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The Origins of Oxidant Stress in Parkinson's Disease and Therapeutic Strategies

Dalton James Surmeier, Jaime N. Guzman, Javier Sanchez-Padilla, and Joshua A. Goldberg

Abstract

Parkinson's disease (PD) is a major world-wide health problem afflicting millions of the aged population. Factors that act on most or all cell types (pan-cellular factors), particularly genetic mutations and environmental toxins, have dominated public discussions of disease etiology. Although there is compelling evidence supporting an association between disease risk and these factors, the pattern of neuronal pathology and cell loss is difficult to explain without cell-specific factors. This article focuses on recent studies showing that the neurons at greatest risk in PD—substantia nigra pars compacta dopamine neurons—have a distinctive physiological phenotype that could contribute to their vulnerability. The opening of L-type calcium channels during autonomous pacemaking results in sustained calcium entry into the cytoplasm of substantia nigra pars compacta dopamine neurons, resulting in elevated mitochondrial oxidant stress and susceptibility to toxins used to create animal models of PD. This cell-specific stress could increase the negative consequences of pan-cellular factors that broadly challenge either mitochondrial or proteostatic competence. The availability of well-tolerated, orally deliverable antagonists for L-type calcium channels points to a novel neuroprotective strategy that could complement current attempts to boost mitochondrial function in the early stages of the disease. *Antioxid. Redox Signal*. 14, 1289–1301.

Pan-Cellular Factors in Parkinson's Disease

STUDIES OVER THE PAST DECADE have made great progress in identifying factors that increase disease risk. The vast majority of these is pan-cellular factors, that is, factors that in principle have a broad, negative impact on neuronal and non-neuronal cell types. The four best documented pan-cellular factors are age, genetic mutations, environmental toxins, and inflammation. The biggest risk factor in Parkinson's disease (PD) is age (20, 25). Disease incidence rises exponentially above the age of 65. Because improvements in healthcare are increasing life expectancy, the number of PD patients is expected to grow dramatically in the coming years, reaching over 2 million in the United States by 2030 (27). Why age is such a strong risk factor is unknown, but it is widely speculated that declining mitochondrial function is a key factor (16, 104).

In the last decade, perhaps the greatest single advance in the PD field has been the identification of genes that increase disease risk (34, 68). Although these still account for <10% of all the cases of PD, in some ethnic populations genetic mutations appear to account for a much larger fraction of cases (68). Unfortunately, most of the PD-associated genes are of unknown or poorly understood function. However, this gap is rapidly closing.

One of the emerging themes in the study of genetic risk factors is mitochondrial dysfunction. Three of the genes associated with a recessive, early onset form of the disease (*DJ-1*, PTEN-induced putative kinase 1 [*PINK1*], and *parkin*) are directly linked to mitochondrial function, providing a potential connection with changes associated with aging (104). DJ-1 is a mitochondrially enriched, redox-sensitive protein, giving it the capacity to signal oxidative challenges and potentially coordinate a variety of mitochondrial oxidative defense mechanisms (2, 58). Parkin and PINK1 also have mitochondrial roles. Fruit flies with functional deletions of *Parkin* have fragmented and apoptotic mitochondria (42); knockout mice have a less dramatic but a clear mitochondrial phenotype (including decreased mitochondrial [respiratory] function, decreased metabolic drive, and increased lipid and protein phosphorylation) (88). *PINK1* deletion leads to a similar phenotype in *Drosophila* as does *Parkin* deletion–fragmented cristae and apoptotic mitochondria; this phenotype can be rescued by Parkin overexpression, suggesting involvement in some common biochemical pathway (22, 89). Although found both in cytosolic and mitochrondrial preparations, PINK1 has an N-terminus mitochondrial targeting sequence (29). Although the functions of the other genes prominently linked to PD (*SNCA*, leucine-rich repeat kinase 2 [*LRRK2*]) remain

Department of Physiology, Feinberg School of Medicine, Northwestern University, Chicago, Illinois.

poorly defined, proteostatic dysfunction resulting in Lewy body (LB) formation is commonly thought to be an essential component of the disease etiology (112).

Another factor linked to PD that should in principle affect all cells is environmental toxin exposure. The proposition that toxins, particularly those that target mitochondria, could be a factor in PD has long been part of the mindset of the field given the ability of 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine neurotoxin (MPTP) and rotenone to reproduce key aspects of the disease phenotype (13, 95). Recent epidemiological studies have found convincing support for a link between pesticide exposure and the risk of developing PD (59, 115).

A fourth pan-cellular factor in PD is inflammation (46, 48, 52). In toxin models of PD, inflammation and resultant oxidant stress are important modulators of cell loss (53, 117–119). In the later stages of the human disease, there are clear signs of microglial activation and inflammation that could contribute to progression (116). Recent work has shown how extrinsic oxidant stress, like that created by inflammation, could result in neuronal death in a cell with high cytosolic calcium levels. Reactive oxygen species (ROS)-mediated activation of protein kinase C beta phosphorylates 66-kDa isoform of the growth factor adapter Shc ($p66^{shc}$), promoting transport into mitochondria, where it alters calcium responses and promotes apoptosis (93).

In the last year, the proposition that a fifth pan-cellular factor—a viral or prion-like infection—is causative in PD has been advanced (47, 86). In the absence of a direct demonstration of an infectious agent, there are two main

