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Neuromelanin in human dopamine neurons: Comparison with peripheral melanins and relevance to Parkinson's disease

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Abstract

Neuromelanin (NM) is a dark polymer pigment produced in specific populations of catecholaminergic neurons in the brain. It appears in greatest quantities in the human brain, in lesser amounts in some other non-human primates, but is absent from the brain in many lower species. Interest in this pigment has seen a resurgence in recent years because of a hypothesised link between neuromelanin and the especial vulnerability of neuromelanin-containing neurons to cell death in Parkinson's disease (PD). Little is known regarding the biology of neuromelanin. As neuromelanin appears to have characteristics in common with the better studied peripheral melanin pigments this review compares what is known about neuromelanin with melanins found in other body tissues. Unlike peripheral melanins, which are produced in specialised cells called melanocytes and may be transferred to other cell types, neuromelanin granules are believed to be stored in the cell in which they are produced. Neuromelanin granules display a unique, more heterogeneous appearance compared with peripheral melanins. Unlike melanin, neuromelanin is traditionally thought to result from a non-enzymatic synthesis pathway with no known pathway for neuromelanin catabolism. More recent data, however, is indicative of some regulation of neuromelanin synthesis and turnover. By analogy with peripheral melanins, neuromelanin may function in vivo to attenuate the effects of damaging stimuli. Among several possible mechanisms suggested, the ability of neuromelanin to interact with transition metals, especially iron, and to mediate intracellular oxidative mechanisms has received particular attention. Recent data from neuromelanin in the Parkinson's disease brain suggests that this proposed function may be compromised, thus rendering pigmented neurons vulnerable to oxidative damage in this disorder.

Keywords: Neuromelanin; Dopamine; Parkinson's disease; Melanin; Human brain

Contents

Abbreviations: ASP, agouti signalling protein; DAM, synthetic dopamine melanin; DHI, 5,6-dihydroxyindole; DHICA, 5,6-dihydroxyindole-2-carboxylic acid; EPR, electron paramagnetic spectroscopy; L-dopa, levodopa; MC1R, melanocortin 1 receptor; MSH, melanocyte stimulating hormone; NM, neuromelanin; NMR, nuclear magnetic resonance; PD, Parkinson's disease; TRP1, tyrosinase-related protein 1; TRP2, tyrosinase-related protein 2; UV, ultraviolet

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1. Introduction

The origin of the name melanin, from the Greek word *melanos* (''dark''), is usually attributed to the Swedish chemist Berzelius (Prota, 1992). Melanin in the brain has a similar appearance and structure to cutaneous melanins, and has thus been designated *neuro*melanin (NM) (Lillie, 1955, 1957). Based on their precursor molecules, melanins are classified into four groups:

- Eumelanin is formed from L-3,4-dihydroxyphenylalanine (L-dopa).
- Pheomelanin is formed by oxidative polymerisation of 5-*S*-cysteinyl-dopa or 2-*S*-cysteinyl-dopa.
- Neuromelanin is thought to be formed by oxidative polymerisation of dopamine or noradrenaline, with the possible involvement of cysteinyl-derivatives.
- Allomelanin is formed by the oxidation of polyphenols, such as catechols and 1,8-dihydroxynaphtalene. They are widely spread in fungi and are often nitrogen-free.

Interest in the black melanin pigment produced within specific catecholamine neurons in the human brain has seen a resurgence in recent years. Although much is known about melanins outside the central nervous system, to which neuromelanin is thought to be related, many basic questions remain to be answered about melanins in the brain. A review of nerve cell pigmentation in 1918 commented that ''there are more opinions than there are investigators'' (Dolley and Guthrie, 1918) and this holds true today. It is unclear why some human dopamine neurons produce an insoluble pigment within their cytoplasm and others do not. There is little information regarding the fate of neuromelanin over the lifespan and little is known about neuromelanin's structure. Consequently, a valid and useful approach to this problem is to consider neuromelanin in terms of what is known about the better-characterised and more prominent peripheral melanins (see Table 1 for comparative summary). For clarity in this review, 'melanin' will be used to refer to melanins occurring within the periphery (i.e., outside the central nervous system), and 'neuromelanin' (NM) will be used to describe melanins occurring within the central nervous system.

2. Neuromelanin

Traditionally, NM is thought to be an inert cellular by-product, produced via a simple autoxidation pathway, a hypothesis supported by the failure to link tyrosinase, the rate-limiting enzyme of peripheral melanin synthesis to NM. Recent evidence, however, suggests some regulation for NM production and a possible physiological role in the cell. Elucidation of these basic biological characteristics of NM may provide clues to the aetiology of Parkinson's disease (PD), a common neurodegenerative disease involving the death of the cells containing this pigment.

2.1. Types of neuromelanin

NM, which is browny-black in appearance, is thought to be a mixture of eumelanin and pheomelanin (Odh et al., 1994). Within the brain, NM is found only in two of the three types of catecholamine synthesising cells—noradrenaline and dopamine but not adrenaline (Bogerts, 1981; Saper and Petito, 1982). Although it has been surmised that the NM in these different cells is likely to be composed of the various neurotransmitters synthesised within that specific cell, no study has proven this due to the relatively small numbers of neurons in the brain that produce these neurotransmitters (one human substantia nigra yields approximately 1 mg isolated NM (Aime et al., 2000)).

2.2. Distribution of neuromelanin

NM-producing neurons are found primarily in the brainstem. In the brainstem pigment is primarily found in the midbrain and pons (Fig. 1), although some NM-containing neurons are found in the hypothalamus and

Table 1
Comparative biology of melanin and neuromelanin

Characteristic		Melanin	Neuromelanin
Occurrence and distribution of pigment	Species	Vertebrates[1]	•Primates, greatest amount in man[14] •Not found in common laboratory animals, such as rat[14]
	Organs specifically within humans	Hair, skin, iris and choroid of eye, inner ear[2]	•Selected catecholamine neurons, predominantly substantia nigra and locus coeruleus of the midbrain[15,16]
Cells that produce pigment		Melanocytes[3]	Neurons[15,16]
Cells that contain pigment		Melanocytes, keratinocytes[3]	Neurons, occasional glial cells[17,18]
Melanosomal	Ultrastructure	•Discrete membrane-bound organelles[4] •Spherical or ellipsoidal in shape[4]	•Indistinctly bordered granules, not membrane-bound, wide size range[19–22] •Three main components: electron dense pigment, electron intermediate component and electron lucent lipid[20,21]
	Maturation	•Internal structure of melanosome visible at first but gradually obscured by deposited melanin in eumelanosomes[2,3] •Structure remains visible in pheomelanosomes[2,3]	Unknown
Regulation of pigment genesis	Genetic	Regulation by 85 distinct gene loci[5]	Unknown
	Cellular	Hormonally regulated by MC1R agonists MSH and ASP[6]	Unknown
	Within melanosome/ granule	•Rate limiting enzyme, tyrosinase[1] •pH of melanosome[7]	•No enzyme currently identified •Effect of pH unknown
Degradation		•Lost via cellular turnover[4]	•Released from dying neurons in PD, catabolism unknown[17]
		•Oxidative breakdown may occur in eye[8]	•Catabolism in healthy brain not investigated
Chemical structure		•Heterologous polymer, basic structural unit generally represented by covalently linked indoles[1] •Integral protein component[9]	•Complex heterologous polymer similar to melanin[23] •Integral protein and lipid component[24–26]
Biological role in humans		•Ocular development[10] •Photoprotective[11] •Binding of iron and other metals[29] •Inner ear–maintenance of ionic composition of endolymph[12] •Absorption of electrons (antioxidant)[30] •Redox properties–antibiotic[1], metal binding[13]	•Interaction with pesticides, toxins[27,28] and neuroleptics

