Cell. Mol. Life Sci. 65 (2008) 1669 – 1682
1420-682X/08/111669-14
DOI 10.1007/s00018-008-7581-9

Cellular and Molecular Life Sciences

Review

The comparative biology of neuromelanin and lipofuscin in the human brain

K. L. Double[a,*], V. N. Dedov[a], H. Fedorow[b], E. Kettle[a], G. M. Halliday[a], B. Garner[a] and U. T. Brunk[c]

[a] Prince of Wales Medical Research Institute, Barker Street, Randwick, NSW 2031 (Australia),
Fax: +61 2 9399 1105, e-mail: k.double@unsw.edu.au
[b] Discipline of Pharmacology, University of Sydney, Sydney, NSW 2006 (Australia)
[c] Department of Pharmacology, University of Linköping, SE-581 85 Linköping (Sweden)

Received 21 December 2007; received after revision 18 January 2008; accepted 25 January 2008
Online First 19 February 2008

Abstract. Neuromelanin and lipofuscin are two pigments produced within the human brain that, until recently, were considered inert cellular waste products of little interest to neuroscience. Recent research has increased our understanding of the nature and interactions of these pigments with their cellular environment and suggests that these pigments may, indeed, influence cellular function. The physical appearance and distribution of the pigments within the human brain differ, but both accumulate in the aging brain and the pigments share some structural features. Lipofuscin accumulation has been implicated in post-mitotic cell aging, while neuromelanin is suggested to function as an iron-regulatory molecule with possible protective functions within the cells which produce this pigment. This review presents comparative aspects of the biology of neuromelanin and lipofuscin, as well as a discussion of their hypothesized functions in brain and their possible roles in aging and neurodegenerative disease.

Keywords. Neuromelanin, lipofuscin, human, brain, structure, function, aging, Parkinson's disease.

Introduction

Melanin and lipofuscin are two pigments found in selected peripheral cells in the human body, as well as in the brain. The presence and physical appearance of these two substances within the brain were described more than a century ago, yet until relatively recently, both pigments were considered inert cellular waste products of little interest to the neuroscience community. Research into brain pigments has been stimulated over the past decade by increased understanding of the interaction of these pigments with their cellular environment and their possible role in aging and neurodegenerative diseases. While separate review articles are available on each of the pigments, a comparison of their nature and their relationships with each other has not yet been addressed. This review presents a comparison of these two pigments and their roles within the brain. Table 1 contains a comparative summary of these two pigments. For clarity, neurolipofuscin will be referred to simply as lipofuscin, while neuromelanin is abbreviated to NM. Unless specified, we use the term NM to refer to the NM of the human substantia nigra, as this is the only human NM intensively studied to date.

* Corresponding author.

Lipofuscin and NM pigment in the human brain

Appearance of the pigments

The most abundant pigment in the human brain is lipofuscin, a protein- and lipid-based pigment with broad distribution. Under light microscopy, lipofuscin appears yellow-brown or translucent and is defined by the histochemical characteristics of sudanophilia, argyrophilia, periodic acid-Schiff positivity and acid fastness [1]. The pigment is osmiophilic, and under the electron microscope, lipofuscin appears as a homogeneously dark, irregularly shaped mass, which is enclosed, often together with small lipid droplets, within a 100-nm-thick lysosomal membrane. Mature, large lipofuscin granula are mainly found in the perinuclear area, but may occasionally be present in the perikaryon and dendrites, axons and even presynaptic areas [2] (Fig. 1A). Lipofuscin-containing granula may reach a maximal size of 3–5 μm but, as lipofuscin accumulates over time, lipofuscin granula in early life are small and are contained within lysosomes of normal size [3]. The most characteristic feature of lipofuscin is autofluorescence, which is revealed by the fluorescence- or laser-scanning microscope (Ex_{max} is around 400 nm that gives a broad Em_{max} between 530 and 650 nm [1], Fig. 1A, B). This phenomenon is commonly believed to be the defining property of lipofuscin, although it is not specific for this pigment. Unlike lipofuscin, which is found in lysosomes, NM exists as dark-colored granules at the opposite end of the neuron to the nucleus, primarily within the cytoplasm (Fig. 1C), but may also extend into the cellular processes. The granules have an amorphic shape, ranging in size from 0.5 to 2.5 μm. In contrast to lipofuscin, NM can be readily seen in the unstained brain macroscopically and at the light microscope level as a dark-colored pigment (Fig. 1C). NM does not immediately fluoresce upon exposure to UV light (Fig. 1D) but does develop fluorescence following extended exposure to UV illumination, a property attributed to fluorescent intermediates of the oxidative degradation of the polymer [4]. The appearance of NM granules is unique among the melanins in that they are composed of three components of different electron density (Fig. 2C). The darkest of these is thought to comprise the melanin polymer itself. This is associated with a second component of intermediate electron density, while the third translucent lipid component is of interest, as it is not found in peripheral melanins [5, 6], nor other non-pigmented central nervous system cells [7]. The three components of NM granules are closely associated, with each granule in cross-section containing variable amounts of each component (Fig. 2C). Given the variable densities of the components making up the NM granule, it might be expected that a membrane enclosing this structure should consistently be at least partially visible in the mature granule. Despite a report of a double membrane enclosing model NM-like pigments produced in cell culture [8], there is no clear evidence to support either the presence or absence of a membrane surrounding NM granules in the human brain *in vivo,* possibly due to the difficulties of preserving membranes in the human brain post-mortem [9]. The question of whether NM granules are membrane bound or not is not just a matter of curiosity, because a pigment uncontained by a limiting membrane would be free to interact with cellular constituents in a manner unavailable to lipofuscin, which is fully enclosed within the lysosomal membrane.

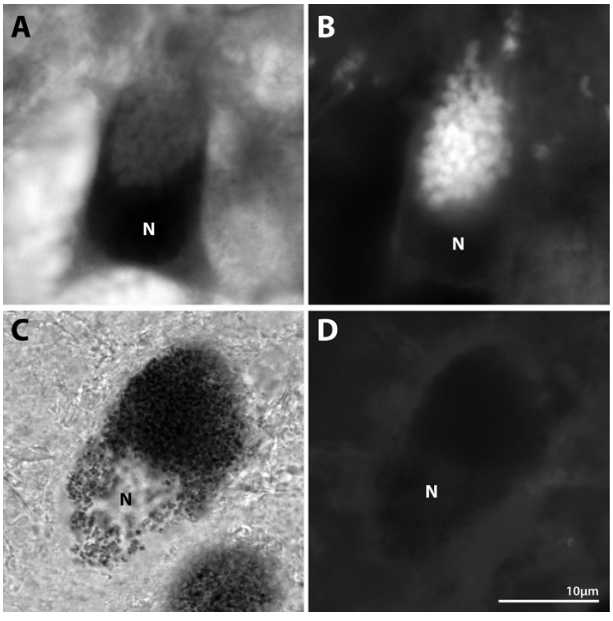

Figure 1. A lipofuscin-containing neuron from the human frontal cortex stained with cresyl violet. The lipofuscin pigment appears translucent viewed with light microscopy (*A*) and fluorescent under UV light (*B*). In contrast, NM pigment within a neuron of the human substantia nigra appears dark under light microscopy (*C*) and is non-fluorescent under UV light (*D*). N, nucleus.

Development of the pigments

While the formation of other melanin pigments in the human body is comparatively well understood, the mechanisms leading to neuromelaninogenesis is a matter of conjecture. In the periphery, melanin synthesis is regulated enzymatically, but as yet no enzyme has been identified which controls the development of NM [9, 10]. Perhaps as a result of the lack of a known enzymatic synthesis pathway, the notion that NM forms as a simple autoxidation product of dopamine is widely accepted but a body of evidence argues against

this idea. For example, the distribution of NM does not correspond with that of dopamine and it is not present in the dopaminergic neurons of the human substantia nigra at birth [11] or indeed at any age in most animal species [12]. Furthermore, the complex ultrastructure of the pigment argues against a production pathway as simple as autoxidation. We have thus argued that NM synthesis is likely to occur via some as yet undefined regulated process, possibly involving α-synuclein [13], which ultimately gives rise to the mature NM granule [9]. Recently, we identified large, elongated and complex aggregations of electron-dense NM pigment, in close proximity to the more typical triphasic NM pigment granules, in a number of neurons within human substantiae nigrae (Fig. 2D). These bodies have not been previously described and, as they consist only of the most electron-dense components of the mature granules, we speculate that these large bodies may be involved in the synthesis of the pigment polymer. The role of these bodies would thus parallel that played by melanosomes in which melanin is formed in the periphery.

The development of NM occurs over three phases in the human substantia nigra [11]. As in most other mammals and lower species, which do not contain NM [12], NM cannot be detected visually using light microscopy methods in neurons of the substantia nigra in the prenatal or infant brain [11]. The pigment first appears within the cytoplasm of the dopaminergic nigral neurons at around the age of 3 years as small, pale granules. The size and number of the granules then increases with age until adulthood (age 20 years) when NM fills, on average, 47 % of the cytoplasm [14]. After the age of 20 the proportion of the cell occupied by the pigment remains stable, but both biochemical measurements of solubilized NM [15] and direct counts of isolated pigment granule numbers using flow cytometry [V. N. Dedov, B. Garner, G. M. Halliday, D. Howells, M. Porritt and K. L. Double, unpublished data] indicate that the amount of pigment progressively increases with age, possibly accounting for the darkening of the pigment noted with aging [11]. These data suggest the term 'age pigment', usually applied to lipofuscin, could apply equally to NM.