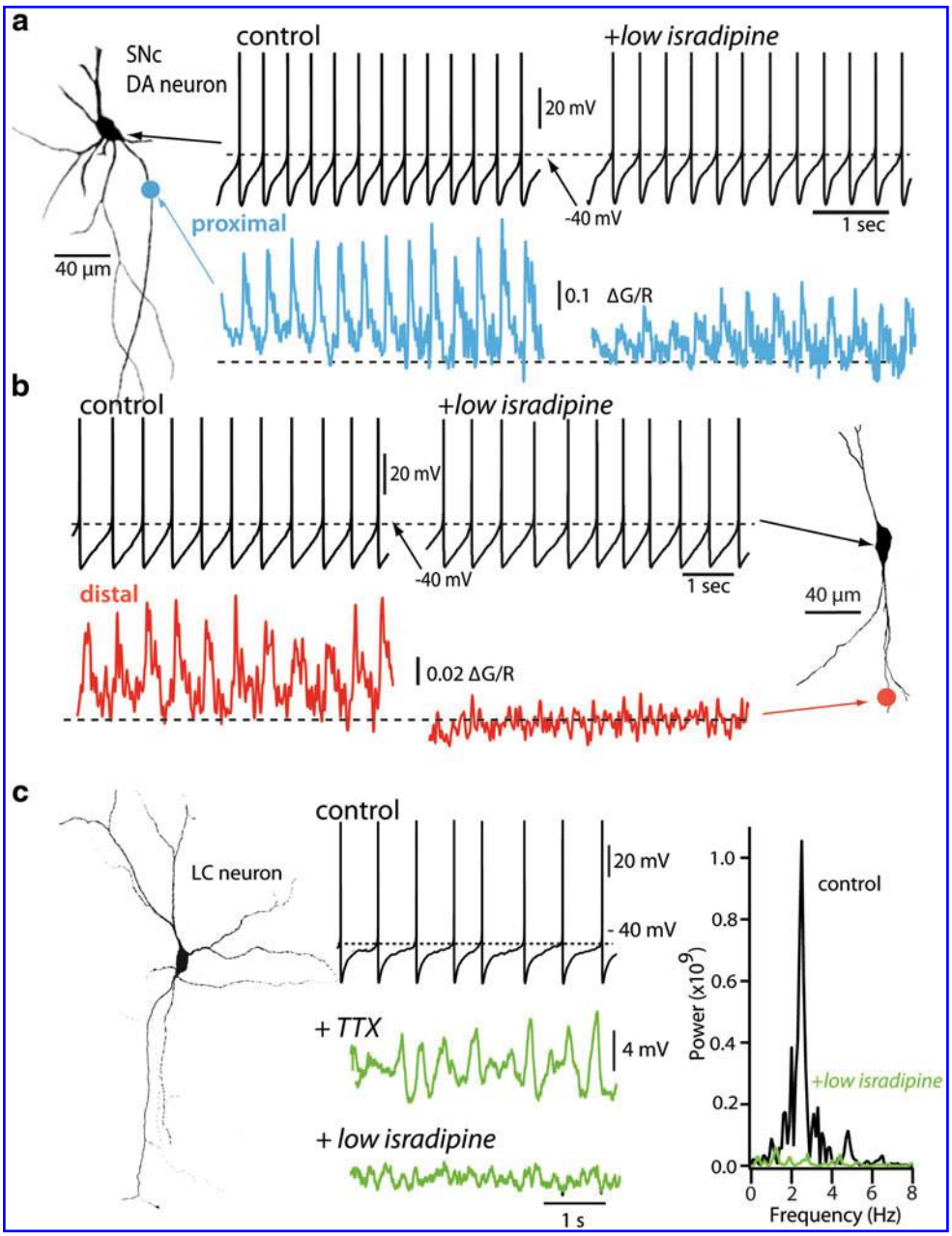

FIG. 1. Physiological properties of substantia nigra pars compacta (SNc) and locus ceruleus (LC) neurons. (a, b) SNc dopamine (DA) neurons pacemaker spikes are coupled to calcium transients sensitive to L-type calcium channel blockers. Shown in the *left*, a reconstruction of an SNc DA neuron filled with the red dye Alexa594 (50 μM) and the calcium-sensitive Fluo4 (200 μM). Shown to the *right* of the DA neuron, representative pacemaking traces before and after bath application of the L-type calcium channel blocker (5 μM isradipine), and below pacemaking traces, time-matched dendritic calcium transients from **(a)** proximal and **(b)** distal dendrite (>80 μm away from soma). Calcium transients in distal dendrites were abolished by application of isradipine, yet the pacemaking firing was insensitive to this calcium channel blocker. **(c)** Shown on the *left* is a biocytin-filled LC neuron illustrating extensive dendritic arbor. *Middle panel* shows pacemaking firing from an LC neuron before and after blockade of sodium spikes with 1 μM tetrodotoxin (TTX). Membrane potential oscillations (green trace) were eliminated by bath application of 5 μM isradipine. **(a, b)** Adapted with permission from earlier published work [© Guzman *et al.* (43)]. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article at www.liebertonline.com/ars).

pieces of evidence that have been used to argue for this type of process. The first is apparent staging of LB pathology in PD (17); the Braak hypothesis asserts that the pathology progresses from peripheral enteric autonomic ganglia, to the caudal medullary autonomic cell groups and then rostrally into the brain. This apparent progression has been taken as evidence of an infection (47). However, it is far from clear that there is this sort of progression in the majority of PD patients, as the whole hypothesis turns on the supposition that patients with medullary and ganglionic pathology alone would have developed PD had they lived longer. Moreover, there is considerable variability in the regional pattern of LB pathology and LBs have an uncertain connection to the pathophysiology underlying the symptoms of the disease (18, 57). The second piece of evidence is derived from grafting embryonic dopamine (DA) neurons into PD patients (65, 69, 77). In some of these grafts, DA neurons had LBs. Since these neurons were relatively young, the appearance of LBs has been taken as evidence of the spread of a virus or of a prion-like agent from the host. However, these data are open to alternative interpretation. The most obvious of which is that DA neurons are particularly susceptible to the stress of grafting, leading to premature proteostatic dysfunction and LB formation. The apparent restriction of LBs to the DA neurons in the graft is certainly consistent with this explanation and not with an infection model. More importantly, at present, there is no compelling evidence that the pattern of neuronal pathology in PD conforms to the predictions of an infection model. The LB pathology in PD does not follow a nearest neighbor rule. Neurons in the nucleus tractus solitarius, for example, show no signs of pathology in PD in spite of being next to neurons in one of the most vulnerable nuclei (dorsal motor nucleus of the vagus [DMV]). There is no evidence that vulnerability is predicted by synaptic connectivity either. Arguments made that connectivity is an issue consistently ignore the fact that every major neuronal population affected in PD is synaptically coupled to a population of neurons that do not display significant pathology. In view of the dearth of hard scientific support, the infection model of PD is difficult to take seriously.

Thus, studies of pan-cellular factors in PD have identified several potential processes in the etiology of PD, the most compelling of which are mitochondrial and proteostatic dysfunction. What is left unexplained by these studies is the pattern of neuronal dysfunction and loss in PD.

