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ORIGINAL ARTICLE

Characterisation of localised pigment accumulation in brains of eastern grey kangaroos (*Macropus giganteus*) after clinical disease due to chronic Phalaris species toxicosis

C El-Hage,[a]* T Chen,[a] L Tatarczuch,[a] J Hufschmid,[a] LF Skerratt,[a] P Whiteley,[a] N Davis[b] and R Ploeg[a]

A progressive tremorgenic syndrome characterised by ataxia and head nodding is well documented in sheep and cattle affected by chronic Phalaris toxicosis (CPT), and is increasingly documented in the eastern grey kangaroo {(EGK), *Macropus giganteus*} in south-eastern Australia. It is characterised on gross necropsy by areas of acquired localised pigment deposits within the brain. This pigment was previously considered a storage disease, but more recently has been determined to be a metabolic breakdown product of tryptamine alkaloids within *Phalaris* species (spp) of introduced grasses. The study included 61 EGKs that were euthanased after a diagnosis of clinically advanced CPT, histopathological studies were performed on all cases and transmission electron microscopic studies on six brains. Histological examination of the brains from EGKs revealed brown pigmentation of neurons, particularly of large motor neurons, with accumulations of discrete granules in the cytoplasm that stained positive with stains used to identify melanin. This feature and the characteristic ultrastructural appearance of the pigment granules leaves little doubt that the pigment is primarily melanin in nature. Specifically, ultrastructural detail of the granules was consistent with neuromelanin present in the brains of higher order primates and humans and has been associated with susceptibility to neurodegenerative diseases in man including Parkinson's disease. Given greater urbanisation and reduced access to native pasture a greater understanding of pathogenesis of CPT is of major importance not only for kangaroo welfare but potentially as a model for neurodegenerative diseases in humans.

Keywords Ataxia; Kangaroos; Melanin; Neuromelanin; Phalaris; Toxicosis; Tryptamine

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A progressive tremorgenic syndrome characterised by ataxia and head nodding in livestock grazing *Phalaris* species (spp) has been well reported and widely termed "Phalaris Staggers" (PS) or chronic Phalaris toxicosis (CPT).[1–6] Documented in sheep and cattle worldwide, reports of CPT in eastern grey kangaroos {(EGKs), *Macropus giganteus*} have also been well published in south eastern Australia.[1,2,4,6–9] In all of these species, the pathogenesis is related to tryptamine alkaloids affecting upper motor neuron pathways in the central nervous system (CNS).[1,2,4,6–8] Although the condition is generally seasonal in temperate climates due to alkaloid levels increasing under certain growth conditions, it can be managed to an extent by prophylactic Cobalt treatment in livestock.[2,3,10] Such prophylactic measures are not as practical for nondomesticated free-grazing wildlife such as kangaroos.

Grossly obvious greyish-green localised discolouration throughout the brains of sheep, cattle and kangaroos is commonly reported in CPT affected animals, and such pigmentation is considered somewhat pathognomonic of the syndrome (Figure 2).[1,2,4,6–8] Until now, this pigment has not been specifically characterized, although it has been reported to likely be melanin, and more recently neuromelanin.[3,6] Our group has previously described the gross and histopathological CNS findings associated with CPT in EGKs. In short, pigment granules observed in neuronal cells stained positively to histochemical stains used to identify melanin.[1] In addition to histological evidence, Magnetic Resonance Imaging (MRI) postmortem studies of EGKs with CPT demonstrate areas of brain pigment consistent with melanin deposits in both T1 and T2 sequencing (increased and reduced respectively).[11]

We report and characterise the gross, histological and ultrastructural appearance of neuronal pigment accumulation in kangaroos afflicted by CPT.

Whist there are several syndromes associated with Phalaris toxicity, including lethal per acute toxicosis in grazing livestock and horses[7,12,13] the focus of this study is on CPT, a chronic neurological affliction of several grazing animal species.[10]

Materials and methods

Sample collection

The study included 61 eastern grey kangaroos (*Macropus giganteus*) observed to be grazing on or near pastures containing *Phalaris* spp. displaying neurological clinical signs consistent with CPT, including head and body tremors, ataxia and hypermetria.

