CHAPTER 4

What causes the death of dopaminergic neurons in Parkinson’s disease?

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Abstract: The factors governing neuronal loss in Parkinson’s disease (PD) are the subject of continuing speculation and experimental study. In recent years, factors that act on most or all cell types (pan-cellular factors), particularly genetic mutations and environmental toxins, have dominated public discussions of disease aetiology. 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 chapter focuses on recent studies showing that the neurons at greatest risk in PD – substantia nigra pars compacta (SNc) dopamine (DA) 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 SNc DA 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.

Pan-cellular risk 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 are 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 strongest risk factor in Parkinson’s disease (PD) is age (Calne and Langston, 1983; de Lau and Breteler, 2006). Disease incidence rises exponentially above the age of 65. Because improvements in health care 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.

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(Dorsey et al., 2007). Why age is such a strong risk factor is unknown, but it is widely speculated that declining mitochondrial function is a key factor (Boumezbeur et al., 2010; Schapira, 2008).

In the last decade, perhaps the greatest single advance in the PD field has been the identification of genes that increase disease risk (Gasser, 2009). At present, seven genes have been clearly linked to familial forms of PD or Parkinsonism (Gasser, 2009; Lees et al., 2009). Although these still account for less than 10% of all the cases of PD, in some ethnic populations genetic mutations appear to account for a much larger fraction of cases (Lees et al., 2009). Unfortunately, most of the PD-associated genes are of unknown or poorly understood function. However, this gap is rapidly closing and there are common themes that are beginning to emerge.

One of these themes is mitochondrial dysfunction. Three of the genes associated with a recessive, early-onset form of the disease (DJ-1, PINK1, Parkin) are directly linked to mitochondrial function, providing a potential connection with changes associated with aging (Schapira, 2008). 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 defence mechanisms (Andres-Mateos et al., 2007; Kahle et al., 2009). Parkin and PTEN-induced putative kinase 1 or PINK1 also have mitochondrial roles. Fruit flies with functional deletions of Parkin have fragmented and apoptotic mitochondria (Greene et al., 2003); 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) (Palacino et al., 2004). 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 over-expression, suggesting involvement in some common biochemical pathway (Clark et al., 2006; Park et al., 2006). Although found both in cytosolic and in mitochondrial preparations, PINK1 has an N-terminus mitochondrial targeting sequence (Exner et al., 2007).

Although the functions of the other genes prominently linked to PD (SNCA, LRRK2) remain poorly defined, proteostatic dysfunction resulting in Lewy body (LB) formation is commonly thought to be an essential component of the disease aetiology (Sulzer, 2007).

The third pan-cellular factor that has been identified 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 (MPTP) and rotenone to reproduce key aspects of the disease phenotype (Betarbet et al., 2000; Przedborski et al., 2004). Recent epidemiological studies have found convincing support for a link between pesticide exposure and the risk of developing PD (Kamel et al., 2007; Tanner et al., 2009).

A fourth pan-cellular factor in PD is inflammation (Hartmann et al. 2003; Hirsch and Hunot, 2009; Hunot and Hirsch, 2003). In toxin models of PD, inflammation and resultant oxidant stress are important modulators of cell loss (Hunot et al., 2004; Teismann et al., 2003). In the later stages of the human disease, there are clear signs of microglial activation and inflammation that could contribute to progression (Tansey and Goldberg, 2010). Recent work has shown how extrinsic oxidative 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-kD isoform of the growth factor adapter Shc (p66SHC), promoting transport into mitochondria where it alters calcium responses and promotes apoptosis (Pinton et al., 2007).

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 (Hawkes et al., 2007; Olanow and Prusiner, 2009). In the absence of a direct demonstration of an infectious agent, there are two main pieces of evidence that have been used to argue for this type of process.
The first is apparent staging of LB pathology in PD (Braak et al., 2004); 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 (Hawkes et al., 2007). 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 (Burke et al., 2008; Jellinger, 2008). The second piece of evidence is derived from grafting embryonic dopamine (DA) neurons into PD patients (Kordower et al., 2008; Li et al., 2008; Mendez et al., 2008). 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 neighbour 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 with 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 aetiology 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.

**Pan-cellular risk factors**

- Age and declining mitochondrial function
- Genetic mutations that compromise mitochondrial or proteostatic function
- Environmental toxins that target mitochondria
- Inflammation (late stage?)