Like NM, lipofuscin formation begins in early childhood and lipofuscin accumulates slowly over time. At 5 years of age, less than 5 % of cortical neurons, but no glial cells, contain lipofuscin [16], although it has been reported that neuronal lipofuscin can be detected as early as 3–4 months of age [1]. By the third decade of life, most cortical neurons contain lipofuscin and, like NM, the number of granules within individual cells becomes more abundant in the aging brain and it can also be found in glial cells [16]. A gradual increase in lipofuscin formation from early childhood has also been reported in the cells of the inferior olivary nucleus [17], and this pigment may occupy up to 75 % of the cell body of some neurons in the aged brain [18]. The increasing amount of lipofuscin seen with brain aging is accompanied by a maturation of the pigment, in that it becomes more heavily polymerized over time. Saccharide residues also appear within the pigment from the fifth decade of life [16]. Unlike NM, which is generally believed to form in the cytoplasm [although see the discussion of a possible NM-defining membrane above and ref. 19], lipofuscin is formed exclusively within lysosomes, the cellular compartment responsible for the turnover of the most long lived proteins and all cellular organelles [3]. The cell recycles many of these useful substances by autophagic degradation to simple molecules, such as amino acids, fatty acids and simple sugars, which may be reused after relocalization to the cytosol. Lysosomal degradation occurs via a variety of lysosomal enzymes, assisted by a mildly acidic environment (pH around 4.5) within the organelle. Lysosomal degradation of material over time is, however, thought to be incomplete [20]. The inability of the lysosome to degrade all incorporated materials results in the peroxidation of non-degraded materials by reactive oxygen molecules, such as the hydroxyl radical. These molecules form in high concentrations within the lysosome via Fenton chemistry in which hydrogen peroxide, which diffuses through the lysosomal membrane, interacts with ferrous iron released from incorporated metalloproteins. This pathway for lipofuscinosis is supported by data demonstrating the stimulation of lipofuscinosis under conditions of oxidative stress, high iron or decreased antioxidant buffer systems and attenuation of pigment formation by iron chelators and antioxidants [reviewed in ref. 20]. The primary constituents of lipofuscin are therefore oxidatively modified protein residues, which are bridged into polymer complexes by acids, and lipid residues such as reactive aldehydes originating from the breakdown of triglyerides, free fatty acids, cholesterol and phospholipids, while carbohydrates form only a minor structural component [3]. These components combine to form the pigment which grows in size via the addition of further precursor components and matures by undergoing intramolecular rearrangement reactions. It is probable that extensive free-radical-mediated cross-linking of proteins and other components in lipofuscin results in the characteristic non-degradable nature of the pigment [21]. The composition of lipofuscin is variable and dependent upon cell type, but in neurons, lipofuscin appears to be derived primarily from autophagocytosed mitochondria [20].

Another term found in the lipofuscin literature is 'ceroid.' This term is used to describe disease-associated lipofuscin, such as that which accumulates in the neuronal ceroid lipofuscinoses, as well as in other disorders [1]. The differences between lipofuscin in the normal brain and ceroid in the diseased brain are unclear. Some authors maintain the distinction is based purely on the circumstances in which the pigment is found [22, 23]. Other authors believe that, while there are many similarities between pigments termed lipofuscin and ceroid, there are sufficient differentiating features to warrant distinct terminology [1, 24].

Distribution of pigments in the brain

Lipofuscin is commonly considered to be a ubiquitous pigment within the brain, the amount of which is correlated with neuronal age. For example, in the inferior olivary nucleus, the anterior horns of the spinal cord [17], the substantia nigra [25] and frontal cortex [Dedov et al., unpublished data], lipofuscin has been shown to accumulate steadily throughout life, except, perhaps, during the very end of the life span when accumulation may accelerate. While it is true that lipofuscin is produced in nearly all cells in the human brain, some cells appear to be devoid of the pigment, even in the aged brain, suggesting that lipofuscin production is not an inevitable consequence of cellular metabolism. For example, von Buttlar-Brentano [26] reported that some hypothalamic nuclei, especially the supraoptic and paraventricular nuclei, do not exhibit lipofuscin accumulation with age. Some non-pyramidal cortical neurons in the aged brain are also reported to be free of lipofuscin [27]. Both the topographical and temporal patterns of lipofuscin production vary throughout the brain and deposition of lipofuscin in early life does not occur at the same time in all parts of the brain [7]. In the adult brain, lipofuscin is most abundant in larger neurons and is prominent in areas of the brain and spinal cord involved in initiating, monitoring and controlling movement, including the inferior olivary nuclei, the dentate nuclei, the globus pallidus and substantia nigra, and motor neurons in the anterior horns of the spinal cord [25]. The pigment may also be deposited in a differential manner in neurons within the same region. For example, pyramidal cells within the aged human cortex contain fine grains of lipofuscin scattered throughout the cell body, while non-pyramidal neurons contain either larger lipofuscin granules or, as mentioned, none at all [27]. Lipofuscin is also produced by glial cells but as these cells, unlike neurons, continue to divide, the absolute amount of lipofuscin is diluted via division, so that a high concentration of glial lipofuscin is suggested to result from the transfer of neuronal lipofuscin to glia, rather than a high rate of intraglial lipofuscin formation [2]. In contrast to the widespread distribution of lipofuscin within the human brain, NM is found in restricted catecholamine-containing nuclei and forms only in neurons. The pigment is produced in 95% of the dopaminergic neurons of the substantia nigra and is just as prevalent in the noradrenergic neurons of the locus coeruleus [28]. The substantia nigra forms part of the basal ganglia motor circuit and thus is important for normal movement control, while the locus coeruleus is involved in the initiation of activity states and sensory information processing. As the absolute number of neurons in the substantia nigra is almost ten times that of the locus coeruleus, the substantia nigra is considered the primary source of NM in the human brain. Interestingly only approximately 50% of the dopaminergic neurons of the ventral tegmental area, lying proximal to the substantia nigra, are pigmented, while other dopamine-containing nuclei, including, for example, the dopaminergic neurons of the olfactory bulb and some hypothalamic nuclei, do not produce this pigment. A number of NM-containing neurons are also found in the dorsal motor nucleus of the vagus nerve, hypothalamus and medulla oblongata [29, 30], in the cerebellum near the fourth ventricle [31] and in spinal and sympathetic ganglia [32]. Three times the number of tyrosine-hydroxylase-positive neurons are present in the medulla oblongata compared with the number of pigmented neurons, and the adrenaline-producing neurons within the medulla oblongata are not pigmented [33]. Furthermore, only 65% of noradrenaline neurons contain NM, suggesting that, like dopamine, the presence of noradrenaline or, especially, adrenaline in neurons is not inevitably linked with pigmentation [33]. The noradrenergic NM-containing neurons are thought to be important for autonomic control of cardiac and respiratory integration and the regulation of hypothalamic hormones [34].

NM is sometimes found within glial cells, but there is no evidence that this pigment is produced within these cells. As NM-containing glia occur more frequently in the aged brain, it is thought that the pigment is phagocytosed into glial cells for removal from the brain following its release from degenerating NM-pigmented neurons within the substantia nigra [35]. More abundant numbers of NM-containing glial cells can be seen in disorders where the NM-pigmented cells markedly degenerate, such as Parkinson's disease.

Fate of pigments in the brain

While the exact composition of lipofuscin varies from cell to cell, a feature shared by lipofuscin in all cells is

the fact that it cannot be degraded by lysosomal hydrolases. This property has been attributed to the presence of peptides in lipofuscin cross-linked by aldehydes into plastic-like structures impervious to biological degradation [36]. Lipofuscin is also unable to be exocytosed. Lysosomes propagate by division, so that lipofuscin, once formed within the lysosome, is divided between daughter organelles. The total cellular load of lipofuscin in postmitotic cells, such as neurons and cardiac myocytes, however, increases over time, as these cell types do not divide and therefore have no mechanism for removal or dilution of this pigment. It is for this reason that lipofuscin is often referred to as 'age pigment', because the total intracellular amount of lipofuscin reflects the chronological age of the cell, although as we have seen, this description could equally be applied to NM. While lipofuscin is, as one would expect, much more abundant in brain cells of aged humans compared with those of children or adolescents, the rate of lipofuscin accumulation in postmitotic cells is species specific and is regulated not only by age but also by mitochondrial activity. The highly active mitochondria of short-lived species, such as rodents, for example, produce more lipofuscin-stimulating metabolic products, such as superoxide and hydrogen peroxide, than those of longer-lived but similarly sized species, such as birds. For this reason, birds have a faster accumulation of lipofuscin than rodents [3]. Like lipofuscin, no mechanism has been identified by which NM is degraded within the cell in which it forms. Given the progressive intracellular increase of the pigment with age, and the fact that the isolated pigment is almost entirely insoluble *in vitro,* it has been assumed that NM, like lipofuscin, once formed *in vivo* is not metabolized. If this is true, however, it appears to be an anomaly that the cellular content of NM does not increase following treatment with the amino acid L-dopa, which may be prescribed at large doses over many years for Parkinson's disease. L-dopa is converted to dopamine in the pigmented neurons and thus should theoretically increase the precursor of NM synthesis within these cells. A further argument to support some system of NM turnover is the observation that the proteinaceous component of NM is altered in parkinsonian NM [37], suggesting that production or degradation of the pigment is altered. While oxidative degradation of peripheral melanins has been proposed [38], and NM degradation by highly oxidative conditions has been demonstrated *ex vivo* [39], it is unknown if the naturally high oxidative environment of the human substantia nigra results in a slow progressive breakdown of the pigment over time.

