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Alpha-synuclein aggregates are excluded from calbindin-D28k-positive neurons in dementia with Lewy bodies and a unilateral rotenone mouse model

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ABSTRACT

α-Synuclein (α-syn) aggregates (Lewy bodies) in Dementia with Lewy Bodies (DLB) may be associated with disturbed calcium homeostasis and oxidative stress. We investigated the interplay between α-syn aggregation, expression of the calbindin-D28k (CB) neuronal calcium-buffering protein and oxidative stress, combining immunofluorescence double labelling and Western analysis, and examining DLB and normal human cases and a unilateral oxidative stress lesion model of α-syn disease (rotenone mouse). DLB cases showed a greater proportion of CB+ cells in affected brain regions compared to normal cases with Lewy bodies largely present in CB− neurons and virtually undetected in CB+ neurons. The unilateral rotenone-lesioned mouse model showed a greater proportion of CB+ cells and α-syn aggregates within the lesioned hemisphere than the control hemisphere, especially proximal to the lesion site, and α-syn inclusions occurred primarily in CB− cells and were almost completely absent in CB+ cells. Consistent with the immunofluorescence data, Western analysis showed the total CB level was 25% higher in lesioned compared to control hemisphere in aged animals that are more sensitive to lesion and 20% higher in aged compared to young mice in lesioned hemisphere, but not significantly different between young and aged in the control hemisphere. Taken together, the findings show α-syn aggregation is excluded from CB+ neurons, although the increased sensitivity of aged animals to lesion was not related to differential CB expression.

1. Introduction

Dementia with Lewy Bodies (DLB) is the most common non-Alzheimer's dementia, showing acetylcholine and dopamine depletion and associated disruption of perception, thinking and behavior. Degeneration of multiple brain systems leads also to atypical Parkinsonism characterized by rigidity of movement combined with cognitive/memory disorder and neuropsychiatric symptoms, including hallucinations and behavioural problems. Factors including oxidative stress, gene mutations and elevated calcium ion concentrations are known to impact on the formation of the intraneuronal protein lesions (Lewy bodies) characteristic of the disease, in which α-synuclein (α-syn) (14 kDa) is a key protein component, leading to its classification as an α-synucleinopathy (Breydo et al., 2012; Cookson, 2009; Jellinger, 2009a; Marques and Outeiro, 2012; Rcom-H'cheo-Gauthier et al., 2014). Unlike in Parkinson's disease (PD), where pathology occurs primarily in the substantia nigra (sn), Lewy body pathology in DLB is widespread throughout the brain, including cortical areas (Jellinger, 2009b; Jenner et al., 2013). Although α-synucleinopathy is predominantly idiopathic, to date, six mutations in PARK1/4, the α-syn gene, have been identified with amino acid substitutions, A30P (Kruger et al., 1998), A53T (Polymeropoulos et al., 1997), E46K (Zarranz et al., 2004), H50Q (Proukakis et al., 2013), G51D (Lesage et al., 2013) and A53E (Pasanen et al., 2014), most linked to PD, but E46K having both PD and DLB variants.

Recent studies have shown that a transient increase in the intracellular free Ca^{2+} concentration induced by chemical treatments caused a significant increase in the proportion of cells bearing microscopically-visible α-syn aggregates (Nath et al., 2011). It was also demonstrated that chelation of intracellular free Ca^{2+} could block α-syn aggregate induction, implicating the direct involvement of raised intracellular free Ca^{2+}. Moreover, *in vitro* protein studies showed that direct binding of Ca^{2+} ion to α-syn promoted rapid oligomer formation. Furthermore, Follett et al. demonstrated that potassium depolarization of the plasma membrane in HEK293T and SH-SY5Y human cell lines resulted in raised intracellular free Ca^{2+} and caused α-syn aggregate formation, with both raised free Ca^{2+} and α-syn aggregation blocked by chelation treatment (Follett et al., 2013).