**Cell-specific risk factors in PD**

Although there are signs of distributed neuropathology in PD (as judged by LB formation) (Braak et al., 2004), the motor symptoms, including bradykinesia, rigidity and resting tremor, are clearly linked to the degeneration and death of substantia nigra pars compacta (SNC) DA neurons (Hornykiewicz, 1966; Riederer and Wuketich, 1976). The palliative efficacy of L-DOPA – a DA precursor – is testament to the centrality of these neurons in the motor symptoms of PD. SNC DA 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 (Greenamyre and Hastings, 2004; Sulzer, 2007). 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 (Damier et al., 1999; Ito et al., 1992; Kish et al., 1988; Matzuk and Saper, 1985; Saper et al., 1991). Second, L-DOPA administration (which relieves symptoms by elevating DA levels in PD patients) does not appear to accelerate disease progression (Fahn, 2005), suggesting that DA is not a significant source of reactive oxidative 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 (Mossharov et al., 2009). 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 produce 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 non-dopaminergic 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 coeruleus [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 (Matsuda et al., 2009). Each axon supports ~370 000 synapses, orders of magnitude higher than the number supported by cortical pyramidal neurons, for example (Arbuthnott and Wickens, 2007). 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 (Galvin et al., 1999). 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 somatodendritic region and lowering spare oxidative capacity, creating an energy crisis (Nicholls, 2008). In fact, mitochondrial density in the somatodendritic region of SNc DA neurons appears to be abnormally low (Liang et al., 2007). 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 (Fig. 1a–d) (Chan et al., 2007; Grace and Bunney, 1984; Guzman et al., 2009; Nedergaard et al., 1993). This pacemaking activity is believed to be important to maintaining ambient DA levels in regions that are innervated by these neurons, particularly the striatum (Romo and Schultz, 1990). While 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 (Bonci et al., 1998; Ping and Shepard, 1996; Puopolo et al., 2007), leading to oscillations in intracellular calcium concentrations (Chan et al., 2007; Guzman et al., 2009; Wilson and Callaway, 2000) (Fig. 1e). The L-type calcium channels used by SNc DA neurons in pacemaking have a distinctive Cav1.3 pore-forming subunit encoded by Cav1.3 (Chan et al., 2007; Striessnig et al., 2006). Cav1.3 calcium channels are relatively rare, constituting only about 10% of the all the L-type calcium channels found in the brain (Sinnegger-Brauns et al., 2009). 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 (Chan et al., 2007; Guzman et al., 2009; Puopolo et al., 2007).
Fig. 1. SNc DA neurons have a distinctive physiology. (a) Representative acute midbrain slice illustrating the SNc DA neurons of the ventral tier region (highlighted in red) selected for electrophysiological recordings. (b) Image depicting a patched SNc DA neuron visualized under infrared differential interference contrast (IR-DIC) microscopy. (c) Whole-cell current clamp recording displaying firing of action potentials for an SNc DA neuron. SNc DA neurons are neuronal pacemakers with firing frequency between 1 and 4 Hz. (d) SNc DA neurons (black trace) display broader action potentials when compared to action potential spikes from medium spiny neurons (green trace). (e) SNc 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 distal dendrite (80 μm away from soma). Calcium transients were abolished by application of isradipine yet the pacemaking firing was insensitive to this calcium channel blocker.
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 nM – 10,000 times lower than the concentration of calcium in the extracellular space (Berridge et al., 2000; Orrenius et al., 2003; Rizzuto, 2001). Calcium entering neurons is rapidly sequestered or pumped back across the steep plasma membrane concentration gradient; this process requires energy stored in Adenosine-5’-triphosphate (ATP) or 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 (Wilson and Callaway, 2000). Preliminary studies using transgenic mice that express a mitochondrially targeted redox-sensitive variant of green fluorescent protein (mito-roGFP) 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. Furthermore, calcium entry (and presumably the concomitant oxidant stress) increases the vulnerability of SNC DA neurons to toxins [MPTP, 6-hydroxydopamine (6-OHDA), rotenone] used to create animal models of PD (Chan et al., 2007).