Cell-Specific Factors in PD

The motor symptoms, including bradykinesia, rigidity, and resting tremor, are clearly linked to the degeneration and death of a small group of neurons in the mesencephalon—substantia nigra pars compacta (SNc) DA neurons (49, 99). The palliative efficacy of levodopa or L-3,4-dihydroxyphenylalanine (L-DOPA)—a DA precursor—is testament to the centrality of these neurons in the motor symptoms of PD. These neurons constitute a tiny fraction of all the neurons in the brain (<0.0001%), arguing that there must be cell-specific factors at work in PD. What might these factors be? DA itself has long been viewed as a culprit, as oxidation of cytosolic DA (and its metabolites) is damaging (40, 112). However, there are reasons to doubt this type of cellular stress alone is responsible for the loss of DA neurons in PD. First, there is considerable regional variability in the vulnerability of DA neurons in PD, with some being devoid of pathological markers (24, 55, 62, 75, 103). Second, L-DOPA administration (which relieves symptoms by elevating DA levels in PD patients) does not appear to accelerate disease progression (30), suggesting that DA is not a significant source of reactive oxidant stress, at least in the short term. Sulzer and colleagues have recently reported that calcium entry through L-type channels stimulates DA metabolism in SNc DA neurons, pushing cytosolic DA concentrations into a toxic range with L-DOPA loading (81). For this mechanism to be relevant to selective vulnerability, one would have to posit that modest elevations in cytosolic DA over decades lead to an accumulation of cellular defects that ultimately produces cell death. If true, treating patients in the early stages of the disease with direct acting agonists, rather than L-DOPA, should lead to a slower progression of the disease. That said, the frank death or phenotypic decline of a variety of nondopaminergic neurons in PD argues that DA itself is not likely to be the principal cell-specific risk factor in the disease.

Another distinctive feature of SNc DA neurons, and many of the other neurons that succumb in PD (*e.g.,* locus ceruleus [LC] neurons), is their enormous axonal field. Recent anatomical work has estimated that a typical SNc DA neuron has mean axonal length of 470,000 μm (74). Each axon supports $\sim$370,000 synapses, orders of magnitude higher than the number supported by cortical pyramidal neurons for example (3). Sustaining such a large field must elevate axonal protein trafficking and proteostatic stress. Given that alpha-synuclein is largely a synaptic protein, its trafficking must be elevated in SNc DA neurons, potentially contributing to the axonal pathology seen in PD patients (33). Moreover, because synaptic terminals are metabolically demanding specializations that typically require mitochondria, sustaining this axonal field could create a mitochondrial sink for these neurons, lowering mitochondrial density in the somodendritic region and lowering spare oxidative capacity, creating an energy crisis (85). In fact, mitochondrial density in the somatodendritic region of SNc DA neurons appears to be abnormally low (71). Diminished oxidative reserve capacity could increase the production of damaging superoxide by mitochondria, contributing to their decline with age.

Our work in the last few years has focused on the potential role of physiological phenotype. Unlike the vast majority of neurons in the brain, adult SNc DA neurons are autonomously active, generating regular, broad action potentials (2–4 Hz) in the absence of synaptic input (21, 39, 43, 84). This pacemaking activity is believed to be important to maintaining ambient DA levels in regions that are innervated by these neurons, particularly the striatum (102). Although most neurons rely exclusively on monovalent cation channels to drive pacemaking, SNc DA neurons also engage L-type ion channels that allow calcium to enter the cytoplasm (15, 92, 96), leading to oscillations in intracellular calcium concentrations (21, 43, 125) (Fig. 1a, b). The L-type calcium channels used by SNc DA neurons in pacemaking have a distinctive Cav1.3 pore-forming subunit encoded by *Cacna1d* (21, 111). Cav1.3 calcium channels are relatively rare, constituting only about 10% of the all the L-type calcium channels found in the brain (107). Channels with this subunit differ from other L-type calcium channels in that they open at relatively hyperpolarized potentials, allowing them to contribute to the mechanisms

driving the membrane potential to spike threshold underlying autonomous pacemaking (21, 43, 96).

The sustained engagement of Cav1.3 calcium channels during pacemaking comes at an apparent metabolic cost to SNc DA neurons. Because of its involvement in cellular processes ranging from the regulation of enzyme activity to programmed cell death, calcium is under very tight homeostatic control, with a cytosolic set point near 100 n*M*—10,000 times lower than the concentration of calcium in the extracellular space (12, 87, 101). Calcium entering neurons is rapidly sequestered or pumped back across the steep plasma membrane concentration gradient; this process requires energy stored in adenosine triphosphate (ATP) or in ion gradients that are maintained with ATP-dependent pumps, like the Na-K ATPase. In most neurons, calcium channel opening is a rare event, occurring primarily during very brief action potentials. This makes the task and the metabolic cost to the cell readily manageable, but in SNc DA neurons, where Cav1.3 calcium channels are open much of the time, the magnitude and the spatial extent of calcium influx are much larger (125). Preliminary studies using transgenic mice that express a mitochondrially targeted redox-sensitive variant of green fluorescent protein under control of the tyrosine hydroxylase promoter have revealed that indeed mitochondria in SNc DA neurons have a high basal oxidant stress that is a direct consequence of opening of L-type calcium channels. Further, calcium entry (and presumably the concomitant oxidant stress) increases the vulnerability of SNc DA neurons to toxins (MPTP, 6-hydroxydopamine, and rotenone) used to create animal models of PD (21).

Another reason to suspect that calcium is an important factor is the inverse correlation between expression of the mobile calcium buffering protein calbindin and vulnerability in PD, as well as in animal models of the disease (35). Calbindin expression is high in DA neurons of the ventral tegmental area as well as the dorsal tier of the SNc, both areas that are relatively resistant in PD. What is less clear is precisely why this should be the case. One possibility is that calbindin reduces calcium entry into the endoplasmic reticulum, holding it for plasma membrane extrusion mechanisms and avoiding double pumping.

