INTERACTION OF VESICULAR MONOAMINE TRANSPORTER 2 (VMAT2) AND NEUROMELANIN PIGMENT AMONG THE MIDBRAIN DOPAMINERGIC NEURONS, IN MAN

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Abstract- Neuromelanin (NM) pigment accumulates with age in catecholaminergic neurons in man, and the ventral substantia nigra dopaminergic neurons that are the most vulnerable to degeneration in Parkinson's disease (PD) contain the greatest amount of this pigment. In vitro data indicate that NM pigment is formed from the excess cytosolic catecholamine that is not accumulated into synaptic vesicles via the vesicular monoamine transporter2 (VMAT2). Using semi-quantitative immunohistochemical methods in human postmortem brain, we sought to examine the relationship between the contents of VMAT2 and NM pigment. The immunostaining intensity (ISI) was measured for VMAT2 in two regions of the midbrain dopaminergic cell complex. The ISI of the cells was related to the density of NM pigment within the cells. We also measured the ISI for tyrosine hydroxylase (TH) and examined the noradrenergic neurons in the locus coeruleus (LC). In brains 22-65 years of age: 1) ventral substantia nigra neurons had the lowest VMAT2 ISI of all neurons in the midbrain cell complex, whereas over 2-fold higher levels are found in most ventral tegmental area neurons; 2) there was an inverse relationship between VMAT2 ISI and neuromelanin pigment in the midbrain dompaminergic neurons; 3) neurons with the highest VMAT2 ISI resided in the LC; 4) neurons with high VMAT2 ISI also had high TH ISI; and 5) in the newborn brain, which has not yet accumulated neuromelanin pigment in the aminergic neurons, the regional distribution of VMAT2 and TH-ISI was similar to that found in the adult brain. These data support the hypothesis that among the midbrain dopaminergic neurons, the ventral substantia nigra dopamine neurons accumulate the highest levels of NM pigment because they have the lowest levels of VMAT2, which thereby renders them especially vulnerable to degeneration in PD.

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INTRODUCTION

Neuromelanin (NM) pigment is a synthetic byproduct of catecholamine synthesis. The golden brown NM pigment granules accumulate with age in catecholaminergic neurons in the primate brain, but not in rodent brain. In man, NM accumulates with age in the substantia nigra dopamine (DA)-containing neurons, and in the locus coeruleus (LC) noradrenergic neurons (1,2). The substantia nigra DA neurons that reside in the ventral portion of the nucleus (SNv) have a high degree of pigmentation in aged humans, and these most-pigmented neurons preferentially degenerate in Parkinson's disease (PD) (3,4). The less pigmented DA neurons in the ventral tegmental area (VTA) are least vulnerable to degeneration in PD (3-5). Some investigators have speculated that NM pigment is toxic to aminergic neurons in that it physically interferes with intracellular communication (6,7). Indeed, the formation of NM pigment in cultured PC12 cells has been shown to interfere with intracellular neurotrophin signaling (8).

The vesicular monoamine transporter 2 (VMAT2) plays an important role in neuronal vulnerability to degeneration in neurotoxin-induced Parkinsonism. VMAT2 sequesters monoamines into synaptic vesicles for action potential-induced release of

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neurotransmitter (9). VMAT2 also sequesters the N-methyl-4-phenyl-1,2,3,6-tetrahydro-pyridine (MPTP) toxic metabolite, N-methyl-4-phenylpyridinium (MPP+) into synaptic vesicles with high affinity (10). MPP+ accumulates within catecholaminergic neurons in direct proportion to the amount of VMAT2 within the neurons (11).

In VMAT2 knockout mice and in mice with VMAT2 pharmacologically inactivated, MPTP produces enhanced toxic effects on substantia nigra DA neurons (12-14). These data suggest that when levels of MPP+ that are accumulated within the cell exceed the storage capacity of the synaptic vesicles, the free cytosolic MPP+ causes toxic effects on mitochondrial respiration at the level of Complex I, and causes cell death (15-17). NM pigment formation is related to VMAT2 function. Sulzer et al demonstrated that NM pigment could be formed in rodent substantia nigra DA neurons in vitro (8). By treating cultured rat DA neurons with L-DOPA for several days, NM pigment was formed. In cells that were made to over-express VMAT2, however, no NM was formed following chronic L-DOPA treatment. These data suggest that NM pigment is formed from oxidation of cytosolic DA and DOPA. Since rodent neurons do not normally contain NM pigment, this implies that most of the DA that is synthesized is sequestered within synaptic vesicles. However, because human aminergic neurons form NM pigment as early as during the first decade of life in the LC (6), it is suggested that the neurons make a greater amount of amine than can be stored in synaptic vesicles.

