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Copper pathology in vulnerable brain regions in Parkinson's disease

Katherine M. Davies [a,b], Sylvain Bohic [c,d,e,**], Asunción Carmona [f,g], Richard Ortega [f,g], Veronica Cottam [a], Dominic J. Hare [h,i], John P.M. Finberg [j], Stefanie Reyes [a,b], Glenda M. Halliday [a,b], Julian F.B. Mercer [k], Kay L. Double [a,b,l,*]

[a] Neuroscience Research Australia, Sydney, Australia
[b] School of Medical Sciences, Faculty of Medicine, University of New South Wales, Sydney, Australia
[c] Inserm, U836, Team 6, Rayonnement Synchrotron et Recherche Médicales, Grenoble Institut des Neurosciences, Grenoble, France
[d] European Synchrotron Radiation Facility, BP220, Grenoble, France
[e] Université Joseph Fourier 1, Grenoble Institut des Neurosciences, Grenoble, France
[f] University of Bordeaux, Centre d'Etudes Nucléaires de Bordeaux Gradignan, Unité Mixte de Recherche 5797, Gradignan, France
[g] Centre National de la Recherche Scientifique, IN2P3, CENBG, UMR 5797, Gradignan, France
[h] Elemental Bio-imaging Facility, University of Technology, Sydney, Australia
[i] The Florey Institute of Neuroscience and Mental Health, University of Melbourne, Parkville, Australia
[j] Faculty of Medicine, Technion, Haifa, Israel
[k] Centre for Cellular and Molecular Biology, Deakin University, Melbourne, Australia
[l] Discipline of Biomedical Science, School of Medical Sciences, Sydney Medical School, The University of Sydney, Australia

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ABSTRACT

Synchrotron-based x-ray fluorescence microscopy, immunofluorescence, and Western blotting were used to investigate changes in copper (Cu) and Cu-associated pathways in the vulnerable substantia nigra (SN) and locus coeruleus (LC) and in nondegenerating brain regions in cases of Parkinson's disease (PD) and appropriate healthy and disease controls. In PD and incidental Lewy body disease, levels of Cu and Cu transporter protein 1, were significantly reduced in surviving neurons in the SN and LC. Specific activity of the cuproprotein superoxide dismutase 1 was unchanged in the SN in PD but was enhanced in the parkinsonian anterior cingulate cortex, a region with α-synuclein pathology, normal Cu, and limited cell loss. These data suggest that regions affected by α-synuclein pathology may display enhanced vulnerability and cell loss if Cu-dependent protective mechanisms are compromised. Additional investigation of copper pathology in PD may identify novel targets for the development of protective therapies for this disorder.

1. Introduction

Neurodegenerative cascades in Parkinson's disease (PD) involve protein aggregation and oxidative stress, although the triggers for these events are unknown. Changes in biometals have long been suspected to play a role in these cascades. Copper (Cu) is an important biometal in the brain, as exemplified by Menkes and Wilson diseases, serious neurological disorders of Cu dyshomeostasis (Schaefer and Gitlin, 1999; de Bie et al., 2007). A significant decrease in total tissue Cu in the degenerating substantia nigra (SN) in PD has been consistently reported over a number of decades (Ayton et al., 2012; Dexter et al., 1989; Loeffler et al., 1996; Popescu et al., 2009; Uitti et al., 1989), and recent evidence suggests that peripheral Cu metabolism is altered in PD (Larner et al., 2013). The complexing of Cu with the PD-associated protein α-synuclein increases aggregation and toxicity of this protein (Rose et al., 2011; Wang et al., 2010), possibly via stimulation of free radical production (Meloni and Vasak, 2011). However, Cu is also a critical cofactor in a range of cuproproteins, including the key protective cellular antioxidant superoxide dismutase 1 (SOD1) (McCord and Fridovich, 1969). Studies in model systems demonstrate that Cu depletion is associated with reduced activity of SOD1

K.M.D. and S.B. contributed equally to this work.

