DOI 10.1007/s00702-006-0450-5
J Neural Transm (2006) 113: 751–756

Journal of Neural Transmission
Printed in Austria

Functional effects of neuromelanin and synthetic melanin in model systems

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Received September 1, 2005; accepted February 24, 2006
Published online May 5, 2006;

Summary. The function of the dark polymer pigment neuromelanin found in catecholaminergic neurons of the human brain is not understood, especially as most published data are based upon a synthetic model melanin which differs structurally to the native pigment. Nevertheless human neuromelanin has been shown to efficiently bind transition metals such as iron, as well as other potentially toxic molecules. The pigment may have a protective function in the healthy brain by, for example, contributing to iron homeostasis within pigmented nuclei. We have demonstrated that synthetic dopamine melanin stimulates cell damage in both cell lines and primary cells in vitro, an effect associated with increased hydroxyl radical production and apoptosis. In contrast, at low iron concentrations native neuromelanin does not induce cell damage but rather protects cells in culture from oxidative stress. This protective function appears to be lost at high iron concentrations where neuromelanin saturated with iron functions as a source of oxidative load, rather than an iron chelator. Changes to neuromelanin and tissue iron load in Parkinson's disease may decrease the protective potential of the pigment, thus increase the potential for cell damage in this disorder.

Keywords: Melanin, neuroprotection, Parkinson's disease.

Neuromelanin (NM) is a complex polymer pigment found primarily in catecholaminergic neurons of the human substantia nigra and locus coeruleus. The pigmentation of the dopaminergic neurons of the human substantia nigra pars compacta appears to underlie the especial vulnerability of these cells in Parkinson's disease (Hirsch et al., 1988). Indeed the most striking pathological characteristic of Parkinson's disease is the relatively specific loss of the pigmented neurons in this brain region and the development of abnormal inclusion bodies primarily within the boundaries of the pigment. An understanding of the natural biology of this pigment and its role in the aetiology of Parkinson's disease will add much to our understanding of this disorder. Surprisingly however, until recently very little has been known about the pigment. Early work on NM in the 1960s was followed by a period of little activity for twenty years until a renewed interested in this molecule and its role in the neurodegeneration of Parkinson's disease stimulated a renaissance in research in this area.

While advances have been made in our understanding of this molecule we still do not understand the physiological function of NM. A useful approach to this problem has been to consider the roles of melanins in other body tissues. We have recently reviewed the comparative roles of melanin in the skin, eye and peripheral organs where, among other postulated roles, melanins are thought to function as endogenous mediators of oxidative mechanisms (Fedorow et al., 2005). By analogy, NM may play a similar role within the brain. Much published literature on the functional role of melanins over the last 15 years, either in peripheral or central tissues, is based however upon data collected using synthetic model melanins, rather than the native pigments. The reason for this is simple; model melanins can be quickly and easily produced in the laboratory while the native melanins are comparatively rare and difficult to isolate. Despite, or perhaps because of, a lack of understanding of the chemical structure of most native melanin pigments (Fedorow et al., 2005) there has been little interest to date in examining the validity of these synthetic melanins as models of the endogenous molecules. It is relevant however, to examine what has been published regarding the putative functional roles of neuromelanin to date. One possible function of the pigment is to prevent the accumulation of catechol derivatives produced as a result of the catabolism of dopamine by incorporating these potentially toxic species into the NM polymer (Zecca et al., 2003). The pigment also binds a range of potentially damaging exogenous molecules, such as pesticides, toxic compounds, (for example 1-methyl-4-phenylpyridinium) and neuroleptics (Fedorow et al., 2005) as well as interacting with some proteins. 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. The most widely discussed role of NM however, is its putative role in binding metals, particularly potentially toxic cations such as iron, zinc, copper, manganese, chromium, cobalt, mercury, lead and cadmium (Zecca et al., 2002). 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. 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 (Berg et al., 2001). A variety of changes in iron regulatory systems occur in Parkinson's disease (Berg et al., 2001). By binding metals, NM may potentiate free radical formation (Youdim et al., 1989). Indeed, the level of redox activity detected in NM-aggregates is reported to be significantly increased in parkinsonian patients and is highest in patients with the most severe neuronal loss (Faucheux et al., 2003). Alternatively, a NM-metal interaction is suggested to reduce hydroxyl radical production, perhaps via metal sequestration (Korytowski et al., 1995). We have shown that 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 *Sepia 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). This data suggests that NM plays a physiological role in intraneuronal iron homeostasis in the healthy brain. A protective role for NM within the vulnerable dopaminergic neurons of the human substantia nigra seems reasonable given the links indicated by epidemiological research between pesticide and transition