The glutamatergic synaptic input to SNc DA neurons could also contribute to their vulnerability. *In vivo*, SNc DA neurons spike in at least two other modes that are created by superimposing synaptic input on the basal pacemaking activity (120). From a functional standpoint as well as from the standpoint of neurodegeneration, the most interesting of these is the burst mode. Because of its association reward prediction errors (105), elevated release of DA in the striatum and the induction of long-term synaptic plasticity, this burst has generated a great deal of experimental attention. A bevy of studies have recently focused on the mechanisms underlying the burst. These studies have established the necessity of N-methyl-D-aspartate (NMDA) receptor activation in burst generation (14, 26, 127). Because pacemaking keeps the membrane potential of SNc DA neurons in a voltage range where magnesium block of NMDA receptors is ineffective, even modest glutamatergic input is capable of producing substantial NMDA receptor currents. Could calcium entry through NMDA receptors synergize with that through Cav1.3 channels engaged by pacemaking to create a metabolic tipping point for mitochondria? Excitotoxicity has long been hypothesized to be a factor in the etiology of PD (6, 41, 108), but the engagement of NMDA receptors and the elevation in cytosolic calcium concentration this brings about has been envisioned to be a relatively late stage event, coming only when cells were unable to maintain a stable, hyperpolarized membrane potential. Nevertheless, SNc DA neurons are pacemakers that do not have a stable hyperpolarized membrane potential when they are healthy, meaning that NMDA receptors should be more easily recruited. Another factor in this equation is the intracellular calcium stores. Metabotropic glutamate receptor (mGluR) activation mobilizes these stores (80), forcing neurons to re-sequester calcium (at the expense of ATP). Because of the sustained calcium entry into SNc DA neurons during pacemaking, these stores are fully charged, which adds to the burden created by mGluR activation. In this way, pacemaking and regular activation of NMDA and mGluRs should create a sustained calcium storm in SNc DA neurons. Although mitochondria appear capable of weathering this storm in the short-term, the elevation in oxidant stress created by the need to supply a steady stream of ATP to pumps should slowly increase damage to their DNA and accelerate their aging (11, 98). Figure 2 summarizes the cell-specific risk factors that could elevate oxidant stress in SNc DA neurons.

PD, ROS, and Aging

One of the oldest and most popular theories of aging is that it is a direct consequence of accumulated mtDNA and organelle damage produced by ROS and related reactive molecules generated by the electron transport chain in the course of oxidative phosphorylation (44, 123). A corollary of this hypothesis is that the rate of aging is directly related to metabolic rate. There is no obvious reason not to extend this organismal postulate to individual cells. The reliance of SNc DA neurons on a metabolically expensive strategy to generate autonomous activity that taxes mitochondria should mean that they age more rapidly than other types of neuron. Is then PD simply a reflection of accelerated aging in neurons that rely too heavily upon Ca^{2+} channels to do their business? Age is undoubtedly the single strongest risk factor for PD (20, 37). Stereological estimates of normal aging related cell death in humans argue that SNc DA neurons at a higher risk than other neurons in the absence of environmental toxins or pathogens, as they are lost at a significantly higher rate than many other types of neurons (some of which show no appreciable loss over a 6–7-decade span) (109). In mammals with significantly shorter lifespans, loss of SNc DA neurons with age has not been seen reliably, but there is a clear decline in phenotypic markers with age that matches that seen in PD, as well as an increased susceptibility to toxins (4, 23, 54, 60, 61, 76). Taken together, these studies make a case that SNc DA neurons age more rapidly than the vast majority of the neurons in the brain.

This wear-and-tear theory suggests that PD is first and foremost a consequence of aging. Why then do some people become symptomatic in their 50s, others in their 60s or 70s, or not at all in an 80- or 90-year life? Genetic factors certainly could account for a large part of this variation (20, 79, 112). These factors could increase the rate at which vulnerable neurons age by compromising mitochondrial function or Ca^{2+} homeostasis. Several of the genetic mutations associated

