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REVIEW

# Neuronal pigmented autophagic vacuoles: lipofuscin, neuromelanin, and ceroid as macroautophagic responses during aging and disease

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**Abstract**

The most striking morphologic change in neurons during normal aging is the accumulation of autophagic vacuoles filled with lipofuscin or neuromelanin pigments. These organelles are similar to those containing the ceroid pigments associated with neurologic disorders, particularly in diseases caused by lysosomal dysfunction. The pigments arise from incompletely degraded proteins and lipids principally derived from the breakdown of mitochondria or products of oxidized catecholamines. Pigmented autophagic vacuoles may eventually occupy a major portion of the neuronal cell body volume because of resistance of the pigments to lysosomal degradation and/or inadequate fusion of the vacuoles with lysosomes. Although the formation of autophagic vacuoles via macroautophagy protects the neuron from cellular stress, accumulation of pigmented autophagic vacuoles may eventually interfere with normal degradative pathways and endocytic/secretory tasks such as appropriate response to growth factors.

**Keywords:** Batten, LC3, L-DOPA, lysosome, mitochondria.

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Mammals possess a limited chromatic palette compared with the hues displayed by plants, birds, fish, crustaceans, and bacteria. Our only pigment synthesized via a specific enzymatic pathway is the melanin (*melania* signifies a black pigment and *melancholia* the mood resulting from black bile) that is responsible for the coloration of eye, hair, and skin. This substance is a classically labeled eumelanin (*true* melanin) if dark brown or black or pheomelanin (*dusky* melanin) if reddish because of its higher sulfur content.

To chemists, melanins are polymers in mammals that are mostly composed of indolequinone and dihydroxyindole carboxylic acid. To cell biologists, melanin is the pigment produced in melanocytes from L-DOPA by tyrosinase within 'melanin granules', a specialized lysosome (Orlow 1995). These lysosomes are secreted from the melanocytes and then endocytosed by the pigmented cells. A variety of depigmentation diseases, such as ocular albinism, occur when steps in these lysosomal functions are defective. Nevertheless, though tyrosinase is expressed at low levels in the brain, no melanin has been reported to be associated with the CNS other than in the non-neuronal retinal pigment epithelium (Gimenez *et al.* 2003).

Mammals, however, display additional pigments associated with aging and disease that are not produced in specialized cells via specific enzymes, but rather as byproducts of the macroautophagy pathway, in which autophagic vacuoles (AVs) engulf intracellular components to later fuse

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*Abbreviations used*: AV, autophagic vacuoles; ERK, extracellular signal-regulated kinase; GFP–LC3, green fluorescent protein–LC3; LF, lipofuscin; mTOR, mammalian target of rapamycin; NCLs, neuronal ceroid lipofuscinoses; NM, neuromelanin; PD, Parkinson's disease; VMAT2, vesicular monamine transporter 2.

with lysosomes in order to degrade their constituents. These pigments, known as neuromelanin (NM), lipofuscin (*dark fat*) (LF), and ceroid (*waxy*) are undigested components within AVs that accumulate over a lifetime.

## Nomenclature

We have recently passed the fiftieth year of research on lysosomes: the term was introduced by De Duve *et al.* (1955) for presumed organelles in fractions with acid phosphatase activity (De Duve *et al.* 1955). The corresponding morphologic structures were shortly after identified by his collaborator Alex Novikoff (Novikoff *et al.* 1956). A classical definition of lysosomes was 'membrane-delimited bodies that contain a characteristic set of acid hydrolases and are capable of participating in intracellular digestion' (Holtzman 1989). Whereas additional roles have been ascribed to lysosomes, their principal occupation is to digest extracellular (heterophagic) and intracellular (autophagic) components, and they provide the dominant means of degrading long-lived proteins.

The term 'autophagy' is used specifically for the lysosomal degradation by a cell of its own components. One form, macroautophagy, in which AVs accumulate cytoslic components including proteins, lipids, and nucleic acids for degradation, provides the only means by which a cell can degrade its own large organelles such as mitochondria. Microautophagy, an AV-independent pathway best characterized in yeast, can envelope smaller organelles and components into existing lysosomes; peroxisomes can be degraded by either macro- or microautophagy (Sakai *et al.* 2006). Chaperone-mediated autophagy is another AV-independent process in which specific proteins are directly transported into the lysosome (Massey *et al.* 2006a).

We refer to the constituents of pigmented AVs as the pigment and the organelles as AVs rather than the classical terms NM or LF granules, which refer to an organelle's resemblance to fine scattered dust in early microscopy studies (Carmichael 1989). AVs refer to all degradative vacuoles that arise via macroautophagy, regardless of whether they are yet to fuse with mature lysosomes, in which case they are often labeled autophagosomes, or if they have accumulated endosomal or lysosomal components via organelle fusion, in which case they are called amphisomes and autophagolysosomes, respectively. Whereas pigmented AVs contain lysosomal constituents, individual pigmented AVs could exist at various stages, *i.e.* with or without multilamellar membrane, an acidic pH gradient or lysosomal hydrolases, particularly when present in neurites that do not have local mature lysosomes available for AV fusion.

## Macroautophagy and the AV life cycle

Numerous forms of cellular stresses activate macroautophagy, and increased AVs are associated not only in aging and ceroid disorders but also in Alzheimer's (Adamec *et al.* 2000), Parkinson's (Anglade *et al.* 1997), and Huntington's (Kegel *et al.* 2000; Petersen *et al.* 2001) diseases, familial diabetes (Davies and Murphy 2002), methamphetamine toxicity (Larsen *et al.* 2002), prion disease (Boellaard *et al.* 1991), and traumatic brain injury (Clark *et al.* 2008).

