). Tissues were dried by using CPD (critical point drying) for removing any extra moisture present in sample and then analysed by using Scanning Electron Microscope (Hitachi S-3400N). Images were analysed from each group and eye phenotypes were scored.

3. Results

3.1. Absorption Titration Shows Binding Affinity of Cu^{2+} with Compound L. The UV-vis titrations with compound L (Figure 1(a)) were carried out in DMSO-water mixture (1 : 9 v/v) solution using standard nitrate salts of Cu^{2+}, Zn^{2+}, and Ag^{+} at room temperature.

UV-vis spectrum of the solution of L (1.0×10^{-5} M) recorded upon the addition of Cu^{2+}, is shown in Figure 2. Upon addition of Cu^{2+} the absorption peak at 315 nm was decreasing, whereas the absorption peak at 352 nm was increasing. The resulting titration revealed an isosbestic point at 290 and 337 nm. The appeared isosbestic point shows that the stable complex (Figure 1(b)) is formed with a definite stoichiometric ratio between L and cation. Interestingly, the addition of other nitrate salts of Zn and Ag did not result in any observable change in the absorption spectrum of L at this wavelength. Colour changes are most probably due to the formation of complex between the amido groups and copper ion. The association constant for copper ion was calculated using equation mentioned in Section 2.4. The value of association constant (K_a) for copper ion was found as 1×10^6 binding in 1 : 1 stoichiometry (Job's plot, S3). Electronic spectra for L remained unchanged in the presence of excess (20 mole equivalents) of other nitrate salts of Zn and Ag.

3.2. Compound L Ameliorates AD Eye Phenotypes. To determine the effect of copper chelator (compound L) on Cu mediated Aβ toxicity, Aβ expressing larvae were cultured separately in copper (500 μM), chelator (250 μM), and Cu + chelator (250 μM) supplemented food (Figure 3). The flies expressing Aβ were also cultured in normal food (NF), and wild type flies were taken as control in each case. Aβ expressing flies showed mild and severe eye degeneration phenotypes (Figure 3(d) ii and iii, resp.) when cultured in normal food. The severity in eye degeneration was enhanced to several folds in Cu treated flies as compared to untreated flies (compare Figure 3(d) iv with iii), while the percentage of flies



(a) (b)

Figure 1: Crystal structure of compound L (a) and structure of compound L with copper binding (b).

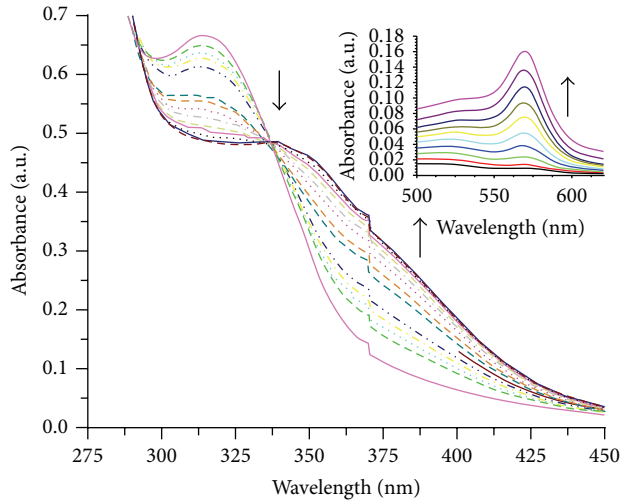


Figure 2: Absorption spectra of compound L = (10^{-5} M, DMSO-water mixture, 1 : 9 v/v) in the absence and presence of increasing amount of Cu^{2+} = (1–10) × 10^{-6} M (in water) at room temperature. Inset of the d-d region appeared at 569 nm after addition of 1 equivalent of $Cu(NO_3)_2$ solution.

showing severe defects was unaltered. The degree of severity was of two types; flies observed form Cu supplemented food showing more severity in eye phenotype with highly degenerative dark patches as compared to flies cultured in normal food (compare Figure 3(d) iv with iii). The statistical significance of these phenotypes was shown in Figure 3. Rescue in severe eye degeneration was found in compound L treated flies at 250 μM concentration (Figure 3(c)), which appears to be the best concentration for copper chelation *in vivo*.

Compound L (chelator) alone was also tested on *UAS*-Aβ_{42}/ey-GAL4 flies (without copper supplementation) and found significant rescue only at 200 μM concentration (compare Figure 4(a)with Figure 3(a)). But chelator at 250 and 300 μM concentrations did not show any apparent rescue (Figures 4(b) and 4(c), resp.). There is no lethality found to be associated with these concentrations of chelator.

3.3. Copper Treatment Induces SOD and Lipid Peroxidation. Cu ion enhances the Aβ toxicity following ROS production. The ROS activity in Aβ expressing flies grown in normal food, copper (Cu) treated, chelator (Che) treated, and copper and chelator (Cu + Che) treated food was assessed indirectly by estimating SOD and MDA levels. The effect of copper treatment on antioxidant markers like SOD (Figure 5) and MDA (Figure 6) in Aβ-driven flies suggests increased ROS activity. We observed a significant ($P < 0.05$, $P < 0.001$) increase in the enzyme activity in Aβ flies fed on normal and copper treated food, respectively (Figure 5), and SOD activity was reduced in chelator treated flies, which is comparable to wild type. A similar trend was observed for MDA activity after copper treatment. There was a significant increase of 1.6- and 1.9fold ($P < 0.05$, $P < 0.001$) in MDA activity in flies grown on normal and copper supplemented food, respectively, as compared to wild type (Figure 6).

3.4. Scanning Electron Microscopy Shows Rescue in Eye Neurodegeneration. Rescue in degenerative eye is clearly evident in digital microscopy imaging (Figure 7(d)). But in order to clearly visualize the internal morphology of the eye, like structural arrangement of ommatidia and bristles, Scanning Electron Microscopy (SEM) is required. SEM has revealed the recovery of normal eye morphology in chelator treated flies (Figure 7(h)) as compared to flies treated with normal and copper supplemented food (Figures 7(f) and 7(g), resp.). This clearly indicates that compound L may be inhibiting Cu mediated Aβ toxicity which causes eye degeneration. Flies expressing the Aβ_{42} transgene in neurons showed severe eye degeneration when grown in normal food (Figures 7(b) and 7(f)), while food supplemented with Cu ions showed highly degenerative ommatidial morphology with complete loss of bristles and reduction of eye size (Figures 7(c) and 7(g)). However, in wild type control, flies smoothly arranged patterns of ommatidia and bristles are found (Figures 7(a) and 7(e)).