* Corresponding author at: Discipline of Biomedical Science, School of Medical Sciences, Sydney Medical School, Cumberland Campus C42, 75 East St. (PO Box 170), Lidcombe, NSW 2141, Australia. Tel.: +61 2 9351 9357; fax: +61 2 9351 9520.
** Alternate corresponding author at: Inserm U-836, Grenoble Institute of Neuroscience, Grenoble Institute of Neuroscience, C42 et Recherches Médicales, ESRF, Rue Jules Horowitz, BP220, F-38043 Grenoble Cedex, France. Tel.: + 33 476 882 852; fax: +33 476 882 852.
E-mail addresses: kay.double@sydney.edu.au (K.L. Double), bohic@esrf.fr (S. Bohic).

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and a concomitant increase in free radical production, which can be normalized by Cu supplementation (Lombardo et al., 2003; Prohaska, 1983). SOD1 activity is reduced in the plasma of PD patients (Torsdottir et al., 2006), and studies in animal models of PD suggest that overexpression of SOD1 increases neuronal survival (Battaglia et al., 2002; Botella et al., 2008; Tripanichkul et al., 2007). These data suggest that a reduction in brain Cu in PD may reduce SOD1-mediated antioxidant defense and contribute to neurodegenerative cascades. In this work, we investigated changes in neuronal Cu levels and transport pathways to determine whether changes in Cu are associated with a reduced antioxidant capacity in regions of neurodegeneration in PD.

2. Methods

2.1. Brain tissue samples

Brain tissue samples were requested and received from the New South Wales Tissue Resource Centre at the University of Sydney and the Sydney Brain Bank at Neuroscience Research Australia. The brain tissue samples were from patients with idiopathic PD and incidental Lewy body disease (ILBD), identified according to the diagnostic criteria of Dickson et al. (2009), and from Alzheimer's disease (AD) and age-matched control subjects, identified according to the diagnostic criteria of Montine et al. (2012) (Supplementary Tables 1–3). All cases of PD had received anti-parkinsonian medications before death, but none of the ILBD, AD, or control cases had received dopamine-replacement therapies. Cases with other neurologic or neuropathologic conditions were excluded, and consent to use autopsy material for research purposes was obtained for all cases.

Brain regions investigated included the SN and locus coeruleus (LC), the brain regions most vulnerable to neuronal loss and Lewy body pathology in PD; the anterior cingulate cortex (ACC), which is affected by Lewy body pathology later in the course of PD and has limited cell loss; and the occipital cortex (OCx), a nondegenerating region in PD (Braak et al., 2003; Harding et al., 2002). Samples were collected within 48 hours of death, dissected using polytetrafluoroethylene-coated blades (ProSciTech, Queensland, Australia), and the fresh tissue samples frozen immediately in liquid nitrogen, then stored at −80 °C before analysis. Fixed tissue samples were stored in formalin before cryomicrotome sectioning. Because of limited tissue availability, not every brain region was used in every experiment.

2.2. Measurement of neuronal and regional Cu levels

Total Cu levels within cells are the sum of different pools of Cu: free Cu and Cu bound to proteins. In the brain, the majority of Cu is not free but is bound to a range of molecules, including Cu transport proteins and other molecules, such as neuromelanin (NM) (Bohic et al., 2008), an intracellular pigment found in vulnerable regions of the PD brain (Hirsch et al., 1988). Total Cu levels were quantified within single neurons, as well as regions in thin sections (20 μm) cut from fixed or frozen tissue samples using synchrotron radiation x-ray fluorescence microscopy (SRXFM) and particle induced x-ray emission (PIXE) microscopy. Experiments were conducted using the microprobes end station at ID22 beamline of the European Synchrotron Radiation Facility and at the Diamond Light Source I18 beamline, as previously described (Antharam et al., 2012; Bohic et al., 2008). Micro-PIXE and micro-backscattering spectrometry (BS) analyses were performed simultaneously using the AIFIRA (Applications Interdisciplinaires des Faisceaux d'Ions en Région Aquitaine) facility to enable quantitative chemical analysis of trace elements in cells and tissues (Carmona et al., 2008). For each case (Supplementary Table 1), 2 fixed tissue sections of the SN, LC, and OCx were investigated: 1 using SRXFM to raster-scanned individual pigmented neurons (6–10 per sections/case) with a step size of 1 μm; the other using PIXE microprobes to raster-scan over a 683 × 683 μm area, corresponding to the larger scan size available for incident protons of 3.0 MeV energy. In the SN and LC, only neurons exhibiting normal pigmented morphology were chosen to avoid measurement of extraneuronal NM pigment. Elemental concentrations derived from single neurons analyzed in each section were averaged, and only 1 value from each patient was used in the analysis. Results from fixed tissue were confirmed in the SN using the same methods on fresh frozen tissue sections from PD and control cases (Supplementary Table 2).