Macroautophagy consists of steps highly conserved from yeast to mammals, than in mammals include (i) a triggering via inhibition of mTOR (*mammalian target of rapamycin*) or regulation of insulin receptor substrate–2 or extracellular signal-regulated kinases (ERKs) that activates the Vps34/beclin complex to promote the formation and elongation of the membrane forming the AV, (ii) sequestration of cytoplasmic content for degradation, and (iii) acidification and fusion of the AV with the lysosome for proteolytic degradation of its content by lysosomal hydrolases.

### Activation of macroautophagy

mTOR activity normally inhibits macroautophagy, and when it is inhibited by rapamycin, macroautophagy is strongly activated in cells including neurons (Ravikumar *et al.* 2002). AV formation by rapamycin is independent of *de novo* transcription and translation, indicating that all of the required components are present and simply await an appropriate trigger. mTOR-independent AV induction pathways in neurons include an activation via insulin receptors that triggers p38 (Yamamoto *et al.* 2006) and an ERK2 pathway identified in dopamine neurons (Zhu *et al.* 2007).

Initiation and elongation of the AV membrane requires phosphoinositide 3 kinase (a step inhibited by 3-methyladenine and wortmannin) and Atg8/LC3 activity. Cytosolic Atg8/LC3 is proteolytically cleaved and then lipidated to a form associated with the AV membrane known as LC3-II (Kabeya *et al.* 2000). A green fluorescent protein green fluorescent protein–LC3 (GFP–LC3) fusion protein is now widely used to visualize AVs in living cells by the appearance of GFP-LC3-II labeled puncta (Kabeya *et al.* 2000). LC3-II dissociates from the AV and/or is degraded following lysosomal fusion.

Macroautophagy can be enhanced by blocking the other main degradative pathways, the proteasome/ubiquitin system, and chaperone-mediated autophagy (Pandey *et al.* 2007; Massey *et al.* 2006b). For example, dopamine-modified α-synuclein can block chaperone-mediated autophagy, resulting in a compensatory activation of macroautophagic degradation in AVs (Martinez-Vicente *et al.* 2008), a step that could contribute to NM AV synthesis.

### Sequestration of cytoplasmic content

Although macroautophagy was long considered a purely non-specific bulk degradation pathway, recent results suggest specific recognition of some cargo. Preferential targeting of

 

proteins to AVs may occur by a form of polyubiquitination in which ubiquitin subunits are linked via their K63 residues, in contrast to polyubiquitination at the K48 site which confers delivery of a substrate to the proteasome (Tan *et al.* 2008). Alternative ubiquitination may explain how aggregates of proteins such as mutant forms of huntington are accumulated in AVs (Tan *et al.* 2008).

Mitochondria may have means for specifically targeting macroautophagic degradation (Tolkovsky *et al.* 2002), which would be particularly important for the health of long axons and distal terminals. 'Mitophagy' of damaged and dysfunctional mitochondria can undergo degradation by an ERK2-dependent signaling pathway independent from mTOR (Zhu *et al.* 2007). There may be multiple substrate recognition/targeting mechanisms that target additional damaged organelles and pigment components to AVs, serving both for degradation and to sequester reactive catecholamine and lipids away from the cyotosol.

### Degradation by AVs

In non-neuronal cells, AVs acquire lysosomal hydrolases within several minutes of formation via fusion with a lysosome (Dunn 1990). Efficient acidification of the AV to provide an effective environment for lysosomal hydrolase function is critical for degradation. The acidification can be observed using weak base fluorescent labels, particularly monodansylcadaverine (Biederbick *et al.* 1995; Bampton *et al.* 2005). Measuring macroautophagic degradation, however, requires a pulse-chase design rather than morphologic analysis as AVs can accumulate either because of more induction or inhibition of their disappearance; indeed, both steps seem to occur with pigmented AVs. Macroautophagic degradation of protein can be measured with specific inhibitors such as 3-methyladenine (Talloczy *et al.* 2002, 2006).

## Lipofuscin

### LF AVs

The yellow/brown autofluorescent pigment LF, also known as *age pigment*, is widely distributed throughout the animal kingdom and has been called 'the most consistent and phylogenetically constant cellular morphologic change of the normal aging process' (Porta 2002). Lipofuscin is most highly expressed in post-mitotic cells, becoming obvious in human neurons at 9 years and at 2–3 months in rat neurons (Oenzil *et al.* 1994), although LF is seen in the adrenal gland at all ages (Holtzman 1989). LF accumulates in neurons and glia throughout life, progressively occupying greater cell body volume (Goyal 1982) (Fig. 1c), particularly in large neurons and in brain regions involved with motor function (Liang *et al.* 2004). The high accumulation of LF in neurons and glia may be related to their apparent inability of these cells to exocytose or secrete lysosomes, a process that occurs normally in melanocytes as mentioned, as well as osteoclasts and possibly many types of cells during membrane resealing following small ruptures (Idone *et al.* 2008).

Neuronal LF is not present at higher than normal levels during 'progeria' disorders (West 1979) such as Hutchinson-Gilford syndrome, in which patients appear to age rapidly because of problems in DNA repair but do not suffer from neurodegeneration. Note also that lentigines or 'aging spots' are associated with melanocytic hyperplasia and contain melanin rather than LF (Porta 2002).

### LF pigment properties

Lipofuscin is highly insoluble and reactive, as indicated by its numerous classical histochemical stains (Glees and Hasan 1976). LF consists of 30–58% protein and 19–51% lipid-like material thought to consist of oxidation products of polyunsaturated fatty acids (Jolly *et al.* 2002). Like NM, LF is highly enriched in iron and other metals.