4. Discussion

We have used a *Drosophila* transgenic model of AD to investigate the therapeutic potential of a novel copper chelator, compound L that might be reducing copper mediated

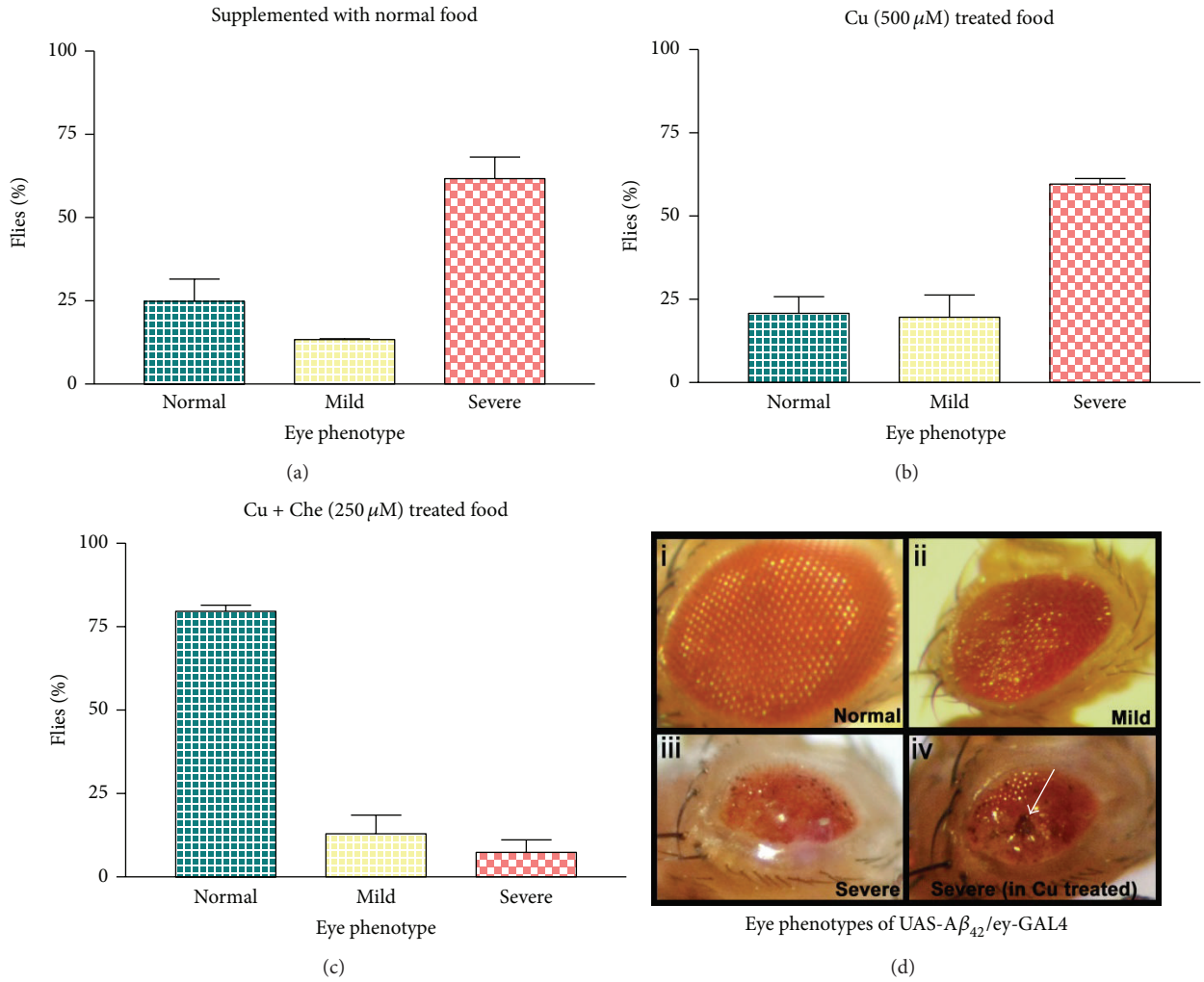


FIGURE 3: Histogram shows the percentage of flies with normal, mild, and severe eye phenotypes in ey-GAL4 driven UAS-Aβ_{42} flies when grown in normal food (a), copper treated food (b), and Cu + chelator treated food (c). The number of flies (n = 100 for (a), (b), and (c)) on y-axes is expressed as % of flies against eye phenotype in each case. Different types of eye degeneration phenotypes of *UAS*-Aβ_{42}/ey-GAL4 like mild ((d) ii) and severe ((d) iii, iv) are shown in panel (d). Note that copper supplemented food resulted in severe ommatidial degeneration as indicated by dark patches in the eye (white arrow in (d) iv).

Aβ toxicity. Many existing compounds aim to reduce Aβ production by blocking β and γ-secretases or by stimulating α-secretase activity of APP. Recent studies have shown that β and γ-secretase inhibitors may cause side effects, because they are important for the cleavage of other biologically important molecules [32]. Alternatively, stimulation of the nonamyloidogenic amyloid precursor protein processing is being developed as a potential therapy against AD [33]. Since metals play a very important role in mediated Aβ toxicity, several researchers are working on the development of chelators that can effectively reduce metal toxicity. Further, Cu mediated Aβ toxicity also results in ROS production, so several others are working on this to prevent ROS generation due to Cu induced Aβ aggregation [34]. Therefore, the main aim of this study is to investigate the neuroprotective efficacy of a newly synthesized copper chelator in an *in vivo Drosophila* AD model and to appraise its use as a potential therapeutic agent.

In this work, a novel copper chelator, compound (L), was synthesized and characterized by MS and EA analyses. In order to test its efficacy, compound L was supplemented through diet to the transgenic *Drosophila* expressing human Aβ. We have used UAS/Gal4 system to express Aβ_{42} specifically in eye tissues. The effect of this novel copper chelator in the rescue of severe neurodegenerative eye phenotype was observed by using statistical (Figures 3 and 4) as well as Scanning Electron Microscopic studies (Figure 7). To see the effect of Cu and Cu chelator on a developmentally induced retinal toxicity phenotypes (the severe rough eye phenotype) generated by Cu induced Aβ_{42}, we have cultured Aβ_{42} expressing transgenic larvae on normal medium, Cu (500 μM) supplemented medium, Cu + chelator (250 μM),