All cases were examined by a veterinarian and were euthanased due to welfare considerations after clinical deterioration. Those kangaroos unable to be safely handled before intravenous injection were anaesthetized using a tranquilizer firearm (Pneudart G2 X caliber®). The anaesthetic protocol included a mixture of Xylazine (2 mg/kg) and Zoletil® (tiletamine & zolazepam at 1.25–2 mg/kg). Body weight was estimated via visual

*Corresponding author.
[a]Melbourne Veterinary School, The University of Melbourne, 250 Princess Highway, Werribee, Victoria 3030, Australia; cmeh@unimelb.edu.au
[b]Parks Victoria, Level 10, 535 Bourke St, Melbourne, Victoria 3000, Australia

assessment. Intravenous pentobarbitone sodium at a dose approximating 100 mg/kg I/V {Lethabarb® (Virbac Australia, 325 mg/mL solution[1,3])} was administered to achieve euthanasia. In addition, six kangaroos euthanased for reasons other than neurological disease were submitted for comprehensive postmortem examinations to confirm the absence of neuropathology, and tissues served as control samples.

Sample processing

Necropsies were performed by the Anatomic Pathology department of the Melbourne Veterinary School, University of Melbourne, Werribee Vic. Australia either immediately on receipt or chilled within 48 h of euthanasia. Major organ systems were examined grossly to rule out other comorbidities. The brain was removed and fixed in 10% formalin.

Histochemical staining

In addition to haematoxylin and eosin other specific staining methods applied include Periodic Acid-Schiff (PAS), Fontana-Masson, Schmorl's special stain, {Toluidine blue, Perl's Prussian blue} and immunohistochemical staining for Melan-A.[14]

Electron microscopy

Tissues were fixed with Karnovsky's fixative, and postfixed in 1% osmium tetroxide (ProSciTech, Australia). The specimens were dehydrated in acetone and embedded in Spurr's resin (ProSciTech, Australia). Semithin sections (1 μm) were stained with 1% methylene blue for light microscopy. Ultrathin sections were double stained with uranyl acetate and Reynold's lead citrate and examined with a Philips CM 10 transmission electron microscope.

Figure 1. Eastern Grey Kangaroo recumbent after ataxic episodes as a result of chronic Phalaris toxicosis (subsequently confirmed at necropsy).

Given logistical issues and financial constraints, four EGKs were submitted for electron microscopy and three diagnosed with CPT and 1 control animal.

Ethics

The collection of tissues of deceased wildlife for this study was conducted under the Flora and Fauna Permit number 10008033 and Wildlife Act 1975 Research Authorization Permit number 10010213 (Department Environment, Land, Water and Planning. Victoria).

Results

Clinical findings

Animals euthanased in this study all presented with similar clinical signs. Head tremor and ataxia were the most prominent findings. Clinical abnormalities were exacerbated when the affected animals were disturbed. The majority also had weak, flaccid ears and were less reactive to external stimuli compared with unaffected kangaroos. The affected animals' obtunded responses often assisted in tranquilization and subsequent capture of these animals. More advanced cases were mostly recumbent and, if able to rise, would do so only for short periods (refer Figure 1).

Gross findings

Multiple, focal to coalescing regions of discolouration ranging from green to greyish brown were noted, and these typically impacted particular regions of the brains of affected kangaroos (Figure 2). The accumulation of pigment was most prominent in the thalamic nuclei and brainstem, with smaller amounts visible in the cortex, cerebellum and dorsal horns of the spinal cord.

In many affected EGKs, the proximal and distal tubular epithelium of the renal cortex and medulla contained pigment that stained positively with Fontana-Masson and brown with HE. Similarly, many retinas examined showed moderate pigmentation of the ganglion cells staining similarly.

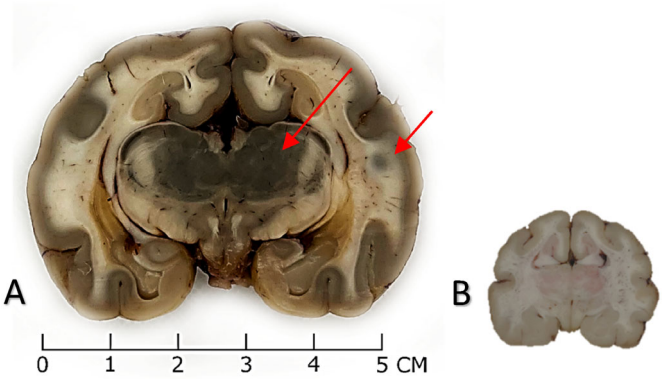

Figure 2. (A) Transverse section through the brain of an eastern grey kangaroo (*Macropus giganteus*) affected by chronic Phalaris toxicosis. Note dark focal pigmentation within the cerebral cortex and thalamic region (arrows). (B) Inset; Transverse section through the brain of a clinically unaffected (control) eastern grey kangaroo, note the lack of focal dark pigmentation. Both specimens preserved in 10% formalin solution.