Superscripts: 1, Riley (1997); 2, Prota (1992); 3, Breathnach (1971); 4, Quevedo and Holstein (1998); 5, Bennett and Lamoreux (2003); 6, Hearing (2000); 7, Ancans et al. (2001); 8, Sarna et al. (2003); 9, Zeise (1995); 10, Oetting (2000); 11, Nakazawa et al. (1998); 12, Tachibana (2001); 13, Szpoganicz et al. (2002); 14, Marsden (1961); 15, Bogerts (1981); 16, Saper and Petito (1982); 17, Forno (1996); 18, Langston et al. (1999); 19, D'Agostino and Luse (1964); 20, Moses et al. (1966); 21, Duffy and Tennyson (1965); 22, Schwyn et al. (1970); 23, Enochs et al. (1993); 24, Double et al. (2000b); 25, Zecca et al. (2000); 26, Fedorow et al. (2005); 27, Lindquist et al. (1988); 28, D'Amato et al. (1986); 29, Zecca et al. (2002b); 30, Korytowski et al. (1995).

medulla oblongata (Bazelon et al., 1967; Rosengren et al., 1985), in the cerebellum near the fourth ventricle (Cowen, 1986), and in spinal and sympathetic ganglia (Hild, 1959). Apart from the spinal and sympathetic ganglia, these brain regions are involved in conscious perception, movement, emotion and memory (Bogerts, 1981). The most rostral group in the midbrain and hypothalamus contain dopamine, those in the pons contain noradrenaline, while those in the medulla oblongata contain noradrenaline and/or adrenaline (Bogerts, 1981; Saper and Petito, 1982).

In the medulla oblongata, three times the number of tyrosine hydroxylase positive cells are present compared to the number of pigmented cells (Halliday et al., 1988). In fact, no pigment is produced in adrenaline-producing neurons within the medulla oblongata (Halliday et al., 1988). Sixty-five percent of noradrenaline neurons contain NM, suggesting that the synthesis and use of noradrenaline and particularly adrenaline does not ensure NM production (Halliday et al., 1988). The noradrenergic NM-containing neurons are thought to be important for autonomic control of

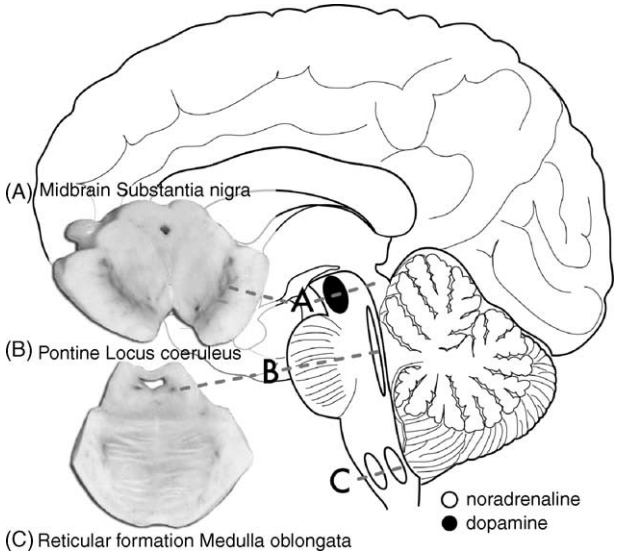

Fig. 1. Within the human brain, three main regions contain NM-producing cells: the substantia nigra of the midbrain (A), the locus coeruleus within the pons (B) and the ventrolateral reticular formation and the nucleus of the solitary tract in the medulla oblongata (C). Of these regions, only the substantia nigra (A) and the locus coeruleus (B) contain a large cluster of pigmented neurons that can be seen macroscopically as a darkened area.

cardiac and respiratory integration and the regulation of hypothalamic hormones (Guyenet, 1991).

Within the pons, catecholamine neurons aggregate in the locus coeruleus (Fig. 1). The neurons in this nucleus produce noradrenaline, and virtually all locus coeruleus neurons contain NM (Baker et al., 1989; German et al., 1988). The locus coeruleus is involved in the initiation of activity states (such as the sleep–wake cycle) and modulation of the collection and processing of sensory information during the attentive state (Berridge and Waterhouse, 2003).

Of the brain centres containing NM-pigmented neurons, the greatest number of pigmented cells is found within the midbrain (Fig. 1) in the substantia nigra pars compacta (Saper and Petito, 1982). Pigmented neurons are also scattered in clusters throughout the medial midbrain and continue rostrally into the arcuate nucleus of the hypothalamus. Pigmented neurons in the various areas of the midbrain differ in the amount of pigment per cell (McRitchie et al., 1995; Saper and Petito, 1982), although the subtlety of the differences being measured and the complexity of the cell clustering pattern in this region has produced conflicting details (Gibb and Lees, 1991; Kastner et al., 1992; McRitchie et al., 1995). Compared with other catecholamine cell groups, neurons in the human substantia nigra consistently contain considerable amounts of NM. Although dopamine cells in the midbrain express tyrosine hydroxylase, the rate limiting enzyme for dopamine synthesis, there is no correlation between degree of pigmentation and tyrosine hydroxylase immunoreactivity (Gaspar et al., 1983). This again suggests that while dopamine/noradrenaline synthesis are required for NM production, the synthesis and use of these catecholamines do not ensure NM production.

2.3. Species differences in neuromelanin

Although NM is macroscopically visible in the adult human brainstem, few other species have NM in the brain, and those that do have significantly less than humans (Fig. 2). NM is absent from all of the commonly used laboratory animals including rodents (Barden and Levine, 1983). It should be noted that all of these species have dopamine and noradrenaline neurons, even though they do not have NM-containing neurons (Fig. 2). This emphasises that the production of NM is not an inevitable consequence of catecholamine synthesis.