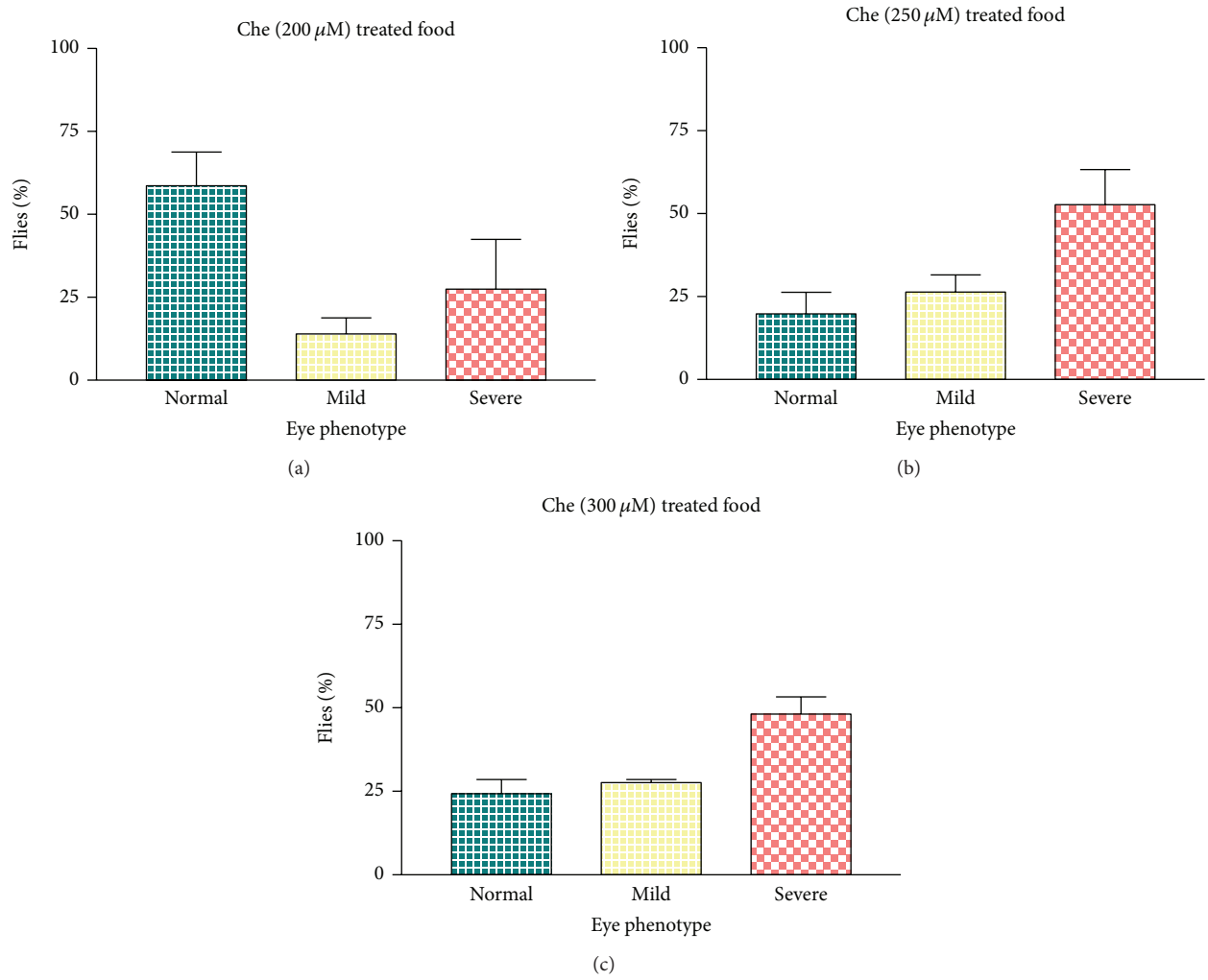


FIGURE 4: Histogram shows the percentage of flies having normal, mild, and severe rough eye phenotypes in ey-GAL4 driven UAS-Aβ_{42} flies, when grown alone in 200 μM (a), 250 μM (b), and 300 μM (c) of chelator (L) containing food. Note that, at 200 μM, eye degeneration phenotype is rescued as evident by increased number of flies with normal eyes (a). The number of flies in each case is 100.

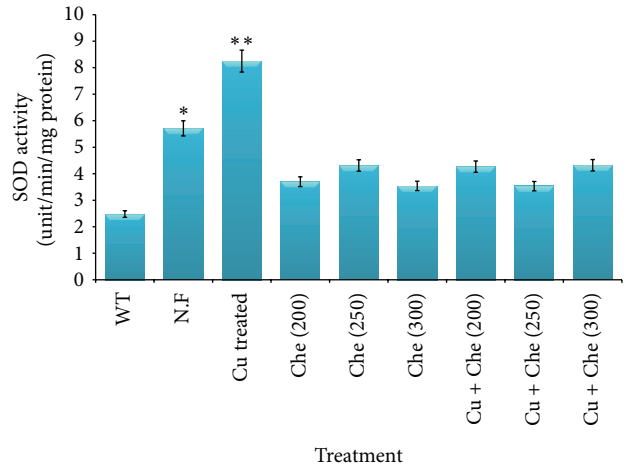


FIGURE 5: Measurement of Superoxide dismutase (SOD) in wild type flies and Aβ expressing flies treated in normal food as well as Cu and chelator supplemented food. Data represented are mean ± SD of normal and drug treated groups experiments made in three replicates. Significance is ascribed as $^{*}P < 0.05$ or $^{**}P < 0.001$ as compared to wild type.

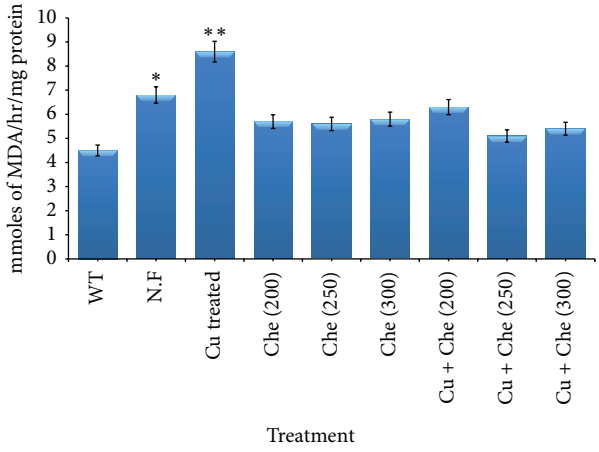


FIGURE 6: Measurement of malondialdehyde (MDA) content in wild type flies and Aβ expressing flies treated in normal food as well as Cu and chelator supplemented food. Data represented are mean ± SD of normal and drug treated groups experiments made in triplicates. Significance is ascribed as $^{*}P < 0.05$ or $^{**}P < 0.001$ as compared to control.

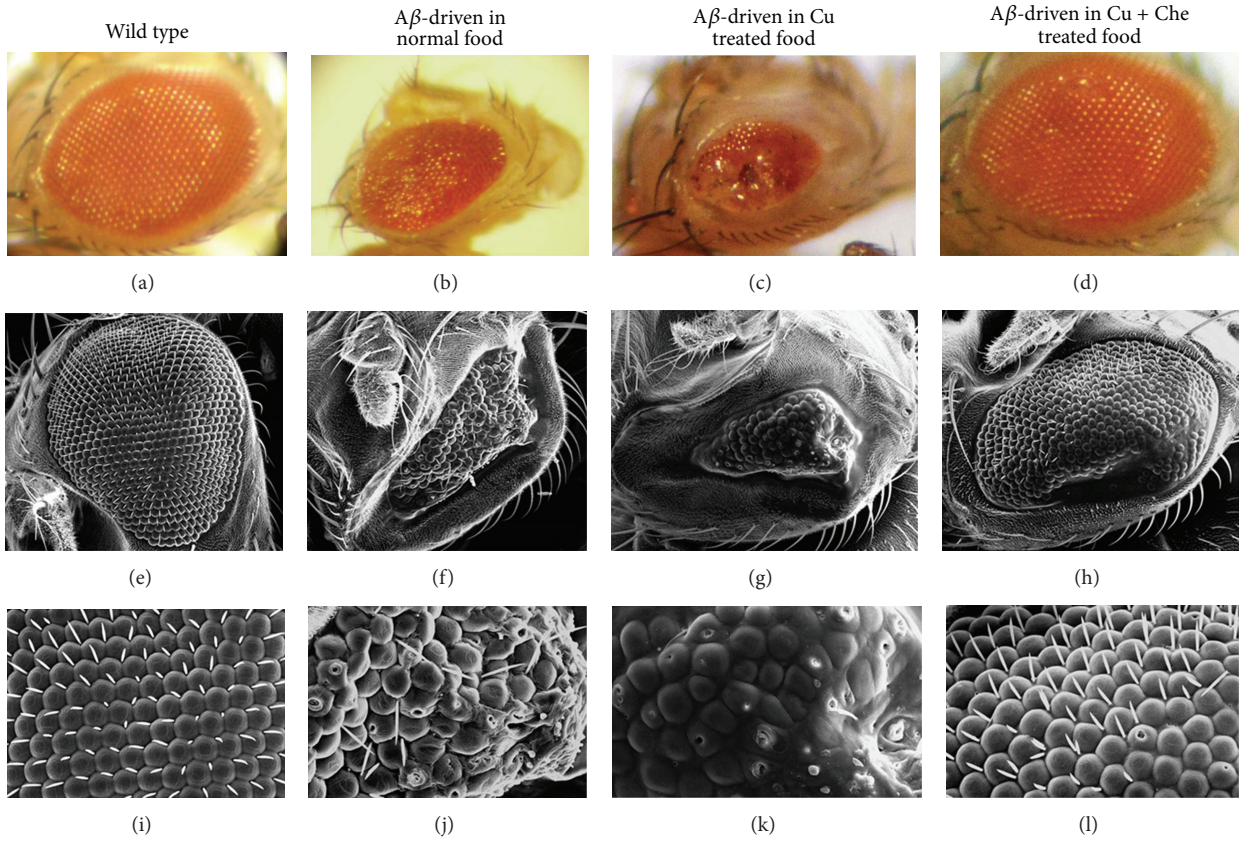


FIGURE 7: Scanning electron micrographs showing eye degeneration and their rescue after compound (L) treatment ((e)–(l)). Upper panel shows the digital images of compound eyes of wild type (a), Aβ expressing (b), Aβ in presence of copper (c), and Aβ with Cu + chelator (d). There is a reduction in size of eye of Aβ expressing fly grown in normal food ((b), (f)) as compared to the wild type ((a), (e)) and more degeneration can be seen after treatment with Cu (500 μM) alone ((c), (g)). Rescue after the treatment with 250 μM of compound (L) ((d), (h)). Magnification is 230x. Lower panel shows the eye phenotypes of corresponding images of middle panels, respectively, ((e)–(h)) showing very distinct pattern of eye degeneration and ommatidia disruption. Magnification is 700x.

