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Dopamine-melanin is actively phagocytized by PC12 cells and cerebellar granular cells: possible implications for the etiology of Parkinson's disease

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Abstract

Neuromelanin in the substantia nigra may be associated with the pathogenesis of nigral cell death in Parkinson's disease. We used synthetic dopamine-melanin (DA-M) as a model compound for neuromelanin and examined its toxic effects on mice cerebellar granule cells and a rat pheochromocytoma cell line (PC12). The DA-M and dopamine-treated cells showed an accumulation of black deposits when examined by light microscopy. Electron microscopy revealed different stages of DA-M phagocytosis, starting with DA-M binding, engulfment of the particles and the formation of phagosomes located in the cytoplasm. Using absorption assays, we found that NaN_3 and low temperature inhibit the internalization of DA-M, pointing to an energy-dependent phagocytosis mechanism. These results suggest that neuromelanin can be phagocytised by neuronal cells which may thus be subjected to its toxic effects. These findings may contribute to our understanding of the formation and disposition of neuromelanin and its possible role in the etiology of Parkinson's disease.

Keywords: Parkinson's disease; Neuromelanin; Dopamine; Dopamine-melanin

In the human substantia nigra (SN), the oxidation products of dopamine polymerize, either enzymatically or via auto-oxidation, to generate neuromelanin [6,9]. The functions of neuromelanin in health and in the pathogenesis of Parkinson's disease (PD) are still an unknown. Neuromelanin may have some anti-oxidative activity and it was shown to inhibit UV and Fe-ascorbic acid-induced lipid peroxidation [4]. Conversely, neuromelanin, in the presence of transitional metals, generates reactive oxygen species (ROS) [10,11,18]. Anatomical studies in PD's SN demonstrate that the dorsal tier, containing high levels of neuromelanin, is less vulnerable than the ventral tier, which contains less neuromelanin and is more affected by the degenerative process [14]. In contrast, the high correlation observed between the percentages of surviving neurons in PD patients and their neuromelanin content, indicates that the heavily melanized neurons are more vulnerable [7,20]. Therefore, the question of whether neuromelanin plays a role in the pathogenesis of the dopaminergic neuronal loss, remains undetermined.

Spectroscopic studies have shown that synthetic dopamine-melanin (DA-M) and the natural nigral pigment have identical absorbance bands [2,8]. Thus, using synthetic DA-M as a model for neuromelanin, we recently found that it is highly toxic and causes typical apoptotic cell death in a neuronal-like PC12 cell line. Furthermore, we demonstrated that the damaging effect of DA-M can be partially inhibited by co-treatment with antioxidants [12]. In the present study, we focused on the morphological changes that take place following incubation of neuronal cultures with DA-M.

Mouse cerebellar granule cells were obtained from cerebella of eight-day old mice as described by Lasar and Zagon (12). The cells were plated at a density of 3×10^6 cells/ml on poly-L-lysine-coated 96-well tissue-culture plates. Rat pheochromocytoma PC12 cells were maintained as described previously (15) and subcultured to poly-L-lysine-

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Table 1
Dopamine and dopamine-melanin (DA-M) toxicity

	0.06 mg/ml DA (%)	0.3 mg/ml DA-M (%)
PC12 cells	33.4 ± 4	37.6 ± 4
Mouse cerebellar granule cells	42.0 ± 6	39.3 ± 5

Cell survival of PC12 cells and mouse CGC treated with synthetic DA-M (0.3 mg/ml) and dopamine (0.06 mg/ml) for 24 h. The percent of cell survival was measured by neutral red assay (mean ± SD, $n = 4$).

coated 96-well microtiterplates (Nunc), 100 μl of 5×10^5 cells/ml, in each well. Cell viability was measured by the Neutral red method.

DA-M was synthesized from DA and separated as described by Das et al. (2).