The present study sought to determine whether there was a relationship between NM pigment and VMAT2 levels in the human postmortem brain. We examined both the midbrain DA neurons and the LC noradrenergic neurons in adult and infant humans.

MATERIALS AND METHODS

The Willed Body Program at the University of Texas Southwestern Medical Center in Dallas provided adult human brains. Infant brain was collected from Children's Medical Center. The Institutional Review Board approved the brain collection protocol. Table 1 describes the demographic information on the brains used in the present study. Brain tissue was fixed in 10% neutral buffered formalin for at least one week. Prior to cutting, tissue blocks containing the substantia nigra and LC were placed in 20% sucrose in formalin for 1-2 weeks. Coronal sections were cut at 40 μm thickness through the midbrain and rostral pons. A standard ABC immunohistochemical staining method was employed. In brief, sections were washed in phosphate buffered saline (PBS), treated with 1% hydrogen peroxide in PBS and blocked in 5% normal goat serum in 0.3% Triton X-100 in PBS (PBST). Staining was performed by incubating sections with diluted primary antibodies over-night at room temperature. This was followed by incubating sections with 1.5 μg/ml biotinylated goat anti-rabbit IgG (Vector Labs, Burlingame, CA) for 30 minutes at room temperature, and with an avidin/biotin/peroxidase reagent (1:250 dilution ABC Elite. Vector Labs) for 1 hour at room temperature. Then sections were reacted in an acetate buffer (pH 6.0) containing 0.035% diaminobenzidine tetrahydro-chloride, 2.5% nickel ammonium sulfate, and 0.00 1% hydrogen peroxide for 5-10 minutes. All of the incubations were done on a shaker.

A polyclonal antibody against TH (Protos Biotech Corp., NY) was used at 1: 1,000 to 1: 6000 concentrations. An affinity purified VMAT2 antibody was made against 13 amino acids in the rat C-terminus of the protein (Alpha Diagnostics, Inc.). An antibody made against the same sequence is also commercially available (CHEMICON, Internatl. Inc., AB 1598P), and has been used previously by us to immunostain VMAT2- containing neurons in the rodent (11). Sections were immunostained with concentrations of 1: 500 to 1: 1000. When either primary antibody was omitted, the catecholaminergic somata did not immunostain. Densitometry methods were used to assess the immunostaining intensity (ISI) for each antibody within the somata of midbrain DA neurons and LC noradrenergic neurons. A Leica DMRE microscope equipped with a Cohu video camera and Stereo Investigator software (Micro-BrightField, Inc.) was employed for densitometry measurements (40 × objectives). On a scale of 0-255, the optical density of cells outlined within the SNv, VTA and LC was determined. The ISI of 10 neurons within each region was determined. In young brain that lacked NM pigment, the ISI was determined by taking the average intensity over the entire somata of cells without an unstained nucleus visible in the plane of section. In cells from older brains that contained NM pigment, the ISI was measured in the portion of the somata that contained little or no NM pigment. The mean intensity was subtracted from the background ISI of the tissue section to calculate the average ISI for the cell region. In this way cells that were stained darkly had high ISI values and cells with a light amount of

immunostaining had low ISI values. The ISI values for the VTA and LC were expressed relative to that of the SNv within each brain, since the SNv cells uniformly contained the lowest ISI values.

RESULTS

Neurons in different regions of the midbrain DA cell complex contained different intensities of VMAT2 and TH immunoreactivity. In all brains examined, cells with the darkest ISI were found within the VTA near the exit of the third nerve rootlets, and also in the retrorubral field, and substantia nigra pars lateralis. Intermediate ISI was observed in dorsal SN neurons. Neurons in the SNv had the lowest ISI values. For cells in the VTA and SNv this relationship was quantified. The specific locations where ISI values were measured in the VTA and SNv are illustrated in Figure 1. VMAT2 ISI was lowest within the SNv and highest within the VTA when observed within the same tissue section, thus controlling the effects of the immunostaining protocol on the intensity of the immunostaining reaction.