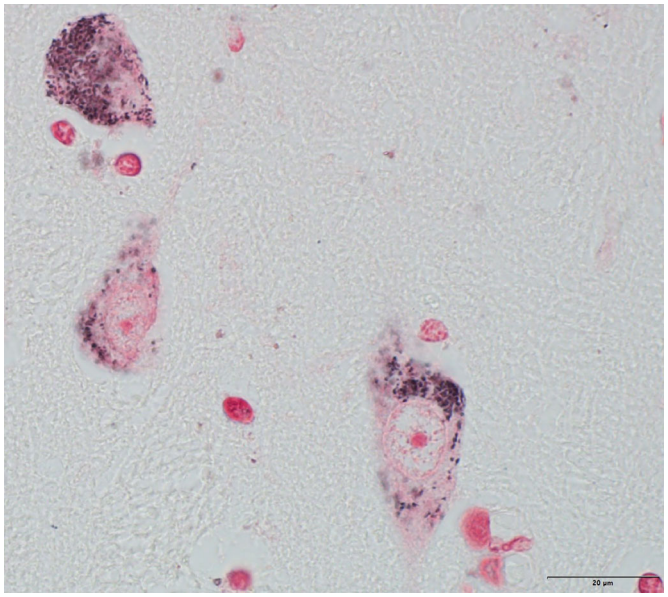

Figure 3. **Histological section from the brain of an eastern grey kangaroo suffering from Chronic Phalaris toxicosis revealing pigment positive for Fontana-Masson stain within the lateral geniculate body in the thalamus. Mag ×100 HP bar 20 µm.**

Histology

Histologic findings of affected animals revealed pigmented granules present in the cytoplasm of neuronal cell bodies, astrocytes and perivascular macrophages (refer Figure 3). Large motor neurons, in particular, were obvious, containing pigment appearing as granules in the cytoplasm, sometimes displacing the nucleus at the periphery (Refer Figure 3). No pigment granules were evident in the cytoplasm of comparable cells examined in control specimens. Although the presence of pigment granules was a consistent finding in CPT cases, there were varying amounts present between cases.

The pigment granules did not stain consistently with periodic acid-Schiff (PAS), although scattered intraneuronal granules did stain (brightly eosinophilic) with this stain.

Histochemical stains to identify melanin were applied to all affected species, the pigment was positive appearing black with Fontana-Masson (Figure 3) and blue with Schmorl's reaction. Other special stains that were negative when exposed to the pigment were toluidine blue (acidic stain positive for nucleic acids and polysaccharides) and Perl's Prussian blue (ferric iron stain). The pigment did not stain positive to the immunological type stain Melan A.

Electron microscopy

In affected neurons, electron dense granules were scattered randomly through the cytoplasm, occasionally in small clusters (demonstrated in Figures 4 and 5). They were variable in number, size and shape, which varied from rounded, oval, slightly irregular or lobulated (Figure 5).

The granules were surrounded by a limiting membrane, and three distinctive structural components were often noted within each: a granular material of medium electron density, a granular material of high electron density and electron lucent vacuoles surrounded by an electron dense edge located in or at the periphery of the granule (Figure 6). Some variation occurred in the relative amount and