The classical defining characteristic of LF pigment is autofluorescence with excitation/emission maxima of 360–420/540–650 nm (Harman 1989; Jolly *et al.* 2002). Spectroscopic studies of individual retinal LF granules reveal heterogeneity in emission maxima (Haralampus-Grynaviski *et al.* 2003), suggesting that a variety of fluorophores are present with varying amounts in individual AVs. Some of this variety is certainly because of compounds relevant to different cells; for instance, the non-neuronal retinal pigment epithelia contains LF primarily derived from retinol in photoreceptor neurons (Yamada *et al.* 2001; Finnemann *et al.* 2002; Tsai *et al.* 1998).

### LF ontogeny

In neurons, LF is generally thought to result from incomplete digestion of mitochondrial products. When mitochondria are exposed to UV, a non-degradable substance can be pelleted that has properties of LF pigment (Gray and Woulfe 2005). A mitochondrial constituent, lipoic acid, is associated with neuronal LF in Alzheimer's disease (Moreira *et al.* 2007).

Lipofuscin's principal lipid components are suggested to stem from reactions of a highly reactive lipid derivative, 4-hydroxy-2-nonenal (Tsai *et al.* 1998), and malonaldehyde as well as an accumulation of dolichols (Ng Ying Kin *et al.* 1983). 4-Hydroxy-2-nonenal reacts with lysine, histidine, and cystine residues to form so-called Michael adducts, Schiff-base cross-links, and fluorescent fluorophores, and has been reported to block proteasome activity (Okada *et al.* 1999). Peroxide and iron-mediated oxidation may play important roles; cell cultures exposed to low oxygen or iron produce LF (or ceroid, see below), whereas iron chelators and anti-oxidants block the pigment synthesis (Brunk and Terman 2002a).

 

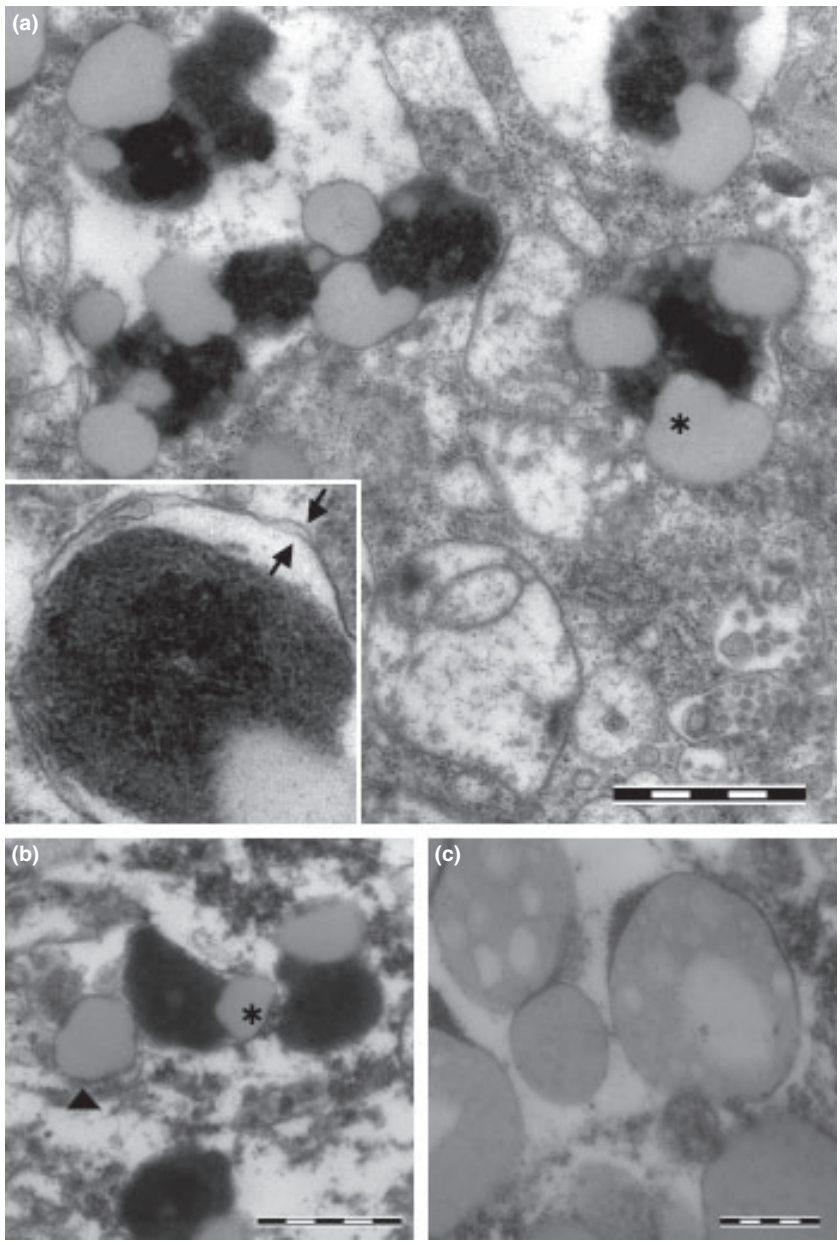


**Fig. 1** Electron micrographs of human brain neuromelanin (NM) and lipofuscin (LF). a) NM autophagic vacuoles (AVs) displaying NM (electron dense matrix) and lipid droplets (*) in a substantia nigra dopamine neuron from a 78-year-old subject. Scale bar = 1 μm. The inset shows a 2.4-fold increased magnification of a single NM AV to clearly display a double membrane that delimits this organelle (arrow heads). b) AVs containing NM and lipid droplets (*) in a norepinephrine neuron of the locus coeruleus from a 81-year-old subject. The arrow indicates an expanse where double membrane can be observed indicating that the organelle is an AV. Scale bar = 1 μm. c) LF bodies in a norepinephrine neuron of locus coeruleus of the same subject. Scale bar = 500 nm.