Also, when high concentrations of VMAT2 antibody were employed (1:500), the SNv neurons exhibited low ISI values, and the VTA neurons exhibited high ISI values (Fig. 2). On the other hand, when low VMAT2 concentrations were employed (1: 1,000), the SNv neurons exhibited no immunoreactivity at all and were only identified by their NM pigment content. At the high VMAT2 staining concentration the VTA DA neurons were even more darkly immunoreactive than at the low staining concentration. For brains 22-65 years of age there was on an average over 2-fold higher VMAT2 ISI in VTA cells than in SNv cells (Table 2). The TH ISI within midbrain DA neurons paralleled the VMAT2 ISI (Table 2). The SNv neurons had low TH ISI and the VTA neurons had on an average over 3-fold higher TH ISI. Thus midbrain DA neurons with low levels of VMAT2 also had low levels of TH, and vice versa. The LC had higher levels of VMAT2 ISI than VTA DA neurons, but did not have the highest TH ISI (Table 2).

Table 1. The demographic information on the brains used in the present study

Brain ID	Age/ gender	Brain weight(g)	PMI (hrs)	TIF (months)	Cause of death
DA-258	0.5/ F	Nd	24	1	CF
DA-132	22/ M	1350	12	144	Gun shot
DA-32	26/ M	1440	11	220	Suicide
DA-243	37/ M	Nd	Nd	72	Gun shot
DA-268	62/ F	1100	15	1	Stroke
DA-257	65/ F	1130	6	1	Stroke

Abbriviations:PMI, post-mortem interval; TIF, time in formalin; CF,Cystic fibrosis

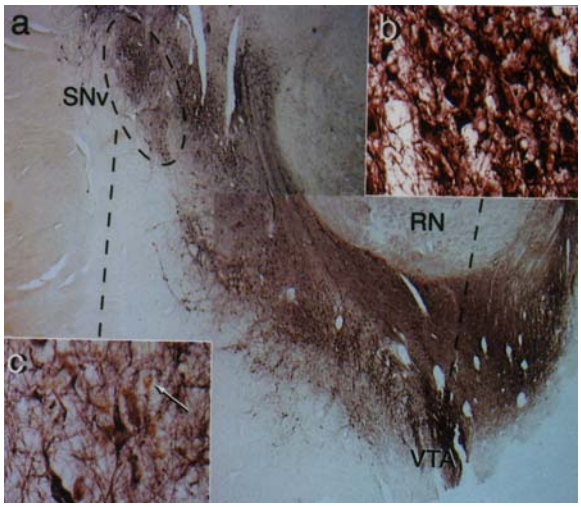

Fig. 1. Midbrain DA neurons were studied in two regions; the ventral substantia nigra (SNv) and the ventral tegmental area (VTA). Neurons are illustrated in a 22-year old male, immunostained for TH. A. Low power micrograph illustrating the location of the SNv and the VTA where cells were analyzed for relative contents of TH and VMAT2. B. High power magnification of VTA neurons. Notice the dark TH immunostaining within the somata and dendrites. C. High power magnification of SNv neurons. Notice the lighter TH immunostaining in these neurons compared to those in the VTA. Also notice that some of the neurons contain no immunostaining but are positive for neuromelanin pigment (golden brown granules) (arrow). RN, red nucleus. Panel A is magnified 16 times, and panels B and C are magnified 200 times.