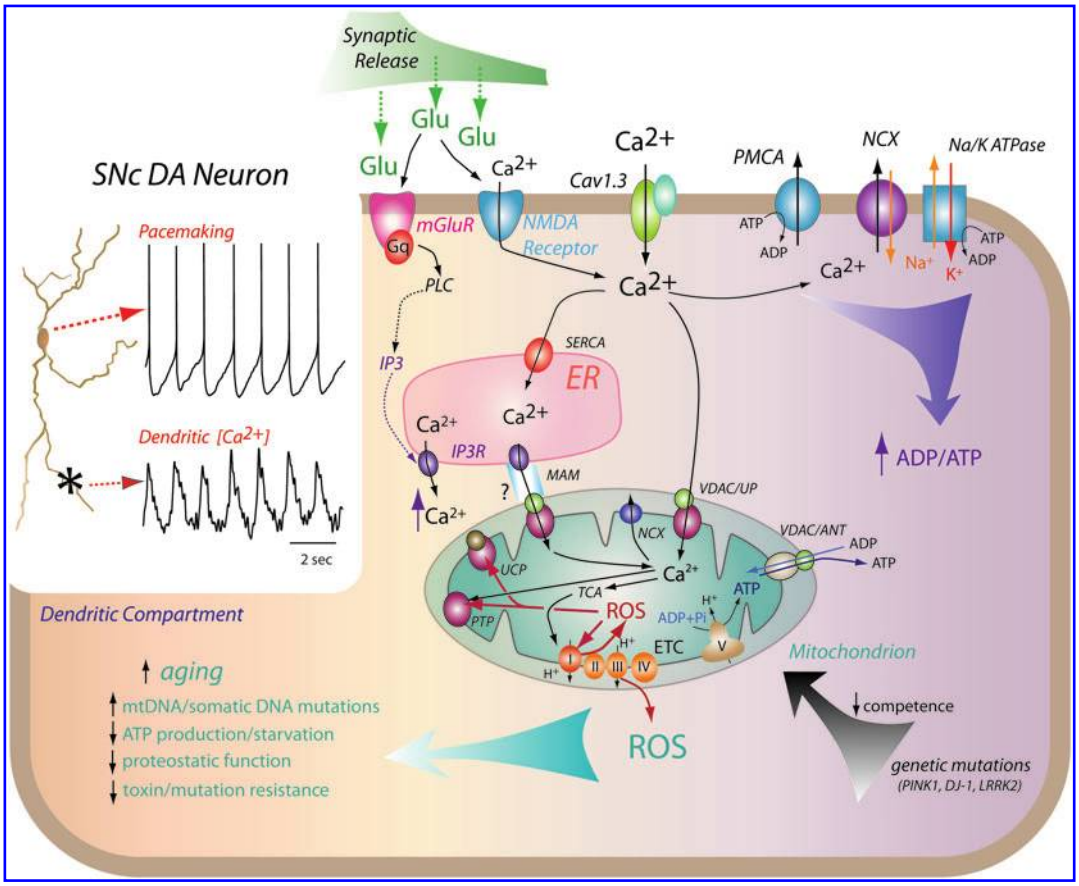

FIG. 2. Calcium signaling in SNc DA neurons could lead to mitochondrial oxidant stress, accelerated aging, and eventual cell death. The steep concentration gradient for calcium allows it to cross the plasma membrane readily into cells through open pores, like L-type Cav1.3 calcium channels, or by synaptic activation of N-methyl-D-aspartate (NMDA) receptors. In addition, the model considers the importance of metabotropic glutamate receptors (mGluRs) as regulators of intracellular calcium *via* endoplasmic reticulum (ER) calcium stores. Once inside neurons, calcium is either transported back across the plasma membrane or sequestered in intracellular organelles. Calcium is transported across the plasma membrane through either the calcium-ATPase (PMCA) or through a sodium/calcium exchanger (NCX) that relies upon the Na^+ gradient. Calcium is rapidly sequestered either by ionic interactions with buffering proteins or by transport into cytosolic organelles (*i.e.*, the mitochondria and the ER). The ER uses high-affinity sarcoendoplasmic reticulum calcium ATPase (SERCA) pumps that depend upon ATP to take calcium from the cytoplasm into the ER lumen. Calcium flows back into the cytoplasm after opening of inositol triphosphate receptors (IP3R) and ryanodine receptors (RyRs) studding the ER membrane. Mitochondria are often found in close apposition to the ER and plasma membrane, creating a region of high (but localized) calcium concentration that drives calcium into the matrix of mitochondria through a calcium uniporter. ER membrane fuse with mitochondria to create mitochondrial-associated membrane (MAM) pores that allows direct communication between the ER and mitochondrion. Calcium can leave the mitochondrion through a number of mechanisms. The dominant mitochondrial calcium efflux path in neurons is through mitochrondrial NCXs. Calcium release through higher conductance ion channels, like the mitochondrial permeability transition pore (mPTP), has also been proposed. The mPTP is posited to have two conductance states: a low conductance state that is reversible and participates in physiological calcium handling, and a high conductance state that is irreversible and leads to mitochondrial swelling and loss of molecules like cytochrome C that trigger apoptosis. Also shown schematically are elements of the tricarboxylic acid (TCA) cycle that produces reducing equivalents for the electron transport chain complexes I–IV. The electrochemical gradient created drives adenosine triphosphate (ATP) synthase and the conversion of ATP from adenosine diphosphate (ADP) delivered to the matrix by the adenine nucleotide transporter (ANT). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article at www.liebertonline.com/ars).

with early onset forms of PD are directly linked to mitochondrial dysfunction (104). Unfortunately, animal models of these forms of PD have failed to recapitulate the disease. For example, mutations of *DJ-1* (*PARK7*) are associated with an early onset form of PD. DJ-1 is redox sensitive, giving it the capacity to signal oxidative challenges and potentially coordinate a variety of mitochondrial oxidative defense mechanisms (58). Although deleting *DJ-1* increases the sensitivity of cells generally to oxidative challenge, it remains to be determined how it compromises SNc DA neurons under more physiological conditions. Studies of *DJ-1* knockouts have not revealed any loss of SNc DA neurons or a fundamental shift in their physiology, only a modest deficit in DA release during burst stimulation that is difficult to tie to an increased vulnerability (38), but there have been no studies in these mice of mitochondrial physiology in mature SNc DA neurons where

Ca^{2+} influx during pacemaking has fully developed. This could prove to be critical to an understanding of the mechanisms that could accelerate the loss of these cells over decades.

Two other genes linked to familial PD have unequivocal linkages to mitochondria. One is Parkin (*PARK2*). Fruit flies with functional deletions of Parkin have fragmented and apoptotic mitochondria (42); knockout mice have a less dramatic but a clear mitochondrial phenotype (including decreased mitochondrial [respiratory] function, decreased metabolic drive, and increased lipid and protein phosphorylation) (88). Another is *PINK1* (*PARK6*). Its deletion leads to an identical phenotype in *Drosophila* as does Parkin deletion–fragmented cristae and apoptotic mitochondria; this phenotype can be rescued by Parkin overexpression, suggesting involvement in some common biochemical pathway (22, 89). Although found both in cytosolic and mitochrondrial preparations, PINK1 has an N-terminus mitochondrial targeting sequence (29). PINK1 deletion also compromises DA release during burst stimulation, like DJ-1 (63). It is not difficult to infer that loss of function mutations in either of these genes should have their biggest impact in a cell type that had a high basal level of metabolic activity, creating a mitochondrial oxidant stress, but to date, this has not been convincingly demonstrated.

PD Is Not Just a Disease of Dopaminergic Neurons in the SNc

In PD loss of neurons in multiple brain regions paralleling the loss of DA neurons in the SNc occurs (17, 32, 36, 56, 121). Two of the cell-specific risk factors standout as common to the other cell types that succumb in PD: autonomous or spontaneous activity with broad action potentials and a depolarized membrane potential that promotes the opening of NMDA receptors. Even though current data set is fragmented, neurons in the DMV, LC, raphe nuclei, pedunculopontine nucleus, lateral hypothalamus, tuberomammillary nucleus, basal forebrain, and olfactory bulb all have these two physiological characteristics, while having widely different transmitters and axonal fields. DMV cholinergic neurons, which are thought to be among the earliest neurons with LBs in PD, are spontaneously active (122); this activity is autonomously generated and depends upon L-type calcium channels (unpublished observations). LC noradrenergic neurons, like SNc DA neurons, have large axonal arbors and are autonomous pacemakers (with broad spikes) that engage L-type calcium channels (124) (Fig. 1c). Serotonergic neurons in the raphe nuclei have broad spikes and are calcium-dependent autonomous pacemakers (19). This is also true of pedunculopontine nucleus cholinergic neurons (114). Tuberomammillary and lateral hypothalamic (orexin expressing) neurons are spontaneously active (70, 110, 126). Tuberomammillary neurons engage L-type calcium channels in this process (110, 113, 124) (this question has not been addressed in orexin neurons). Basal forebrain cholinergic are lost in PD (17). These neurons have large axonal terminal fields, are spontaneously active in brain slices, and have prominent calcium channel currents (83). Moreover, with aging there are significant and deleterious changes in calcium homeostasis in these neurons (82). DA neurons in the olfactory bulb are a slightly different case from the standpoint of pathology. These neurons are calcium-dependent autonomous pacemakers (91). However, there are no signs of cell loss in the olfactory bulb in spite of deficits in olfaction being a harbinger of the motor symptoms in PD (50, 94). This does not mean that a reliance upon calcium is a bad thing though, as this region is capable of adult neurogenesis (90). Although much needs to be done, the shared physiological characteristics of these seven vulnerable cell types points to a common mechanism underlying their slow functional decline with age: calcium mediated stress.