## Neuromelanin

### NM AVs

Neuromelanin AVs fit the classic definition of a lysosome according to De Duve *et al.* 1955, although it may be that their ability to digest is poor (see below). NM AVs in the substantia nigra (*black substance*) were shown nearly 40 years ago to contain lysosomal hydrolases (Barden 1970) using the histochemical techniques that Novikoff used to originally identify lysosomes. The identification of lysosomal components was recently seconded by proteomic analysis (Tribl *et al.* 2006). Electron micrographs from human autopsy material (Duffy and Tennyson 1965; Sulzer *et al.* 2000) show that NM organelles are membrane delimited. In some cases, these organelles possess a double membrane (Fig. 1a), which identifies the organelles as AVs; the only other candidate organelle would be mitochondria. Often only a single membrane is obvious in NM AVs or no membrane is observed. In the latter case, this is likely because of postmortem breakdown of the autopsy material, but in the case of a single membrane, this may be because of the breakdown of the inner membrane by lysosomal hydrolases, as is typical for autophagolysosomes. Whereas external LC3-II has not been detected in NM vacuoles in autopsy, it can be observed in neuronal culture models of induced NM (see below). In any case, LC3-II is thought to be removed from late stage AVs (see

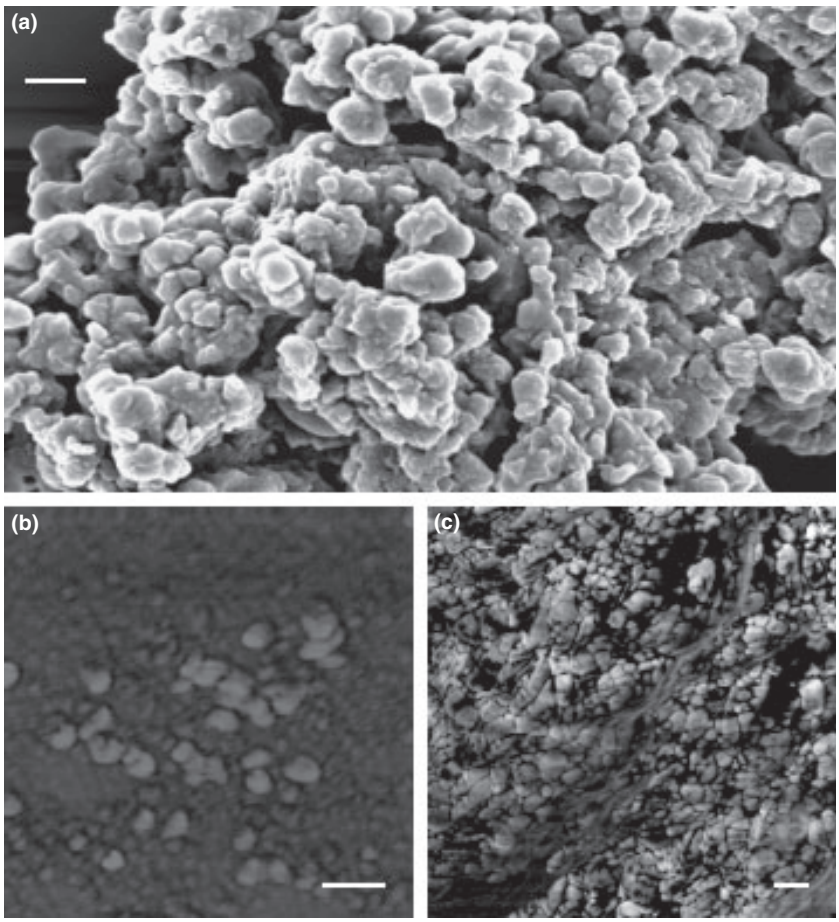


**Fig. 2** (a) Scanning electron microscopy image of dried neuromelanin (NM) pigment isolated from human substantia nigra (Bush *et al.* 2006) that appears to be composed of smaller spherical particles. Scale bar = 500 nm. (b and c) Atomic force microscopy confirms that NM aggregates are composed of approximately 30 nm diameter spheres, both after isolation (b) and within aggregated structures (c). Scale bar = 150 nm.

above). Thus, all evidence identifies NM AVs as autophagolysosomes.

## NM pigment

Neuromelanin is an electron-dense brown/black pigment that within the AV is composed of aggregates of approximately 30 nm diameter spheres with a pheomelanin core and eumelanin surface (Fig. 2). The eumelanin/pheomelanin ratio is 3 : 1 (Wakamatsu *et al.* 2003) and so the spheres apparently contain an approximately 9 nm pheomelanin core encased in a approximately 6 nm eumelanin shell.

The eumelanin is characterized by dihydroxyindole groups formed by dopamine oxidation, which is attributed to the metal chelating ability of NM (Zecca *et al.* 1996). Catecholamine oxidation and the formation of cysteinyl-dopamine products are enhanced by iron; this apparently pertains to NM synthesis, as the iron chelator desferrioxamine inhibits NM formation within AVs in neuronal culture (Sulzer *et al.* 2000). The identification of oxidized dopamine and cysteinyl-dopamine as NM building blocks suggests that NM AVs prevent accumulation of these toxic products in the cytosol (Wakamatsu *et al.* 2003). The ability of NM to chelate metals also contributes to classical histochemical techniques used to label the compound. In addition to the NM melanic component derived from the oxidized dopamine, there are also incompletely characterized peptide and aliphatic components (Zecca *et al.* 2000).

In electron micrographs, NM AVs exhibit both electron dense portions that correspond to NM pigment and 'lipid droplets' that contain acyglycerols, phospholipids, and dolichol and dolichoic acid (J. D. S. and L. Z., unpublished results; Ward *et al.* 2007). Lipid droplets are far less electron dense than NM pigment (Fig. 1a and b), and NM AVs that contain both could result from the fusion of multiple pigmented AVs.