and with chelator alone (200 μM, 250 μM, 300 μM). The Aβ_{42} expressing flies in normal and Cu supplemented food showed mild and severe eye degeneration phenotypes in both the cases (Figures 3(a) and 3(b), resp.). However, the severity in eye degeneration in Cu treated flies is more as compared to the flies grown in normal food though the % number of flies did not vary in both the cases (Figure 3(d)). Interestingly, Cu chelator at 250 μM concentration showed very good rescue against Cu induced severe neurodegeneration phenotype (Figure 3(c)). Further, compound L alone at 200 μM concentration also showed significant rescue against the severe rough eye phenotypes of Aβ_{42} expressing flies (Figure 4(a)). However, 250 μM and 300 μM concentrations did not show such recovery in eye phenotypes (Figures 4(b) and 4(c)), though there is no lethality associated with these doses of chelator. It is unclear why high concentration of chelator did not show better recovery. Perhaps further studies on concentration dependent Aβ_{42} chelator *in vitro* binding assays will shed light on this aspect. We have also checked the effect of copper complex ($[Cu(L)]\cdot 2NO_3$) on eye phenotypes at the same concentrations used for chelator. But, in this case, there is a lethality observed at both embryonic and early larval stages (data not shown). The lethality is caused due to the presence of high levels of Cu in the complex itself. Similarly, in the SEM analysis, we found that ommatidial irregularity was recovered by the treatment of compound L (Figure 7). We observed the best rescue (~75–80%) of the rough eye phenotype in Aβ_{42} expressing flies when treated with 250 μM of compound (L). Hua et al., showed ameliorating the Aβ-associated toxicity using Cu and Zn chelators [35], thus preventing and/or delaying the progression of AD. Our results show that supplementation with this novel copper chelator reduces copper mediated neurodegeneration by inhibiting Aβ aggregation in *Drosophila* eye. However, the mechanism of action is not clear, and future work should also address the mechanism of action of this novel copper chelator in reducing the copper mediated Aβ toxicity. It is now widely accepted that Cu promotes the Aβ mediated ROS production [17] that causes toxicity to cells. In this context, we have checked some *in vivo* ROS markers like SOD and MDA in treated as well as control flies including wild type and found significant increase in SOD and MDA activities in flies fed on normal as well as copper treated food. This suggests that copper has a role in Aβ toxicity via ROS production. SOD is an antioxidant enzyme and primarily acts to protect oxygen-metabolizing eukaryotic cells from the adverse effects of superoxide ions [36]. Transgenic flies over expressing SOD show a decreased level of oxidative damage and a 33% increase in life span compared to the controls [37]. Generally, SOD activity is observed to be elevated in case of any therapeutic drug, but in our case, we found significantly increased activity of SOD in case of flies fed on normal and copper treated food, and reduction in SOD activity was found after compound L treatment. This observation of elevated SOD activity in copper supplemented flies and reduced activity of SOD in chelator treated flies could be due to Cu and Zn cofactor mediated SOD activity. However, further studies related to SOD and other marker enzymes in this context are required to address this intriguing issue. Thus, our results indicate that this novel copper chelator plays a role in protecting against Cu mediated Aβ aggregation and neurotoxicity in an *in vivo* model system. Our results provide support for the neuroprotective effect of this novel compound L as a potential therapeutic agent for AD.

5. Conclusion

In conclusion, we show that the Cu mediated toxicity of Aβ peptides can be reduced through chelation of aggregation-promoting Cu metal ions by suitable chelating agent as developed here (L). In addition, further investigations on the mechanism of action of this novel copper chelator are required.

Acknowledgments

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Review Article

Cholesterol and Copper Affect Learning and Memory in the Rabbit

Bernard G. Schreurs[1,2]

[1] *Blanchette Rockefeller Neurosciences Institute, West Virginia University, Morgantown, WV 26505, USA*
[2] *Department of Physiology and Pharmacology, West Virginia University, P.O. Box 9302, Morgantown, WV 26506, USA*

Correspondence should be addressed to Bernard G. Schreurs; bschreurs@hsc.wvu.edu

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A rabbit model of Alzheimer's disease based on feeding a cholesterol diet for eight weeks shows sixteen hallmarks of the disease including beta amyloid accumulation and learning and memory changes. Although we have shown that feeding 2% cholesterol and adding copper to the drinking water can retard learning, other studies have shown that feeding dietary cholesterol before learning can improve acquisition and feeding cholesterol after learning can degrade long-term memory. We explore the development of this model, the issues surrounding the role of copper, and the particular contributions of the late D. Larry Sparks.

1. Introduction

In 2001, we were looking for nontransgenic animal models of Alzheimer's disease (AD) in which we could study the effects of potential treatments on AD deficits in learning and memory. A review of the literature revealed very few options other than aged animals that would take many months or even years to reach a point at which they could be studied [1–4]. One exception was a cholesterol-fed rabbit model of AD that Sparks and colleagues showed had several hallmarks of Alzheimer's pathology, particularly beta amyloid accumulation, that developed in as a little as 8 weeks of being fed a 2% cholesterol diet [5–8]. Surprisingly, given the well-characterized rabbit eyeblink conditioning preparation first published by Gormezano and colleagues in the 1960s [9–13], there were no studies in the literature examining learning and memory in these cholesterol-fed rabbits. We contacted Larry Sparks to ask why no one had published learning and memory studies with this model and the answer was as clear and emphatic as only Larry Sparks could make it: he had tried to convince researchers for years to do the experiments but no one seemed to be interested.

One possible reason for this apparent lack of interest in studying learning and memory in a rabbit model of AD was the fact that standard rabbit eyeblink conditioning experiments in which a tone preceded and overlapped with a puff of air to the eye was mediated in large part by the cerebellum [14, 15], and the cerebellum is the last and least affected brain structure in patients with AD [16]. However, this mediation of learning by the cerebellum is only true for the most basic of classical conditioning paradigms known as delay conditioning in which the stimuli overlap [17]. If there is a substantial trace between the two stimuli and the tone and air puff do not overlap, there is good evidence that the hippocampus and prefrontal cortex are engaged and become critical to successful learning and memory [18–30]. The hippocampus and cortex are among the areas that are the first and most profoundly affected structures in patients with AD [16, 31].

2. The Effects of Cholesterol on Learning

In collaboration with Sparks, we sought to assess the effects of a cholesterol diet on trace conditioning of the rabbit nictitating membrane response [32]. The results of these first experiments with the nictitating membrane response (NMR) were to start us on a ten-year odyssey that still continues to challenge us and has been deeply affected by the untimely death of our colleague Larry Sparks.