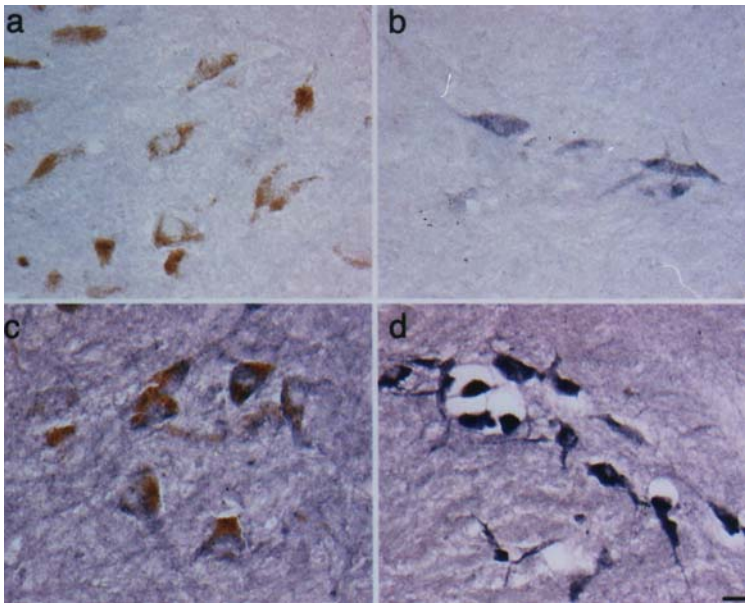

Fig. 2. Midbrain DA neurons in the SNv and VTA stain with different intensities for VMAT2. Neurons are illustrated from a 37-year-old male. A. Neurons in the SNv do not stain for VMAT2 using an antibody concentration of 1: 1,000. The neurons are identifiable by their content of NM pigment (golden brown). B. Neurons in the VTA, rostral level, stain for VMAT2 with the 1: 1,000 concentration (black reaction product). No NM pigment is observed in these neurons. C. Neurons stain faintly in the SNv using a VMAT2 concentration of 1:500. Notice the black-VMAT2 reaction produce with golden brown NM pigment granules within the cytoplasm. D. Neurons in the VTA stain even more darkly using the 1:500 concentration of VMAT2 antibody than at the lower concentration. Marker, 20 μm.

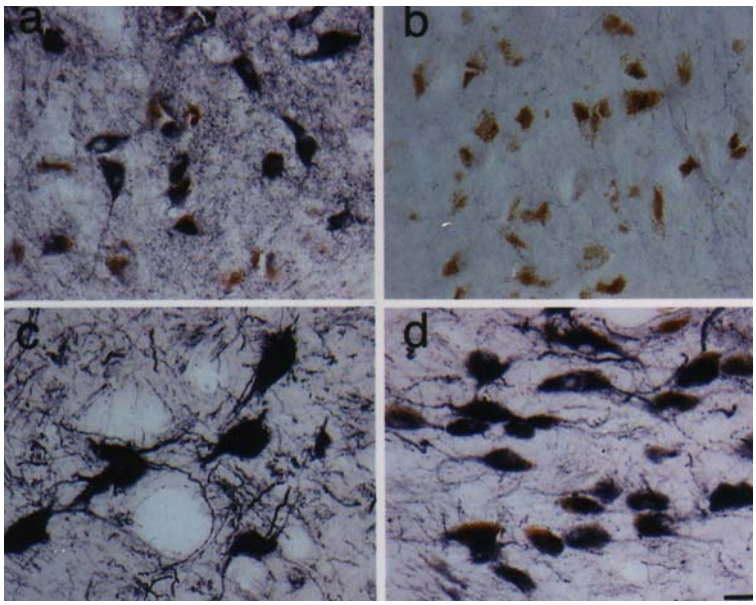

Fig. 3. There is an inverse relationship between VMAT2 immunoreactivity and neuromelanin pigment content. Neurons are illustrated from a 65-year-old female. A. Ventral tegmental area DA neurons stain darkly for VMAT2, and NM pigment is visible within some of the same immunostained cells. B. Ventral substantia nigra neurons do not stain for VMAT2 but contain many NM pigment granules (golden brown), in the same tissue section as in panel 'A'. C. LC neurons stain very darkly with the VMAT2 antibody. Panels A-C were stained with an antibody concentration of 1:500. D. LC neurons still stain darkly using an antibody concentration of 1: 1,500, and NM pigment is visible in many neurons. Marker, 30 μm.

In all brains examined the TH ISI for LC was lower than that for the VTA neurons. On the other hand, the VMAT2 ISI for LC was higher than that of the VTA (Fig. 3). The levels of VMAT2 ISI were inversely related to the amount of NM pigment in midbrain DA neurons. In the brain of a 37-year-old male, the SNv neurons contained moderate levels of NM pigment and faint amounts of VMAT2 ISI whereas neurons in the VTA exhibit 2.9-fold higher VMAT2 ISI than the SNv and had no detectable NM pigment (Fig. 2). This can also be observed in the brain of a 22-year old male, in which VTA neurons exhibited NM pigment within the somata of cells with high VMAT2 ISI, but in the SNv there are many cells that only contain NM pigment and no VMAT2 ISI using an antibody concentration of 1:500 (Fig. 3).