## NM ontogeny

Substantia nigra neurons contain large amounts of NM AVs in older people. NM becomes detectable 3–5 years after birth (Zecca *et al.* 2002) and accumulates until the ninth decade of life (Fig. 3), suggesting that NM, like LF, is poorly degraded by neurons. There are differences in NM accumulation between nationalities, with an equivalent number of substantia nigra neurons that possess NM in Nigerian and British subjects, whereas subjects from India have lower numbers (Muthane *et al.* 2006, 1998). While possibly due to diet, the reason for these different levels of NM is unknown. The exception to NM pigment accumulation over a lifetime is in

 

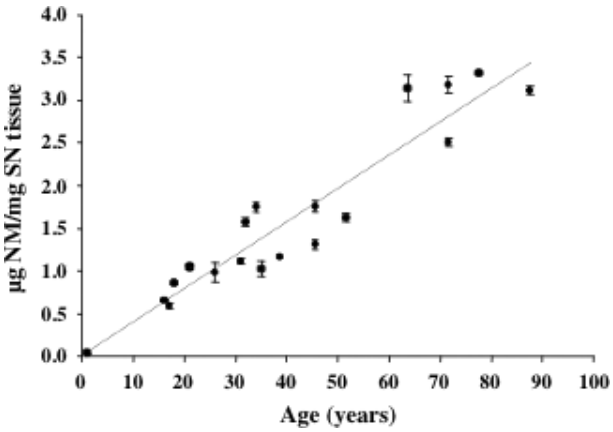


**Fig. 3** Increased levels of neuromelanin (ng/mg wet tissue) in substantia nigra of human normal subjects during aging (Zecca *et al.* 2002). The values are expressed as mean ± SEM of 3–5 measurements.

Parkinson's disease (PD), where substantia nigra NM is decreased following its release from dying cells and phagocytosis and degradation by microglia (D. S. and L. Z. *et al.*, under submission).

It has been reported that levels of NM within individual substantia nigra neurons are decreased in PD compared with control subjects (Kastner *et al.* 1992). This could be a consequence of increased oxidative processes, so that NM is bleached in stressed neurons, although an alternate explanation is that lower levels of NM are synthesized because of a shift of dopamine metabolism to homovanillic acid instead of generating cysteinyl-dopamine derivates that are the precursors of NM. If there is a slow degradation of the pigment in lysosomes, the net result over time would be lower NM levels within the neuron.

## NM as an example of pigmented AV induction

In postnatal substantia nigra neuronal culture, L-DOPA begins to be detectably converted to dopamine within 90 s (Pothos *et al.* 1998), leading to rapid increases in substantia nigra cytosolic dopamine from less than 100 nM to approximately 10 μM (Mosharov *et al.* 2003) (E. V. M. *et al.*, manuscript in submission). L-DOPA in cultures of substantia nigra neurons also induces AVs, as observed by GFP-LC3 redistribution from a diffuse cytosolic pool to puncta, and from the induction of putative late AVs labeled by monodansylcadaverine (Fig. 4). The dark, electron-dense NM pigment within these AVs was clearly visible by both light and electron microscopy following 1 week of L-DOPA exposure (Sulzer *et al.* 2000).

The catecholamine oxidation underlying NM synthesis in substantia nigra cultures occurred within the neuronal cytosol as over-expression of the synaptic vesicle transporter for catecholamines, vesicular monamine transporter 2 (VMAT2), powerfully inhibited NM synthesis (Sulzer *et al.* 2000). VMAT2 over-expression enhances dopamine accumulation into organelles including synaptic vesicles (Pothos *et al.* 2000; Larsen *et al.* 2002) and decreases cytosolic dopamine resulting from L-DOPA exposure (E. V. M., unpublished results). Thus, an L-DOPA induced elevation of cytosolic

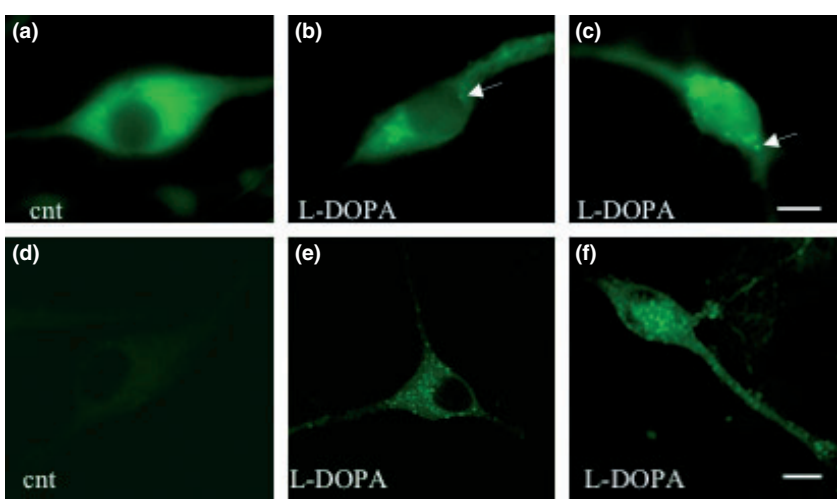


**Fig. 4** Induction of autophagic vacuoles (AVs) by L-DOPA. a–c) GFP-LC3 indicates L-DOPA induction of AVs. Ventral midbrain neuronal cultures derived from green fluorescent protein–LC3 (GFP–LC3) transgenic mice were treated with vehicle (cnt) or 100 μM L-DOPA for 48 h. Note that the control exhibits diffuse cytosolic fluorescence, whereas puncta (arrows) are visible in the L-DOPA-treated neurons. d–f) Monodansylcadaverine label is consistent with L-DOPA induction of AVs. Wild-type ventral midbrain neuron cultures were treated with 100 μM L-DOPA (e: 24 h, f: 1 week) or vehicle labeled with 50 μM monodansylcadaverine for 1 h. AVs appear to be absent in controls and are markedly enhanced by L-DOPA. Scale bars = 10 μm.

dopamine was apparently responsible for NM biosynthesis. Consistent with this hypothesis, human ventral midbrain dopamine neurons with low levels of VMAT2 display the highest levels of NM (Liang *et al.* 2004).