In order to study NMR conditioning in cholesterol-fed rabbits, we instituted a standard set of procedures that began

with rabbits being fed 2% cholesterol or standard Purina rabbit chow (0% cholesterol) for eight weeks and then presented the rabbits with pairings of a brief tone (100 ms, 82 dB, 1 kHz) as the conditioned stimulus (CS) followed by an eyeblink-eliciting air puff (100 ms, 4 psi) or periorbital electrical pulse (100 ms, 2.0 mA, 60 Hz) as the unconditioned stimulus (US). In some experiments, half of the rabbits received explicitly unpaired presentations of the CS and US to assess nonassociative contributors to responding [9, 13, 33–36]. Importantly, the interval between the CS and US for paired rabbits was more than 500 ms creating a significant trace which previous studies by a number of groups have shown made classical conditioning dependent on the hippocampus [18–20, 37–40] and prefrontal cortex [23, 25, 27, 29, 41–45] in addition to the cerebellum. In each of our trace conditioning experiments, acquisition of a conditioned response was a function of the trace interval and usually took many days of training to reach asymptote, and this asymptote tended to be lower than that seen using delay conditioning [17]. Importantly, subsequent delay conditioning and sensory thresholds were always the same for cholesterol-fed rabbits and normal chow controls [32, 46]. Cholesterol-fed unpaired control subjects showed low levels of responding that were consistent with previous observations in rabbits fed normal chow [9, 33, 47]. In all of these experiments, the cholesterol diet continued throughout the course of the behavioral manipulations.

With the previously well-documented accumulation of intracellular beta amyloid induced by feeding 2% cholesterol for 8 weeks reported by Sparks and his colleagues [5, 6, 8], the first experiments we conducted were surprising because of the expectation that we would see a beta amyloid-induced deficit in learning when, in fact, we saw a facilitation of NMR conditioning [32]. This is a finding we have seen in many of our subsequent experiments [48–50]. The facilitated conditioning was indexed by higher levels of responding to the CS [32, 48] and heightened responsivity to the US measured after conditioning, and known as a conditioning-specific reflex modification [32, 48–50]. When the levels of beta amyloid accumulation in our cholesterol-fed rabbits were examined by the Sparks laboratory, the immunoreactivity was relatively light although significantly higher than in the rabbits fed normal chow. At that point, we discussed the results with Sparks and it became clear that there was an as yet untold part of the story involving the drinking water given to rabbits.

3. The Contents of Tap Water

The effects of drinking water on beta amyloid accumulation have been discussed at length in a number of articles by Sparks and colleagues and will only be summarized here [51–53]. The original finding that the contents of tap water might be important to the level of beta amyloid immunoreactivity began with the observation by Sparks that after moving his laboratory from Kentucky to Arizona, cholesterol-fed rabbits showed significantly less intense beta amyloid immunoreactivity. Upon investigation, and ruling out other potential causes, it came to light that the rabbits in the Arizona facility were being given distilled water to drink and, when they were returned to tap water, the beta amyloid immunoreactivity became more intense [51]. An analysis of trace metals in Morgantown tap water by an independent laboratory showed virtually no detectable levels of copper [32]. In contrast, rabbits that had been switched to and maintained on tap water in Arizona showed high levels of beta amyloid immunoreactivity and high levels of copper in the tap water [32, 51]. Subsequent manipulation of copper in distilled drinking water showed that the level of beta amyloid immunoreactivity was, indeed, a function of copper in the drinking water [46, 51–54]. In his own inimitable style, Sparks initially broached the entire subject of the role of drinking water in beta amyloid accumulation when he began a conversation about our initial light beta amyloid staining results with "And now for the rest of the story…".

4. The Effects of Copper on Learning

We next conducted a seminal experiment [46] in which we added 0.12 parts per million (ppm) copper as copper sulfate to distilled drinking water and found that the levels of beta amyloid in cholesterol-fed rabbits had increased over previous levels to the point of generating extracellular plaques and, importantly, these rabbits showed a deficit in trace conditioning relative to controls. The photo montage in Figure 1 shows evidence of extracellular plaque-like structures as well as dense intracellular immunoreactivity to the beta amyloid antibody 10D5 shown in detail at the bottom left of Figure 1. Figure 1 also shows a thioflavin-S stained neuron in detail (top left) in a rabbit fed cholesterol and given copper in its drinking water. Figure 2 shows that the level of trace conditioning acquired by rabbits given cholesterol and copper was significantly lower than rabbits fed cholesterol and given distilled water and rabbits fed normal chow and given copper in their drinking water. Although there is a suggestion that the cholesterol-fed animals drinking distilled water might have had higher terminal levels of responding than rabbits fed normal chow, the differences were not significant. As noted above, all rabbits were able to acquire a simple delay conditioning task in which the tone and air puff overlapped and all showed very similar auditory thresholds indicating that the cholesterol and copper did not affect sensory processing or simple delay conditioning [46]. The essential aspects of these initial findings of increased beta amyloid and lower levels of trace eyeblink conditioning were subsequently replicated by another rabbit conditioning laboratory [55, 56]. The beta amyloid accumulation resulting from rabbits being fed cholesterol has also been independently confirmed by the Ghribi group who have gone on to study some of the underlying molecular mechanisms [57–60].

To further explore the role of copper and tap water, we conducted a simple experiment in which we fed rabbits 2% cholesterol and provided them with Morgantown tap water which had been supplemented with 0.12 ppm copper. These rabbits were compared to rabbits that were fed normal chow and given tap water supplemented with 0.12 ppm copper. The data in Figure 3 show both the level of responding during trace conditioning and the number of beta amyloid

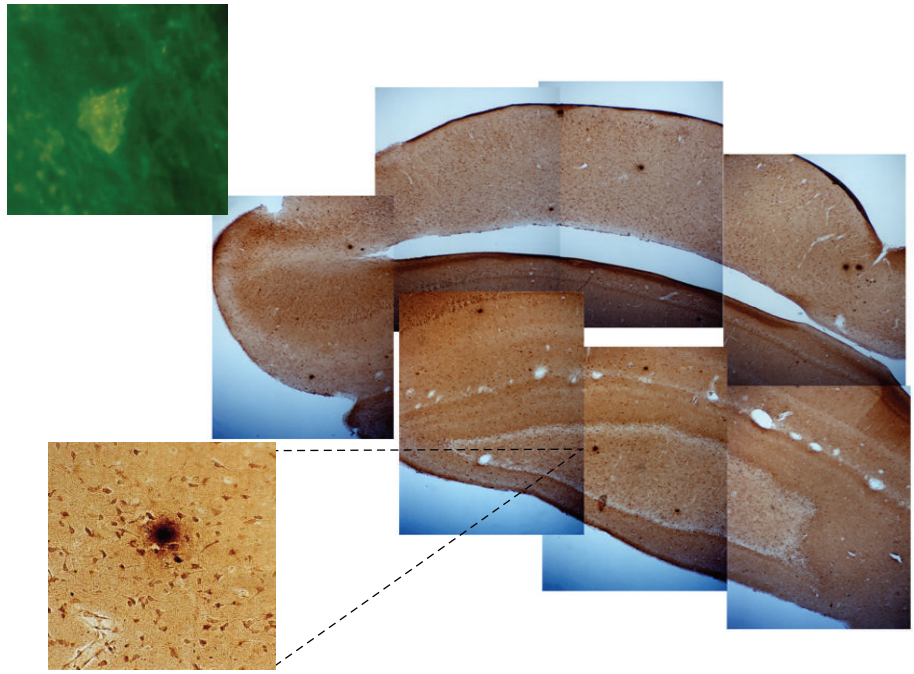

Figure 1: Photo montage of temporal lobe and hippocampus for a cholesterol-fed rabbit given distilled water supplemented with 0.12 parts per million copper as copper sulfate (right). Note the numerous dark spots that appear to be plaque-like structures that are shown in detail at bottom left. The top left image shows a thioflavin-S stained neuron from a cholesterol-fed rabbit given distilled water supplemented with 0.12 parts per million copper.