Table 2. Relative TH and VMAT2 Immurioreactivity Intensity in Midbrain Dopamine Neurons and Locus Coeruleus Noradrenergic Neurons

Brain ID	Antibody	SNv	VTA	LC
DA-258	TH	1*	4.2	3.5
	VMAT2	1	2.1	2.7
DA-132	TH	1	1.4	nd
	VMAT2	nd	nd	nd
DA-32	TH	1	2.6	2.6
	VMAT'2	1	1.6	1.7
DA-243	TH	nd	nd	nd
	VMAT2	1	2.9	nd
DA-257	TH	1	5.4	3.5
	VMAT2	1	3.1	6.1
DA-268	TH	1	1.5	1.4
	VMAT2	1	1.6	2.1

Abbreviation: nd, not determined due to lack of tissue.
*Immunoreactivity intensity is expressed relative to the staining darkness of the SNv within each brain. The higher the number, the darker the immunostaining intensity.

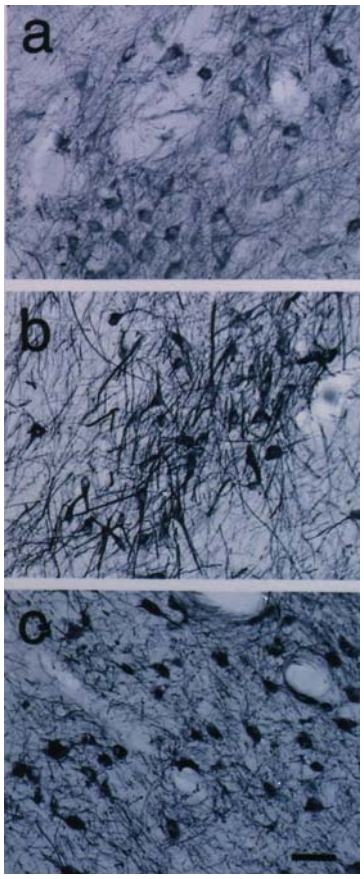

Fig. 4. In young brain, where there is little neuromelanin pigment, ventral substantia nigra neurons stain faintly with VMAT2 immunoreactivity whereas ventral tegmental area and locus coeruleus neurons stain more intensely. Neurons are illustrated from a 6month old female. A. Neurons in the ventral substantia nigra contain low levels of VMAT2 immunoreactivity. B. Neurons in the ventral tegmental area contain high levels of VMAT2 immunoreactivity. C. Neurons in the locus coeruleus stain intensely for VMAT2. Marker, 70 μm.

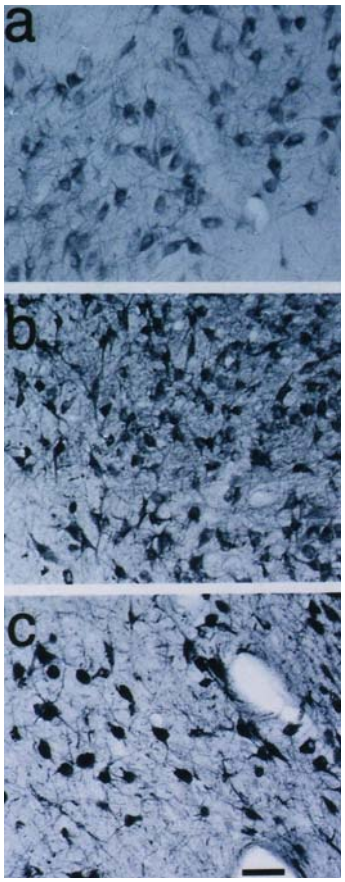

Fig. 5. In young brain, where there is little neuromelanin pigment, ventral substantia nigra neurons stain faintly with TH immunoreactivity whereas ventral tegmental area and locus coeruleus neurons stain more intensely. Neurons are illustrated from a 6-month old female. A. Neurons in the ventral substantia nigra contain low levels of TH immunoreactivity. B. Neurons in the ventral tegmental area contain high levels of TH immunoreactivity. C. Neurons in the locus coeruleus also stain intensely for TH. Marker, 70 μm.