A number of pathological disorders, most notably Parkinson's disease, and accidental administration of the toxin 1-methyl-4-phenyl-1,2,5,6 tetrahydropyridine (MPTP), are characterized by the dramatic death of the NM-pigmented neurons and a subsequent loss of NM from this tissue [15, 40; Dedov et al., unpublished data]. The exact fate of NM after neuronal death is unclear, but the pigment can be seen within glial cells in these disorders [40, 41] and it is presumed that glia phagocytose extracellular NM released from dying neurons and remove it from the brain, via mechanisms not yet understood. A number of studies suggest that the NM-containing neurons of the substantia nigra are progressively lost with aging in the healthy brain [42–46], although other studies report the preservation of nigral neurons in the aged brain [47]. Extracellular NM is more commonly seen in the aged compared with the young adult brain [35] and is thought to be removed via glial mechanisms.

Chemical structure of lipofuscin and NM

NM is a heterologous organic polymer made up of a complex of dihydroxyindole and benzothiazine units [48, 49] believed to arise primarily from the products of dopamine metabolism. Comparisons with other types of melanins have revealed that NM shares some structural features with other natural melanins but is identical to none of them [reviewed in ref. 9]. Following hydrolysis with hydroiodic acid, we have demonstrated the presence of small amounts of dopa and reduced dopamine in NM, in addition to unidentified structural components we suggest derive from the oxidation of dopamine [50]. These data, in addition to the observation that dopamine oxidizes *in vitro* to form a dark-colored pigment called dopamine melanin (often employed as a model of the endogenous pigment [51]), support the hypothesis that NM forms *in vivo* from the oxidation of dopamine. Unlike the model pigment, however, 5–15% of the native pigment consists of a proteinaceous component and cytosolic proteins, as well as proteins normally associated with various cellular organelles such as lysosomes, mitochondria and endoplasmic reticulum [19, 50, 52]. A third of the NM granule by area consists of the lipidic bulbs depicted in Figure 2C [14]. Unusually high quantities of the isoprenoid dolichol, as well as small amounts of other lipid compounds, such as ubiquinone-10, α-tocopherol and cholesterol, are reported in the lipidic component of pigment isolated from the human brain [52–54]. The function of NM-associated lipids is unknown but they may play a role in the regulation of NM granule size, as removal of lipids from NM granules results in larger pigment aggregates [55].

The association of NM granules with significant amounts of lipid suggests a lysosomal and/or mitochon-

drial origin; indeed, lysosomal marker proteins have been reported in NM [19]. If lipofuscin were a precursor of NM, lipofuscin granules would be expected to occur commonly in NM-containing neurons. Several early workers, however, reported that melanin and lipofuscin are not colocalized within brain cells [56–57] and our recent data suggests that neurons in the substantia nigra contain either NM or lipofuscin, but not both of these pigments, as nigral levels of NM, but not lipofuscin, are significantly decreased in Parkinson's disease [Dedov et al., unpublished data]. Nevertheless, there are occasional references in the literature to lipofuscin being part of, or associated with, NM [for example, see ref. 58]. Perhaps this belief has arisen from the fact that NM granules contain lipidic bulbs which may be mistaken for lipofuscin (Fig. 2C); however these bulbs do not display autofluorescence, a characteristic feature of lipofuscin.

Another characteristic feature of NM is its ability to bind a number of elements and other molecules which may also contribute to its structure. Of most interest has been the association of NM with a variety of transition metals, including physiologically relevant ions such as iron, copper and zinc [59–68]. We have recently demonstrated that a range of biologically essential trace elements, such as iron and selenium, are associated with NM throughout the entire human lifespan, while others, such as manganese, are only found in NM in the aged brain [S. Bohic, K. Murphy, W. Paulus, P. Cloetens, M. Salomé, J. Susini and K. Double, unpublished data]. The pigment has also been shown to bind a number of exogenous substances, including toxic substances such as pesticides, neuroleptics and the 1-methyl-4-phenylpyridinium ion used to produce dopaminergic cell death in the substantia nigra in experimental models of Parkinson's disease [69–72].

It is clear from the above discussion that the structure of NM is as yet poorly defined. This also holds true for lipofuscin, and both pigments tend to be defined in terms of their physical appearance and the presence or absence of autofluorescence, rather than their chemical nature. Analyses of isolated lipofuscin have demonstrated the presence of a variety of compounds, primarily of protein (30–70%) and lipid (20–50%) origins [22]. The protein component of lipofuscin is poorly characterized and the amino acid content is variable but electron immunocytochemistry suggests that, like NM, lysosomal enzymes are associated with the pigment [73], consistent with an intralysosomal location for lipofuscin. The lipid component of lipofuscin contains triglycerides, free fatty acids, cholesterol, phospholipids, dolichol and phosphorylated dolichol [22]. It is unknown if lipofuscin in the brain contains the high ratio of dolichol to cholesterol demonstrated in NM [53], but lysosomes, the organelle in which lipofuscin forms, are rich in dolichol [74], and lipofuscin in the thyroid contains approximately equivalent amounts of dolichol and cholesterol [75]. A small amount (4–7%) of carbohydrate is also present [22, 23], although carbohydrates are not present in lipofuscin prior to the fifth decade of life [16]. The insoluble fraction of lipofuscin has been reported to exhibit a similar infrared spectrum to that of nigral NM, suggesting some shared structural features [76]. As discussed above and demonstrated in Figure 1B, a characteristic feature of lipofuscin is autofluorescence. The basis of this feature has not been determined, however, *in vitro* experiments implicate the involvement of Schiff bases, 1,4-dihydropyridines and 2-hydroxy-1,2-dihydropyrrol-3-ones resulting from lipid peroxidation reactions in lipofuscin fluorophores [22].

A feature common to both NM and lipofuscin is the association of metals with the pigments. Both pigments contain primarily iron while, like NM, copper, zinc, aluminum, calcium and manganese have also been identified within lipofuscin [23, 77]. The binding of iron to NM occurs at two distinct binding sites in a manner similar to that in which ferritin binds iron [78]. Given that ferritin is not present within pigmented neurons [79] and the naturally high iron content of these neurons, we have proposed that the iron-binding capabilities of NM may represent a physiological role in controlling iron homeostasis within NM-containing neurons [80]. The manner in which iron, and other metals, bind to lipofuscin has not yet been investigated in such detail, and the interaction of iron with lipofusin is suggested to impose a negative influence on the cell [22] rather than the protective role proposed for NM. Indeed the incorporation of metal-containing proteins, such as those from mitochondria, into lipofuscin is suggested to drive production of the pigment itself, via metal-stimulated lipid peroxidation and protein oxidation [81].

Like NM, a synthetic model of lipofuscin can be produced *in vitro* by UV cross-linking purified mouse liver mitochondria which demonstrates some of the spectral and physical properties of the endogenous pigment [23, 82]. The insoluble fraction of the pigment can be isolated from the human brain using methods widely used to isolate NM [Dedov et al, unpublished data]. Reports that NM can be isolated from brain regions not known to contain this pigment [83] perhaps result from the isolation of lipofuscin from these areas.

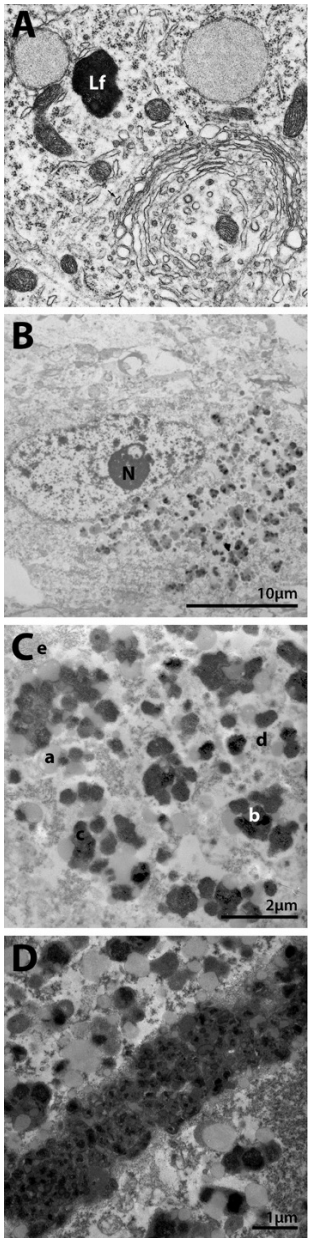

Figure 2. (*A*) Appearance of lipofuscin (Lf) as a dark, irregularly shaped mass surrounded by a 10-nm, lysosomal-type membrane within a cerebella neuron of a Rhesus monkey under the electron microscope. Magnification, × 27000 [reproduced from ref. 7 with permission]. (*B*) Appearance of NM pigment within the cytoplasm of a dopaminergic neuron in the human substantia nigra under the electron microscope. N, nucleolus; (*C*) At higher magnification it becomes apparent that NM granules have an irregular shape and size and exhibit a complex ultrastructure comprising three components of differing electron density: an electron-lucent (a) and an electron-dense (b) component and an electron intermediate component (c). d, and e, indicate ribosomes and endoplasmic reticulum, respectively. (*D*) An unusual feature of several substantiae nigrae examined were large bodies which appeared as coalescences of primarily electron-dense NM pigment. These bodies have not been previously described and their function is unknown.