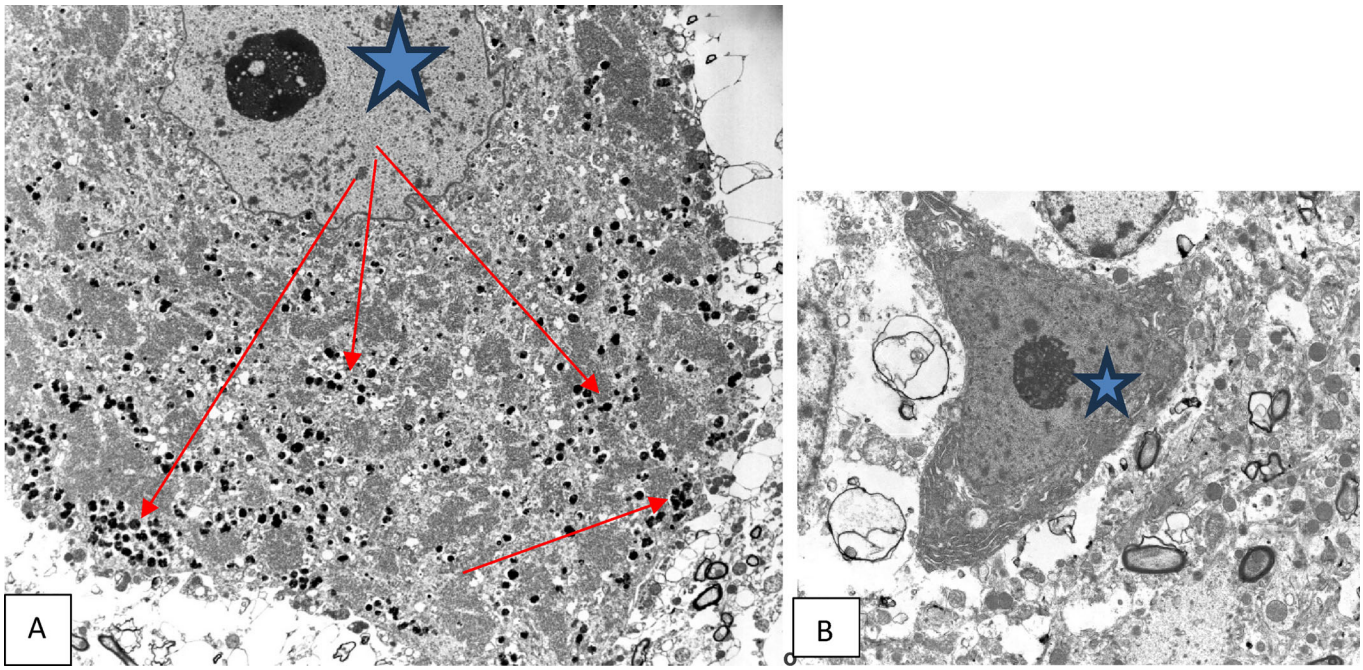

Figure 4. **(A) Low power electron micrographic image from the brain of an Eastern Grey Kangaroo demonstrating clinical signs of chronic Phalaris toxicosis; electron dense (black) granules (red arrows indicate several) are present scattered throughout the cytoplasm (note nucleus left of field marked with blue asterisk) (B) Control image from the brain of an Eastern Grey kangaroo without clinical signs of disease; no granules evident. Magnification ×2000; 1 mm = 0.5 µm.**