The regional differences in VMAT2 and TH ISI in midbrain DA and LC noradrenergic neurons are not related to the presence of NM pigment. In the brain of a 6-month old female, the VTA neurons contain over 4-fold higher TH ISI than the SNv neurons and over 2-fold higher VMAT2 ISI (Fig. 4 and 5). Likewise, the LC contained the highest VMAT2 ISI and the TH ISI was second to that of VTA DA neurons (Table 2). These data indicate that the presence of NM pigment in brains from 22-years to 65-years of age do not change the relative ISI for either protein.

DISCUSSION

In midbrain DA neurons, there is an inverse relationship between NM pigment content and VMAT2 immunoreactivity. Neurons with high levels of VMAT2 immunoreactivity are located in the VTA, dorsal portion of the substantia nigra, and in the retrorubral field. The latter neurons have relatively low levels of NM pigment in brains in the 6th decade of life and older (3), when NM pigment has reached its greatest concentration in the nigral DA neurons (18). On the other hand, neurons in the SNv have low levels of VMAT2 immunoreactivity and high levels of NM pigment. A similar distribution of TH immunoreactivity was also found in the human midbrain DA neurons. Mann and Yates reported decreased cytoplasmic RNA levels in the neurons with high concentrations of NM pigment (18). Because the SNv neurons contain high densities of NM pigment, perhaps the pigment somehow lowers the expression of both TH and VMAT2 in the neurons. However, this does not appear to be the case since in the present study a 6-month old infant brain, with little-to-no NM pigment in the DA neurons, still had relatively low levels of TH and VMAT2 in the SNv vs. the VTA neurons. These data indicate that the relatively low protein levels that were observed are not the result of NM pigment in some way interfering with their synthesis.

In the midbrain DA cell complex, the levels of the rate-limiting enzyme in catecholamine synthesis, TH, and VMAT2 are directly correlated. Neurons with high levels of TH also have high levels of VMAT2. The lowest levels of TH and VMAT2 are found in the SNv somata. Intermediate-to-high levels of both markers were found in the neurons of the VTA, and were also observed in the retrorubral field, dorsal substantia nigra and substantia nigra pars lateralis. These data suggest that aminergic neurons that contain high levels of the catecholamine synthetic enzyme, TH, has a greater vesicular storage capacity for the amine than neurons expressing low levels of TH. In vitro studies have found that when synaptic vesicles contain more VMAT2 molecules, they can store and release more catecholamine (19). Since VTA neurons tend to fire in bursts that release more neurotransmitter than the SN neurons which often fire in a non-burst, regular fashion (20-22), the VTA neurons may need more TH and VMAT2 protein than the SN DA neurons.

If catecholamine synthesis and vesicular storage are positively correlated within catecholaminergic neurons in man, why do some neurons form more NM pigment than others? In rodent midbrain DA neurons, TH and VMAT2 ISI are also positively correlated (German et al., unpublished observation), but there is normally no NM formation in the rodent neurons. In rodents, it may be that the animals do not live long enough to accumulate sufficient amounts of NM pigment to be visible at the light microscopic level. Also, it may be that most of the catecholamine that is synthesized is stored within synaptic vesicles, whereas in man some of the neurons make disproportionately more amine than can be stored in vesicles. Although the SNv neurons possess relatively low levels of TH, they must also have proportionately lower levels of VMAT2 such that there is still a pool of DA that does not get stored in synaptic vesicles, which can be oxidized to ultimately form NM pigment. The substantia nigra neurons, compared to other midbrain DA neurons, contain the highest level of DA transporter mRNA (23). The SNv neurons have dendrites that extend ventrally into the substantia nigra pars reticulata, and the dendrites release DA (24) from a non-vesicular pool via reversal of the plasma membrane DA transporter (25). This non-vesicular pool of DA, used for dendritic communication with DA and non-DA neurons, may provide an important source of amine that is ultimately metabolized to form NM pigment.