Functional significance of the pigments

The physiological roles of lipofuscin and NM in the human brain are contentious. For many decades it was assumed that both pigments represented cellular waste products of little interest to neuroscientists. More recently, however, arguments have been presented that both pigments influence cell function and survival, resulting in an upsurge of interest in these overlooked materials. The incorporation of undigested cellular breakdown products into lipofuscin may benefit the cell, in that potentially damaging metabolites are inactivated by incorporation into the pigment [22, 23]. The majority of studies of this pigment have argued, however, that the accumulation of lipofuscin with aging is likely to be detrimental to the cell. Enhanced lipofuscin accumulation is suggested to be linked with increased oxidative stress, decreases in antioxidative defense systems, lysosomal iron overload and mitochondrial dysfunction [2, 22]. Experimental evidence of a link between lipofuscin accumulation and reduced cellular function has been reported in lipofuscin-loaded fibroblasts in which proteasome activity is impaired [84], possibly as a result of a lipofuscin-mediated reduction of proteasome recycling, as these are degraded by lysosomes [85]. Further evidence that progressive lipofuscin accumulation may have negative physiological effects and is associated with lysosomal dysfunction can be drawn from the neuronal ceroid lipofuscinoses. These are a group of rare, terminal degenerative neurological diseases, characterized pathologically by the buildup of lipofuscin, usually in early life, as the result of reduced lysosomal function [23]. In contrast, other reports suggest that accumulation of lipofuscin has no detrimental effect upon the surface area of rough endoplasmic reticulum and the formation of neurosecretory granules [86] or protein synthesis [87]. These studies utilized cellular systems in which, however, only modest amounts of lipofuscin were present, while neuronal lipofuscin may occupy up to 75 % of the perikaryon [18]. This suggests that modest quantities of lipofuscin may not have discernible effects upon the cell, but that a heavy lipofuscin load induced by disease or advanced age may compromise normal cellular functions. Indeed, one of the current authors (U.B.) attributes age-related decreases in the ability of the cell to maintain cellular functions to lipofuscin-associated changes as described below. Nevertheless, it has also been observed that some neurons which contain abundant lipofuscin, such as the neurons of the lateral geniculate nucleus, are relatively resistant to age-related loss [23], suggesting that even heavy accumulation of lipofuscin is not

inevitably detrimental to the cell and other factors may be required to trigger cellular dysfunction.

Like lipofuscin, NM was also considered for many years to be a simple cellular waste devoid of any physiological function. In hindsight this view appears a little naive given that other melanins in the body, for example those in the skin and the eye, are considered to play a functional role within the cells in which they appear. Peripheral melanins are believed to act as a buffer against oxidative stress [9], a role which would also be useful within catecholaminergic neurons which experience a high oxidative load produced by the enzymatic breakdown of catecholamines and via the autoxidation of these molecules. We [80] and others [88, 89] have suggested that NM may modify oxidative load by directly interacting with and inactivating free radical species by virtue of its catechol structure, analogous to suggestions for peripheral melanins (such as that for melanin in the eye [90]). Little experimental data are available to support or disprove this point, but we have shown that NM significantly reduces cell death *in vitro* in a highly oxidative environment, although the mechanism of this apparent protection is unclear [51]. NM has also been suggested to act as a trap for toxic catechol derivatives which are effectively inactivated by incorporation into the pigment polymer [91]. As mentioned previously, NM binds a number of biologically relevant metals and other elements, including a range of toxic cations [68]. One NM-associated metal of particular interest, because of its posited role in Parkinson's disease (discussed below), is iron. Two iron-binding sites have been identified on NM [78] and the pigment appears to bind iron in oxyhydroxy-clusters in the ferric form, much like the binding of iron to ferritin [78, 92], an iron-binding protein not found in pigmented neurons [79]. NM appears to be only partially saturated with iron *in vivo,* suggestive of a residual chelating ability [65]. Given that the primary iron-binding protein ferritin is not found within pigmented neurons [93], we [80] and others [59, 94–96] have suggested that NM functions as an endogenous iron-binding molecule to regulate iron homeostasis within pigmented neurons. In contrast, while lipofuscin also contains high concentrations of iron, the pigment is not thought to assume a functional role in modifying cellular levels of this metal. While there are some published data on the interaction of lipofuscin with metals [75] and the functional consequences of this [22], the relationship between lipofuscin and metals has not yet been as well studied as that in NM. By binding iron, and possibly other metals, in a manner in which they are no longer redox active, NM may thus attenuate free radical production within pigmented neurons [78]. In support of this hypothesis, we have shown that NM significantly reduces primary mesencephalic cell death following exposure to an iron-mediated oxidative environment [51]. The hypothesis that NM acts as a safe intracellular haven for toxic catechol metabolites and potentially toxic metal species underlines an emerging conceptual difference between these two pigments. The accumulation of lipofuscin is, in general, still considered to be a potentially negative event for the cell, while emerging data suggest NM may be considered a cellular protectant system in the normal brain.

The role of pigments in human brain aging and disease

Intracellular concentrations of both NM and lipofuscin progressively accumulate with age, yet it is commonly believed that increasing amounts of these pigments do not significantly influence cellular function. We have previously argued that the accumulation of lipofuscin in lysosomes over time results in a diversion of lysosomal enzymes into lipofuscin-loaded lysosomes in an attempt by the cell to degrade this non-degradable material. The resultant lack of lysosomal enzymes for autophagy then leads to reduced ability to recycle other cellular organelles, such as mitochondria, which continue to function beyond their optimal life span. A compromised mitochondria population subsequently results in a lower rate of ATP production and increased free radical production by these aged organelles. Furthermore, the release of the abundant iron from the aged intralysosomal compartment by free-radical-mediated membrane damage will also stimulate free radical production via Fenton chemistry, possibly leading to apoptotic cell death. This process has been termed the mitochondrial-lysosomal axis theory of aging [20]. The progressive accumulation of misfolded and aggregated proteins deleterious to neuronal survival is a mechanism accepted for normal aging and neurodegenerative diseases, many of which are characterized pathologically by an abundance of insoluble inclusions, such as Lewy bodies or amyloid plaques [97]. Indeed the possible reversal of this phenomenon has received considerable attention as a potential therapeutic approach for Alzheimer's disease [98]. There is now increasing interest in the idea that the removal or prevention of lipofuscin accumulation from the brain may slow age- or disease-related decline in human brain function [20, 23, 99, 100], although to date no mechanism to achieve this goal has been proposed. A direct relationship between lipofuscin accumulation and brain dysfunction is seen in the neuronal ceroid lipofuscinoses described above, but a role for lipofuscin accumulation has also been proposed in other

Table 1. Comparative characteristics of lipofuscin and neuromelanin in the human brain.

Characteristic		Lipofuscin	Neuromelanin
Appearance	At light microscope level	Yellow-brown or translucent under visible light [1] Autofluorescent under UV light [1]	Dark brown to black under visible light Non-fluorescent under UV light
	At electron microscopy level	Homogeneously dark, irregularly shaped granules (3–5 μm) Produced in lysosomes Lysosomal membrane bound Primarily perinuclear but not associated with the nucleus [3]	Irregularly shaped granules of varying electron density and size (0.5–2.5 μm) Cytoplasmic The presence or absence of a surrounding membrane is unclear [9] Present at opposing end of neuron to the nucleus
Distribution in human brain		Most neurons and glia	Selected catecholamine neurons only [9]
Temporal pattern of development		First appearance within first year of life [1] Progressively increases over lifespan	First appearance around 3 years of age [11] Progressively increases over lifespan
Chemical structure		Aldehyde-linked protein residues (30–70 %) and lipid (20–50 %) residues, some carbohydrates; contains functionally active, loosely bound metals, such as iron Derived from autophagocytosed mitochondria and other intracellular structures [3, 22]	Organic polymer of dopamine metabolic products [48–50], 5–15 % proteins [19, 50, 52] and one –third by mass lipids [52–54] Binds a range of functionally active ions, including iron, and exotoxins
Formation and degradation		Iron-catalyzed peroxidation of materials under degradation in lysosomes No enzymatic regulation mechanism identified Non-degraded *in vivo*	No enzymatic regulation mechanism identified [9] Non-degraded *in vivo*; released from dying neurons and removed from brain via immune pathways
Functional roles in health and disease		High lipofuscin load suggested to negatively influence cellular functions by hampering autophagy Hypothesized to underlie postmitotic cell aging [20]	May inactivate cellular metabolites and toxic cations, in particular iron Possible role as a free radical scavenger May function as an intracellular iron regulatory system [59, 94–96] Changes in neuromelanin may underlie cell vulnerability in Parkinson's disease