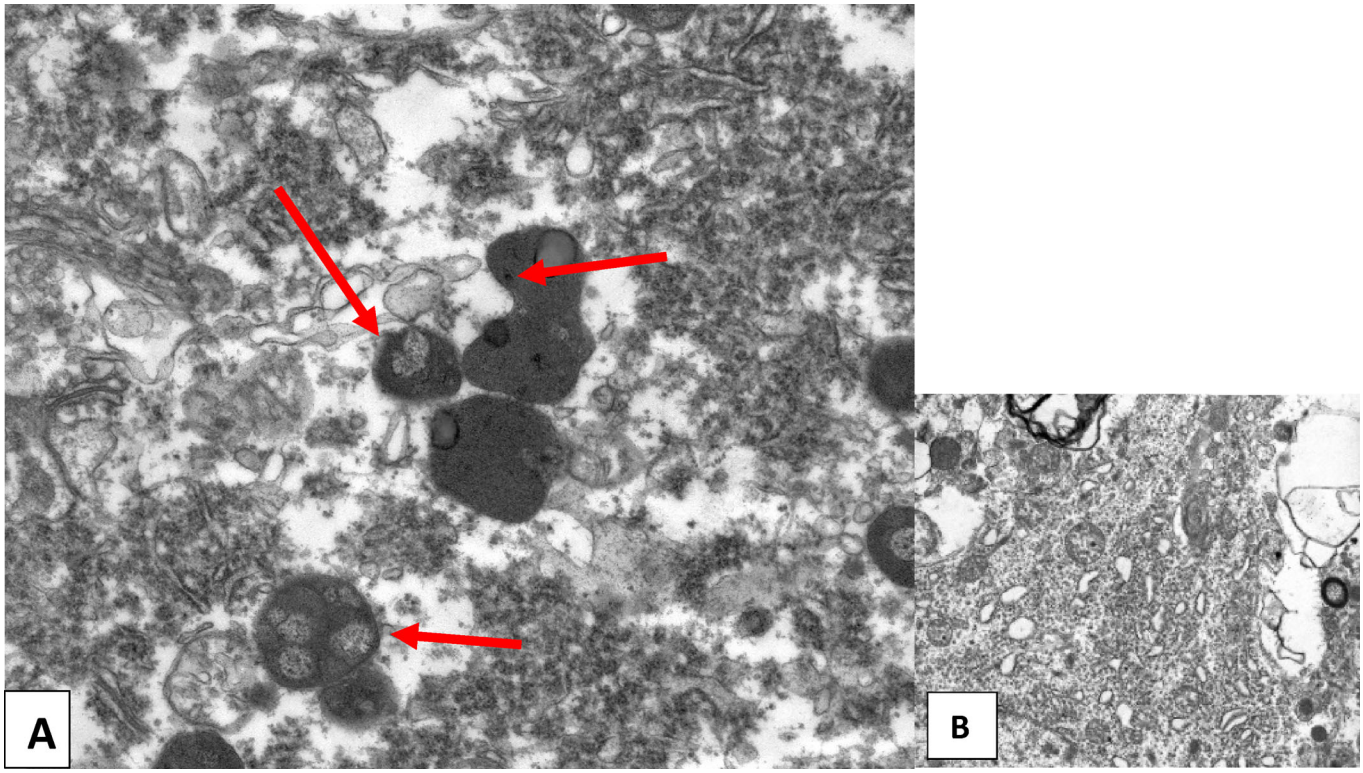

Figure 5. (A) Medium Power electron micrographic image from the brain of an Eastern Grey Kangaroo demonstrating clinical signs of chronic Phalaris toxicosis. Electron-dense granules are magnified; note variation in contents, number, size and shape, some indicated by red arrows. (B) Image from Eastern Grey Kangaroo brain without clinical signs of disease (control) note lack of characteristic Neuromelanin granules. Magnification: ×10,000, 1 mm = 0.10 µm.

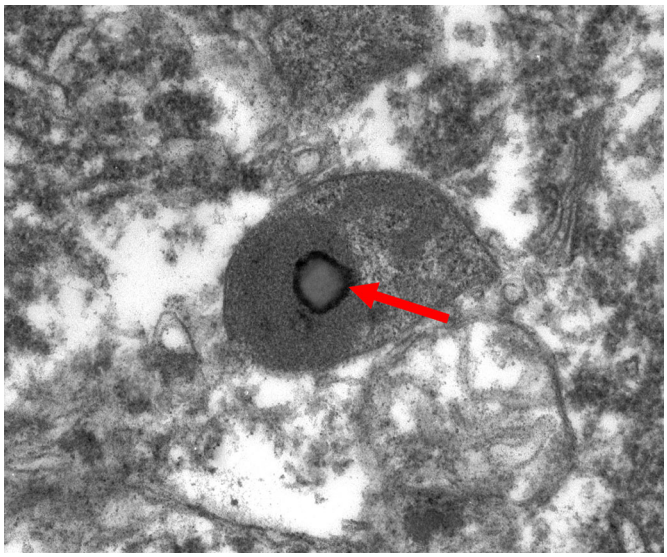

Figure 6. High power electron micrograph image of a typical granule (centre) within an affected neuron from the brain of an Eastern Grey Kangaroo demonstrating clinical signs of chronic Phalaris toxicosis. Note the ring granule is surrounded by a limiting membrane and contains material of varied electron density and a lipid droplet (red arrow) which are characteristic of neuromelanin granules. Magnification ×25,000, 1 mm = 0.04 µm.

distribution of these components. The amount of the dense material was generally scant or moderate, but occasionally so abundant that the medium dense granular material was almost completely obscured. Vacuoles also varied in number and size between granules. No such granules were evident in the brain of the control EGKs.

Discussion

Our investigation has revealed the pigment accumulating in CNS tissue of EGKs suffering from CPT to be consistent with melanin polymers as postulated by previous researchers.[3,7] Based upon clinical signs, history of exposure to Phalaris spp and histological examination of the CNS, we are confident that the melanin pigment in the brains of EGKs was the result of CPT after ingestion of high levels of Tryptamine in Phalaris spp. Furthermore, common neurological infectious agents and toxicoses of Kangaroos were able to be ruled out after careful assessment of history and environment, gross and histological examination of tissues, including protozoal, bacterial, viral and fungal organisms.