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 (German et al., 1992a). 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 (ER), 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 (Tepper et al., 1987). 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 (Schultz, 2007), 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 (Blythe et al., 2009; Deister et al., 2009; Zweifel et al., 2009). 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 aetiology of PD (Beal, 1998; Greenamyre and O’Brien, 1991; Sonsalla et al., 1998). 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. But 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 (Morikawa et al., 2003), 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 mGluR receptors 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 (Bender et al., 2008; Reeve et al., 2008).

**Cell-specific risk factors for SNc DA neurons**

- Oxidation prone neurotransmitter
- Profuse axonal terminal field
- Slow, autonomous pacemaking with broad action potentials; engagement of L-type Cav1.3 calcium channels in pacemaking, leading to an elevation in cytosolic calcium concentration
- Lack of mobile calcium binding proteins, like calbindin
- Depolarized membrane potential that promotes the opening of calcium permeable NMDA receptors

**Do other types of neuron that succumb in PD share these risk factors?**

There are a number of regions of the brain that have cell loss paralleling that of the SNc (Braak et al., 2004; Fronczek et al., 2007; German et al., 1992b; Jellinger, 2009; Thannickal et al., 2007). Of the cell-specific risk factors discussed, two 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. Although the available data set is fragmented, neurons in the DMV, LC, raphe nuclei (RN), pedunculopontine nucleus (PPN), lateral hypothalamus, tuberomammillary nucleus, basal forebrain (BF) and olfactory bulb and 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 (Travagli and Gillis, 1994); 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 (Williams et al., 1984). Serotonergic neurons in the RN have broad spikes and are calcium-dependent autonomous pacemakers (Burlhis and Aghajanian, 1987). This is also true for PPN cholinergic neurons (Takakusaki and Kitai, 1997). Tuberomammillary and lateral hypothalamic (orexin expressing) neurons are spontaneously active (Li et al., 2002; Stevens and Haas, 1996; Yamanaka et al., 2003). Tuberomammillary neurons engage L-type calcium channels in this process (Stevens and Haas, 1996; Taddese and Bean, 2002; Williams et al., 1984) (this question has not been addressed in orexin neurons). BF cholinergic neurons are lost in PD (Braak et al., 2004). These neurons have large axonal terminal fields, are spontaneously active in brain slices and have prominent calcium channel currents (Murchison and Griffith, 1995). Moreover, with aging here are significant and deleterious changes in calcium homeostasis in these neurons (Murchison and Griffith, 2007). DA neurons in the olfactory bulb are a slightly different case from the standpoint of pathology. These neurons are calcium-dependent, autonomous pacemakers (Pignatelli et al., 2005). 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 (Huisman et al., 2008; Postuma et al., 2006). This does not mean that a reliance upon calcium is a bad thing though, as this region is capable of adult neurogenesis (Pignatelli et al., 2009). Although much needs to be done, the shared physiological characteristics of these seven vulnerable cell types point to a common mechanism underlying their slow functional decline with age: calcium-mediated stress.

**Is there an interaction between risk factors?**

Is there an interaction between pan-cellular and cell-specific processes that produce the pattern of degeneration seen in PD (Fig. 2)? 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 mitochondrial DNA (mtDNA) and organelle damage.
produced by ROS and related reactive molecules generated by the electron transport chain (ETC) in the course of oxidative phosphorylation (Harman, 2003; Wallace, 2005). 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 are at a higher risk than many other types of neurons (Stark and Pakkenberg, 2004). 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 match that seen in PD, as well as an increased susceptibility to toxins (Backman et al., 2000; Collier et al., 2007; Ishikawa et al., 1996; Kanaan et al., 2008; McCormack et al., 2004). There is also an aging-related decline in SNc mitochondrial function (Beal, 1995), some of which could easily be attributed to the accumulation of mtDNA mutations with normal aging (Bender et al., 2006).

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 result in a selective pattern of degeneration. Consider the loss of DJ-1 function that compromise mitochondrial oxidant defences. In most neurons, oxidant defences engagement is likely to be mild, episodic and readily endured even without a fully functional defence system. But in cell types, like SNc DA neurons, where oxidant stress appears to be sustained, compromising oxidant defences could come at higher long-term cost. Mutations that diminish proteostatic competence (e.g. alpha-synuclein over-expression) could also exert their primary effects on cellular viability through increasing ATP utilization. It would be of considerable interest to see if over-expression of alpha-synuclein increased mitochondrial oxidant stress. Furthermore, 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 damaging reactive oxygen species in and around surviving neurons. If these neurons are already over-producing ROS because of cell-specific factors, their oxidant defences could be overwhelmed, leading to apoptosis.