neurodegenerative diseases. The rate of lipofuscin formation in the frontal, but not superior temporal gyri, is increased in Alzheimer's disease [101], and large amounts of lipofuscin and lysosomal enzymes are seen in amyloid-containing Alzheimer neurons [102], suggesting that the formation of amyloid may involve lipofuscin-associated mechanisms. Interestingly, in contrast to the findings of increased lipofuscinogenesis in Alzheimer's disease, reduced concentrations of intraneuronal lipofuscin, attributed to reduced mitochondrial function, are reported in the degenerating inferior olivary nucleus in another dementia disorder, dementia with Lewy bodies [103]. NM has been discussed as a critical factor underlying neuronal vulnerability in Parkinson's disease for several decades [see, for example, refs 104, 105]. This hypothesis has been difficult to test empirically, as animal models of Parkinson's disease do not produce NM within dopaminergic substantia nigra neurons. A feature of the parkinsonian brain is the presence of unusually high amounts of extracellular NM released from the dying neurons, and studies using cell cultures incubated with NM report activation of microglia [106] and inhibition of the ubiqitin-proteasome system [107], although we found no effect of NM on indices of cell morphology or survival of primary rat mesencephalic cocultures [51]. In 1992, Gibb [108] noted that the ventral tier of the substantia nigra, in which cell loss is most marked in Parkinson's disease, contains less NM than the heavily pigmented and relatively preserved cells of the dorsal tier. This finding was interpreted as suggesting that NM may increase cell survival, an idea also proposed by Kastner and colleagues [109], who observed that very heavily melaninized neurons tend to be preserved in Parkinson's disease. Also of interest is the fact that Lewy bodies, the pathological inclusion body found in synucleinopathy disorders such as Parkinson's disease, form only in close physical association with either NM or lipofuscin [110, 111], suggestive of a link between these pigments and Lewy body formation. Our recent data using flow cytometry to quantify pigment granule number, however, demonstrate that only NM is lost in Parkinson's disease, with lipofuscin preserved in both the degenerating midbrain and in the frontal cortex in which cell loss is not seen [Dedov et al., unpublished data]. Given that lipofuscin is present in nearly every neuronal type in the human brain and that Parkinson's disease is characterized by a dramatic loss of pigmented nigral neurons (over 85 % nigral cell loss in advanced disease confirmed using histochemical methods [14]), the continuing

presence of lipofuscin in the midbrain in this disorder supports our observations that NM-containing neurons in the midbrain do not contain lipofuscin. Furthermore, these data link the pathogenesis of Parkinson's disease inextricably with NM-pigmented neurons. One possibility that has not yet been fully explored, due to the technical difficulties of doing so, is that NM is altered in the parkinsonian brain, and available data suggest that this may be the case. Pigmented neurons in the parkinsonian brain are reported to contain less NM than in the healthy brain [109, 112], while the optical density of the pigment is increased [14]. ^{13}C-NMR spectral analysis of NM isolated from the parkinsonian brain exhibits a decreased melanin signal, compared with NM from the normal brain, suggested to reflect a protease-resistant, lipoproteic material not seen in the healthy brain [112, 113]. Furthermore, pigment-associated cholesterol is reduced in NM in the parkinsonian brain, a change that appears to occur early in the disease process [14]. α-Synuclein, the synaptic protein which forms Lewy bodies in the parkinsonian brain, is cross-linked to NM isolated from the parkinsonian brain [37] and aggregates specifically on the vulnerable pigmented cells of the substantia nigra *in vivo* [14], suggesting the pigment plays a vital role in the neurodegenerative cascade in this disorder [14, 114]. Dysfunction of NM's proposed role as an endogenous iron regulator has also been suggested in Parkinson's disease. Iron levels are widely reported to be significantly increased within the parkinsonian substantia nigra [115–121], including at the level of single dopaminergic nigral neurons [122], yet it has also been reported that less iron is bound to NM in this disorder, compared with the healthy brain [123, 124]. Should the iron chelation ability of NM be reduced in Parkinson's disease, this may increase levels of intraneuronal free iron which are potentially damaging for the cell via the role of iron in stimulating free radical pathways.

Are lipofuscin and NM related or distinct pigments in the human brain?

Current concepts regarding NM and lipofuscin do not suggest any relationship between the pigments. It is interesting, however, that formation of these pigments in the human brain occurs at approximately the same age and that both increase with age. Furthermore, as indicated above, while lipofuscin is present in the substantia nigra in the aged brain, dopaminergic NM-pigmented neurons of this nucleus do not form visible quantities of lipofuscin [Dedov et al., unpublished data]. This interesting observation suggests that the presence of NM in dopaminergic nigral neurons is incompatible with the development of significant quantifies of lipofuscin. Indeed, a possibly analogous situation occurs within the retinal pigment epithelium cells, where melanin is suggested to protect these cells from the development of damaging lipofuscin accumulation [90]. The lack of lipofuscin in melanized brain cells may reflect a functionally important role for melanin in these brain cells that is worthy of further investigation. A possible explanation for the apparent lack of lipofuscin in NM-pigmented neurons is that the formation of oxidatively modified protein and lipid residues within the lysosome, which make up the primary substrates of lipofuscin, is significantly reduced in NM-pigmented neurons due to the proposed role of the pigment as a radical scavenger and/or as a chelator of transition metals described above. Arguing against this idea, however, is our observation that lipofuscin is also not visible in surviving pigmented neurons in neurodegenerative disorders such as Parkinson's disease where the substantia nigra is thought to exist in a state of heightened oxidative stress [125]. A second possibility is the incorporation of the main substrate for lipofuscin in the brain, damaged mitochondria, into developing NM either prior to, or following autophagocytosis of the mitochondria into lysosomes, thus preventing the cross-linking reactions underlying lipofuscin development. Mitochondrial and lysosomal proteins have been identified in NM isolated from the human brain [19], and NM and lipofuscin appear to share a subset of lipid species [22, 52–54]. As discussed above, however, visible mitochondrial and/or lysosomal features, such as membranes, have not been observed in NM at the electron microscope level in the human brain [9].

Conclusions

Interest in lipofuscin and NM has been stimulated by emerging evidence that the pigments are not purely cellular junk but have the capacity to influence intracellular events and may have important, albeit yet poorly understood, roles in human aging and disease. The current challenge is to complete our understanding of the biology of these pigments and their roles in health and disease. Therapeutic modulation of brain pigment pathways may provide novel approaches to slow or halt dysfunction within the aged or diseased human brain.

Acknowledgements. This work was funded by the National Health and Medical Research Council (NHMRC) of Australia. B.G., G.H. and K.D are Fellows of the NHMRC of Australia. Tissues were received from the Prince of Wales Medical Research Institute Tissue Resource Centre which is supported by the NHMRC and

the Prince of Wales Medical Research Institute. We thank H. Cartwright for assistance preparing the figures.

