

Dark microglia: Why are they dark?

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ABSTRACT

Using transmission electron microscopy (TEM) we recently characterized a microglial phenotype that is induced by chronic stress, fractalkine receptor deficiency, aging, or Alzheimer disease pathology. These 'dark' microglia appear overly active compared with the normal microglia, reaching for synaptic clefts, and extensively engulfing pre-synaptic axon terminals and post-synaptic dendritic spines. From these findings we hypothesized that dark microglia could be specifically implicated in the pathological remodeling of neuronal circuits, which impairs learning, memory, and other essential cognitive functions. In the present addendum we further discuss about the possible causes of their dark appearance under TEM.

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Introduction

Using immunohistochemical TEM we recently uncovered the existence of an ultrastructurally distinct microglial phenotype that is predominantly associated with pathological states.¹ These cells are rare under steady state conditions, but become prevalent upon chronic unpredictable stress, aging, or Alzheimer disease (AD) pathology, accounting for almost one third of the normal microglial population in APP-PS1 mice. We named these cells 'dark' microglia because of their characteristic dark appearance under TEM.¹

The physiological significance of these dark microglia remains to be elucidated, but they appear extremely active. They frequently reach for synaptic clefts, while extensively encircling axon-terminals, dendritic spines, and entire excitatory synapses with their highly ramified and thin processes. They strongly express CD11b, which forms the complement receptor 3 involved in developmental pruning,² as well as myeloid-cell specific TREM2 when associated with the amyloid- β plaques.¹ In AD pathology, TREM2 positive cells were recently shown to express the phagocytic effectors MERTK and AXL.³ These findings suggest that dark microglia could represent a subset of cells that become stressed as a result of their hyperactivity under adaptive pressure, leading to abnormal (or perhaps specialized) interactions with synapses.

Below we discuss about the possible causes of their darkness, considering what is known about 1) how contrast is generated in biological samples prepared for TEM, and 2) the literature describing at the ultrastructural level changes in the electron density of cells across functional states.

Contrast in transmission electron microscopy

The contrast in TEM is generated by scattering of the electrons that interact with the specimen.⁴ The 2 factors that determine this scattering are the thickness of the sample (more electrons are scattered in thicker samples) and its molecular composition: the higher the atomic number, the more scattered are the electrons, and the greater is the contrast. Since thickness of the ultrathin tissue sections examined by TEM is very uniform, as they are generated at high precision with an ultramicrotome, their contrast primarily results from the nature and concentration of the cellular constituents being visualized. Considering that biological tissues are composed of atoms of low atomic number, mainly carbon, oxygen, nitrogen, and hydrogen, contrast is greatly enhanced by their staining with heavy metals.⁴

The dark microglia we uncovered typically display an electron-dense cytoplasm and nucleoplasm giving them a 'dark' appearance in electron microscopy. Our

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observations were made in brain tissue fixed with acrolein (or glutaraldehyde) and paraformaldehyde, post-fixed with osmium tetroxide and embedded in a hydrophobic plastic resin as required for ultrathin sectioning. Whether these steps aimed at altering the tissue-constituents to prevent autolysis and decay through the action of micro-organisms, as well as the distortions obtained by tissue processing, could result in their dark appearance is analyzed below.

Neither lipids nor carbohydrates are essential for cohesion of the protoplasm and nucleoplasm. For this reason, tissue fixation primarily stabilizes proteins constituting the framework of its cells. Aldehydes mainly link proteins, while osmium tetroxide acts both on lipids and proteins. In addition, the rapid penetration and cross-linking abilities of acrolein quickly stabilize proteins, preserving even small peptides which can be successfully immunolabeled.^{5,6} The fixatives that we used in our preparation for TEM are considered “non-coagulant” fixatives, given that gels of membranous and fibrous proteins retain their original appearance under their action. Osmium tetroxide also renders proteins non-coagulable by ethanol,⁷ a solution used with ascending concentrations to dehydrate our samples prior to embedding in resin.