Transmission electron microscopic analysis of granules confirmed a previous report from several of the authors (LT, CME, PAW)[6] that they displayed ultrastructural characteristics consistent with those of neuromelanin granules.[15–18]

Although special histochemical staining revealed that the pigment has melanin properties, ultrastructural characterization of neuromelanin characteristics provides greater specificity. Although negative for the immunohistochemical melanin stain Melan A, it is possible that the nonmelanosomal formation in CPT cases may be poorly antigenic for these antibodies.[19]

Although melanin is not normally present in the brains of most nonprimate mammals, in humans, it is a feature in dopaminergic nuclei including the substantia nigra named due to the dark appearance of (neuro) melanin.[16,20,21]

Intraneuronal granules that contain neuromelanin differ structurally from those within melanosomes of cutaneous melanocytes and may contain unique structural components such as lipid.[22] Furthermore, despite ultrastructural characteristics consistent with melanin, no structures resembling melanosomes of cutaneous melanocytes were evident.[22–24]

The pathway of neuromelanin synthesis differs to that of peripheral melanin which results from the enzymatic conversion of tyrosine to melanin within melanosomes. Neuromelanin is found in higher order primate brains[16,20,21] and is considered to be derived from monoamine compounds.[17,20,21,24] The larger size and complexity of the polymer is believed to account for its formidable cytoprotective properties including Ultraviolet light absorption, antioxidant properties and metal ion scavenging.[21,23–25] Identification of melanin using Schmorl's and Fontana-Masson stains in fact relies on melanin's reductant properties with the capacity to reduce iron and silver respectively.[26]

Paradoxically, melanin is considered neuroprotective, and several well-described human neurodegenerative syndromes, including Parkinson's disease, demonstrate reduced levels of melanin in the brains of affected patients.[16,17,20]

Given the well-established cytoprotective properties of neuromelanin, EGKs affected with CPT, and subsequent cytoplasmic neuronal melanin, often sustain long-term neurological impairment. The relationship of the presence of neuronal pigment to the clinical signs remains unclear.[27] An association has been identified where neuromelanin-containing neurons were considered more susceptible to oxidative damage in patients suffering from Parkinson's disease (PD).[20,28] In addition, Tayebi et al. established that EGKs suffering from CPT demonstrated α-synuclein aggregates closely associated with neuromelanin deposits.[6] These factors may contribute to explaining much that remains unknown regarding the ongoing neurological derangements often noted in EGKs with CPT. Investigation of the pathophysiology associated with neuromelanin accumulation in EGKs after CPT may be of great benefit not just in terms of animal welfare, but potentially providing a valuable model for human diseases including PD.

This work advances our understanding of CPT, which remains a health and welfare issue not only in exposed kangaroos but also in grazing livestock. Given the complex aetiology and ongoing encroachment on wildlife grazing associated with urbanisation, facilitating greater insight into this condition is vital and may provide more tools to prevent and manage the disease and assist understanding of neurodegenerative conditions in humans.

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Conflicts of interest and sources of funding

The authors declare no conflicts of interest or sources of funding for the work presented here.

Data availability statement

The data that support the findings of this study are available from the corresponding author upon reasonable request.