Like humans, catecholamine neurons in many other primates also contain NM. However, the presence of NM in these species is not as visible (Fig. 2). Pigmentation is most consistent in the substantia nigra in primates and within primates, the degree of pigmentation is maximal in the human (Marsden, 1961). For example, in adult hominoidea (apes with no tails) NM is macroscopically visible by the grey color of the substantia nigra, while in platyrrhini (new world primates some of which have long tails) there is less NM and it can only be seen under the microscope (Adler, 1942; Scherer, 1939). Although less NM is present in the catecholamine neurons of non-human primates, the pigment itself is similar in ultrastructure (Moses et al., 1966; Schwyn et al., 1970). There are conflicting reports of pigmentation in other non-primate species, due to different staining techniques and differing definitions of NM, but pigmented nigral neurons have been reported in species as varied as the horse (Cozzi et al., 1988; Scherer, 1944), giraffe (Scherer, 1944) and the frog (Lindquist et al., 1988), while in the dog NM has been reported not only in the substantia nigra but also in the hypothalamus (Barden and Barrett, 1973; DeMattei et al., 1986; Grünthal, 1929). A detailed study of pigmentation in the substantia nigra of 10 mammalian orders found some degree of pigmentation in at least one representative of 7 of these orders, although the amount of pigmentation varied between the orders and also between members within an order (Marsden, 1961).

2.4. The importance of neuromelanin in Parkinson's disease and other disorders

PD and other parkinsonian disorders are characterised by motor dysfunction which arises primarily as a result of the death of the dopamine cells of the substantia nigra (Forno, 1996). Although regions other than the pigmented substantia nigra exhibit pathology in PD (Marsden, 1983), the loss of NM-containing cells within this area represents a cardinal pathologic diagnostic criteria for the disease (Fig. 2). The presence of NM as a basic characteristic of this group of vulnerable cells suggests a role for this pigment in neurodegeneration. This was confirmed in 1988 by Hirsch and colleagues who reported an inverse relationship between the amount of NM contained within the dopaminergic neurons of the midbrain and the relative vulnerability of

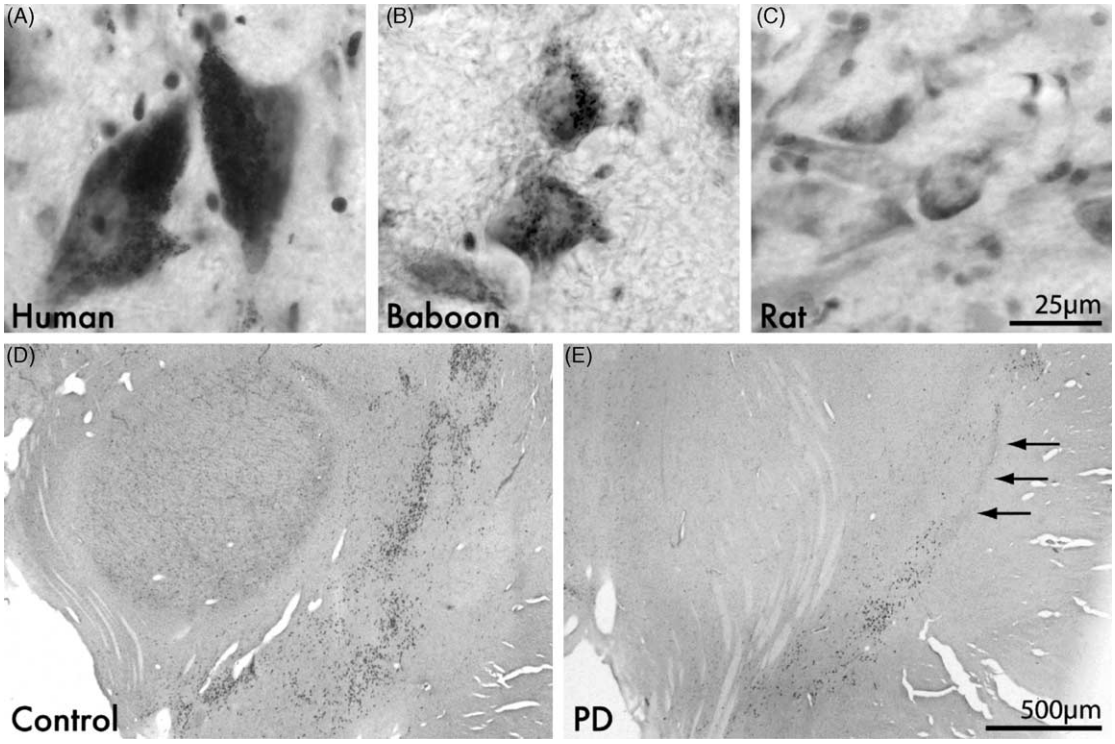

Fig. 2. Cresyl violet stained sections from the substantia nigra (A-E). Human dopamine neurons (A) contain a greater quantity of NM compared with the pigmented dopamine neurons of the baboon (B). Rat dopamine neurons (C) do not contain NM. The pigmented dopamine neurons in the PD substantia nigra (E) have degenerated compared to the control substantia nigra (D), especially in the lateral region (arrows).

these cells to PD (Hirsch et al., 1988). In addition to the degree of NM pigmentation, cell position within the nigral complex was also found to be a key factor for neuronal survival in PD, with the ventrolateral tier being most severely affected (Damier et al., 1999; Gibb, 1992; Halliday et al., 1996; Kastner et al., 1992)

The related parkinsonian syndromes such as multiple system atrophy (Ozawa et al., 2004), progressive supranuclear palsy (Halliday et al., 2000), corticobasal degeneration (Dickson, 1999) and dementia with Lewy bodies (Iseki, 2004) also have pigmented cell loss in the nigra, although the pattern of cell loss may vary (Fearnley and Lees, 1991). Disorders characterised by reduced levels of NM include dopa-responsive dystonia, a disease with parkinsonian features in early childhood (Bandmann and Wood, 2002; Jeon, 1997), and Alzheimer's disease (Kemppainen et al., 2002; Reyes et al., 2003). The degree of cellular depigmentation is considerably more severe in dopa-responsive dystonia than in Alzheimer's disease. In both disorders, the substantia nigra neurons appear otherwise healthy (Kemppainen et al., 2002; Rajput et al., 1994; Reyes et al., 2003).

3. Other types of cellular melanins

Melanin is widely distributed throughout the plant and animal kingdoms. The black pigment found in fungi, plants and bacteria, although termed allomelanin, is structurally different to the dopa-derived melanins found in animals. In humans, these heterogenous, macromolecular pigments occur naturally in the hair, the skin, the inner ear, and the iris, choroid and retinal pigmented epithelium of the eye. In vivo, melanins occur as an ill-defined heteropolymer of both eumelanin and pheomelanin and do not generally occur in a pure form of one type (Ito, 1993).