The transmission electron microscopy (TEM) study was done on culture cells fixed with 2.0% paraformaldehyde and 2.5% glutaraldehyde for 1 h at room temperature and post-fixed with 1% osmium tetroxide for an additional hour at 4°C, dehydrated in alcohol and embedded in Epon. Thin sections were stained with uranyl acetate and lead citrate and photographed using a JEOL 1200EX transmission electron microscope. Statistical significance was evaluated using the two-tailed unpaired *t*-test.

Viability of PC12 cells and cerebellar granule cells treated with synthetic DA-M (0.3 mg/ml) and dopamine (0.06 mg/ml) was markedly reduced, as shown in Table 1. Light microscopy revealed that black granules accumulated in the cytoplasm in the DA-M treated cells. In order to characterize this phenomenon, CGC were incubated for 3 h with DA-M, and the unbound DA-M was washed-out by centrifuge with 5 ml Histopaque (Pharmacia). The band containing the cells was lysed with water, and the supernatant was measured at 650 nm. As seen in Fig. 1, the lysates of cells incubated with DA-M at 37°C for 3 h showed absorption of 0.188 ± 0.040 OD (untreated cells were used as blank). This massive internalization of DA-M in the cells was found to be temperature-dependent since incubation of the cells with DA-M at a lower temperature (4°C), reduced the absorption by 76% ($P < 0.05$, Fig. 1). Addition of 0.1% sodium azide (NaN_3), a toxin that blocks ATP production, reduced the absorption by 94% ($P < 0.01$). These experiments indicate a temperature and energy dependent internalization process.

Electron microscopy of CGC and PC12 cells following DA-M treatment revealed different stages of DA-M phagocytosis. The DA-M treated cerebellar granule cells showed vesicle-enclosed electron dense DA-M aggregates in the cytoplasm, especially near the outer membrane (Fig. 2A). Different stages of DA-M phagocytosis were detected in the cells, starting with DA-M attachment to the cells, engulfment of the particles and formation of phagosomes in the cell periphery. The phagosomes contained clusters of DA-M aggregates of various sizes ranging from 0.5 μm for a single particle to multiple clusters measuring 3 μm (Fig. 2B). Some of the cells displayed typical apoptotic morphology, including formation of apoptotic bodies, chromatin condensation and membrane blebbing (Fig. 2C, upper cells), whereas others revealed an undamaged ultrastructure (Fig. 2C, lower cells).

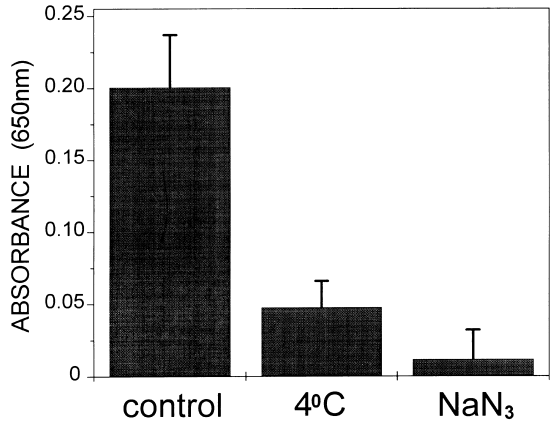


Fig. 1. Absorption of CGC lysates following DA-M treatment. Cells were treated for 3 h with 0.3 mg/ml DA-M at 37°C (control), 4°C and at 37°C in the presence of 0.1% NaN_3. Lysates of the washed cells were measured by spectrophotometer (650 nm) and the untreated cell lysates used as a blank. Bars represent mean ± SE ($n = 4$).