The Interplay Between Pan-Cellular and Cell-Specific Risk Factors in PD

Age is the strongest of the pan-cellular risk factors. One of the oldest and most popular theories of aging is that it is a direct consequence of accumulated mtDNA and organelle damage produced by ROS and related reactive molecules generated by the electron transport chain in the course of oxidative phosphorylation (45, 123). A corollary of this hypothesis is that the rate of aging is directly related to metabolic rate. There is no obvious reason not to extend this organismal postulate to individual cells. The reliance of SNc DA neurons on a metabolically expensive strategy to generate autonomous activity that taxes mitochondria should mean that they age more rapidly than other types of neuron—creating a positive interaction between cell-specific and pan-cellular risk factors. This perspective predicts that there should be functional impairment or loss of SNc DA neurons with normal aging. Stereological estimates of normal aging-related cell death in humans argue that SNc DA neurons at a higher risk than many other types of neuron (109). In mammals with significantly shorter lifespans, loss of SNc DA neurons with age has not been seen reliably, but there is a clear decline in phenotypic markers with age that matches that seen in PD, as well as an increased susceptibility to toxins (4, 23, 54, 61, 76). There is also an aging-related decline in SNc mitochondrial function (5), some of which could easily be attributed to the accumulation of mitochondrial DNA mutations with normal aging (10).

There could be a positive interaction between cell-specific mitochondrial stress and the other pan-cellular risk factors. As with aging, an interaction of this sort could help create a tipping point in the pathogenesis of PD that would results in a selective pattern of degeneration. Consider the loss of DJ-1 function that compromise mitochondrial oxidant defenses. In most neurons, oxidant defense engagement is likely to be mild, episodic, and readily endured even without a fully functional defense system, but in cell types like SNc DA neurons, where oxidant stress appears to be sustained, compromising oxidant defenses could come at higher long term cost. Mutations that diminish proteostatic competence (*e.g.*, alpha-synuclein overexpression) could also exert their primary effects on cellular viability through increasing ATP utilization. It would be of considerable interest to see if overexpression of alpha-synuclein increased mitochondrial oxidant stress. Further, broadly acting environmental toxins that partially compromise mitochondrial function should have a bigger impact on cell types that have high mitochondrial demands. Lastly, inflammation in the late stages of the disease should significantly increase the production of ROS in and around surviving neurons. If these neurons are already overproducing ROS because of cell-specific factors, their oxidant defenses could be overwhelmed, leading to apoptosis.

Targeting Dihydropyridine-Sensitive L-Type Calcium Channels as a Potential Therapeutic Strategy for PD

Current therapeutic strategies are ostensibly targeting the pan-cellular factors (*e.g.,* Coenzyme Q10). Attacking the cell-specific factors is another strategy. Certain risk factors, like DA and axonal terminal field, are not malleable, at least not without compromising brain function. One factor that does appear to be a viable target is calcium entry through L-type calcium channels during autonomous or spontaneous spiking. These channels are antagonized by dihydropyridines (DHPs) that are approved for human use. DHPs have a very modest side-effect profile and have been used for decades to treat hypertension (28).

There are two basic questions that have to be answered before moving ahead with this kind of a neuroprotective strategy in the early stages of PD. The first is whether antagonizing L-type calcium channels will significantly impair the ability of SNc DA neurons to perform their duties. The second is whether neuroprotective concentrations of DHP can be achieved in the brain of PD patients.

Are L-type calcium channels necessary for pacemaking?

Several groups over the last 15 years have argued that voltage-dependent calcium channels were necessary (1, 78, 84, 125). Considering the sensitivity of pacemaking to DHPs, it has been inferred that these channels were L-type channels. In support of this view, SNc DA neurons robustly express L-type channels having a Cav1.3 pore-forming subunit with the kind of gating properties necessary to drive a sub-threshold membrane potential oscillation thought to underlie pacemaking (21, 43, 66). However, the concentrations of DHPs necessary to slow or stop pacemaking are more than three orders of magnitude higher than the equilibrium binding constant of most DHPs for Cav1.3 channels—raising basic questions about the necessity of these channels for pacemaking.

As a first step toward understanding what concentration of DHP is sufficient to antagonize L-type calcium channels in SNc DA neurons, a modulated receptor model was constructed using the framework proposed by Bean (8). In this model, the channel was assumed to have high and low affinity states governed by voltage-dependent inactivation (Fig. 3a). The macroscopic balance between these states was governed by voltage-dependent inactivation of the Cav1.3 calcium channel. Estimates of this parameter were taken from the work of Koschak *et al.* (66), in which channels were heterologously expressed in a cell type that was readily voltage-clamped (Fig. 3b). Because isradipine has a relatively high affinity for the Cav1.3 calcium channels thought to underlie pacemaking, our initial calculations modeled its actions (107). The apparent dissociation constant (K_{app}) of isradipine as a function of membrane voltage was computed using the formula proposed by Bean (1984) by assuming that the dissociation constant (K_D) for high affinity state was equal to that estimated from equilibrium binding studies (0.48 n*M*) and the dissociation constant for the low affinity state was a thousand fold higher (480 n*M*) (107) (Fig. 3c). Next, an all-points histogram of membrane potential during pacemaking was generated (Fig. 3d); this distribution had two modes: one near −60 mV and another near −40 mV. Considering this result, the relationship between isradipine concentration and the fraction of Cav1.3 channels available (not antagonized) was computed at − 50, −60, and (for comparison) −90 mV (Fig. 3e). This calculation showed that at either −50 or −60 mV, more than 90% of the Cav1.3 channels should be antagonized by 100 n*M* isradipine, whereas at −90 mV only about 30% of the channels should be antagonized. For the purposes of comparison, the dose–response curve for another commonly used, but less potent, DHP (nifedipine) was calculated at a potential of −60 mV. Considering this calculation, at equilibrium one micromolar nifedipine should antagonize >90% of the Cav1.3 channel population during pacemaking (Fig. 3f). These calculations strongly argue that at equilibrium, submicromolar concentrations of isradipine and other DHPs should effectively suppress Cav1.3 calcium channel currents and disrupt pacemaking that depends upon them. These calculations also show that serum concentrations of isradipine found in patients taking the medication for hypertension (~5 n*M*) should antagonize roughly half of the Cav1.3 channels in SNc DA neurons if we assume equilibrium across the blood–brain barrier (BBB).

Based upon these results, we re-examined the role of Cav1.3 channels in pacemaking. The problem is that we needed a measure of Cav1.3 channel function that was independent of pacemaking. We turned to calcium imaging using two photon laser scanning microscopy, allowing us to monitor pacemaking neurons deep in a brain slice from the mesencephalon. In these dual recordings, the dendritic calcium concentration oscillate in phase with somatic spiking. Bath application of 200 n*M* isradipine for 20 min (to allow an equilibrium to be achieved 50–100 μm below the surface of the slice) eliminated the dendritic calcium oscillation, but had no impact on pacemaking rate—providing a clear dissociation between Cav1.3 channel opening and pacemaking.