3.1. Comparison between melanins

The peripheral melanins are very diverse in the proportion and amount of eu- to pheomelanin. Some peripheral melanins are more diverse than others, e.g., hair melanin. The difference between black, brown and blond hair depends more on melanin quantity than the ratio of eu- to pheomelanin, while red hair contains a similar amount of eumelanin to blond hair, with a greater proportion of pheomelanin (Borges et al., 2001). Similarly to hair, variations in skin pigmentation result mainly from differences in the levels of melanin pigment, i.e., darkly pigmented skin contains more of both melanin types than lightly pigmented skin, but relative ratios of eumelanin and pheomelanin do not seem to contribute (Ito and Wakamatsu, 2003). Melanin within the retinal pigment epithelium is mostly eumelanin, regardless of skin colour, while the pigment in the iris can be either eu- or pheomelanin (Prota et al., 1998). There are no conclusive studies in humans

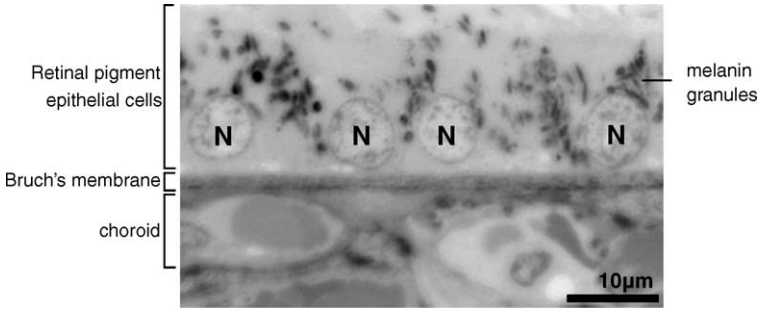

Fig. 3. Retinal pigment epithelial (RPE) cells from a toludine blue stained section of a normal eye from a 47 year old male. The melanin granules can be seen in the cytoplasm near the nuclei (N) of the cells. The retina is a layered structure, and below the RPE cells, the choroid can be seen.

investigating the types of melanin within the stria vascularis of the inner ear, but in both black and yellow mice it is eumelanin (Bartels et al., 2001).

3.2. Cell types that produce and contain melanins

Peripheral melanins are produced in melanosomes, discrete regularly sized membrane-bound organelles, by specialised dendritic cells called melanocytes (Fig. 3). Although melanin is produced by melanocytes, it either remains in the melanocyte or is transferred to other different cell types. In the skin, melanocytes transfer melanin to keratinocytes or to the hair cells, and the majority of pigment is found within these cells. There is no difference between the density of melanocytes in African–American and Caucasian skin (Staricco and Pinkus, 1957; Szabo, 1954). Retinal pigment epithelial cells retain their melanin granules within the cytoplasm (Fig. 3) and do not secrete them. Retinal pigment epithelial cells phagocytose the tips of photoreceptor outer segments and as terminally differentiated cells are thought to have a life span of up to 70 years (Marshall, 1987). In the stria vascularis of the inner ear, it is also the melanocyte that contains the melanosomes (Quevedo and Holstein, 1998). Therefore extracutanous melanocytes are distinct from epidermal melanocytes in that they synthesise melanin during the short period after arriving in the target tissue, and retain their melanosomes instead of transferring them to adjacent cells. NM exists as dark-coloured granules in the cytoplasm of the catecholaminergic neurons where it is synthesised (Fig. 2), as NM in non-neuronal cells is associated with neurodegeneration or following toxin-induced neuron death (Forno, 1996; Langston et al., 1999).

3.3. Comparison between the intracellular distribution of melanins

Melanin is contained within organelles called melanosomes (Fig. 4), which differ structurally depending on which type of melanin they produce. Cutaneous melanosomes are mostly arranged in membrane-bound clusters in light skin, while in dark skin they are usually larger, and distributed individually (Thong et al., 2003). Melanosomes from darkly pigmented skin contain more melanin (van Nieuwpoort et al., 2004). Current evidence suggests that a melanosome can produce either type of melanin (van Nieuwpoort et al., 2004).

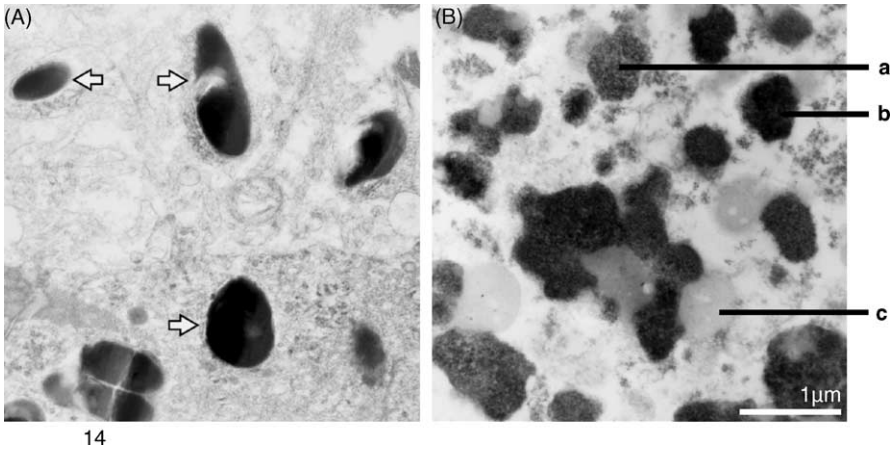

Fig. 4. Ultrathin sections stained with uranyl acetate and lead citrate. (A) High magnification electron micrograph of black melanin granules (arrows) in a retinal epithelial cell in the normal eye of a 47 year old male. The melanin granule has a smooth appearance and a regular boundary. (B) High magnification electron micrograph of NM granules from a dopamine neuron within the normal human substantia nigra of a 20 year old male. The three components of the granules are indicated: (a,b) electron dense (b) and electron intermediate (a) components of NM polymer (c) an electron lucent pigment-associated lipid. NM granules have an irregular boundary and no defining membrane enclosing the granules is apparent.

Differences occur in the size and arrangement of melanosomes in skins of different colour (Thong et al., 2003). Melanosomes that produce eumelanin are large (~0.9 × 0.3 μm diameter) and ellipsoidal in shape with a highly ordered glycoprotein matrix. Pheomelanin producing melanosomes are smaller (~0.7 μm), spherical, and composed of a coarsely aggregated and disordered glycoprotein matrix (reviewed in Quevedo and Holstein, 1998). Although both melanin and NM are situated in the cytoplasm of their respective cells, the ultrastructure of NM appears quite different to that of peripheral melanins (Fig. 4).

NM pigment granules, as they are called, exhibit a wider size range than that of melanosomes (0.5–2.5 μm) (Moses et al., 1966). From the current research, it is not clear whether NM granules are membrane-bound. The first ultrastructural work on NM by D'Agostino (D'Agostino and Luse, 1964) suggested that NM was occasionally delimited by a membrane while Moses (Moses et al., 1966) observed that a membrane was often present. Other studies describe NM granules having an indefinite boundary but do not describe any membranes (Duffy and Tennyson, 1965; Schwyn et al., 1970) suggesting that this feature was not observed. The lack of clear evidence for a limiting membrane surrounding NM granules may result purely from methodological grounds. Descriptions of NM in the Japanese monkey include a clear limiting membrane (Hirosawa, 1968). Given that the perfusion of brain tissue in the experimental situation may have allowed for more efficient preservation of the membrane than that achievable in post-mortem human brain any membrane apparent may have been better preserved. Although membranes surrounding artificially-induced pigment have been described in in vitro systems (Sulzer et al., 2000), neither the cells used nor the resultant pigment granules are similar to that which occurs in the human brain.