1 Porta, E. A. (2002) Pigments in aging: an overview. Ann. N.Y. Acad. Sci. 959, 57–65.
2 Riga, D., Riga, S., Halalau, F. and Schneider, F. (2006) Brain lipopigment accumulation on normal and pathological aging. Ann. N.Y. Acad. Sci. 1067, 158–163.
3 Terman, A. and Brunk, U. (2004) Lipofuscin. Internat. J. Biochem. Cell Biol. 36, 1400–1404.
4 Elleder, M. and Borovansky, J. (2001) Autofluorescence of melanins induced by ultraviolet radiation and near ultraviolet light: a histochemical and biochemical study. Histochem. J. 33, 273–281.
5 Duffy, P. E. and Tennyson, V. M. (1965) Phase and electron microscope observation of Lewy bodies and melanin granules in the substantia nigra and locus coeruleus in Parkinson's disease. J. Neuropath. Exp. Neurol. 24, 398–414.
6 Moses, H. L., Ganote, C. E., Beaver, D. L. and Schuffman, S. S. (1966) Light and electron microscopic studies of pigment in human and rhesus monkey substantia nigra and locus coeruleus. Anat. Rec. 155, 167–184.
7 Peters, A., Palay, S. L. and Webster, H. D. (1991) The Fine structure of the Nervous System, 3rd ed., Oxford University Press, New York.
8 Sulzer, D., Bogulavsky, J., Larsen, K. E., Behr, G., Karatekin, E., Kleinman, M. H., Turro, N., Krantz, D., Edwards, R. H, Greene, L.A. and Zecca, L. (2000) Neuromelanin biosynthesis is driven by excess cytosolic catecholamines not accumulated by synaptic vesicles. Proc. Natl. Acad. Sci. USA 24, 11869–11874.
9 Fedorow, H., Tribl, F., Halliday, G., Gerlach, M., Riederer, P. and Double, K. L. (2005) Neuromelanin in human dopamine neurons: comparison with peripheral melanins and relevance to Parkinson's disease. Prog. Neurobiol. 75, 109–124.
10 Tribl, F., Arzberger, T., Riederer, P. and Gerlach, M. (2007) Tyrosinase is not detected in human catecholaminergic neurons by immunohistochemistry and Western blot analysis. J. Neural Transm. Suppl. 72, 51–55.
11 Fedorow, H., Halliday, G. M., Rickert, C., Gerlach, M., Riederer, P. and Double, K. L. (2004) Evidence for specific phases in the development of human neuromelanin. Neurobiol. Aging 27, 506–512.
12 Barden, H. and Levine, S. (1983) Histochemical observations on rodent brain melanin. Brain Res. Bull. 10, 847–851.
13 Bisaglia, M., Mammi, S. and Bubacco, L. (2007) Kinetic and structural analysis of the early oxidation products of dopamine: analysis of the interactions with α-synuclein. J. Biol. Chem. 282, 15597–15605.
14 Halliday, G. M., Ophof, A., Broe, M., Jensen, P. H., Kettle, E., Fedorow, H., Cartwright, M., Griffiths, F. M., Shepherd, C. E. and Double, K. L. (2005) α-Synuclein redistributes to neuromelanin lipid in the substantia nigra early in Parkinson's disease. Brain 128, 2654–2664.
15 Zecca, L., Fariello, R., Riederer, P., Sulzer, D., Gatti, A. and Tampellini, D. (2002) The absolute concentration of nigral neuromelanin, assayed by a new sensitive method, increases throughout the life and is dramatically decreased in Parkinson's disease. FEBS Lett.510, 216–220.
16 Benavides, S. H., Monserrat, A. J., Farina, S. and Porta, E. A. (2002) Sequential histochemical studies of neuronal lipofuscin in the human cerebral cortex from the first to the ninth decade of life. Arch. Gerontol. Geriatr. 34, 219–231.
17 Mann, D. and Yates, P. (1974) Lipoprotein pigments: their relationship to aging in the human nervous system. II. The melanin content of pigmented nerve cells. Brain 97, 489–498.
18 Terman, A. and Brunk, U. T. (1998) Lipofuscin: mechanisms of formation and increase with aging. APMIS 106, 265–276.
19 Tribl, F., Gerlach, M., Marcus, K., Asan, E., Tatschner, T., Arzberger, T., Meyer, H. E., Bringmann, G. and Riederer, P. (2005) 'Subcellular proteomics' of neuromelanin granules isolated from the human brain. Mol. Cell Proteom. 4, 945–947.
20 Terman, A. and Brunk, U. (2006) Oxidative stress, accumulation of biological "garbage" and aging. Antioxid. Redox Signal 8, 197–204.
21 Kikugawa, K., Kato, T., Beppu, M. and Hayasaka, A. (1989) Fluorescent and cross-linked proteins formed by free radical and aldehyde species generated during lipid oxidation. Adv. Exp. Med. Biol. 266, 345–357.
22 Brunk, U. T. and Terman, A. (2002) Lipofuscin: mechanisms of age-related accumulation and influence on cell function. Free Radic. Biol. Med. 33, 611–619.
23 Gray, D. and Woulfe, J. (2005) Lipofuscin and aging: a matter of toxic waste. Sci. Aging Knowl. Environ. 5, 1–5.
24 Katz, M. L. and Robison, W. G. J. (2002) What is lipofuscin? Defining characteristics and differentiation from other autofluorescent lysosomal storage bodies. Arch Gerontol. Geriatr. 34, 169–184.
25 Mann, D. M. A. and Yates, P. O. (1987) Ageing, nucleic acids and pigments. In: Recent Advances in Neuropathology, pp. 109–137. Cavanagh,, J. B. (ed.), Livingston, Edinburgh.
26 von Buttlar-Brentano, K. (1954) Zur Lebensgeschichte des Nucleus basalis, tuberomillaris, supraopticus, und paraventricularis unter normalen und pathogenen Bedingungen. J. Hirnforsch. 1, 337–419.
27 Braak, H. (1984) Architectonics as seen by lipofuscin stains. In: Cerebral Cortex, vol 1, pp. 59–104, Peters, A. and Jones, E. G. (eds), Plenum, New York.
28 Berridge, C. W. and Waterhouse, B. D. (2003) The locus coeruleus-noradrenergic system: modulation of behavioural state and state-dependent cognitive processes. Brain Res. Brain Res. Rev. 42, 33–84.
29 Bazelon, M., Fenichel, G. M. and Randall, J. (1967) Studies on neuromelanin. I. A melanin system in the human adult brainstem. Neurology 17, 512–519.
30 Rosengren, E., Linder-Eliasson, E. and Carlson, A. (1985) Detection of 5-S-cysteinyldopamine in human brain. J. Neural. Transm. 63, 247–253.
31 Cowen, D. (1986) The melanoneurons of the human cerebellum (nucleus pigmentosus cerebellaris) and homologues in the monkey. J. Neuropathol. Exp. Neurol. 197, 63–80.
32 Hild, W. (1959) Das Neuron.. In: Handbuch das Mikroskopischen Anatomie des Menschen, pp. 1–144, W. v. A. B. Möllendorf. (ed.) Springer, Berlin.
33 Halliday, G. M., Li, Y. W., Joh, T. H., Cotton, R. G., Howe, P. R., Geffen, L. B. and Blessing, W. (1988) Distribution of monoamine-synthesising neurons in the human medulla oblongata. J. Comp. Neurol. 273, 301–317.
34 Guyenet, P. G. (1991) Central noradrenergic neurons: the autonomic connection. Prog. Brain Res. 88, 365–380.
35 Beach, T. G., Sue, L. I., Walker, D. G., Lue, L. F., Connor, D. J., Caviness, J. N., Sabbagh, M. N. and Adler, C. H. (2007) Marked microglial reaction in normal aging human substantia nigra: correlation with extraneuronal neuromelanin pigment deposits. Acta Neuropathol. 114, 419–424.
36 Kikugawa, K., Kato, T., Beppu, M. and Hayasaka, A. (1989) Fluorescent and cross-linked proteins formed by free radical and aldehyde species generated during lipid oxidation. Adv. Exp. Med. Biol. 266, 345–357.
37 Fasano, M., Giraudo, S., Coha, S., Bergamasco, B. and Lopiano, L. (2003) Residual substantia nigra neuromelanin in Parkinson's disease is cross-linked to alpha-synuclein. Neurochem. Int. 42, 603–606.
38 Sarna, T., Burke, J. M, Korytowski, W., Rozanowska, M., Skumatz, C. M, Zareba, A. and Zareba, M. (2003) Loss of melanin from human RPE with aging: possible role of melanin photooxidation. Exp. Eye Res. 76, 89–98.
39 Kayatz, P., Thumann, G., Luther, T. T, Jordan, J. F., Bartz-Schmidt, K. U, Esser, P. and Schraermeyer, U. (2001) Oxidation causes melanin fluorescence. Invest. Opthalmol. Vis. Sci. 42, 241–246.