Does this mean that Cav1.3 channels are completely superfluous? Modeling the pacemaking process revealed that there is a rich interplay of ionic conductances that lead to autonomous spiking. Many of the conductances play similar roles, so that deficiencies in one can be compensated for to maintain the correct spiking rate. For example, when Cav1.3 channels are blocked in our model, outward currents through small conductance calcium-dependent potassium channels decline and the trajectory of the after hyperpolarization changes leading to stronger engagement of cationic hyperpolarization activated cyclic nucleotide (HCN) channels. The net effect on pacemaking is minimal. Similarly, if HCN channels are blocked by themselves, Cav1.3 current increases to compensate. However, if both HCN and Cav1.3 channels are blocked, pacemaking stops because the cell cannot generate enough inward current near spike threshold.

This behavior of the model was verified experimentally in SNc DA neurons. The takeaway message is that SNc DA neurons are well designed, with a robust network of ion channels to support pacemaking. It is a fail-safe system because pacemaking is so important to the functioning of the basal ganglia.

Our contention that Cav1.3 channels are not necessary for pacemaking has been challenged in a recent article (97). The crux of their argument is that they can experimentally restart pacemaking that has been halted with high concentrations of DHP by using dynamic current clamp to reintroduce Cav1.3 channels into the soma. The basic problem with this argument is that there is nothing unique about the inward conductance created by the dynamic clamp; introducing a Nav1

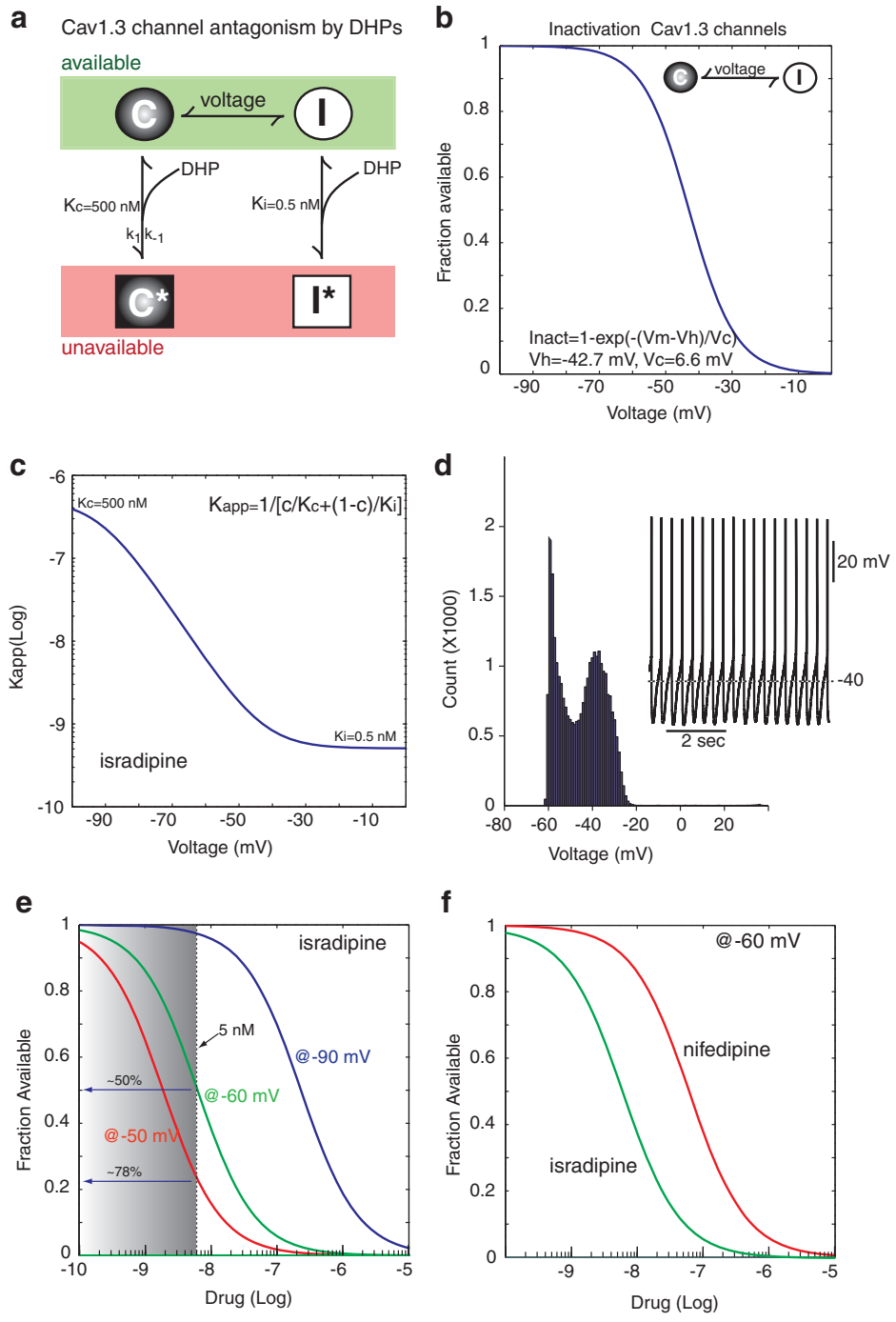

FIG. 3. Nanomolar concentrations of isradipine should produce a significant antagonism of Cav1.3 calcium channels. (a) Model of the modulated receptor model of dihydropyridine drug action. C stands for a closed state of the channel; C* stands for a closed, drug-bound state. The dissociation constant (K_c) for this reaction is assumed to be 500 n*M*. I stands for the inactivated state of the channel. Transitions between C and I states are controlled by membrane voltage. I* stands for the inactivated, drug-bound state; the dissociation constant (K_i) for this reaction is assumed to be 0.5 n*M*. **(b)** Steady-state inactivation of Cav1.3 channels as a function of membrane voltage; this describes the transition of channels from the C to I states in **(a)**. **(c)** Plot of the apparent dissociation constant as a function of transmembrane voltage computed from the equation $K_{app} = 1/[C/K_c + (1 - C)/K_i]$, where C is the fraction of the channels in the closed state from **(b)** and Kc and K_i are from **(a)**. **(d)** Recording of an SNc DA neuron and an all-points histogram of membrane potential showing that these neurons reside at potentials more depolarized than −60 mV. **(e)** Plot of the fraction of available channels as a function of drug concentration for three different membrane potentials. The plots reveal that with a holding potential of −60 mV, roughly half the channels are antagonized by 5 n*M* isradipine; only a small percentage of the channels will be antagonized by this drug concentration if the membrane potential is at −90 mV (as would be the case in a striatal medium spiny neuron). **(f)** Plot of the fraction of Cav1.3 channels available as a function of isradipine concentration (*green line*) at −60 mV or of a lower affinity antagonist nifedipine (*red line*). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article at www.liebertonline.com/ars).

channel or an HCN channel would produce the same outcome. So, in the end all the authors demonstrate that pacemaking is robust in the sense that it can be generated in several ways.