For quantification of whole tissue Cu content, samples of fresh frozen brain (35–60 mg) from the SN, ACC, and OCx were subjected to closed-vessel microwave digestion (Milestone MLS1200) in 4 mL of concentrated nitric acid and 1 mL 35% hydrogen peroxide (both Seastar Chemicals, Sidney, Canada). Digests were diluted w/w to c.a. 50 g with 1% nitric acid, and total metal concentrations were determined using an Agilent Technologies 7500cs inductively coupled plasma mass spectrometer (Forrest Hill, Victoria, Australia), as previously described (Davies et al., 2013). Specificity for Cu was confirmed by measuring both m/z 63 and 65. Cu levels in the SN (subsequently referred to as TH-associated Cu levels) were calculated relative to nigral tyrosine hydroxylase determined by Western blot as an index of dopamine neuron loss.

2.3. Preparation of brain tissue proteins for Western blotting and activity assays

Samples from fresh frozen brain (62–84 mg) from the SN, ACC, and OCx were homogenized in 17 volumes of buffer solution (10 mM Tris-HCl, 0.25 M sucrose and 1 mM ethylenediaminetetraacetic acid [EDTA], pH 7.4) using a handheld electric homogenizer with a polycarbonate probe (OmniTH, Kelly Scientific, Sydney, NSW, Australia). For assaying SOD activity, homogenates of brain tissue proteins were centrifuged at 1500 *g* for 5 minutes at 4 °C. For Western blot analysis, 1% sodium dodecyl sulfate (SDS) and 1:50 dilution of protease inhibitor cocktail (Sigma, St Louis, MO) was added to homogenates of brain tissue proteins before being centrifuged at 10,000 *g* for 15 minutes at 4 °C. Supernatants were collected and stored at −80 °C. Pierce BCA Protein Assay Kit (Thermo Scientific, Scoresby, Vic, Australia) was used to determine sample protein concentration.

2.4. Primary antibodies used for detection of Cu transport proteins using Western blotting and immunohistochemistry

Primary antibodies used are detailed in Supplementary Table 4. Specificity of each antibody was confirmed by including no primary antibodies and, for Atox1, by preadsorption of the Atox1 antibody with the antigen against which the primary antibody was raised (the antigen was not available for the other antibodies used). The ATPase antibodies used in this study have been well characterized in previous reports, and their specificity confirmed (Cater et al., 2007; Ke et al., 2006). Horseradish peroxidase–conjugated antibodies used for detection of proteins by Western blotting include mouse anti-goat immunoglobulin (Ig)G, goat anti-mouse IgG, and donkey anti-sheep/goat IgG (all Millipore, Bellerica, MA, USA). Fluorescent antibodies used for detection of proteins include donkey anti-goat Alexa Fluor 594 IgG (H+L), donkey anti-sheep Alexa Fluor 594 IgG (H+L), donkey anti-sheep Alexa Fluor 488 IgG (H+L), donkey anti-mouse Alexa Fluor 594 IgG (H+L), and donkey anti-rabbit Alexa Fluor 488 IgG (H+L), all purchased from Invitrogen (Eugene, OR, USA).