Whereas the molecular steps by which high levels of cytosolic dopamine induce AVs remain unknown, it could be via an ERK-related pathway, as observed with 1-methyl-4-phenylpyridinium in dopamine neurons (Zhu *et al.* 2007). It is also possible that autophagy of non-cytosolic catecholamine such as that accumulated within synaptic vesicles or amphisomes contributes to NM if these organelles are accumulated within AVs.

## Ceroid

### Ceroid AVs

Ceroid AVs are classically considered to arise from disease, in contrast to age-associated LF. In the brain, ceroid AVs are particularly linked to a set of neurodegenerative lysosomal disorders known as neuronal ceroid lipofuscinoses (NCLs) or Batten disease. These common childhood diseases are characterized by progressive psychomotor retardation, dementia, progressive blindness, and premature death (Tynela *et al.* 2000; Weimer *et al.* 2002; Wisniewski *et al.* 2001). There is an adult dominant form (Nijssen *et al.* 2003). Pathologically, NCLs are characterized by accumulation of ceroid in AVs, particularly of the CNS, preceding progressive cell death (Dawson and Cho 2000).

The mutations that underlie NCLs are autosomal recessive lysosomal genes; the proteins encoded by CLN1, 2, 3, 5, 6, 8 (and possibly 7) reside within the lysosome (Weimer *et al.* 2002). These include the lysosomal enzymes palmitoyl protein thioesterase 1 (CLN1), tripeptidylpeptidase 1, a tripeptidyl serine protease (CLN2), and cathepsin D (CLN8), and two lysosomal membrane proteins of unknown function, CLN3 and CLN5 (Kyttala *et al.* 2003; Weimer *et al.* 2002).

Additional lysosomal disorders, including mucopolysaccharidosis types I, II, IIIA, polysulfatase deficiency, mucolipidosis I and GM2 and GM1 gangliosidoses (Elleder *et al.* 1997; Itoh *et al.* 2001; Karten *et al.* 2002), and the lysosomal disorder aspartylglucosaminuria (Kyttala *et al.* 1998) also display AV ceroid. Niemann-Pick disease results from mutations blocking normal lysosomal processing of cholesterol or sphingomyelin, and there is an accumulation of cholesterol and reports of ceroid within lysosomes in Niemann-Pick type C disease (Cheruku *et al.* 2006).

There are also ceroid AVs in disorders that are not clearly caused by lysosomal dysfunction, including Huntington's Disease (Tellez-Nagel *et al.* 1975) and methamphetamine toxicity (Teuchert-Noodt and Dawirs 1991). These diseases are additionally associated with elevated AVs (Petersen *et al.* 2001; Kegel *et al.* 2000; Larsen and Sulzer 2002). Ceroid AVs are also present in 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine models of PD, where they may include α-synuclein (Meredith *et al.* 2002), which appears consistent with the enhancement of macroautophagy reported in PD models (Martinez-Vicente *et al.* 2008).

### Ceroid pigment properties and ontogeny

Whereas the principal components of LF pigment are thought to derive from mitochondria, the evidence is even stronger for ceroid diseases. The major protein component of ceroid in at least six forms of Batten Disease (the exception is CLN1) has been identified as the highly hydrophobic subunit C (a.k.a. subunit 9) of the F0 portion of mitochondrial ATP synthase (a.k.a. F1-F0 or F-ATPase) (Hall *et al.* 1991; Buzy *et al.* 1996). This subunit is often referred to as a proteolipid because it is extracted with lipids in chloroform–ethanol.

Subunit C plays an important role in proton translocation through mitochondria and ATP synthesis (Fillingame *et al.* 2003; Bockmann and Grubmuller 2002; Tsunoda *et al.* 2001; Kaim *et al.* 2002; Sambongi *et al.* 1999; Itoh *et al.* 2004). It is normally found only in the inner mitochondrial membrane, where it accounts for 2–4% of protein, but may compromise 40% of ceroid in Batten disease and in cattle and horse NCLs (Martinus *et al.* 1991).

Subunit C is also present at variable levels in the ceroid associated with Niemann-Pick type A and C disease, and a variety of other lysosomal disorders, including mucopolysaccharidosis types I, II, IIIA, polysulfatase deficiency, mucolipidosis I, and GM2 and GM1 gangliosidoses (Elleder *et al.* 1997; Itoh *et al.* 2001; Karten *et al.* 2002).

In adult dominant Batten disease, subunit C expression is variable in ceroid, but there may be high levels of saposin D (sphingolipid activating protein D) (Nijssen *et al.* 2003). The phospholipids in ceroid diseases include bis-(mono-acylglycero) phosphate, a component of lysosomal membranes (Jolly *et al.* 2002).

## Why do pigmented AVs accumulate with age and disease?

In contrast to the short lifetime of typical AVs, pigmented AVs accumulate over a lifetime. There are three non-exclusive hypotheses for why; an *accumulation of indigestible material*, an *inhibition of lysosomal fusion with the vacuoles*, and *lysosomal dysfunction*.

### Evidence that pigment is indigestible

Neuromelanin is a notoriously difficult substance to breakdown in the test tube. The purification technique entails multiple exposure to sodium dodecyl sulfate, proteinase K, methanol, hexane, high salt, and repeated washing; and then NM is the stuff that survives in the pellet!