Can dihydropyridines be therapeutically effective?

Given the existence of both cell-specific and pan-cellular risk factors in PD, how should the development of a neuroprotective therapeutic move forward? Current therapeutic strategies are ostensibly targeting the pan-cellular factors (e.g. co-enzyme Q10). Attacking the cell-specific factors is another strategy. Certain risk factors, like dopamine 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 (Eisenberg et al., 2004).

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 channels necessary for pacemaking?

Several groups over the last 15 years have argued that voltage-dependent calcium channels were necessary (Amini et al., 1999; Mercuri et al., 1994; Nedergaard et al., 1993; Wilson and Callaway, 2000). 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 (Chan et al., 2007; Guzman et al., 2009; Koschak et al., 2001). 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 towards 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 (1984). 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. (2001) 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 modelled its actions (Sinneger-Brauns et al., 2009). 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 nM) and the dissociation constant for the low-affinity state was a 1000-fold higher (480 nM) (Sinneger-Brauns et al., 2009) (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 mV and (for comparison) −90 mV (Fig. 3e). This calculation showed at either −50 or −60 mV, more than 90% of the Cav1.3 channels should be antagonized by 100 nM 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 1 μM nifedipine should antagonize more than 90% of the Cav1.3 channel population during pacemaking (Fig. 3f). These calculations strongly argue that at equilibrium, sub-micromolar 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 nM) should antagonize roughly half of the Cav1.3 channels in SNc DA neurons if we assume equilibration 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 (Fig. 1e, left). In these dual recordings, the dendritic calcium concentration oscillates in phase with somatic spiking. Bath application of 200 nM 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 (Fig. 1e, right).

Does this mean that Cav1.3 channels are completely superfluous? Modelling 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 activated-potassium (SK) 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 behaviour 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 paper (Putzier et al., 2009). 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 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.

**Epidemiological support for cell-specific risk factors**

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 (Becker et al., 2008). More recently, a large Danish data set has been examined (Ritz et al., 2010). 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) (Eisenberg et al., 2004; Kupsch et al., 1996; Mannhold, 1995), 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 (Sinnegger-Brauns et al., 2009). At the doses used to treat hypertension, isradipine has relatively minor side-effects (Fitton and Benfield, 1990). 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-OHDA toxicity are very close to those achieved in humans with a very well-tolerated daily dose (10 mg/day,
Cav1.3 channel antagonism by DHPs

\[ \text{Inact} = 1 - \exp\left(-\frac{(V_m-V_h)}{V_c}\right) \]

\[ V_h = -42.7 \text{ mV}, \quad V_c = 6.6 \text{ mV} \]

\[ K_c = 500 \text{ nM}, \quad K_i = 0.5 \text{ nM} \]

\[ k_1 \quad k_{-1} \]

\[ \text{DHP} \]

\[ C^* \quad I^* \]

unavailable

Fig. 3. (Continued)
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, co-enzyme Q10, and other antioxidant supplements have been disappointing (Hung and Schwarzchild, 2007), they share the hypothesis that oxidative stress exacerbates the symptoms and progression of PD. Co-enzyme Q10 is an electron acceptor for complexes I and II that appears compromised in PD patients (Shults, 2005) and is neuroprotective in animal models of PD (Beal, 1998). Creatine is a substrate for ATP production that can both improve mitochondrial efficiency and reduce oxidative stress by buffering fluctuations in cellular energy production (Klivenyi et al., 1999). Both approaches are aimed at improving mitochondrial function rather than attacking the source of stress on mitochondria. Rasagiline or deprenyl also could prove to have neuroprotective effects by virtue not of its ability to inhibit monoamine oxidase B, but by their ability to its ability to induce the expression of antioxidant defences (Magyar and Szende, 2004). Because their sites of action differ within the chain of events leading to oxidative stress and mitochondrial dysfunction, a combination therapy could prove more effective than any one therapy alone.

**Unanswered questions**

There are a number of questions concerning the role of cell-specific risk factors in the neuronal pathology seen in PD. Answering these questions not only could provide novel therapeutic strategies for preventing or slowing the progression of the disease but could also help to create strategies for ‘successful aging’ generally.