Ca^{2+} regulation is vital for cell survival and function and there are specific proteins responsible for Ca^{2+} buffering in neurons, including

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calbindin-D28K (CB), calretinin and parvalbumin. Yamada et al. found that dopaminergic neurons of the sn that were high in CB were preferentially spared in PD patients, indicating that increased free Ca^{2+} may be a major factor in the pathogenesis of α-synucleinopathies (Yamada et al., 1990). Bu et al. found decreases in both calretinin and CB in aged compared to young cortical neurons, put no difference in parvalbumin positive neurons (Bu et al., 2003). Moreover, German et al. found in both MPTP monkey or C57BL mouse models that CB+ neurons were spared whilst neurons in CB− regions were lost (German et al., 1992). In other studies, calretinin expressing dopaminergic neurons of the sn were more protected against 6-hydroxydopamine (Kim et al., 2000; Tsuboi et al., 2000). Ca^{2+} blockade was performed by Chan et al. using brain slices prepared from a MPTP mouse model, who found that by blocking L-type $Ca_v1.3$ Ca^{2+} channels with isradipine, they could recover dopaminergic neural activity (Chan et al., 2007).

There is evidence that oxidative stress is increased in normal aged brain however the level of oxidative stress is greatly increased in patients with neurodegenerative diseases (Sims-Robinson et al., 2013). Moreover, Kume et al. found urinary 8-OHdG levels were significantly higher in DLB cases compared to controls, indicating systemically increased oxidative stress (Kume et al., 2012). The major contribution to oxidative stress in ageing primates originates from mitochondrial complexes I and III of the electron transport chain leading to greater mitochondrial DNA damage compared to nuclear DNA damage (Castro Mdel et al., 2012). Indeed, widespread mtDNA damage occurs at early stages of Lewy body disease (Lin et al., 2012). Oxidative stress characterized by α-syn lipoxidation precedes the formation of α-syn aggregates and the development of neocortical Lewy body pathology in DLB (Dalfó and Ferrer, 2008). Furthermore, Quilty et al. showed that when mouse primary neocortical cells were incubated in the absence of antioxidants, mild oxidative stress caused raised α-syn accumulation in a subset of neurons (Quilty et al., 2006). Indeed, recent studies have found the oxidized form of the endogenous oxidative stress sensor, DJ-1, progressively increased in the later stages of PD and more highly oxidized forms were likely present in DLB (Saito et al., 2014). Furthermore, Surmeier et al. have shown that calcium influx can interact with α-syn to mediate increased oxidative stress (Dryanovski et al., 2013) and Goodwin et al. demonstrated a synergistic effect of combined oxidative stress and raised intracellular free calcium on α-syn aggregation (Goodwin et al., 2013). In animal models, the complex I inhibitor, rotenone, results in oxidative stress and replicates behavioural, anatomical and biochemical characteristics of α-synucleinopathy (Betarbet et al., 2000; Vila et al., 2001; Sanders and Greenamyre, 2013). Unilateral stereotactic injection of rotenone into the medial forebrain bundle (MFB) at low dose resulted in widespread α-syn inclusion bodies, primarily in neurons, and permits pair-wise-comparison between brain hemispheres of protein expression between treated and untreated hemispheres, with a more severe phenotype apparent in aged over young adult animals (Weetman et al., 2013; Radford et al., 2015). Whereas acute, targeted rotenone injection, rather than systemic exposure, allowed for diffusion of rotenone whilst avoiding gross physical trauma resulting from the surgery and produced a gradient of α-syn and neuroinflammation pathology (Radford et al., 2015).

