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Neuromelanin-bound ferric iron as an experimental model of dopaminergic neurodegeneration in Parkinson's disease☆

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Abstract

This article briefly reviews findings from studies on neuromelanin (NM)-bound ferric iron, which provide unique insights into the physiological functions of NM and possible pathophysiological mechanisms underlying dopaminergic neuronal cell death in Parkinson's disease (PD). NM is considered an endogenous iron-binding molecule of pigmented neurons and is believed to play a physiological role in intraneuronal iron homeostasis. In PD, where nigral iron levels are increased, saturation of high-affinity iron-binding sites on NM may overwhelm the protective capacity of this molecule, leading instead to an increase in redox-active iron, and subsequent cellular damage both *in vitro and in vivo*. Available data also suggest that the iron released from NM affects the ubiquitin−proteasome system in mitochondria, leading to the failure to clear proteins such as α-synuclein and to the development of abnormal α-synuclein-immunopositive Lewy bodies that contribute to dopaminergic nerve cell death in PD.

NM-bound ferric iron mimics certain characteristic features of the human disease *in vitro* and *in vivo* (face validity), in conformity with the theoretical rationale for PD (construct validity) and predicts aspects of PD behaviour and neurobiology (predictive validity) that makes it a valid experimental model with which to study the mechanisms of dopaminergic neurodegeneration in PD.

Keywords: Parkinson's disease; Animal models; Cell culture; Neuromelanin; Iron; Proteasome

1. Introduction

Neuromelanin (NM) is a dark polymer pigment produced by specific populations of catecholaminergic neurons in the brain such as the substantia nigra (SN) and the locus coeruleus. It appears in greatest quantities in the human brain and in lesser amounts in some other non-human primates, but it is absent from the brain of lower species [1]. Interest in this pigment has seen resurgence in recent years because of a hypothesized link between NM and the particular vulnerability of NM-containing neurons of the SN pars compacta (SNpc) to cell death in Parkinson's disease (PD) [2]. Indeed, the most striking neuropathological characteristic of PD is the relatively specific loss of the pigmented neurons of the SNpc and the resulting pallor of the midbrain, as well as the development of abnormal α-synuclein-immunopositive inclusion bodies within the boundaries of the pigment [3,4].

Histopathological, biochemical and *in vivo* brain imaging techniques have revealed a consistent increase of SN iron in PD [5−7]. Interestingly, the increased iron was shown to be restricted to the SNpc and occurs in the ferric (Fe^{3+}), rather than ferrous (Fe^{2+}), form of the metal [8]. 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 [5−7]. As ferritin

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is primarily located in the glia, rather than in neurons, it is unlikely that this protein could regulate neuronal iron levels. NM, an abundant iron-binding molecule, is thought to act as an endogenous iron-binding molecule in pigmented neurons [9]. It may therefore play a physiological role in intraneuronal iron homeostasis. Support for this theory has come from the observation that the PD brain has significantly less iron bound to NM than the normal brain [10]. This suggests that changes in iron-binding to NM result in increased levels of intraneuronal free iron with the subsequent cell damage observed in PD.

This article briefly reviews findings showing that NM-bound ferric iron represents an iron pool that, under certain circumstances, can be released extracellularly and that, by interacting with free-radical-producing pathways and the ubiquitin–proteasome system, may ultimately lead to dopaminergic nerve cell death. NM-bound ferric iron can therefore be used as an experimental model for studying the mechanism leading to dopaminergic neurodegeneration in PD.

2. Effects of neuromelanin on free-radical-producing mechanisms *in vitro*

NM isolated from the human SN significantly decreased membrane damage in rat cortical homogenates *in vitro* as measured by lipid peroxidation [11]. When NM was added together with ferrous iron that catalyses the generation of hydroxyl radicals via the Fenton reaction, the amount of lipid peroxidation measured was significantly less than that induced by ferrous iron alone. These results support the hypothesis that NM protects the cell membrane from free-radical-induced damage either by oxidizing ferrous to ferric iron that can be bound by NM, or inactivation of free hydroxyl radicals. Indeed, in iron-binding studies using NM isolated from the human SN, we demonstrated that NM contains high-affinity ($K_d = 7.18 \pm 1.08$ nM) and low-affinity binding sites ($K_d = 94.31 \pm 6.55$ nM) for ferric iron [12].