 

Neuromelanin can, however, be degraded by oxidizing agents including hydrogen peroxide, which bleaches the pigment to produce characteristic products (Wakamatsu *et al.* 2003). Microglia can phagocytose extracellular NM released from dying substantia nigra neurons and degrade the pigment within minutes (Haralampus-Grynaviski *et al.* 2003) via reaction with hydrogen peroxide within the phagosome (D. S. and L. Z. *et al.*, unpublished results).

Similarly, LF may become undigestible because the proteins are 'fixed' via aldehyde bridges between amino groups, a form of cross-link that is not a good substrate for lysosomal hydrolases (Gray and Woulfe 2005). A trimethyl lysine modification in subunit C is proposed to contribute to the resistance of ceroid pigment to degradation in Batten disease (Katz *et al.* 1995).

It has been suggested that with advancing age there may be some slow 'slippage' as the rate of free radical damage increases, which could block AV/lysosome fusion, while protease activity decreases (Harman 1989; Lynch and Bi 2003), leading to a buildup of indigestible material.

### Evidence for lysosomal dysfunction

There is much evidence for disrupted lysosomal function over aging (Gray and Woulfe 2005; Massey *et al.* 2006c), which may further contribute to pigment accumulation. As mentioned, Batten disease is caused by mutations of lysosomal proteases, e.g. CLN8 (cathepsin D) (Koike *et al.* 2000; Tyynela *et al.* 2000). The CLN2 mutation of a lysosomal serine protease appears to specifically inhibit lysosomal degradation of subunit C (Ezaki *et al.* 1999) and possibly an analogous subunit of the secretory vesicle ATPase (Tanner *et al.* 1997). In addition to mutations of lysosomal enzymes, pharmacologic inhibition of lysosomal proteases with leupeptin (Nunomura and Miyagishi 1993) produce AVs.

There is also evidence for an alternate form of lysosomal dysfunction associated with AV accumulation due to collapse of acidic pH gradients. Collapse of lysosomal pH gradients inhibits lysosomal protein degradation because of the acidic pH requirements of their degradative enzymes. Membranophilic weak bases that collapse lysosomal pH gradients including methamphetamine, ammonia, and chloroquine (Cubells *et al.* 1994; Mahon *et al.* 2004; Larsen *et al.* 2002; Teuchert-Noodt and Dawirs 1991) each induce AVs and inhibit overall lysosomal proteolytic degradation in neurons (Z. Talloczy and Ana Maria Cuervo, unpublished results). These AVs are labeled by monodansylcadaverine and LC-3 (Z. T., unpublished results), as well as endocytic tracers (Cubells *et al.* 1994), and thus appear to be amphisomes.

A related case occurs in the CLC-3 knockout mouse. CLC-3 is a chloride channel expressed in lysosomes and secretory vesicles, and is important in the regulation of acidic pH, transmitter accumulation, and organelle fusion (Stobrawa *et al.* 2001; Barg *et al.* 2001). CLC-3-deficient mice showed developmental retardation, high mortality, blindness, motor coordination deficit, and spontaneous hyperlocomotion. In histologic analysis, the mice showed progressive degeneration of the retina and hippocampus similar to the phenotype of CLN8/cathepsin D knockout mice (Stobrawa *et al.* 2001). There is also lysosomal accumulation of ceroid LF subunit C and an elevation in the endosomal pH of the knockout mice (Yoshikawa *et al.* 2002). This is obviously similar to the ceroid neuronal LF diseases, but implies a role for lysosomal pH gradients as well as proteolytic enzymes.

### Inhibition of lysosomal fusion

The number of AVs increase when their fusion with lysosomes is disrupted *e.g.* when microtubules are disrupted by colchicine. This may occur if organelle traffic through the axon is overcrowded, resulting in stearic hindrance or problems in organelle transport (Duncan and Goldstein 2006). We have observed many instances in video microscopy of labeled AVs that appear to be stuck in axons, sometimes bouncing off each other as they attempt retrograde transport (D. S. and Z. T., unpublished results). Given the cross-talk between degradation pathways, so that inhibition of one pathway stimulates another (Massey *et al.* 2006a), it may not be surprising that proteasome inhibition also induces LF (Terman and Sandberg 2002; Sullivan *et al.* 2004), perhaps via enhancing AV formation. The fusion of endosomes or AVs with lysosomes could be blocked by alkalinization within these organelles, as the acidic pH and the ATP-driven vacuolar proton pump is required for the fusion of yeast endocytic structures with the central vacuole (Peters *et al.* 2001).

## Evidence for neuroprotection by pigmented AVs

Autophagic vacuole induction is classically a protective response, serving to clear reactive and damaged compounds and organelles from the cytosol and provide amino acids during starvation. The formation of pigmented AVs could also be protective by sequestering potentially toxic compounds. For instance, NM may sequester reactive dopamine products and dopamine-modified proteins, perhaps including dopamine-modified α-synuclein which is not broken down by the protein's normal chaperone-mediated autophagic degradative pathway and promotes macroautophagy (Martinez-Vicente *et al.* 2008). It is possible that NM AVs provide a means to protect neurons with high cytosolic dopamine against PD.

There is evidence that neurons with higher levels of pigmented AVs are relatively spared during age-related stress (Kanaan *et al.* 2007; Gray and Woulfe 2005, although see Liang *et al.* 2004), and that in rhesus monkeys there is more LF in dopamine neurons corresponding to those spared in PD, which suggests that it could be protective (Kanaan *et al.* 2007).

 

## Evidence for disruption of neuronal function by pigmented AVs

There are, however, numerous possible downstream effects that would adversely affect cellular health: for example, the common age-related disease, macular degeneration is widely thought to result from accumulation of LF.