immune-positive neurons in the cortex and hippocampus of the two groups. Figure 3 shows and statistical analysis confirmed that the level of trace conditioning acquired by rabbits given cholesterol and copper in tap water was significantly lower than rabbits fed chow and given tap water with copper ($P < .005$). Once again all rabbits were able to acquire delay conditioning and showed identical auditory thresholds indicating that the cholesterol and copper did not affect sensory processing or simple response acquisition. The inset of Figure 3 shows that the number of beta amyloid immune-positive cells was significantly higher in the cortex and hippocampus of rabbits fed cholesterol and given copper in tap water than those fed chow. These data help confirm the original findings shown in Figure 2 and suggest that there may be more to the effects of water than first thought. For example, a subsequent analysis of the tap water supplemented with 0.12 ppm copper stored in standard carboys for four-five weeks yielded a large number of components similar to our original analysis of the tap water in Morgantown [32] but, surprisingly, the level of copper was only 0.085 ppm. The level of copper in tap water supplemented with 0.12 ppm copper and stored for only two weeks was 0.104 ppm. Clearly, storage in polypropylene carboys caused significant changes in copper concentration as a function of time and, as a result, we instituted weekly preparation of fresh copper-supplemented water that was administered to rabbits in glass bottles.

5. Cholesterol, Copper, and Beta Amyloid

At this point, we began a series of parametric experiments in which we manipulated the concentration [48] and duration [50] of cholesterol and routinely included copper in the distilled drinking water. We continued to see the facilitating effects of cholesterol on NMR conditioning [48, 50] but, surprisingly, we did not see the debilitating effects on NMR conditioning with the addition of copper to the cholesterol. Importantly, although we continued to see higher levels of beta amyloid immunoreactivity with copper added to the drinking water of cholesterol-fed rabbits compared to those on distilled water, there was no evidence of extracellular beta amyloid plaques. This was even true when we doubled the copper concentration in the drinking water to 0.24 ppm although, in that case, the cortical levels of beta amyloid immunoreactivity were significantly higher in chow-fed rabbits given 0.24 ppm copper compared to those given distilled water suggesting that copper by itself was having an effect on beta amyloid accumulation [61].

During this second phase of behavioral experiments, a heightened sensitivity by veterinary staff to the hepatotoxic effects of the cholesterol diet [53, 62–64] meant that animals were being given supplementary feeding or withdrawn from studies earlier and more often than had occurred in our original studies. It is possible that the beta amyloid load was not as severe and the consequent extracellular plaque formations were no longer being detected because of this earlier withdrawal from the studies. In a separate development, the Sparks laboratory began to notice a decrease in the intensity of beta amyloid staining with the 10D5 antibody and the problem became worse with succeeding batches of antibody. Nevertheless, in an unpublished study, we continue to find beta amyloid immunoreactivity with a commercial human beta amyloid enzyme linked-immunosorbent assay

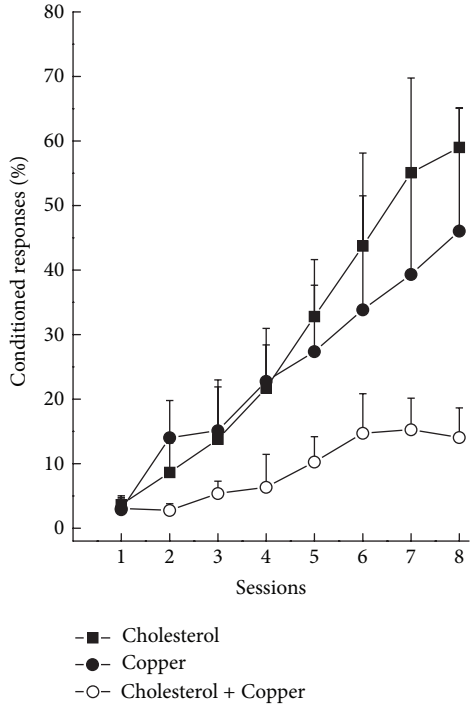

Figure 2: Mean percent (±SEM) conditioned responses to a tone conditioned stimulus as a function of eight days of pairings (sessions) of the tone and air puff to the eye for rabbits fed 2% cholesterol in their rabbit chow (Cholesterol), fed normal chow and given 0.12 parts per million copper as copper sulfate in their distilled drinking water (Copper), or fed 2% cholesterol and given 0.12 parts per million copper in their distilled drinking water (Cholesterol + Copper). The data show lower levels of trace conditioning of the nictitating membrane response in rabbits fed cholesterol and given copper in their drinking water. The data are modified from Sparks and Schreurs [46].

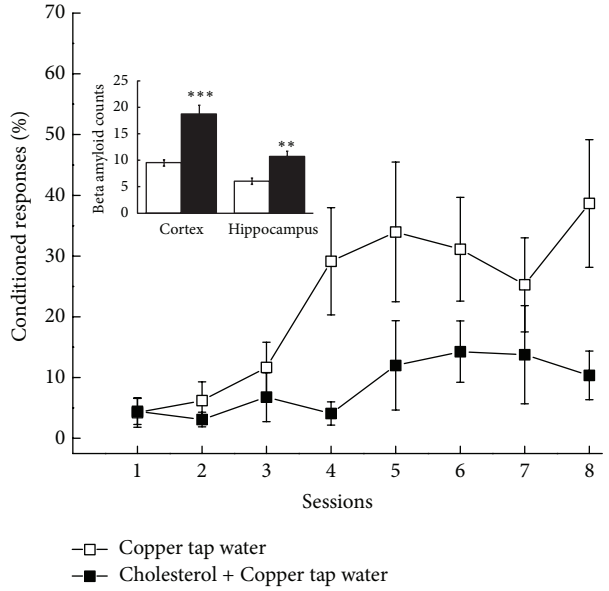

Figure 3: Mean percent (±SEM) conditioned responses to a tone conditioned stimulus as a function of eight days of pairings (Sessions) of the tone and air puff to the eye for rabbits fed normal rabbit chow and given 0.12 parts per million copper as copper sulfate in their tap water (Copper Tap Water) or fed 2% cholesterol and given 0.12 parts per million copper in their tap water (Cholesterol + Copper Tap Water). The inset shows the mean (±SEM) number of counts of beta amyloid immunoreactive cells in the cortex and hippocampus. The data show lower levels of trace conditioning of the nictitating membrane response and number of beta amyloid positive cells in rabbits fed cholesterol and given copper in their tap water.

and immunofluorescent labeling based on the 6E10 beta amyloid antibody. Moreover, other groups have also reported significant elevations in beta amyloid as a result of feeding rabbits cholesterol [55, 58, 65, 66].