NM exhibits three components of different electron density (Fig. 4). Most apparent is an electron dense pigment component. This is associated with a second component of intermediate electron density as well as a third component of a lesser electron density, which was suggested to be lipid (Duffy and Tennyson, 1965; Moses et al., 1966). The lipid component in NM granules is not found in peripheral melanin pigments (Moses et al., 1966). The association of NM granules with lipid suggests a lysosomal and/or mitochondrial origin, possibly from another lipid-containing pigment lipofuscin which accumulates intracellularly with age (Barden, 1969; Schwyn et al., 1970). If lipofuscin were a precursor of NM, lipofuscin granules would be expected to commonly occur in NM-containing neurons; however, the opposite had been noted in the brain (Foley and Baxter, 1958; Hirosawa, 1968) and recently also in melanin-containing retinal pigment epithelial cells (Nilsson et al., 2003). While the insoluble fraction of lipofuscin exhibits a similar infrared spectrum to that of nigral NM (Van Woert and Ambani, 1974), both pigments may contain lipids in different oxidation states.

4. Chemistry of melanins

Although NM is the focus of this review, a general understanding of melanogenesis and chemistry can be provided by investigation of the synthesis pathway of peripheral melanins and comparison to what is known about NM. The first steps in the investigation of melanin synthesis were not undertaken by molecular biologists, rather by organic chemists. Genetic and enzymatic regulation of melanin production in the periphery has been primarily characterised by the study of fur pigmentation in the mouse. Similar experiments cannot be used to elucidate the pathway of NM synthesis as NM does not occur in rodents.

4.1. Comparison of the chemical structure of melanins

The chemical structure and composition of the members of the melanin family are still not known in detail but they appear to be heterologous polymers. Two complementary strategies have been employed to elucidate the core components of melanins (Ito, 1986; Mason, 1967; Nicolaus and Piatelli, 1962; Novellino et al., 2000; Piatelli et al., 1962; Swan, 1974). Firstly, melanogenesis has been mimicked in vitro and intermediate compounds have been investigated. Secondly, compounds liberated by the degradation of synthetic or naturally occurring melanins have been analysed. Using either strategy, L-dopa (L-3,4-dihydroxyphenylalanine) appears to be the principal melanin precursor molecule with intermediates including 5,6-dihydroxyindole (DHI) and 5,6-dihydroxyindole-2-carboxylic acid (DHICA) (Raper, 1927). Pheomelanogenesis incorporates the precursor cysteine and the oxidative polymerisation of 5-*S*-cysteinyl-dopa and 2-*S*-cysteinyl-dopa (Bjorklund et al., 1972) with the formation of the intermediate 1,4-benzothiazine subunit (Di Donato et al., 2002; Napolitano et al., 2001).

Consistent with NM exhibiting properties of both eu- and pheomelanin, 21–25% consists of the additional precursor 5-*S*-cysteinyl-dopa (Odh et al., 1994; Rosengren et al., 1985; Wakamatsu et al., 2003). Comparative nuclear magnetic resonance (NMR) studies of NM and synthetic L-dopa melanin or other natural melanins have revealed some characteristics (Aime et al., 2000, 1996, 1994; Zecca et al., 2000) with NM resembling synthetic cysteinyl-dopa melanin more closely than the simpler eumelanins (Aime et al., 1996, 1994; Double et al., 2000b). In contrast to conventional histochemical detection methods, electron paramagnetic spectroscopy (EPR) permits a more reliable discrimination between eumelanins and sulphur containing pheomelanins. Although the EPR spectrum of NM is closer to that of pheomelanin than that of eumelanin, the presence of heterocyclic 1,4-benzothiazine-subunits typical of this melanin subtype could not be demonstrated (Enochs et al., 1993). A recent study using pyrolysis–gas chromatography/mass spectrometry analysis also suggests that NM does not contain benzothiazine-type monomer units originated from

cysteinyl conjugates of dopamine (Dzierzega-Lecznar et al., 2004). The presence of compounds characteristic of eumelanins in the pyrolysates of NM samples may be indicative of dopa-derived indole-type monomers in natural pigment, supporting the generally accepted view on dopamine involvement in the formation of the NM macromolecule in vivo (d'Ischia and Prota, 1997).

L-Dopa polymerisation creates the melanin backbone and two models have been proposed to describe this structural characteristic. Studies employing matrix-assisted laser desorption/ionisation mass spectrometry (MALDI) to investigate the structure of squid ink melanin from *Sepia officinalis* led to the polymer model which suggests that melanins are composed of polymers with variable molecular masses (Pezzella et al., 1997; Prota, 1992, 2000). Based on X-ray scattering data and structural modelling of eumelanin, the planar model has been put forward, suggesting that melanins are an assembly of planar molecules stacked in a graphite-like manner (Cheng et al., 1994a,b; Gallas et al., 2000; Zajac et al., 1994). Similar information for other melanins is currently unavailable.

Of course natural melanins have some intrinsic variability in composition and structure depending on local environmental factors, especially melanins from different origins (i.e., from hair, feathers, irises, (Novellino et al., 2000). It is therefore likely that studies on artificial melanins may not allow direct comparisons with naturally occurring biocompounds. For example dopamine, the suggested precursor of NM, autoxidises at a physiological temperature and pH. This results in a dark pigment-like substance in vitro, which is often called ''dopamine melanin'' or DAM. Given the rarity of NM, isolation of native pigment from human brain in sufficient quantities required for most experimental procedures is difficult, thus DAM is used widely as an experimental model of NM (Nguyen et al., 2002; Offen et al., 1999, 1997; Stepien et al., 2000). Spectral studies suggest, however, that this model melanin differs structurally to the native pigment (Aime et al., 2000; Double et al., 2000b), making the validity of this model unclear.

In addition to the melanin backbone, NMR studies demonstrate that cholesterol and other uncharacterised lipid components are closely associated with NM granules (Double et al., 2000b; Zecca et al., 2000) and lipids may even be part of the polymer itself (Aime et al., 1994). A lipid component chemically bound to the NM macromolecule was also shown in a more recent study using pyrolysis–gas chromatography/mass spectrometry (Dzierzega-Lecznar et al., 2004). We have recently demonstrated that the primary lipid species associated with NM is dolichol, accounting for 14% of the pigment mass, while other hydrophobic compounds such as ubiquinone, ω-tocopherol and cholesterol together account for less than 0.5% of NM lipid mass (Fedorow et al., 2005). Electron microscopic studies suggest that lipids are not found in peripheral melanins in similar quantities (Moses et al., 1966).