metals and the development of Parkinson's disease. A reduction in the amount of tissue pigment, such as that occurring in Parkinson's disease, or an intrinsic change in the pigment's iron-binding capacity in this disorder, may render the cell more susceptible to oxidative damage. While little is known about the status of the pigment in Parkinson's disease there is some data which suggests that NM pigment in the parkinsonian brain differs to that in the healthy brain (Bolzoni et al., 2002; Lopiano et al., 2000), although the consequences of these changes are yet to be investigated.

Despite the body of data which suggests a protective role for NM within the substantia nigra, other published data using a synthetic dopamine melanin (DAM) has not supported this idea. The addition of DAM to PC12 cells in culture resulted in significant cell death via apoptotic pathways (Offen et al., 1997) suggesting that DAM is intrinsically toxic. Biochemical data also suggests that under certain conditions DAM can stimulate free radical production (Pilas et al., 1988; Zareba et al., 1995). Given the demonstrated differences between the chemical structures of DAM and native NM pigment (Double et al., 2000) it is important to understand what facets of NM the synthetic pigment models, and whether the functional effects of the model pigment reflect those seen for the endogenous pigment. We have compared the functional effects of NM isolated from the human substantia nigra and DAM in a variety of in vitro systems. The addition of isolated human NM and DAM to the human-derived neuronal cell line SK-N-SH resulted in the incorporation of the melanins into the cytoplasm of these cells via phagocytosis, resulting in an in vitro model mimicking the endogenous pigmentation of the dopaminergic cells of the substantia nigra (Li et al., 2005). Similarly, the human-derived glial cell line U373 also incorporated the melanins, mimicking the phagocytosis of NM released from dying dopaminergic neurons in the parkinsonian brain. Using a concentration of 100 ng/mL of each melanin type we showed that DAM, but not NM, significantly increased the activity of lactate dehydrogenase in the neuronal cell line. DAM, but not NM, also massively increased the amount of lipid peroxidation measurable in the neuronal cultures. Further, spin-trapping experiments demonstrated that DAM, but not NM, significantly stimulated hydroxyl production in SK-N-SH cells and was associated with increased cell death via apoptotic pathways (Li et al., 2005). In contrast with a recent report in microglia (Wilms et al., 2003), neither melanin type induced any functional changes in the glial cell line, perhaps reflecting the apparent resistance of these cells to degeneration in the parkinsonian brain. The divergent functional effects of the native and synthetic melanins in the neuronal cell line were also seen in experiments in primary mesencephalic rat co-cultures. Functionally, the inhibition of ^{3}H-dopamine uptake in the primary cultures by increasing concentrations of DAM was significantly greater than that of NM. Total cell death in DAM-treated primary cultures was also significantly greater than that seen in cultures treated with NM. A detailed morphological analyses of the tyrosine hydroxylase immunoreactive cells within the primary cultures demonstrated that the morphology of the DAM-, but not NM-treated, cultures was indicative of cell damage consistent with apoptotic mechanisms (Li et al., 2005, Fig. 1). The lack of toxicity of NM seen in our cell culture models is consistent with the reported lack of toxicity of human NM injected into the rat substantia nigra in vivo (Aimi et al., 1996).

Of particular interest were experiments where the primary cells were cultured in the presence of a high oxidative stimulus created by the addition of Fenton reagent. While equivalent cell death occurred in the cultures treated with Fenton reagent alone or with DAM and Fenton reagent, cell death in cultures treated with NM and Fenton reagent was sig-

A	Control cultures	DAM-treated cultures	NM-treated cultures
Mean cell body area (mm^2)	$16.91 \pm 0.45 \times 10^{-5}$	$17.13 \pm 0.61 \times 10^{-5}$	$16.41 \pm 0.59 \times 10^{-5}$
Mean neurite number	2.96 ± 0.15	2.07 ± 0.14 $p<0.0001$	3.24 ± 0.19
Total neurite length (mm)	0.241 ± 0.01	0.10 ± 0.01 $p<0.0001$	0.24 ± 0.02
Total cell death (percent)	9.12 ± 0.58	15.11 ± 1.15 $p<0.0001$	9.20 ± 0.86