6. Imaging the Effects of Cholesterol and Copper

At the conclusion of many of our behavioral experiments, we began structural MRI imaging of the rabbits' brains to explore the effects of cholesterol and copper on rabbit ventricular volume [61, 67] and cerebrovascular diameter [67]—indices that have been noted to change in patients with AD [68–71]. The four panels of Figure 4 show structural MRI scans of rabbits that received normal chow and distilled water (a), normal chow and 0.12 ppm copper added to the distilled water (b), 2% cholesterol and distilled water (c), and 2% cholesterol and copper (d), with insets that show the area of the third ventricle. The data in Figure 4 illustrate clearly that a cholesterol diet significantly increased the area of the third ventricle and consequently, when the entire rabbit brain was analyzed, the volume of the third ventricle was found to be higher for the cholesterol-fed rabbits than the normal chow-fed controls [72]. This was true regardless of whether the rabbits were given copper in the drinking water or whether the concentration of that copper was 0.12 ppm [72] or 0.24 ppm [61]. In all of these experiments, the levels of beta amyloid immunoreactivity to the 10D5 antibody was always higher in cholesterol-fed rabbits than normal chow controls, and the addition of copper tended to increase the intensity of that immunoreactivity even further although this copper-induced increase was not always significantly higher [48–50, 61]. Figure 5 shows the blood vessels in a rabbit brain that were visualized by time-of-flight angiography during our MRI studies and they include the common carotid arteries, the basilar artery, the internal carotids, and the posterior communicating arteries [67]. Figure 6 shows that the basilar, internal carotid, and posterior communicating arteries were all narrowed by a 2% cholesterol diet compared to normal chow controls and that the addition of 0.12 ppm copper to the drinking water did not significantly increase the narrowing [72]. There were no significant differences in diameter of the common carotid arteries.

7. The Effects of Cholesterol on Other Forms of Learning

As important as the behavioral effects of cholesterol were with the rabbit NMR, there were two broader questions. First,

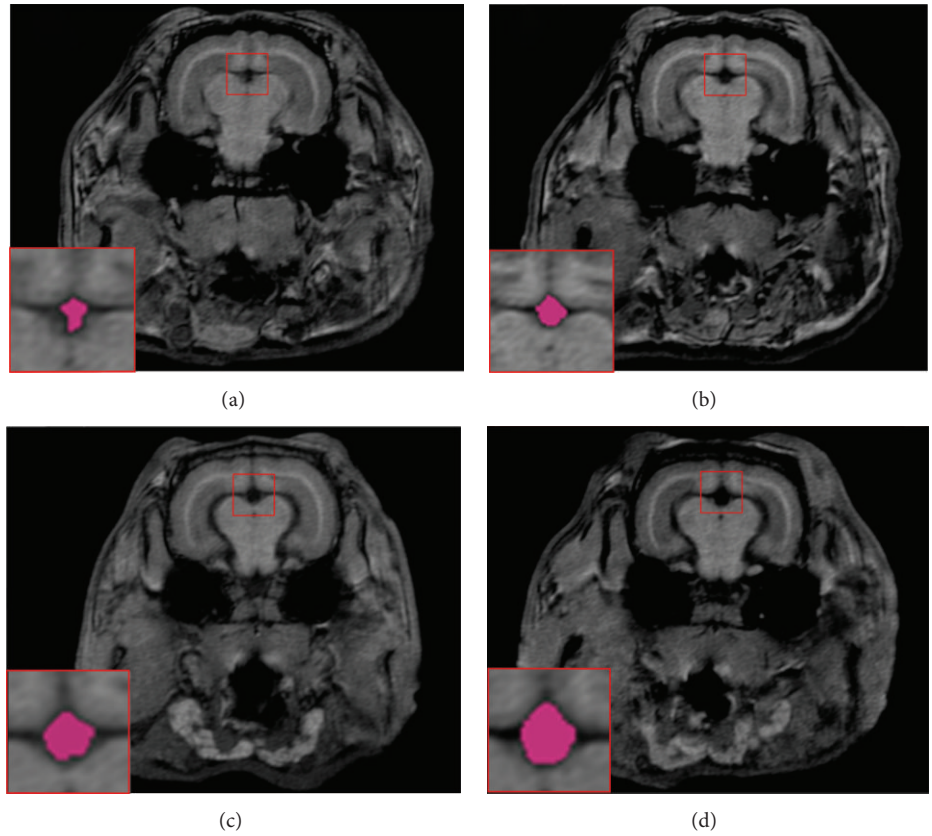

Figure 4: Structural MRI scan of a rabbit that received normal chow and distilled drinking water (a), normal chow and 0.12 ppm copper added to the distilled drinking water (b), 2% cholesterol and distilled drinking water (c), or 2% cholesterol and copper added to the distilled drinking water (d). The inset shows the area around the third ventricle in each rabbit and the red shading illustrates the significantly larger area of the third ventricle for cholesterol-fed rabbits ((c) and (d)) compared to the chow-fed control rabbits ((a) and (b)). The data show significant increases in the area of the third ventricle as a function of being fed cholesterol. Data are modified from [72].

would the effects of cholesterol on acquisition of the rabbit NMR hold true for other forms of learning, and second, what were the effects of cholesterol on memory? Although the majority of the research into learning in rabbits is based on changes in skeletal responses, particularly the closure of the upper eyelid and the sweep of the nictitating membrane [35], a significant body of research has examined an autonomic response—deceleration of heart rate [73–80]. The adult rabbit typically shows an unconditioned increase in heart rate (HR) to electrical stimulation and an unconditioned decrease in HR to tones. The unconditioned HR acceleration to electrodermal stimulation is an acute response to a stressful stimulus and has been used as a measure of the animal's "defense reaction" [80]. The unconditioned deceleration in HR to a tone is an orienting response that can be habituated by tone-alone presentations. Heart rate classical conditioning occurs when rabbits that receive pairings of tone and shock show a conditioned deceleration in HR to the tone relative to rabbits that show little or no change in HR when they receive explicitly unpaired tone and shock presentations [73, 81, 82]. This type of autonomic conditioning usually occurs within a relatively few pairings that consist of tones that are separated from shock by a second or more [83]. Using a trace conditioning paradigm, we found that cholesterol facilitated rabbit HR conditioning and that the unconditioned HR response to shock was also modified by conditioning [49]. The significant facilitation of HR conditioning suggests that the effects of cholesterol on learning were not specific to one form of conditioning involving a skeletal response but to an autonomic response as well. Importantly, a major anatomical locus for rabbit HR conditioning is the amygdala [75, 84–86] and we found that, in addition to the hippocampus and cortex, the level of beta amyloid staining in the amygdala was higher in cholesterol-fed rabbits than in controls [49].

Our findings of cholesterol-induced facilitated learning are consistent with experiments in a number of other animal models that have reported that modifying dietary cholesterol can improve learning [87]. For example, increasing cholesterol in mutant mice in which hippocampally dependent spatial learning is normally impaired improves performance in the Morris water maze [88, 89]. Feeding cholesterol to young, normal rats also improves performance in the Morris water maze [90, 91]. Feeding cholesterol to rats that are either deficient in cholesterol or have cholesterol synthesis blocked reverses problems with learning in the water maze and acquisition of eyeblink conditioning [92–95]. These animal data are also consistent with some human literature showing that higher cognitive functioning is correlated with high

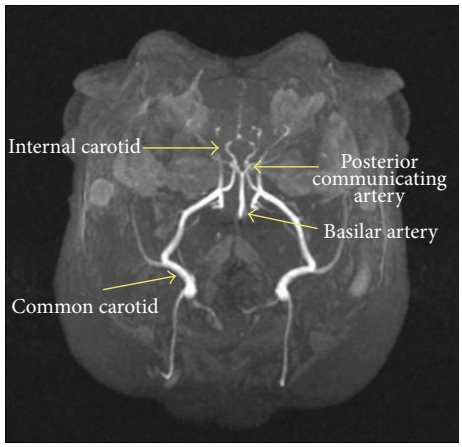

Figure 5: Blood vessels in the rabbit brain that were visualized by time-of-flight magnetic resonance angiography during our MRI studies and analysis of vessel diameters focused on the left and right common carotid arteries, the basilar artery, the left and right internal carotids arteries, and the left and right posterior communicating arteries.