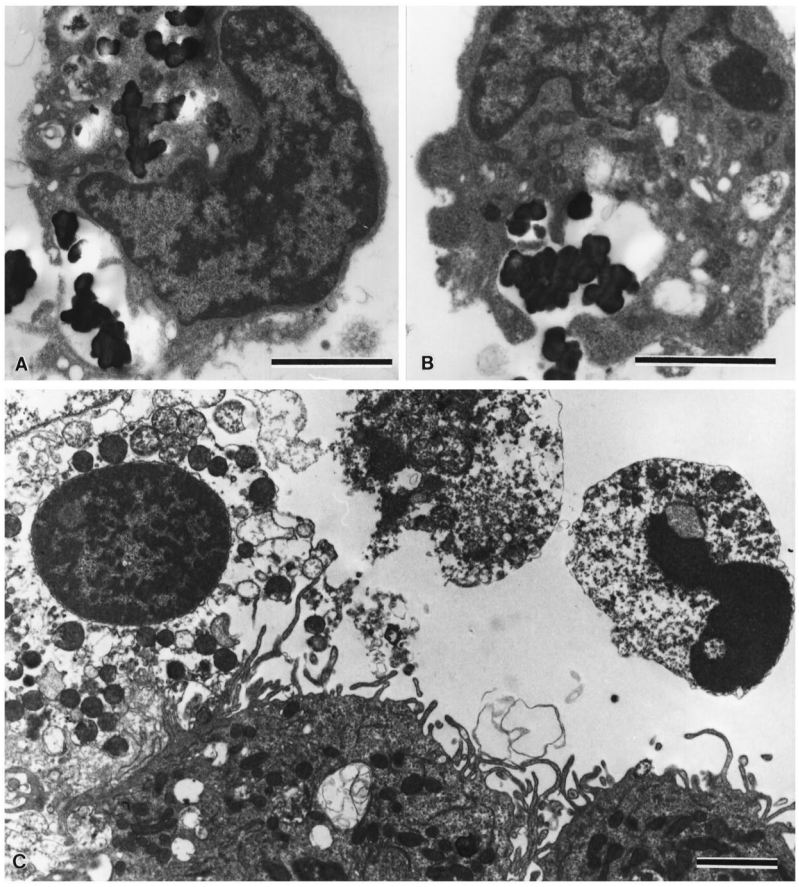
The neuronal-like PC12 cells treated by DA-M, demonstrated a similar degree of ultrastructural damage and cell death (Fig. 3A–C). In Fig. 3A, single DA-M particles are shown shortly after internalization in the periphery of the outer membrane while in Fig. 3B, clusters of DA-M particles are detected in large phagosomes. Fig. 3C shows the nucleus of a cell undergoing late stages of apoptotic death. Chromatin condensation is seen at the margins of the nuclear envelope, whereas the cytosol was completely lysed. Similar characteristic apoptotic features could be seen in both CGC and PC12 cells exposed to dopamine (0.5 mM for 24 h), but were not seen in untreated cells.

Our study shows that synthetic DA-M can be phagocytized by PC12 cells and CGC that contain more than 90% neurons [15]. The electronmicroscopy analysis demonstrated formation of typical phagosomes in the cytoplasm. Moreover, suppression of DA-M internalization by NaN_3 and temperature lowering indicates that this process of phagocytosis is typically energy- and temperature-dependent.

Fig. 2. Electron microscopy of CGC treated with DA-M. Different stages of DA-M phagocytosis were detected in the cells: engulfment of the particles and formation of phagosomes in the cell periphery (A,B) and cell death (C). Scale bar, 2 μm.

Fig. 3. Electron microscopy of PC12 cells incubated with DA-M. Phagocytosis of DA-M particles begins with engulfment of DA-M clusters (A,B) and formation of phagosomes (A). Cell death of the DA-M-effected cells seen in the upper portion of (C) in comparison to the intact cells seen in the lower part. Scale bar, 2 μm.