Why do the SNv and LC neurons die in PD? The SNv neurons are the most vulnerable neurons to degenerate in the midbrain DA cell complex (4,5,26). These DA neurons appear to be the first ones to degenerate in the disease (4). There is also extensive degeneration of the LC neurons in PD (27,28). The aminergic cell death in PD may depend upon several factors. Cells with relatively high levels of NM, like the LC and SNv neurons, may reflect neurons with relatively large pools of cytosolic amine that exist in addition to those in synaptic vesicles. This pool can be

used for dendro-dendritic communication in the SN (25), and a similar amine pool in the LC may also exist (29). It can be hypothesized that when a sizeable non-vesicular pool of amine exists in the cytoplasm for a prolonged period of time, reactive oxygen species (ROS) are formed along with toxic DA-adducts. The ROS and adducts are stored in lysosomal structures which constitute the NM pigment granule (8). It may be that after the neuron has accumulated an excessive amount of NM pigment, additional ROS and toxic DA-adducts can no longer be stored in the form of NM pigment, and once the toxins are in the cytoplasmic compartment the neuron becomes "poisoned". Consistent with this hypothesis, old monkeys treated with the DA neurotoxin MPTP exhibit a preferential loss of NM-containing DA neurons vs. DA neurons without NM (i.e., cells that stain positively for TH) (30).

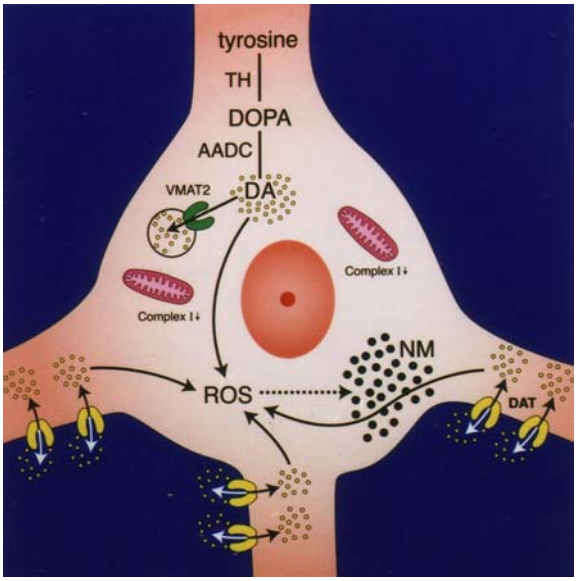

Fig. 6. Model to explain the interaction of VMAT2, dopamine transporter (DAT), neuromelanin (NM) pigment and Complex I in the selective degeneration of ventral substantia nigra DA neurons in PD. DA is synthesized by tyrosine, via tyrosine hydroxylase (TH) to L-DOPA, and via the enzyme aromatic amino acid decarboxylase (AADC) to DA. DA can be accumulated within synaptic vesicles via VMAT2. Some DA remains in the cytoplasm, which becomes oxidized via enzymes in the mitochondria (purple) to reactive oxygen species (ROS), and these DA-related products are stored within a lysosome-like structures as NM. Notice that the DA that is taken up into the cytoplasm via the plasma membrane DAT may also be a source for NM pigment formation. In PD, because Complex I of mitochondria respiration is often compromised, it is proposed that the resultant decrease in ATP formation causes VMAT2 to sequester lower than normal amounts of DA within synaptic vesicles. When there is more cytoplasmic DA, which ultimately leads to the formation of NM pigment, the cells become more vulnerable to degeneration.

Another important factor that contributes to aminergic cell death in PD relates to mitochondrial function. The SN DA neurons require sufficiently high levels of ATP formation to provide energy for cellular function. When mitochondrial respiration is inhibited pharmacologically, using malonate (31,32) and rotenone (33), there is a disproportionate damage to SN DA neurons. In rats, systemic administration of rotenone, a pesticide that is an inhibitor of mitochondrial Complex I, causes degeneration of SN DA neurons, a sparing of VTA DA neurons, and the formation of Lewy body-like structures (33). Individuals with sporadic PD have a defect in Complex I of the mitochondrial respiratory chain (34). Since VMAT2 requires ATP to pump amine into synaptic vesicles, individuals with a Complex I defect may have higher than normal levels of cytoplasmic DA, which hastens NM pigment formation, α-synuclein-containing Lewy body formation (35), and leads to premature cell death (Fig. 6). If this scenario proves to be correct, then therapeutic intervention could be accomplished by the early identification of individuals with a Complex I defect, and pharmacological treatments aimed at lowering cytoplasmic amine levels, which should prolong the life of the remaining midbrain aminergic neurons.

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