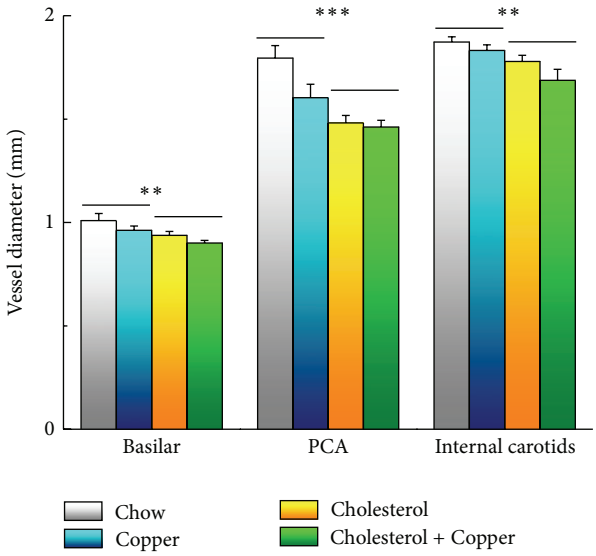

Figure 6: Mean (±SEM) vessel diameter of the left and right internal carotid arteries, basilar artery, and left and right posterior communicating arteries (PCA) for rabbits fed normal chow and given distilled drinking water (Chow), 2% cholesterol in their rabbit chow and given distilled drinking water (Cholesterol), fed normal chow and given 0.12 parts per million copper as copper sulfate in their distilled drinking water (Copper), or fed 2% cholesterol and given 0.12 parts per million copper in their distilled drinking water (Cholesterol + Copper). Data are modified from [72].

cholesterol [96, 97] and that cholesterol may protect against cognitive decline especially in the elderly [97–100].

8. The Effects of Cholesterol on Memory

The majority of research with humans suggests strongly that cholesterol is detrimental to memory. A significant number of studies show that elevated serum cholesterol is a risk factor for mild cognitive impairment [101–105] and dementia [106, 107] and that cholesterol levels are correlated with measures of intelligence [103, 108–111] except in the very elderly [99, 112]. Low HDL cholesterol has been correlated with deficits and declines in memory in midlife [113]. A study of cholesterol synthesis showed the level of the cholesterol precursors lanosterol and lathosterol are correlated with low memory performance as subjects age [114]. It is to this second question—the effects of cholesterol on memory—that we next turned our attention.

In an experiment by Darwish and colleagues, we trained rabbits to asymptotic levels of NMR tracing conditioning and then instituted an 8-week diet of 2% cholesterol or normal chow before assessing the memory of trace conditioning by presenting the tone alone over a period of days during extinction [115]. Rabbits fed normal chow showed response levels of about 60% at the beginning of extinction which was very consistent with a previous assessment of long-term memory for trace conditioning of the rabbit NMR after 8 weeks [116]. In contrast, cholesterol-fed rabbits showed significantly lower response levels of only 30%—a level that was not significantly higher than cholesterol-fed unpaired control rabbits [115]. We were able to replicate this finding with different concentrations of cholesterol that all showed lower levels of responding during extinction than a normal chow control group [117]. Importantly, neither of these experiments involved the addition of copper to the distilled drinking water suggesting that cholesterol by itself can degrade a previously acquired memory.

9. The Effects of Cholesterol and Copper on Both Learning and Memory

In a recent study, we combined our behavioral procedures and cholesterol feeding regimens into a single paradigm. We used discrimination reversal conditioning to assess the effects of a cholesterol diet and copper in the water on the memory of a previously acquired association and the ability of the rabbits to acquire a new and opposite association. In brief, rabbits were trained to discriminate between two tones of different frequency (1 kHz and 8 kHz), then placed on an 8-week cholesterol diet with or without 0.12 ppm copper in their drinking water, then tested for their memory of the original discrimination, and subsequently trained to reverse that discrimination [118–122]. The data showed that cholesterol and distilled water degraded the ability of rabbits to remember the original discrimination but facilitated their ability to learn the reversal of that discrimination. Interestingly, the addition of copper to the water of cholesterol-fed rabbits had the opposite effect on both phenomena—the rabbits were able to recall the original discrimination but were less able to learn the reversal of that discrimination. Importantly, a rabbit's ability to successfully reverse a discrimination is dependent on an intact, functioning hippocampus which allows the rabbit to inhibit responding to a previously paired stimulus [118, 120]. We found that cholesterol-fed rabbits were better able to inhibit responding than cholesterol-fed rabbits given copper

and have shown elsewhere that the membrane excitability of hippocampal neurons is increased by cholesterol feeding that is decreased by the addition of copper to the drinking water [123]. Membrane excitability has been shown to increase as a function of learning [124–128]. Taken together, these data show that cholesterol by itself and cholesterol supplemented by copper have opposite effects on behavior as well as on one of the underlying neural mechanisms associated with learning and memory.

10. Effect of Cholesterol and Copper on Beta Amyloid Accumulation and Learning and Memory in This Model

It is clear that feeding rabbits cholesterol increases the level of beta amyloid in the brain at the same time that it has significant systemic effects particularly in the liver and vasculature [87, 129–133]. Our research shows that against a backdrop of increased beta amyloid immunoreactivity in the cortex and hippocampus, there are replicable effects of feeding cholesterol on learning and memory. Given the essential role of the cortex and hippocampus in the acquisition and recall of trace conditioning in both eyelid and heart conditioning [26, 39, 55, 134–138], it is tempting to draw causal inferences from the accumulation of beta amyloid in those structures and the observed changes in learning and memory particularly given our findings of cholesterol-induced changes in the membrane properties of hippocampal neurons [123]. In fact, this recapitulates the inferences the field continues to make concerning beta amyloid and Alzheimer's disease. However, the recent very public failures of clinical trials designed to mitigate the effects of beta amyloid have given some researchers grounds to revisit other potential mechanisms that may play a part in the development of Alzheimer's disease including the role of vascular factors, inflammation, oxidative stress, and the role of trace metals including copper, to name a few. For example, there is strong evidence that human cognitive impairment is correlated with the extent of cholesterol-induced atherosclerosis both in peripheral arterial disease [139] and in carotid atherosclerosis [140]. There is also a growing awareness of the effects of inflammation on cognitive decline [141–144]. Similarly, there are a significant number of peripheral effects of cholesterol in the rabbit that may have effects on learning and memory including atherosclerosis [145–147], inflammation [5, 133, 148, 149], oxidative stress [150–152], and copper [153–155]. With the compromise of the rabbit's blood brain barrier that occurs with cholesterol feeding [5, 58, 156–158], these peripheral effects may very well also become central effects. Finally, beta amyloid is present in the brain from birth to death and, in normal concentrations, is critical for cell function, synaptic plasticity, and memory [159]. In other words, there are a number of complex effects of cholesterol, copper, and beta amyloid and it is probably a combination or interaction of several of these effects that can best explain their influence on learning and memory.

11. Summary

From our first published rabbit NMR study [32], the majority of experiments have shown that cholesterol facilitates learning and the addition of copper, which increases the level of beta amyloid immunoreactivity, reverses this facilitation, and, in at least two cases, makes it significantly worse than controls [46]. More recently, we have found that cholesterol degrades long-term memory both of simple acquisition as well as discrimination learning, and, in the latter case, copper returns responding to control levels. On the other hand, as noted above, the addition of copper to cholesterol tends to exacerbate the level of beta amyloid immunoreactivity but only slightly increases other indices of pathology including increases in ventricular volume and cerebrovascular diameter beyond those induced by cholesterol alone [61, 67]. Taken together, these results have raised a number of important questions including the nature of the effects of cholesterol on learning and memory, the potential mechanisms of these effects, and the role copper may have in modifying the effects of cholesterol and in elevating beta amyloid.

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