A proteinaceous component makes up approximately 5–15% of the isolated NM molecule and has been suggested to represent an integral component of the polymer (Double et al., 2000b; Zecca et al., 2000). Peripheral melanins also have a bound proteinaceous component, which seems to have a regulatory role in binding and regulating melanin polymerisation (Sharma et al., 2002). Interestingly, the proteinaceous component of an in vitro enzymatically formed eumelanin was found to have the same amino acid composition as the enzyme used to synthesize it (Zeise, 1995), suggesting that the enzyme was trapped by the forming polymer. A similar phenomenon may contribute to the proteinaceous component of NM, thus characterisation of this protein or proteins may hold some clues to the synthesis and/or regulation pathways of NM.

4.2. Comparisons between melanin synthesis

Pioneering work on the formation pathway of eumelanin was performed by Raper (1927, 1928), who proposed an enzymatic action of tyrosinase on the substrate tyrosine, resulting in an insoluble, dark coloured compound. He proposed an oxygen-consuming reaction pathway beginning with L-tyrosine, which is oxidised via L-dopa to dopachinone, subsequently forming dopachrome after spontaneous cyclisation. For readers with an interest in this work, we recommend the most recent summary by Ito and Wakamatsu (2003). Succeeding the work of Raper, Prota showed for the first time that formation of alkali-soluble pheomelanin occurred on incubation of tyrosine and tyrosinase in the presence of cysteine (Prota et al., 1970). This discovery was a considerable contribution to the understanding of pheomelanin formation. More recently, molecular biology has had a significant part to play in the characterisation of the enzymes of the melanin synthesis pathway.

Melanogenesis begins with the amino acid L-tyrosine. The production of melanin by mammalian pigment cells involves a series of cellular events within a specialised organelle, the melanosome, and involves the enzymes tyrosinase, tyrosinase-related protein 1 (TRP-1) and tyrosinase-related protein 2 (TRP–2), as well as structural proteins (Hearing, 2000). Tyrosinase (EC 1.14.18.1; EC 1.10.3.1) (Prota, 1992) is a copper-containing membrane-bound glycoprotein located in melanosomes. Tyrosinase is the rate-limiting enzyme for melanogenesis and catalyses the oxidation of L-tyrosine via L-dopa to L-dopaquinone. The disproportionation of dopaquinone reforms L-dopa and, via spontaneous cyclisation, also results in the melanogenic precursor DHICA (Cooksey et al., 1997; Riley, 1997). Within the melanosome, melanin production and the ratios of eu- to pheomelanin are primarily regulated by the amount of tyrosinase present (Cooksey et al., 1997; Ozeki et al., 1997), although other regulating factors remain to be elucidated. Tyrosinase has a lower activity and expression during pheomelanogenesis compared with eumelanogenesis (Kobayashi et al., 1995).

Mammalian melanogenesis is regulated at multiple levels by over 127 distinct gene loci (Bennett and Lamoreux, 2003). This includes the regulation of migration of melanocytes during melanogenesis, as well as the regulation of melanin synthesis at cellular, within the organelle, and at enzymatic levels. At a cellular level, the main physiological factors that regulate the quantity and type of melanin are melanocyte-stimulating hormone (MSH), agouti signalling protein (ASP) and ultraviolet (UV) light. These factors modulate the expression of genes encoding the melanosomal enzymes. The MSH receptor is termed the melanocortin 1 receptor (MC1R) and is controlled by the agonist MSH and the agonist ASP in regulation of the eumelanin/pheomelanin switch. MSH results in the production of a greater proportion of eumelanin, while ASP stimulates the production of pheomelanin (Hearing, 2000). No hormones or receptors have been investigated for a role in NM synthesis.

During the maturation of melanin within a melanosome, the internal structure of the melanosome is at first visible but gradually becomes obscured by deposited melanin. Stages of the maturation of the melanosome have been investigated using electron microscopy and biochemical examination of isolation of subcellular melanocyte fractions (Prota, 1992; Seiji et al., 1961). The structural proteins and tyrosinase are incorporated into the premelanosomes in consecutive stages. The structural proteins are assembled in the smooth endoplasmic reticulum or multivesicular bodies connected to the rough endoplasmic reticulum. Concurrently, tyrosinase is synthesised and inserted into the endoplasmatic reticulum, leaving the Golgi apparatus by formation of small vesicles which transfer the enzyme into the premelanosomes, which already contain the structural proteins. The melanosome then gradually transforms into a uniformly dense particle with no visible structure. The stages of NM development have not been investigated in detail.

It has long been debated whether NM synthesis is enzymatically controlled, like all melanins in the periphery, or whether NM arises from a simple autoxidation process. In 1958, Foley et al. noted the presence of NM in the substantia nigra of albino individuals that stained negative for tyrosinase, the rate-limiting enzyme in the melanin synthesis pathway in the periphery (Foley and Baxter, 1958). Foley thus suggested that melanin within the brain formed in the absence of the tyrosine-tyrosinase enzymatic system. The involvement of dopamine as a precursor of NM was first suggested in 1963 (Vander Wende et al., 1963), and given the lack of evidence for enzymatic involvement in NM synthesis, it was later suggested that NM arose through simple autoxidation of this substrate (Mann and Yates, 1974; Rodgers and Curzon, 1975). Soon afterwards, suggestions appeared in the literature that NM was a waste product of catecholamine metabolism (Bogerts, 1981; Graham, 1979; Mann and Yates, 1983; Mann et al., 1977). However, if NM results from the autoxidation of dopamine it is pertinent to ask why NM doesn't have the same cellular distribution as dopamine.

Unlike cutaneous melanin, and melanin in other systems such as the retina, NM is not present during foetal development or at birth, but develops over the first few decades of life. An early descriptive study suggested that NM is routinely present in nigral dopaminergic neurons at the age of five (Fenichel and Bazelon, 1968) exhibiting the complex ultrastructure characteristic of the polymer in the adult brain (Moses et al., 1966). More recently, quantification of the concentration of NM in the substantia nigra measured biochemically demonstrated an increase over the lifespan (Zecca et al., 2002a), although the absolute number of pigmented neurons in the nigra is suggested to decrease from midlife by an average of 9.8% per decade (Ma et al., 1999). These data argue for increasing pigmentation in each neuron with age. This increase in intracellular NM with age may suggest that the pigment is indeed simply a cellular byproduct of dopamine metabolism formed via autoxidation and, in the absence of any mechanism to remove it, accumulates intracellularly throughout the lifespan.