40 Langston, J. W., Forno, L. S., Tetrud, J., Reeves, A. G., Kaplan, J. A. Karluk, D. (1999) Evidence of active nerve cell degeneration in the substantia nigra of humans years after 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine exposure. Ann. Neurol. 46, 598–605.
41 Forno LS. (1996) Neuropathology of Parkinson's disease. J. Neuropathol. Exp. Neurol. 55, 259–272.
42 Cabello, C. R., Thune, J. J., Pakkenberg, B. (2002) Ageing of substantia nigra in humans: cell loss may be compensated by hypertrophy Neuropathol. Appl. Neurobiol. 28, 283–291.
43 Chu, Y., Kompoliti, K., Cochran, E. J., Mufson E. J Kordower J. H. (2002) Age-related decreases in Nurr1 immunoreactivity in the human substantia nigra. J Comp. Neurol. 450, 203–214.
44 Fearnley, J. and Lees, A. (1991) Ageing and Parkinson's disease: substantia nigra regional selectivity. Brain 114, 2283–2301.
45 Ma, S. Y., Roytt, M., Collan, Y. and Rinne, J. O. (1999) Unbiased morphological measurements show loss of pigmented nigra neurones with ageing. Neuropathol. Appl. Neurobiol. 25, 394–399.
46 McRitchie, D. A., Cartwright, H. and Halliday, G. M. (1997) Specific A10 dopaminergic nuclei in the midbrain degenerate in Parkinson's disease. Exp. Neurol. 144, 202–213.
47 Kubis, N., Faucheux, B. A., Ransmayr, G., Damier, P., Duyckaerts, C., Henin, D., Forette, B., Le Charpentier, Y., Hauw, J. J., Agid, Y. and Hirsch, E. C. (2000) Preservation of midbrain catecholaminergic neurons in very old human subjects. Brain 123, 366–373.
48 Odh, G., Carstam, R., Paulson, J., Wittbjer, A., Rosengren, E. and Rorsman, H. (1994) Neuromelanin of the human substantia nigra: a mixed-type melanin. J. Neurochem. 62, 2030–2036.
49 Wakamatsu, K., Fujiwara, H., Zucca, F. A., Zecca, L. and Ito, S. (2003) The structure of neuromelanin as studied by chemical degradative methods. J. Neurochem. 86, 1015–1023.
50 Double, K., Zecca, L., Costo, P., Mauer, M., Greisinger, C., Ito, S., Ben-Shachar, D., Bringmann, G., Fariello, R. G., Riederer, P. and Gerlach, M. (2000) Structural characteristics of human substantia nigra neuromelanin and synthetic dopamine melanins. J. Neurochem. 75, 2583–2589.
51 Li, J., Scheller, C., Koutsilieri, E., Griffiths, F., Beart, P. M., Mercer, L. D., Halliday, G., Kettle, E., Rowe, D., Riederer, P., Gerlach, M., Rodriguez, M. and Double, K. L. (2005) Differential effects of human neuromelanin and synthetic dopamine melanin on neuronal and glial cells. J. Neurochem. 95, 599–608.
52 Zecca, L., Costi, P., Mecacci, C., Ito, S., Terreni, M. and Sonnino, S. (2000) Interaction of human substantia nigra neuromelanin with lipids and peptides. J Neurochem. 74, 1758–1765.
53 Fedorow, H., Pickford, R., Hook, J. M., Double,, K. L., Halliday, G., Gerlach, M., Riederer, P. and Garner, B. (2005) Dolichol is the major lipid component of human substantia nigra neuromelanin. J. Neurochem. 92, 990–995.
54 Ward, W. C., Guan, Z., Zucca, F. A., Fariello, R. G., Kordestani, R., Zecca, L., Raetz, C. R. and Simon, J. D. (2007) Identification and quantification of dolichol and dolichoic acid in neuromelanin from substantia nigra of the human brain. J. Lipid Res. 48, 1457–1462.
55 Dedov, V., Griffiths, F., Garner, B., Halliday, G. and Double, K. L. (2007) Lipid content determines aggregation of neuromelanin granules in vitro. J. Neural. Transm. Suppl. 72, 35–38.
56 Foley, J. and Baxter, D. (1958) On the nature of pigment granules in the cells of the locus coeruleus and substantia nigra. J. Neuropathol. Exp. Neurol. 17, 586–598.
57 Hirosawa, K. (1968) Electron microscopic studies on pigment granules in the substantia nigra of locus coeruleus of the Japanese monkey (Macaca fuscata yakui). Z. Zellforsch. Mikrosk. Anat. 88, 187–203.
58 Hong, L. and Simon, J. (2007) Current understanding of the binding sites, calacity, affinity and biological significance of metals in melanin. J. Phys. Chem. B 111, 7938–7947.
59 Youdim, M., Ben-Shachar, D. and Riederer, P. (1989) Is Parkinson′s disease a progressive siderosis of substantia nigra resulting from iron and melanin induced neurodegeneration? Acta Neurol. Scand. 126, 47–54.
60 Ben-Shachar, D., Riederer, P. and Youdim, M. B. H. (1991) Iron-melanin interaction and lipid peroxidation: implications for Parkinson′s disease. J. Neurochem. 57, 1609–1614.
61 Jellinger, K., Kienzel, E., Rumpelmair, G., Riederer, P., Stachellberger, H., Ben-Shachar, D. and Youdim, M. B. H. (1992) Iron-melanin complex in substantia nigra of parkinsonian brains: an X-ray microanalysis. J. Neurochem. 59, 1168–1171.
62 Perl, D., Good, P. and Olanow, C. (1993) Iron (Fe) and aluminium (Al) accumulate in the neuromelanin granules of the substantia nigra pars compacta (SNC) of idiopathic Parkinson′s disease (PD). J. Neuropathol. 76, 254–258.
63 Zecca, L., Pietra, R., Goj, C., Mecacci, C., Radice, D. and Sabbioni, E. (1994) Iron and other metals in neuromelanin, substantia nigra and putamen of human brain. J. Neurochem. 62, 1097–1101.
64 Zecca, L., Shima, T., Stroppolo, A., Goj, C., Battiston, A., Gerbasi, R., Sarna, T. and Swartz, H. M. (1996) Interaction of neuromelanin and iron in substantia nigra and other areas of human brain. Neuroscience 73, 407–415.
65 Shima, T., Sarna, T., Swartz, H., Stroppolo, A., Gerbasi, R. and Zecca, L. (1997) Binding of iron to neuromelanin of human substantia nigra and synthetic melanin: an electron paramagnetic resonance spectroscopy study. Free Radic. Biol. Med. 23, 110–119.
66 Aime, S., Bergamasco, B., Biglino, D., Digilio, G., Fasano, M., Giamello, E. and Lopiano, L. (1997) EPR investigations of the iron domain in neuromelanin. Biochem. Biophys. Acta 1361, 49–58.
67 Double, K. L., Ben-Shachar, D., Youdim, M. B. H., Zecca, L., Riederer, P. and Gerlach, M. (2002) Influence of neuromelanin on oxidative pathways within the human substantia nigra. Neurotoxicol. Teratol. 24, 621–628.
68 Zecca, L., Tampellini, D., Gatti, A., Crippa, R., Eisner M, Sulzer, D., Ito, S., Fariello, R. and Gallorini, M. (2002) The neuromelanin of the human substantia nigra and its interaction with metals. J. Neural Transm. 109, 663–672.
69 D'Amato, R. J., Lipman, Z. P. and Snyder, S. H. (1986) Selectivity of the parkinsonian neurotoxin MPTP: toxic metabolite MPP+ binds to neuromelanin. Science 231, 987–989.
70 Lindquist, N. G., Larsson, B. S. and Lyden-Sokolowski, A. (1988) Autoradiography of (14C)paraquat or (14C)diquat in frogs and mice: accumulation in neuromelanin. Neurosci. Lett. 93, 1–6.
71 Naoi, M., Maruyama, W. and Dostert, P. (1994) Binding of 1,2(N)-dimethyl-6,7-dihydroxy-isoquinolium ion to melanin: effects of ferrous and ferric ion on the binding. Neurosci. Lett. 171, 9–12.
72 Ostergren, A., Annas, A., Skog, K., Lindquist, N. G. Britlebo, E. B. (2004) Long-term retention of neurotoxic beta-carbolines in brain neuromelanin. J. Neural Transm. 111, 141–157.
73 Essner, E. and Novikoff, A. V. (1960) Human hepatocellular pigments and lysosomes. J. Ultrastruct. Res. 3, 374–391.
74 Löw, P., Peterson, E., Edlund, C., Brunk, U. T. and Appelkvist, E. L. (1992) Nonmembrane associated dolichol in rat liver. Lipids 27, 1–9.
75 Jolly, R. D., Palmer, D. N. Dalefield, R. R. (2002) The analytical approach to the nature of lipofuscin (age pigment). Arch. Gerontol. Geriatr. 34, 205–217.
76 Van Woert, M. H. and Ambani, L. M. (1974) Biochemistry of neuromelanin. Adv. Neurol. 5, 215–223.
77 Jolly, R. D., Douglas, B. V., Davey, P. M. and Roiri, J. E. (1995) Lipofuscin in bovine muscle and brain: a model for studying age pigment. Gerontology 41, 283–295.
78 Double, K. L., Gerlach, M., Schünemann, V., Trautwein, A. X., Zecca, L., Gallorini, M., Youdim, M. B. H., Riederer,