2



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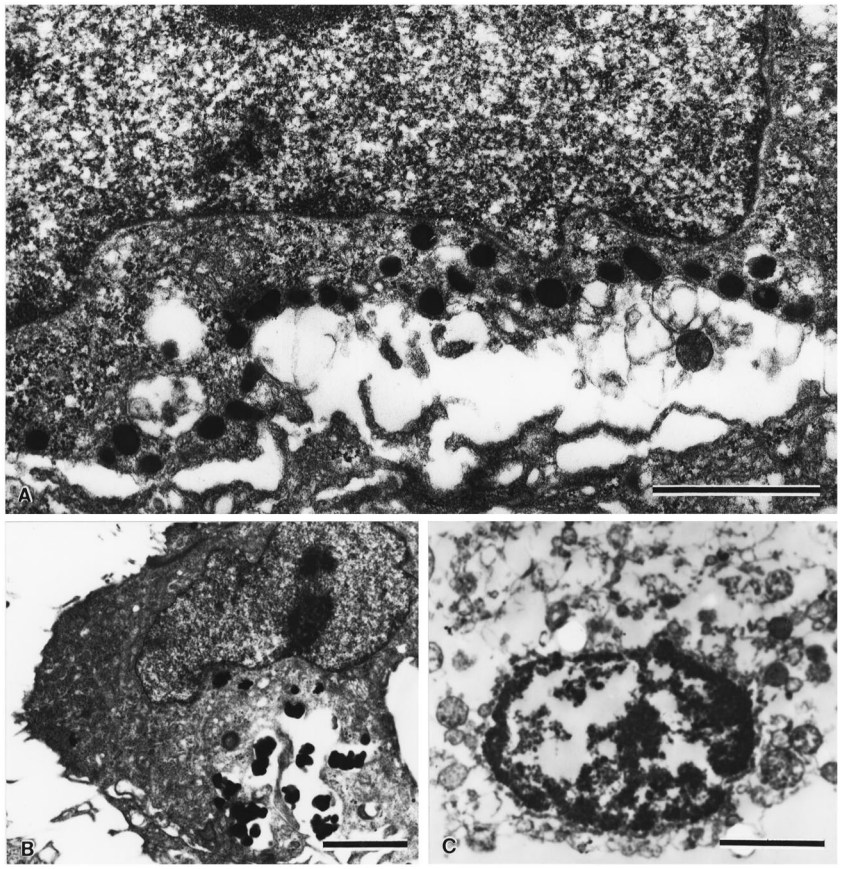

Natural neuromelanin is found mainly in the substantia nigra, where dopamine is produced. It has therefore been postulated that the pigment is an aggregate of the auto-oxidation product of endogenous intracellular or released extracellular dopamine, internalized into the cell through the dopamine re-uptake transport channels [13]. Our observations provide evidence for active DA-M internalization in neurons and suggest an additional (or alternative) process for the pigmentation in the SN. Thus, neuromelanin aggregates might also be derived from polymerized extracellular dopamine which is then phagocytized by the dopaminergic neurons. The hypothetical sources of extracellular DA-M may derived from dopamine that is released physiologically from the nerve terminals (axon, dendrites) or from a spillout from neurons damaged during a pathological process.

One of the interesting questions concerning neuromelanin in the normal SN, is whether it has physiological importance or it is a toxic by-product. Several in-vitro experiments demonstrate possible protective features of neuromelanin [4,11], however, many more observations indicate that it is a toxic product of the DA metabolism. Firstly, neuromelanin is not conserved during evolution: it appears only in humans and few other species (i.e. primates and dogs) [1,5]. Secondly, neuromelanin accumulates in adults, and is not found in infants [5,17,16]. Thirdly, overload of neuromelanin is neurotoxic both, in vitro and in vivo [1,7,15,16,19,20].

The reason for the accumulation of neuromelanin in the SN, but not in other brain nuclei containing dopaminergic neurons, is still a mystery. It can be postulated that this selectivity is a result of dopamine autooxidation, promoted by large quantities of iron which are present in the SN [18]. Alternatively, it might be a product of an unidentified biochemical pathway unique to the SN or some failure of the system to efficiently metabolize the dopaminergic neuromelanin [3]. Our experiments indicate that the neuromelanin phagocytosis, in vitro, is not specific for the dopaminergic neurons and it might be found in other neuronal cultures. Further in vivo experiments are needed to explain the selective accumulation of DA-M in the dopaminergic neurons.

In conclusion, our data suggest that phagocytosis of neuromelanin by neurons may be one of the pathways of accumulation of intracellular neuromelanin which then might promote selective neurodegeneration in PD. However, further studies are needed to confirm if neuromelanin phagocytosis indeed occurs in the human substantia nigra by the dopaminergic neurons.

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