References

1. Bacci B, Whiteley PL, Barrow M et al. Chronic phalaris toxicity in eastern grey kangaroos (*Macropus giganteus*). *Aust Vet J* 2014;92:504–508.
2. Binder EM, Blodgett DJ, Currin JF et al. *Phalaris arundinacea* (reed canarygrass) grass staggers in beef cattle. *J Vet Diagn Invest* 2010;22:802–805.
3. Bourke CA, Carrigan MJ, Dixon RJ. The pathogenesis of the nervous syndrome of *Phalaris aquatica* toxicity in sheep. *Aust Vet J* 1990;67:356–358.
4. East NE, Higgins RJ. Canary grass (*Phalaris* sp) toxicosis in sheep in California. *J Am Vet Med Assoc* 1988;192:667–669.
5. Hartley W, Kater JC. Diseases of the central nervous system of sheep. *Aust Vet J* 1965;41:107–111.
6. Tayebi M, El-Hage CM, Pinczowski P et al. Plant poisoning leads to alpha-synucleinopathy and neuromelanopathy in kangaroos. *Sci Rep* 2019;9:16546.
7. Gallagher CH, Koch JH, Hoffman H. Diseases of sheep due to ingestion of *Phalaris tuberosa*. *Aust Vet J* 1966;42:279–286.
8. Jolly RD, Hartley WJ. Storage diseases of domestic animals. *Aust Vet J* 1977;53:1–8.
9. Chen T, Hufschmid J, Whiteley P et al. Chronic phalaris toxicity in macropods is widespread and peaks in July in Victoria, Australia. *Aust Vet J* 2024;102(7):331–338. https://doi.org/10.1111/avj.13327.
10. Radostits OM, Gay CC, Hinchcliff KW et al. *Veterinary medicine*. 10th edition. London, Saunders Elsevier, 2007;1869–1871.
11. El-Hage CM. Pigmentary neurodegenerative disease in kangaroos exposed to pastures containing *Phalaris* spp. In: Ordidge R, editor. *Melbourne brain imaging symposium Ian potter auditorium*. Melbourne, Melbourne Brain Centre, The University of Melbourne, 2015.
12. Bourke CA, Colegate SM, Rendell D et al. Peracute ammonia toxicity: a consideration in the pathogenesis of Phalaris aquatica 'Polioencephalomalacia-like sudden death' poisoning of sheep and cattle. *Aust Vet J* 2005;83:168–171.
13. Bourke CA, Colegate SM, Slattery S et al. Suspected *Phalaris paradoxa* (paradoxa grass) poisoning in horses. *Aust Vet J* 2003;81:635–637.
14. Orchard GE. Pigments and minerals. In: Suvarna SK, Layton C, Bancroft JD, editors. *Bancroft's theory and practice of histological techniques*. Churchill Livingstone, London, 2013;239–270.
15. Duffy PE, Tennyson VM. Phase and electron microscopic observations of Lewy bodies and melanin granules in the substantia Nigra and locus Caeruleus in Parkinson's disease. *J Neuropathol Exp Neurol* 1965;24:398–414.
16. Hirosawa K. Electron microscopic studies on pigment granules in the substantia nigra and locus coeruleus of the Japanese monkey (*Macaca fuscata yakui*). *Z Zellforsch* 1968;88(2):187–203. https://doi.org/10.1007/BF00703907.
17. Zecca L, Bellei C, Costi P et al. New melanic pigments in the human brain that accumulate in aging and block environmental toxic metals. *Proc Natl Acad Sci USA* 2008;105:17567–17572.
18. Moses HL, Beaver DL, Ganote CE. Electron microscopy of the trigeminal ganglion. I. comparative ultrastructure. *Arch Pathol* 1965;79:541–556.
19. Chen YT. Stains and CD markers MelanA/MART1 Pathology Outlines. 2022.

20. Fedorow H, Tribl F, Halliday G et al. Neuromelanin in human dopamine neurons: comparison with peripheral melanins and relevance to Parkinson's disease. *Prog Neurobiol* 2005;75:109–124.
21. Zucca FA, Giaveri G, Gallorini M et al. The Neuromelanin of human substantia Nigra: physiological and pathogenic aspects. *Pigment Cell Res* 2004;17:610–617.
22. Slominski A, Tobin DJ, Shibahara S et al. Melanin pigmentation in mammalian skin and its hormonal regulation. *Physiol Rev* 2004;84:1155–1228.
23. Lin JY, Fisher DE. Melanocyte biology and skin pigmentation. *Nature* 2007;445:843–850.
24. Solano F. Melanins: skin pigments and much more—types, structural models, biological functions, and formation routes. *New J Sci* 2014;2014:1–28.
25. Lai C, Healy E. The Warthin–starry stain for detection of cutaneous melanin: more than a historical curiosity. *Exp Dermatol* 2016;25:763–764.
26. Jones ML. Histochemical stains for melanin: the classics. *J Histotechnol* 2003;26:233–237.
27. Summers BA. Degenerative diseases of the central nervous system. In: Summers BACJF, de Lahunta A, editors. *Veterinary neuropathology*. Mosby, St Louis, Missouri, USA, 1994;263.
28. Zucca FA, Capucciati A, Bellei C et al. Neuromelanins in brain aging and Parkinson's disease: synthesis, structure, neuroinflammatory, and neurodegenerative role. *IUBMB Life* 2023;75:55–65.

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