There are several factors, however, that argue against the idea that NM is simply a byproduct of dopamine metabolism. Firstly, the lack of pigment in the infant brain and the restriction of the pigment to some, but not all, dopaminergic neurons in humans and the lack of pigment in the midbrains of many other species suggest that the formation of NM is unlikely to result simply as a consequence of dopamine metabolism. Secondly, the ultrastructure of NM granules is complex and comprises three distinct parts, including a high concentration of at least one molecule not usually found in these quantities in the human brain (Fedorow et al., 2005). These data point to NM synthesis being in some way a regulated process, although the intracellular pathways involved remain largely obscure.

Tyrosinase mRNA has been identified in both adult and embryonic mouse brain (Tief et al., 1996; Tief et al., 1998), although tyrosinase gene expression in mouse brain was not detected (Gimenez et al., 2003). The relevance of these findings for NM synthesis is questionable, however, as NM is not found in the mouse brain. Low levels of tyrosinase mRNA have been reported in human substantia nigra (Xu et al., 1997), but tyrosinase protein does not appear to be expressed in the human substantia nigra (Ikemoto et al., 1998). The current evidence thus suggests that tyrosinase does not appear to have a role in the synthesis of human brain NM. To date, no other enzyme system has been demonstrated to be involved in neuromelanogenesis, although studies have investigated enzymes as various as macrophage inhibitory factor, prostaglandin H synthase and peroxidase. A role for monoamine oxidase (MAO) has also been put forward, but the presence of this enzyme in the substantia nigra dopamine cells of the rat, another species that does not produce a coloured pigment, is not consistent with this hypothesis (Rabey and Hefti, 1990).

In the apparent absence of a role for tyrosinase in neuromelaninogenesis, the search for an enzyme associated with NM production has yielded no likely candidates to date.

It is noteworthy that PD patients treated with large quantities of L-dopa do not exhibit increased quantities of NM within their surviving nigral neurons as might be expected to be the case if NM represents a process of autoxidised dopamine. It has also been noted that non-pigmented human foetal dopaminergic cells implanted into the striatum of patients affected by PD as an experimental therapy show an adult level of pigmentation three years after implantation (Check, 2002). This observation suggests that some factor or factors within the adult, but not the foetal, striatum allow for, or stimulate, the development of NM.

Another important regulator of melanogenesis in the periphery appears to be the maintenance of a relatively acidic melanosomal environment (Brilliant and Gardner, 2001). The murine 'p' protein, the human homologue of which is known as 'P' protein, has recently been suggested to act as an anion pump to maintain melanosomal pH in the mouse (Brilliant and Gardner, 2001; Puri et al., 2000). Recent work on human amelanotic cells demonstrated that normal processing of tyrosinase can become dysregulated by changes in pH, thus modulating cell pigmentation (Watabe et al., 2004). The pH of NM granules has not yet been examined.

A factor known as *stablin* in melanoma cells is thought to prevent autoxidation of the precursors of melanin to melanin (Pawelek et al., 1992; Solano et al., 2000). In addition, ''novel'' proteins isolated from melanoma cells inhibit melanin polymerisation in vitro (Sharma et al., 2002). To date, however, it is unknown if *stablin*, or similar proteins are present in the brain or whether they play a role in NM synthesis.

4.3. Comparisons between melanin degradation and turnover

Within the lysosome, the ultrastructure of melanin granules disintegrate and non-melanin constituents of melanin undergo enzymatic degradation (Ohtaki and Seiji, 1971). However, the process by which the melanin itself is degraded has not been identified (Borovansky and Elleder, 2003). In the eye, oxidative degradation of melanin is suggested to be a mechanism by which the pigment is degraded (Sarna et al., 2003), and interestingly, it appears that melanin-associated proteins reduce the photoreactivity of melanin toward oxidisable substrates (Kayatz et al., 2001), suggesting a protective role. Correspondingly, exposure of NM to harsh oxidative conditions, such as light or hydrogen peroxide, results in the almost complete degradation of the melanin (Kayatz et al., 2001), although it is currently unknown if NM is degraded in vivo.

Another aspect of pigment degradation is cell turnover. Cutaneous melanin is contained in specialised cells called keratinocytes, the upper layers of which are continuously exuviated. Thus de novo melanogenesis is required to replace these cells. NM-containing neurons are, however, post-mitotic and do not divide. Given that NM-containing neurons in L-dopa treated PD patients do not appear to contain increased quantities of pigment, some system of pigment quantity control may exist. The recent observation that α-synuclein protein is entrapped within NM granules extracted from PD patients but not controls, supports this hypothesis (Fasano et al., 2003).

Although a possible pathway for NM degradation in vivo is unknown, it is interesting that the pigmented dopaminergic cells are characterised by a strongly oxidising neurochemical environment suggesting that oxidation of NM may also occur in vivo, possibly resulting in a slow breakdown of NM over time. In PD or after chronic toxin administration, glial cells, in addition to neurons in the substantia nigra, can also sometimes be seen containing NM, which is assumed to have been phagocytosed from dying neurons. The fate of NM in glial cells is also unknown (Forno, 1996; Langston et al., 1999), but these cells may possess a similar or alternative mechanism of degrading NM or may transport the pigment to another cell type for degradation. Recently it was reported that free NM activates microglia in vitro, leading these authors to suggest that NM released from dying neurons may contribute to degenerative processes within the parkinsonian brain via a chronic inflammatory process (Wilms et al., 2003). Activated NM-containing microglia found in the brains of PD and 1-methyl-4-phenyl-1,2,5,6 tetrahydropyridine (MPTP)-intoxicated patients post-mortem support this idea (Zucca et al., 2004).

5. Biological roles of melanins

In peripheral tissues, melanins are thought to function as endogenous mediators of oxidative mechanisms. Thus by analogy, NM may play a similar role within the brain (Double et al., 2002).

5.1. Skin melanins

The level of skin pigmentation significantly affects the incidence of skin cancer, and several of the properties of melanin are thought to be highly significant in providing this protective effect (Tadokoro et al., 2003). The most well-known characteristic of cutaneous melanin is its ability to absorb much of the visible and UV light spectrum (Riley, 1997). Melanin also acts as a physical shield to minimise the penetration of UV into the subcutaneous layers (Krol and Liebler, 1998). In vitro melanin inhibits UV induced lipid peroxidation suggesting that it has antioxidant properties, with this effect inhibited by iron (Fe III; Krol and Liebler, 1998). Melanocytes stimulated by exposure to UV light and heat (considered destructive stimuli) proliferate and produce more melanin, suggesting a photoprotective role for melanin (Nakazawa et al., 1998). There is little information on other non-photoprotective roles for melanin in the skin.

5.2. Eye melanins

Eyes lacking melanin are slower to adapt to light after periods of darkness, as shown in experiments comparing albino with control rats (Behn et al., 2003). This may be through a cellular antioxidant mechanism, consistent with its photoprotective role in the retinal pigment epithelial layer of cells (Sarna et al., 2003). It is known that certain drugs can induce oculotoxicity and melanin may be protective against some basic and lipophilic drugs (Leblanc et al., 1998). This protection is afforded as melanin can act as a depository through its physicochemical binding properties. Melanin has a postulated role in the development of the retina (Oetting, 2000) through an as yet undetermined cellular mechanism.