In the current study, we investigated the interplay between α-syn aggregation, expression of CB and oxidative stress by examining CB-positive neurons and α-syn inclusion bodies in DLB and normal cases and in the unilateral rotenone mouse model. DLB cases showed an increased proportion of CB+ neurons compared to normal cases, whilst Lewy bodies, that were widespread in DLB tissue, were not detected in CB+ neurons. In the mouse model, CB+ neurons were over-represented in the rotenone lesioned compared to control hemisphere, especially proximal to the lesion site, whilst α-syn aggregates were increased and occurred almost exclusively in CB− cells. Comparing aged (1.75 years-plus) animals with young (6–12 months) animals, both the number of CB+ cells and total CB level was greater in the lesioned tissue, especially of the aged animals, but not significantly different in unlesioned tissue between aged and young animals, indicating that decreased CB expression does not underlie the increased susceptibility to lesion observed for aged animals.

2. Materials and methods

2.1. Human tissue

Human brain tissue was acquired from the South Australian Brain Bank. Diagnosis of disease was conducted at autopsy by neuropathologist Professor Peter Blumbergs according to disease specific criteria. Autopsies were conducted 4–17 hour post-mortem where the brains were bisected into hemispheres with one hemisphere formalin fixed, whilst the other was stored at −80 °C. The formalin fixed tissue was then embedded in paraffin, sectioned (5 μm) and mounted on gelatin-coated glass slides. Six DLB (age at death: 70 ± 7, post-mortem interval, PMI 8 ± 3 h), and six age matched normal cases (age at death 73 ± 9; PMI 14 ± 8 h) were analyzed, six brain regions in each case.

2.2. Animals

Experiments conducted under the approval of the Griffith University Ethics Committee comprised of 17 aged (1.75 year-plus), 15 young adult (6–12 months) and two young (8 weeks) wild-type (WT) C57BL/6 mice. Mice were housed in standard cages, on a 12-hour light cycle with *ad libitum* access to food and water. Surgery involved stereotaxic injection of a rotenone solution into the medial forebrain bundle (MFB) which allows for the diffusion of rotenone into the *substantia nigra pars compacta*, as described (Weetman et al., 2013). A medial-sagittal incision was made, to locate the physiological markers of lambda and bregma, and these sutures were used to calibrate and zero the stereotaxic apparatus for positioning at the injection point as given by x (1.25 mm) and y (−0.94 mm) axis. A single burr hole was drilled gaining access to the right hemisphere and allowing for placement of a 5 μL Hamilton syringe connected to a dental 27G needle directly above the target point. The needle was then slowly advanced along the z axis 5.35 mm then retracted to 5.25 mm creating a cavity into which, the treatment could be injected. The toxin solution (0.25 mg/mL of rotenone in 1:1 DMSO:PEG) or vehicle only was injected over a period of 4 min into the brain at a rate of 0.5 μL/s which gives a final volume injected of 2 μL. The needle was slowly retracted over a period of 4 min to prevent rotenone from being drawn out of the treatment cavity.

Animals were sacrificed after two weeks *via* injection with Ketamine (320 μL) and Xylazine (80 μL), then mice were perfused transcardially with 0.5% sodium nitrite in 0.1 M phosphate buffered saline to eliminate blood and extraneous material followed by Zamboni's fixative.

2.3. Immunofluorescence

Human tissue sections were deparaffinized by xylene (2 washes for 10 min), then rehydrated in 100%, 95%, and 70% ethanol. Heat induced antigen retrieval was performed in 1 mM EDTA pH 8 solution at 100 °C. After allowing the slides to cool (<50 °C), non-specific binding sites were blocked by immersing the tissue in 20% normal horse serum (NHS) in TBS for 1 h then incubated with rabbit anti-calbindin-D28K (1:200; Swant) and mouse anti-α-synuclein (1:200; LB 509 invitrogen) primary antibodies and Alexafluor 488 and 568 (1:200; Life Technologies) secondary antibodies and imaged using an Olympus FV1000 confocal laser scanning microscope. Negative control slides incubated only with secondary antibodies were imaged to set microscope settings (laser voltage, electronic gain and offset) to appropriate levels. The 60× oil immersion lens was used for image acquisition. Neuronal α-syn staining was confirmed by use of the doublecortin (DCX) antibody marker (1:50; Abcam).