P. and Ben-Shachar, D. (2003) Iron binding characteristics of neuromelanin of the human substantia nigra. Biochem. Pharmacol. 66, 489–494.
79 Mirza, B., Hadberg, H., Thomsen, P. and Moos, T. (2000) The absence of reactive astrocytosis is indicative of a unique inflammatory process in Parkinson's disease. Neuroscience 95, 425–432.
80 Double, K., Riederer, P. and Gerlach, M. (1999) The significance of neuromelanin in Parkinson's disease. Drug News Dev. 12, 333–340.
81 Jung, T., Bader, N. and Grune, T. (2007) Lipofuscin: formation, distribution and metabolic consequences. Ann. N. Y. Acad. Sci. 1119, 97–111.
82 Nilsson, E. and Yin, D. (1997) Preparation of artifical ceroid/lipofuscin by UV-oxidation of subcellular organelles. Mech. Ageing Dev. 99, 61–78.
83 Zecca, L., Pietra, R., Goj, C., Mecacci, C., Radice, D. and Sabbioni, E. (1994) Iron and other metals in neuromelanin, substantia nigra and putamen of human brain. J. Neurochem. 62, 1097–1101.
84 Sitte, N., Huber, M., Grune, T., Ladhoff, A., Doecke, W. D., Von Zglinicki, T. and Davies, K. J. (2000) Proteasome inhibition by lipofuscin/ceroid during postmitotic aging of fibroblasts. FASEB J. 14, 1490–1498.
85 Cuervo, A. M., Palmer, A., Rivett, A. J. and Knecht, E. (1995) Degradation of proteosomes by lysosomes in rat liver. Eur J. Biochem 227, 792–800.
86 Davies, I., Fotheringham, A. and Roberts, C. (1983) The effect of lipofuscin on cellular function. Mech. Ageing Dev. 23, 347–356.
87 Ferland, G., Audet, M. and Tuchweber, B. (1992) Effect of dietary restriction on lysosomal bodies and total protein synthesis in hepatocytes of aging rats. Mech. Ageing Dev. 64, 49–59.
88 Korytowski, W., Sarna, T. and Zareba, M. (1995) Antioxidant action of neuromelanin: the mechanism of inhibitory effect on lipid peroxidation. Arch. Biochem. Biophys. 319, 142–148.
89 Zareba, M., Bober, A., Korytowski, W., Zecca, L. and Sarna, T. (1995) The effect of a synthetic neuromelanin on yield of free hydroxyl radicals generated in model systems. Biochem. Biophys. Acta 1271, 343–348.
90 Peters, S. and Schraermeyer, U. (2001) Characteristics and functions of melanin in retinal pigment epithelium. Ophthalmolge 98, 1181–1185.
91 Zecca, L., Zucca, F. A., Wilms, H. and Sulzer, D. (2003) Neuromelanin of the substantia nigra: a neuronal block hole with protective and toxic characteristics. Trends Neurosci. 26, 578–580.
92 Gerlach, M., Trautwein, A. X., Zecca, L., Youdim, M. B. H. and Riederer, P. (1995) Mössbauer spectroscopic studies of purified human neuromelanin isolated from the substantia nigra. J. Neurochem. 65, 923–926.
93 Connor, J., Synder, B., Beard,, J. L., Fine, R. and Mufson, E. (1992) Regional distribution of iron and iron-regulatory proteins in the brain in aging and Alzheimer's disease. J. Neurosci. Res. 31, 327–335.
94 Youdim, M., Ben-Shachar, D. and Riederer, P. (1993) Iron-melanin interaction and Parkinson's disease. News Physiol. Sci. 8, 45–49.
95 Zecca, L., Mecacci, O., Seraglia, R. and Parati, E. (1992) The chemical characterization of melanin contained in substantia nigra of human brain. Biochim. Biophys. Acta 1138, 6–10.
96 Thomas, M. and Jankovic, J. (2004) Neurodegenerative disease and iron storage in the brain. Curr. Opin. Neurol. 17, 437–442.
97 Keller, N. J., Dimayuga, E., Chen, Q., Thorpe, J., Gee, J. and Ding, Q. (2004) Autophagy, proteasomes, lipofuscin and oxidative stress in the aging brain. Int. J. Biochem. Cell Biol. 36, 2376–2391.
98 Woodhouse, A., Dickson, T. C. and Vickers, J. C. (2007) Vaccincation strategies for Alzheimer's disease: a new hope? Drugs Aging 24, 107–119.
99 Fonseca, D. B., Sheehy, M. R. J., Blackman, N., Shelton, P. M. J. and Prior, A. E. (2005) Reversal of a hallmark of brain ageing; lipofuscin accumulation. Neurobiol. Aging 26, 69–76.
100 Riga, S., Riga, D., Schneider, F. and Halalau, F. (2006) Processing, lysis and elimination of brain lipopigments in rejuvenation therapies. Ann. N. Y. Acad. Sci. 1067, 383–387.
101 Mountjoy, C. Q., Dowson, J. H., Harrington, C., Cairns, M. R. and Wilton-Cox, H. (2005) Characteristics of neuronal lipofuscin in the superior temporal gyrus in Alzheimer's disease do not differ from non-diseased controls: a comparison with disease-related changes in the superior frontal cortex. Acta Neuropathol. 109, 490–496.
102 Adamec, E., Mohan, P., Cataldo, A., Vonsattel, J. and Nixon, R. (2002) Up-regulation of the lysosomal system in experimental models of neuronal injury: implications for Alzheimer's disease. Neuroscience 100, 663–675.
103 Drach, L. M., Bohl, J., Wach, S., Schlote, W. and Goebel, H.-H.. (1989) Reduced intraneuronal lipofuscin content in dementia with Lewy bodies compared with Alzheimer's disease and controls. Dement. Geriatr. Cogn. Disord. 9, 1–5.
104 Mann, D. M. A. and Yates, P. O (1982) Ageing, nucleic acids and pigments. In: Recent Advances in neuropathology, pp. 109–138, Smith, W. T. and Cavanagh, J. B. (eds), Churchill Livingston, Edinburgh.
105 Hirsch, E., Graybiel, A. and Agid, Y. (1988) Melanized dopamine neurons are differentially susceptible to degeneration in Parkinson′s disease. Nature 28, 345–348.
106 Wilms, H., Rosenstiel, P., Sievers, J., Deuschl, G., Zecca, L. and Lucius, R. (2003) Activation of microglia by human neuromelanin is NF-κB-dependent and involves p38 mitrogen-activated protein kinase: implications for Parkinson's disease. FASEB J. 17, 500–502.
107 Maruyama, W., Shamoto-Nagai, M., Akao, Y., Riederer, P. and Gerlach, M. (2006) The effect of neuromelanin on the proteasome activity in human dopaminergic SH-SY5Y cells. J. Neural Transm. Suppl. 70, 125–132.
108 Gibb, W. (1992) Melanin, tyrosine hydroxylase, calbindin and substance P in the human midbrain and substantia nigra in relation to nigrastriatal projections and differential neuron susceptibility in Parkinson′s disease. Brain Res. 581, 283–291.
109 Kastner, A., Hirsch, E., Lejeune, O., Javoy-Agid, F., Rascol, O. and Agid, Y. (1992) Is the vulnerability of neurons in the substantia nigra of patients with Parkinson′s disease related to their neuromelanin content? J. Neurochem. 59, 1080–1089.
110 Braak, H., Rub, U., Sandmann-Keil, D., Gai, W. P., de Vos, R. A., Jansen Steur, E. N., Arai, K. E. B. (2000) Parkinson's disease: affection of brain stem nuclei controlling premotor and motor neurons of the somatomotor system. Acta Neuropathol. (Berl.) 99, 489–495.
111 Braak, E., Sandmann-Keil, D., Rüb, U., Gai, W. P., de Voa, R. A., Jansen Steur, E. N. H., Arai, K. and Braak, H. (2001) Alpha-synuclein immunopositive Parkinson's disease-related inclusion bodies in lower brain stem nuclei. Acta Neuropathol. 101, 195–201.
112 Aime, S., Bergamasco, B., Casu, M., Digilio, G., Fasano, M., Giraudo, S. and Lopiano, L. (2000) Isolation and 13C-NMR characterization of an insoluble proteinaceous fraction from substantia nigra of patients with Parkinson's disease. Mov. Disord. 15, 977–981.
113 Fasano, M., Bergamasco, B. and Lopiano, L. (2006) Is neuromelanin changed in Parkinson's disease? Investigations by magnetic spectroscopies. J. Neural Transm. 113, 769–774.
114 Double, K. L. and Halliday, G. M. (2006) New face of neuromelanin. J. Neural Transm. Suppl. 70, 119–123.
115 Sofic, E., Riederer, P., Heinsen, H., Beckman, H., Reynolds, G. P., Hebenstreit, G. and Youdim, M. B. H. (1988) Increased iron (III) and total iron content in post mortem substantia nigra of parkinsonian brain. J. Neural Transm. 74, 199–205.
116 Riederer, P., Sofic, E., Rausch, W. D., Schmidt, B., Reynolds, G. P., Jellinger, K. and Youdim, M. B. (1989) Transition metals, ferritin, glutathione, and ascorbic acid in parkinsonian brains. J. Neurochem. 52, 515–520.

117 Jellinger, K., Paulus, W., Grundke-Iqbal, I., Riederer, P. and Youdim, M. B. H. (1990) Brain iron and ferritin in Parkinson′s disease and Alzheimer′s disease. J. Neural Transm. 2, 327–340.
118 Dexter, D. T., Carayon, A., Javoy-Agid, F., Agid, Y., Wells, F. R., Daniel, S. E., Lees, A. J., Jenner, P. and Marsden, C. D. (1991) Alterations in the levels of iron, ferritin and other trace metals in Parkinson′s disease and other neurodegenerative diseases affecting the basal ganglia. Brain 114, 1953–1975.
119 Mann, V. M., Cooper, J. M., Daniel, S. E., Srai, K., Jenner, P., Marsden, C. D. and Schapira, A. H. (1994) Complex I, iron, and ferritin in Parkinson′s disease substantia nigra. Ann. Neurol. 36, 876–881.
120 Double, K. L., Gerlach, M., Youdim, M. B. H. and Riederer, P. (2000) Impaired iron homeostasis in Parkinson's disease. In: Advances in Research on Neurodegeneration, pp. 37–58, Riederer, P., Calne, D. B. (eds), Springer, Vienna.
121 Berg, M. D., Gerlach, M., Youdim, M. B. H., Double, K. L., Zecca, L., Riederer, P. and Becker, G. (2001) Brain iron pathways and their relevance to Parkinson's disease. J. Neurochem. 79, 225–236.
122 Oakley, A. E., Collingwood, J. F., Dobson, J., Love, G., Perrott, H. R., Edwardson, J. A., Elstner, M. and Morris, C. M. (2007) Individual dopaminergic neurons show raised iron levels in Parkinson's disease. Neurology 68, 1820–1825.
123 Bolzoni, F., Giraudo, S., Lopiano, L., Bergamasco, B., Fasano, M. and Crippa, P. R. (2002) Magnetic investigations of human mesencephalic neuromelanin. Biochem. Biophys. Acta 1586, 210–218.
124 Lopiano, L., Chiesa, M., Digilio, D., Giraudo, G., Bergamasco, B. and Fasano, M. (2000) Q-band EPR investigations of neuromelanin in control and Parkinson's disease patients. Biochem. Biophys. Acta 1500, 306–312.
125 Reynolds, A., Laurie, C., Mosley, R. L. and Gendelman, H. E. (2007) Oxidative stress and the pathogenesis of neurodegenerative disorders. Int. Rev. Neurobiol. 82, 297–325.