In contrast, when ferric iron-saturated NM was added to the membrane homogenate, cell damage was increased to 264% of that induced by NM alone; this damage was attenuated by the addition of the iron chelator desferrioxamine (DFO) [11]. In summary, these results support the hypothesis that NM can have a protective influence on the cell, but can be detrimental when iron levels rise above a certain level, leading instead to an increase in redox-active iron, and subsequent cellular damage.

Isolated human NM consists of 2.8% iron as estimated by Mössbauer spectroscopy [13], while the concentration of ferric iron in the SN has been determined using electron paramagnetic resonance at 11.3 μg iron/mg isolated NM [14]. Our Mössbauer spectroscopy data demonstrated also that the iron bound to NM is purely ferric iron bound to ferritin-like oxyhydroxide clusters [13]. This NM increased the formation of hydroxyl radicals as determined fluorometrically by the use of 2′,7′-dichlorodihydrofluorescein diacetate in mitochondrial homogenate isolated from the human dopaminergic neuroblastoma cell line SH-SY5Y, but synthetic dopamine melanin (DAM) did not [15]. Superoxide dismutase and DFO completely suppressed this increase, indicating that superoxide radicals produced by a Fenton-mediated reaction have a central role. This suggests that NM-bound ferric iron is released and reduced to ferrous iron, most likely by electrons produced by the mitochondrial respiratory chain.

3. Effects of neuromelanin in cell culture systems

NM and DAM are incorporated into both the glial and the human-derived dopaminergic neuronal cell lines by a phagocytosis-like mechanism [16]. Consistent with the physiological functions of glial cells, both melanins were incorporated by a larger number of U373 cells than by SK-N-SH cells, although equal concentrations of NM and DAM were incorporated within each individual cell. In contrast to the incorporation of DAM into mouse cerebellar granule cells [17], we could not detect membrane-bound vesicles containing melanin within our SK-N-SH or U373 cells at the electron microscope level [16]. This is consistent with the lack of a persistent membrane-bound organelle associated with NM granules reported in the human midbrain [1].

Consistent with previous reports [17,18], we demonstrated a significant toxicity of synthetic DAM via apoptotic mechanisms, other than caspase-dependent signalling [16], by using biochemical and morphological analyses of the neuronal SK-N-SH cell line and primary rat mesencephalic cultures. In contrast, exposure to the native pigment did not stimulate indices of cell damage or death. Indeed, evidence from primary cultures was suggestive of a protective role of NM under conditions of high oxidative load. Our data provide new evidence for a physiological role of NM *in vivo*, and highlight the caution with which data based on model systems such as DAM, which may not accurately represent the complexities of the human brain, should be interpreted.

Under the conditions of our cell culture experiments, both NM and DAM were unable to attenuate the toxic effects of an added oxidative stimulus, including a total of 224 μg iron/mg NM. Measurements of iron contained in iron-laden NM by atomic absorption experiments suggest that NM may be saturated at 100 μg iron/mg NM; thus the added iron probably exceeded the chelating capacity of the added NM. The iron chelator DFO effectively reduced Fenton-induced indices of cellular damage, suggesting a pivotal role of iron in the observed toxicity. In contrast, in the primary culture experiments, 17 μg iron/mg NM was added, a concentration under the apparent saturation threshold of the pigment. In these experiments, Fenton-induced cell death was significantly attenuated by NM, but not by DAM. While the mechanism for this apparent protective effect is unclear, we have previously demonstrated that NM has approximately 10-fold the iron-binding capacity of DAM [12], suggesting that chelation of iron by NM represents a reasonable explanation for the efficacy of NM, but not of DAM, to attenuate Fenton-induced cell death in cellular systems. Interestingly, in our system, Fenton-associated cell death – apparently as a NM-mediated protective effect – was expressed in the non-dopaminergic cell population; the data are nevertheless consistent with a possible protective role of NM *in vivo*.