### Inhibition of lysosomal function

The loss of lysosomal function during aging may in part result from pigmented AVs. Undigestible material in lysosomes could bind up or otherwise inhibit proteases. The pigmented AVs could dilute or divert the delivery of lysosomal proteases from performing degradation efficiently. As proteasome is thought to be turned over by autophagy (Cuervo *et al.* 1995), blockade of proteasome turnover could disrupt additional degradative pathways.

Neurons may spend too much energy into constructing new lysosomes as a response to stress. There is an accumulation of lysosomes in Alzheimer's Disease that may be a compensatory response to problems in degradation (Adamec *et al.* 2000). Similarly, more lysosomes are present following inhibition of cathepsins B and L (Bednarski *et al.* 1997) or if β-galactosidase is disrupted (Itoh *et al.* 2001).

Finally, whole organelles including peroxisomes and mitochondria are degraded by macroautophagy. The destruction of damaged mitochondria makes room for new ones. If blocked by pigmented AVs, the damaged mitochondria may gradually accumulate (Brunk and Terman 2002a,b).

### Inhibition of other secretory pathways

The build up of pigmented AVs could inhibit secretory pathways that provide a host of additional secretory tasks, including nutrient uptake and response to growth factors or recovery from stress. For instance, indigestible material in AVs could inhibit organelle fusion or block the supply of amino acids from autophagic or heterophagic protein breakdown, inducing cell starvation.

One example of apparent blockade of normal endocytic/secretory function by pigmented AVs is that accumulation of NM in PC12 cells significantly delays axon induction by nerve growth factor (Sulzer *et al.* 2000). It may be that too large of a population of AVs occludes trafficking of other organelles on microtubules, inhibiting axonal and dendritic transport underlying normal secretory operations and response to exogenous factors such as growth factors, as well as trafficking of mitochondria and other organelles. Alternatively, pigments may interfere with the normal processing or recycling of receptors and ligands within lysosomes.

### Pigment reactions

If pigments escaped AVs, they could directly damage intracellular components. Pigment-induced disruption of proteasomal (Okada *et al.* 1999) or other house-keeping enzymatic pathways would further inhibit normal protein processing. NM in culture was found to inhibit 26S proteasome (Shamoto-Nagai *et al.*, 2004) as has loading cells with LF (Sitte *et al.* 2000).

Nevertheless, while clearly subject to oxidation/reduction reactions, NM under vacuum has a surface oxidation potential of −100 mV, which does not appear sufficient to directly generate a high level of oxidative stress for cells (Bush *et al.* 2006). Indeed, we have observed that isolated human NM does not obviously damage neuronal membrane after even days of direct exposure (D. S., unpublished results). A related model is that extracellular pigment could cause damage following cell death, perhaps via triggering an immune response. When substantia nigra neurons die, the released extracellular NM apparently causes proliferation of microglia with consequent neuronal stress (Wilms *et al.* 2003; Zecca *et al.* 2003).

## Can the accumulation of pigmented AVs be reversed?

If lysosomes do not degrade the pigments, how might the accumulation of pigmented AVs be slowed or reversed? One approach is to use drugs to inhibit cytosolic precursors, as seen with cysteamine, a small aminothiol that may decrease substrate accumulation resulting from the CLN1 mutation (Lu and Hofmann 2006), although this compound may work additionally as an inhibitor for transglutaminase and other enzymes. The drug centrophenoxine, also a thiol anti-oxidant (Nehru and Bhalla 2006) and structurally similar compounds were found long ago to inhibit LF in neurons (Nandy and Bourne 1966) and have been used to treat Alzheimer's and senile dementia. Anti-oxidants including red wine – but for some reason not port wine! (Assuncao *et al.* 2007) – seem to block LF formation at steps prior to AV accumulation, as do lipoic acid and carnitine dietary supplementation (Savitha *et al.* 2007). As mentioned, VMAT2 over-expression decreases cytosolic precursors to halt L-DOPA induced NM synthesis by decreasing dopamine in the cytosol.

For some lysosomal diseases featuring accumulated AV constituents susceptible to normal lysosomal hydrolases, there are already replacement therapies to enhance lysosomal function. In the 1960s, Elizabeth Neufeld *et al* reported the lysosomal mucopolysaccharidosis disorders could be corrected by factors secreted from normal cells (Fratantoni *et al.* 1968). This was later found to result from endocytosis of lysosomal enzymes that bind the Golgi apparatus mannose-6-phosphate receptor and are targeted to the lysosome. The first successful lysosomal enzyme replacement therapy was for Gaucher's disease, which consisted of intravenous injection of recombinant β-glucocerebrosidease enzymes that bind to these receptors (Sly *et al.* 2006). A variety of enzyme replacement therapies using both i.v. injection of recombi-

 

nant enzyme and gene therapies are now thought to operate because of cellular recognition and lysosomal targeting (Beck 2007), This approach may, however, be ineffective at late stages when the intralysosomal environment is already severely disturbed, *e.g*. if the pH gradient is collapsed.

Amino acid starvation or caloric restriction is well known to enhance macroautophagy within lysosomes and could contribute to degrading AV pigments that are still subject to lysosomal breakdown, as could macroautophagy activators including the mTOR inhibitor rapamycin (Ravikumar *et al.* 2002). Vitamin C expedites lysosomal protein degradation and reverses the accumulation of AVs in astrocytes (Martin *et al.* 2002) apparently via enhancement of lysosomal acidification and enhancement of fusion, whereas dietary $\omega$-3 polyunsaturated fatty acids are reported to enhance lysosomal degradation and possibly prevent pigmented AVs and macular degeneration in monkeys (Elner 2002).

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