Osmium tetroxide oxidizes the fatty acid's unsaturated double bonds and gets reduced to a black metallic osmium which is electron dense and adds contrast to especially lipids.⁸ Unsaturated fatty acids are considered to have a high affinity for osmium tetroxide.⁹ Osmium tetroxide also links proteins, especially the oxidizable moieties of lysine, arginine, histidine, proline and tryptophan's amino acid chains.¹⁰ This explains the differing affinity of this heavy metal for various organelles. Friend and Brassil suggested steroid biogenesis to be, at least partially, responsible for the intense osmium staining of mitochondria, the endoplasmic reticulum and the Golgi apparatus.¹¹ Biological membranes enclosing cells and organelles are typically delineated by the metallic osmium. Structures enriched in proteins for instance the nuclear heterochromatin, which has a high concentration of histones (and DNA) similarly show an increased electron density.¹² In addition, osmium straining is considered to result from the bonding to hydrogen of the reduced osmium forms. Ferrocyanide-reduced osmium fixation,¹³ a modification of the conventional glutaraldehyde-osmium tetroxide fixation technique developed by Karnovsky, indeed provides contrast enhancement, allowing a better delineation of cellular boundaries and visualization of glycogen.^{14,15}

Together, these pieces of information suggest that the dark microglia's electron density might result from the particular nature and distribution of their cytoplasmic

and nucleoplasmic proteins and lipids, especially those having high affinity for osmium tetroxide.

An image of cellular phenotypes?

The contrast in biological samples observed under TEM is not only determined by the structural constituents of the cells being observed, but also by their phenotypic transformations.

Various changes in cellular composition following tissue damage are evidenced under TEM, and are reflected by modifications in the electron density of cells. It was recently demonstrated that after ischemic stroke, perivascular astrocytes are filled by glycogen granules of small sizes (~20 nm), with their cytoplasm changing from a characteristic electron lucent appearance to a denser and punctiform aspect.¹⁶ Astrocytes also display well-defined morphological alterations under TEM in the traumatic human edematous cerebral cortex, where they can be differentiated by the electron density of their cytoplasm, becoming either dense reactive hypertrophic astrocytes characterized by an electron lucent cytoplasm, dilated endoplasmic reticulum, and the appearance of swollen mitochondria, or phagocytic astrocytes engulfing degenerated presynaptic endings and remnants of dead nerve cells.¹⁷ Oligodendrocytes also dramatically change their morphology following injury. In human, it was reported that oligodendrocytes acquire distinct ultrastructural properties upon traumatic brain edema, adopting phenotypes that were designated as reactive, anoxic-ischemic, or hypertrophic phagocytic. The post-traumatic reactive type displays enlargement of their endoplasmic reticulum, accompanied by increased numbers of free ribosomes and swollen mitochondria, while hypertrophic phagocytic oligodendrocytes display lobulated nucleus, swollen mitochondria, dense inclusion bodies and increased numbers of free ribosomes, all clearly distinctive under TEM.¹⁸

The dark microglia's electron density was accompanied by endoplasmic reticulum dilation which is the best characterized sign of oxidative stress at the ultrastructural level.¹⁹ There was also mitochondrial disruption and a nearly complete loss of heterochromatin pattern, especially in the context of amyloid- β pathology. Oxidative stress is a condition where the levels of toxic reactive oxygen or nitrogen species are higher than the levels of counteracting antioxidant molecules, caused by elevated production of free radicals or decreased antioxidant activity.²⁰ The excessive reactive species generated have detrimental effects on nucleic acids, lipids and proteins, leading to cellular dysfunction due to disrupted physiological processes and metabolic pathways.²¹ Oxidative stress as a result of psychological stress, infection, lesion,

toxicity or aging is accompanied by distinctive ultrastructural changes. Aside from endoplasmic reticulum dilation, one of the earliest manifestations of oxidative stress in several cell types that include glial cells and aging red blood cells, is the rapid and substantial decrease in cell volume, likely affecting a range of cellular functions.^{22,23} Oxidative stress is causally linked to the induction of apoptosis,²⁴ but can also be rescued depending on the organism's interaction with its environment. Cells undergoing apoptosis typically display shrinkage, blebbing, rounding and fragmentation of their nucleus, as well as condensation and margination of their heterochromatin. By contrast, necrosis refers to cellular death associated with the loss of control of ionic balance, uptake of water, swelling, and cellular lysis.²⁵

The dark microglia display ultrastructural features of cells undergoing oxidative challenge. In addition to their electron-dense cytoplasm and nucleoplasm, they have a dilated endoplasmic reticulum and loss of heterochromatin pattern. The cellular shrinkage induced by oxidative stress likely induces condensation of their cytoplasmic and nucleoplasmic contents, including lipids and proteins visible with osmium tetroxide, which could explain why the dark microglia are dark. Previously, 'dark' neurons and oligodendrocytes were also documented,^{26,27} notably by Peters.^{26,27} Although dark microglia show several features of oxidative stress, they do not appear to be apoptotic considering their lack of blebbing, rounding and fragmentation of their nucleus, and neither necrotic cells given their overall ultrastructural characterization. The dark microglia abundantly contact synapses with their highly ramified processes, which most probably reflect an extreme phagocytic or stripping activity.

Disclosure of potential conflicts of interest

No potential conflicts of interest were disclosed.

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