5.3. Melanins in peripheral organs

The appearance of hair derives from both the structural and colour properties of melanin. Hair originates from keratinocytes within the scalp, but these cells are no longer alive in hair outside the scalp. The functional role of melanin in hair derives from its structural rather than physicochemical properties. Melanin of the hair physically blocks sun from the scalp, and acts as a thermal insulator by trapping radiant heat (Van Neste and Tobin, 2004).

It is also known that the melanocytes in the stria vascularis of the inner ear maintain normal ionic composition in this tissue through the Na^+K^+-ATPase and potassium channels and are essential for hearing in mammals (Tachibana, 2001).

5.4. Neuromelanin

Although NM was traditionally considered to be an inert substance having no physiological role, this is not supported by recent investigations.

It has been suggested that synthesis of NM could play a protective role within the cell by preventing the accumulation of toxic catechol derivatives by incorporation into the polymer (Zecca et al., 2003). NM may play a further chemoprotective role by interacting with a variety of potentially damaging molecules such as pesticides (e.g., paraquat) and toxic compounds (e.g., 1-methyl-4-phenylpyridinium (MPP+), beta-carbolines and 1,2(*N*)-dimethal-6-7-dihydroxyisoquinolinium) (D'Amato et al., 1986; Lindquist et al., 1988; Naoi et al., 1994; Ostergren et al., 2004), and neuroleptics. As epidemiological research has linked pesticides to the development of PD (Lai et al., 2002), this implicates NM in the aetiology of neurodegeneration in this disease.

Another suggested role for NM is in binding metals, particularly potentially toxic cations such as iron, zinc, copper, manganese, chromium, cobalt, mercury, lead and cadmium (Zecca et al., 2002b). The ability of NM to bind a variety of metal ions seems to be shared by at least some other synthetic and natural eumelanins (Ben-Shachar et al., 1991; Liu et al., 2004), indeed the binding of metal ions to peripheral melanins in humans has been suggested to play a role in the transcutaneous excretion of these species (Szpoganicz et al., 2002). The interaction between iron and NM has been a focus of research for neurochemists, as a marked accumulation of iron related to disease severity is reported in the parkinsonian substantia nigra (Becker et al., 1995; Dexter et al., 1991; Jellinger et al., 1990; Mann et al., 1994; Riederer et al., 1989; Sofic et al., 1988). The cellular location of this apparent increase in iron is unclear but a variety of changes in iron regulatory systems, such as the major iron-binding protein ferritin, occur in PD (Berg et al., 2001). As ferritin is primarily located in glia, rather than neurons (Connor et al., 2003), it is unlikely that this protein could regulate neuronal iron levels. By binding metals, NM may potentiate free radical formation (Youdim et al., 1989) or assist with protective scavenging of hydroxyl radicals (Korytowski et al., 1995) via metal sequestration (Double et al., 2000a; Gibb, 1992; Youdim et al., 1994). Reductions in NM in PD are likely to render the cell more susceptible to oxidative damage. Indeed, the level of redox activity detected in NM-aggregates was significantly increased (+69%) in parkinsonian patients and was highest in patients with the most severe neuronal loss (Faucheux et al., 2003). This change was not observed in tissue in the immediate vicinity of melanized-neurones.

NM can also be considered as an endogenous iron-binding molecule in pigmented neurons (Ben-Shachar et al., 1991; Double et al., 2002, 2000a; Jellinger et al., 1992; Youdim et al., 1989; Zecca et al., 2001; Zecca and Swartz, 1993). In fact, iron-binding studies using Mössbauer spectroscopy show that iron sites in human NM are similar to human ferritin (Gerlach et al., 1995). Like ferritin, NM appears to bind iron in oxyhydroxy clusters in the ferric form, although the iron core in NM may be simpler, smaller and less regular than that in ferritin (Double et al., 2003). NM contains both high- and low-affinity iron-binding sites and Mössbauer studies suggest that additional iron is added to existing iron clusters in NM, analogous to the formation and growth of the ferritin iron core (Double et al., 2003). Like the melanin of *S. officinalis* (Liu et al., 2004), NM is proposed to be only partially saturated with iron in vivo, thus maintaining a residual chelating capacity to protect the substantia nigra against iron toxicity (Shima et al., 1997). NM may therefore play a physiological role in intraneuronal iron homeostasis. Support for this theory comes from changes in NM in the PD brain where significantly less iron is bound to NM than that seen in the normal brain (Bolzoni et al., 2002; Lopiano et al., 2000). This suggests that changes in iron-binding to NM result in increased levels of intraneuronal free iron and the subsequent cell damage observed in PD.

In addition to binding metals, NM also contains and interacts with proteins. Recent characterization of NM using ^{13}C NMR demonstrated that the spectral pattern of NM isolated from the PD brain exhibits a comparatively

decreased melanin signal than that measurable from control NM. This appeared to be due to the presence of a novel protease-resistant, lipoproteic material not seen in the healthy brain (Aime et al., 2000). In particular, recent work has shown that α-synuclein, one of the components of Lewy bodies, is covalently bound to NM in the parkinsonian brain (Fasano et al., 2003), suggesting an important interaction between NM and this lipoprotein. α-Synuclein is a lipoprotein thought to be important in intracellular trafficking of diverse proteins and lipids (Perez and Hastings, 2004). Indeed it has been suggested that in PD the fate of cytosolic dopamine is diverted from NM production and into alternative metabolic pathways resulting in increased levels of dopamine quinone production, which may stimulate a variety of potentially toxic events, including increased oxyradical formation and protein aggregation in these cells (Sulzer and Zecca, 2000) Further, it was recently demonstrated that NM is capable of compromising the activity of the ubiquiton-proteasome system (Shamoto-Nagai et al., 2004), a relevant finding in light of the proposed contribution of the ubiquitin-proteasome system to degeneration in PD. It will be important to determine all protein/NM interactions.

6. Conclusions

Although difficult to analyse, the chemical structure of both peripheral and central melanins has been significantly advanced. Many aspects of the normal biology of NM remain to be clarified, particularly the regulation of NM formation and turnover. For peripheral melanins, enzymatic synthesis and turnover is highly regulated. There is insufficient current evidence to support either enzymatic synthesis or simple autoxidation as the main pathways regulating NM formation. At present there is no information on NM turnover. The chemical properties of NM have now been sufficiently investigated to reveal some functions of this pigment. Although it is unlikely that NM is involved in the primary initiation of PD, the role of NM in iron binding and protein interactions may contribute to the progression of degeneration. Clearly, an increased understanding of the normal development of NM in the human substantia nigra and changes that occur in PD will advance our understanding of this disorder and may provide targets for the development of novel interventions or treatments for this disease.

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