On the basis of findings from our group and others, the question that still remains is, could antagonizing L-type calcium channels prevent or slow PD in a normal lifespan? Calcium channel antagonists (CCAs), including the DHPs used in animal studies, are commonly used in clinical practice to treat hypertension, creating a potential database to be mined. A case–control study of hypertensive patients found a significant reduction in the observed risk of PD with CCA use, but not with medications that reduce blood pressure in other ways (9). More recently, a large Danish data set has been examined (100). The authors agreed with the main conclusions of the Becker *et al.* study but extended their findings by showing that only DHPs that cross the BBB are associated

with reduced PD risk (~30%). Given the short period of treatment in many cases (~2 years), variable dosing, and low relative affinity of DHPs for Cav1.3 calcium channels (compared to Cav1.2 channels) (28, 67, 73), this is a surprisingly strong association and lends further credence to the proposition that a BBB permeable and potent Cav1.3 antagonist could be a very effective neuroprotective agent.

That said, these studies are not a substitute for a controlled clinical trial. In the absence of a selective Cav1.3 CCA, the DHP isradipine is the most attractive drug for such a trial. Isradipine has a relatively higher affinity for Cav1.3 calcium channels than the other known DHP and has good brain bioavailability (107). At the doses used to treat hypertension, isradipine has relatively minor side effects (31). The question is whether it will prove neuroprotective at doses tolerated by the general population. Pharmacokinetic studies by our group have found that serum concentrations of isradipine achieved in mice that are protected (~1–2 ng/ml) against MPTP and 6-hydroxydopamine toxicity are very close to those achieved in humans with a very well-tolerated daily dose (10 mg/day, Dynacirc CR). As shown above, these isradipine concentrations should antagonize around half of the Cav1.3 channels in a pacemaking neuron, suggesting that neuroprotection is achievable.

It is also worth considering how DHPs might be used in combination with other drugs that are being tested in clinical neuroprotection trials for PD. Although early trials with creatine, coenzyme Q10, and other antioxidant supplements have been disappointing (51), they share the hypothesis that oxidant stress exacerbates the symptoms and progression of PD. Coenzyme Q10 is an electron acceptor for complexes I and II that appears compromised in PD patients (64, 106) and is neuroprotective in animal models of PD (6, 7). Creatine is a substrate for ATP production that can both improve mitochondrial efficiency and reduce oxidant stress by buffering fluctuations in cellular energy production (64). Both approaches are aimed at improving mitochondrial function rather than attacking the source of stress on mitochondria. Rasagline or deprenyl also could prove to have neuroprotective effects by virtue not of their ability to inhibit monoamine oxidase B, but by their ability to induce the expression of antioxidant defenses (72). Because their sites of action differ within the chain of events leading to oxidant stress and mitochondrial dysfunction, a combination therapy could prove more effective than any one therapy alone.

Conclusions

Even though pan-cellular risk factors dominate current thinking about the etiology of PD, there are compelling reasons to believe that cell-specific factors are important as well. From a theoretical standpoint, it is very difficult to explain the pattern of neuronal pathology in PD without these factors. While transmitter or anatomical phenotype might contribute to the vulnerability of SNc DA neurons, the trait with the clearest mechanistic path to cellular aging and degeneration is the engagement of L-type calcium channels in the generation of autonomous spiking. The sustained entry of calcium undoubtedly taxes the ATP-dependent pumps responsible for keeping its concentration low, and in so doing creates a burden on mitochondrial oxidative phosphorylation. An inevitable consequence of oxidative phosphorylation is the production of superoxide capable of damaging DNA and proteins. Although this metabolic stress is not sufficient to disable SNc DA neurons in the short run, it is possible that in the long run it exacerbates the normal aging-related decline in mitochondrial function, resulting in persistent energy shortages that compromise proteostatic competence. Because L-type channels are not necessary for SNc DA neurons to do their job, L-type channel antagonists seem to be a viable neuroprotective strategy. These drugs are well tolerated and safe, and their use is associated with a diminished risk of PD. Because this physiological phenotype is not unique to SNc DA neurons but appears to be shared by many of the neurons that succumb in PD, these antagonists could confer protection well beyond the SNc.

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Address correspondence to:
Dr. Dalton James Surmeier
Department of Physiology
Feinberg School of Medicine
Northwestern University
303 E. Chicago Ave.
Chicago, IL 60611

E-mail: j-surmeier@northwestern.edu

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Abbreviations Used

ADP = adenosine diphosphate
ANT = adenine nucleotide transporter
ATP = adenosine triphosphate
BBB = blood–brain barrier
CCAs = calcium channel antagonists
DA = dopamine
DHP = dihydropyridines
DMV = dorsal motor nucleus of the vagus
ER = endoplasmic reticulum
ETC = electron transport chain
Glu = glutamate
HCN = hyperpolarization activated cyclic nucleotide
IP3R = inositol triphosphate receptors
LB = Lewy body
LC = locus ceruleus
L-DOPA = levodopa or L-3,4-dihydroxyphenylalanine
LRRK2 = leucine-rich repeat kinase 2
MAM = mitochondrial-associated membrane
mGluR = metabotropic glutamate receptors
MPTP = 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine neurotoxin
mPTP = mitochondrial permeability transition pore
Na-K ATPase = sodium/potassium ATPase
NCX = sodium/calcium exchanger
NMDA = N-methyl-D-aspartate
PD = Parkinson's disease
P_i = inorganic phosphate
PINK-1 = PTEN-induced putative kinase 1
PLC = phospholipase C
PMCA = plasma membrane calcium ATPase
ROS = reactive oxygen species
RyRs = ryanodine receptors
SNc = substantia nigra pars compacta
SNCA = gene name for alpha-synuclein
TCA = tricarboxylic acid
TTX = tetrodotoxin
UCP = uncoupling proteins
UP = mitochondrial calcium uniporter
VDAC = voltage-dependent anion channel

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