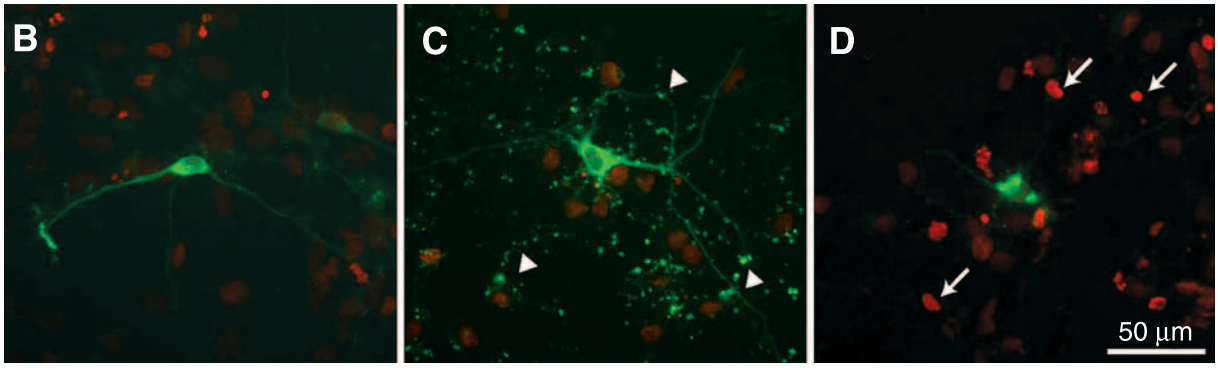

Fig. 1. **A** Morphological analyses of tyrosine hydroxylase-positive cells in primary rat mesencephalic cultures and percentage cell death within total cell number. P values given are compared with control values. **B** Typical TH-positive neuron in an untreated culture. **C** Typical TH-positive neuron in an NM-treated culture demonstrating normal morphology. Isolated NM, by virtue of its autofluorescence, can be seen lying close to the cell body and neurites (arrowheads). **D** Typical TH-positive cell in a DAM-treated culture demonstrating significantly abbreviated neurite formation. Small nuclei staining intensely with propidium iodide (arrows) were counted as dead cells. NM: 100 ng/mL neuromelanin, DAM: 100 ng/mL synthetic dopamine melanin. Incubation time was 48 hours. Data are mean values ± S.E.M from quadruplicate wells as described in Li and colleagues (Li et al., 2005)

nificantly less than that occurring in cultures treated with Fenton reagent alone, suggesting the presence of NM can significantly attenuate oxidative cell death (Li et al., 2005). The mechanism of this apparent protective effect of NM, but not DAM, is unclear but given we have previously demonstrated that NM has approximately ten-fold the iron binding capacity of DAM (Double et al., 2003), we suggest the iron chelating ability of NM represents a reasonable explanation for these findings (Li et al., 2005). While an apparent protective effect of NM demonstrated in our culture system might seem at odds with the proposal that iron binding by NM in vivo might have negative consequences for the substantia nigra in Parkinson's disease, there is also experimental evidence to support this viewpoint. We have shown that NM saturated with iron can stimulate significant lipid peroxidation of rat brain membranes in vitro (Double et al., 1999). Further, we have shown in a rat model that NM can act as a vehicle to introduce iron into the substantia nigra with subsequent dopaminergic cell death (Double et al., 2003). Thus a negative influence of NM upon the cell which contain this pigment appears to be dependent upon the amount of iron bound to the pigment, as originally suggested by Ben-Shachar and Youdim in 1990 (Ben-Shachar et al., 1990). While the origin of this increased iron is yet uncertain there is growing evidence for the importance of this phenomenon, if not as a primary trigger for the

disorder then as an important factor in the progressive nature of the neurodegeneration.

Considered together this body of data suggests that NM, unlike DAM, does not impinge negatively upon the pigmented nigral neurons in the healthy substantia nigra and may in fact play a protective role within these cells by virtue of its chelating abilities. DAM appears to be a poor model for the native pigment. In the diseased brain a localised increase in nigral iron concentrations may significantly increase the amount of iron bound to NM, potentially stimulating iron-mediated damage within these cells. Alternatively, or additionally, a change in the structure of NM in Parkinson's disease may decrease the ability of the pigment to chelate iron, thus increasing the potential for cell damage. Further studies on possible changes in the structure of NM in the parkinsonian brain are required to answer these questions.

Acknowledgements

The author is a R.D. Wright Fellow of the National Health and Medical Research Council of Australia.

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