One major question is whether activity-dependent calcium entry into neurons creates a significant mitochondrial oxidant stress. In spite of its plausibility, there is no direct evidence that plasma membrane calcium influx elevates mitochondrial oxidative phosphorylation and the production of superoxide. Limiting plasma membrane calcium influx certainly diminishes the sensitivity of SNc DA neurons to mitochondrial toxins, but this effect could be indirect. The development of redox-sensitive optical probes (Desireddi et al., 2010; Hanson et al., 2004; Wang et al., 2008) and two-photon laser scanning microscopy to allow imaging of mitochondria in situ puts this question within reach. Being able to directly assess mitochondrial function, living SNc DA neurons could shed light on the intriguing observation that mitochondrial uncoupling proteins (UCPs) enhance their resistance to toxins (Andrews et al., 2005, 2006). Mitochondrial UCPs are hypothesized to form part of a negative feedback loop to reduce the production of superoxides by the electron

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**Fig. 3.** Nanomolar concentrations of isradipine should produce a significant antagonism of Cav1.3 calcium channels. (a) 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 nM. 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 nM. (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 K_c 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 nM 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).
transport chain (Brand et al., 2004). There are five known UCPs (UCP1–5); UCP2, 4 and 5 are robustly expressed in the SNC (Andrews et al., 2005). While UCP2 has been implicated in the response to MPTP, understanding the role of these proteins in regulating physiological calcium stress could point to novel therapeutic strategies.

A related albeit more difficult question is whether the role of mitochondria in intracellular calcium buffering contributes to neuronal apoptosis or necrosis in slowly progressing neurodegenerative diseases, like PD. Although mitochondria do not normally flux calcium from the cytoplasm at physiological concentrations, calcium released from the ER through inositol trisphosphate or ryanodine receptors can enter mitochondria at points of apposition between the organelles, which form functional calcium microdomains in which calcium concentrations can rise into the micromolar range (Csordas et al., 2006; Rizzuto and Pozzan, 2006). Through these junctions, ‘dumping’ of ER calcium stores into mitochondria could trigger apoptosis in marginally competent mitochondria (Hajnoczky et al., 2003). However, the vast majority of the studies demonstrating the existence of close interactions between mitochondria and the ER have been performed in cell lines; none have been performed in SNC dopaminergic neurons where the functional relationship between these organelles could be quite different. That said, mechanisms like this seem to be in play in Alzheimer’s disease (Bezprozvanny, 2009).

A second major set of questions is how genetic mutations associated with PD interact with cell-specific risk factors like calcium entry to trigger neuronal pathology and death. It is easy to imagine that a negative dominant mutation of a gene like DJ-1 could exacerbate the basal oxidative stress in an SNC DA neuron and accelerate mitochondrial and proteostatic collapse, leaving neurons lacking the same basal stress largely unaffected. But these types of interaction between mutations and cellular phenotype have not been pursued. All too often, the effects of genetic mutations are pursued solely in cell lines, immature neurons or in neurons that are not vulnerable to the disease.

A third set of questions is whether the non-dopaminergic neurons that are vulnerable in PD share a common cellular phenotype that is therapeutically manipulable. As mentioned above, there is evidence that a large subset of the neurons that die or functionally decline are spontaneously active, have broad spikes and flux lots of calcium. However, the phenotypic characterization must be explored more systematically in those cell types at greatest risk (DMV, LC, BF cholinergic neurons, dorsal raphe, etc.). Moreover, this should be done in situ (either with in vivo recording or in brain slices from adult mice) where the behaviour of the neurons is as close as possible to that found in humans.

Conclusions

Although pan-cellular risk factors dominate current thinking about the aetiology 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.

**Abbreviations**

- ATP: adenosine triphosphate
- BF: basal forebrain
- BBB: blood brain barrier
- CCAs: calcium channel antagonists
- DA: dopamine
- DHP: dihydropyridines
- DMV: dorsal motor nucleus of the vagus
- ETC: electron transport chain
- ER: endoplasmic reticulum
- 6-OHDA: 6-hydroxydopamine
- HCN: hyperpolarization activated cyclic nucleotide channel
- LH: lateral hypothalamus
- LRRK2: leucine rich repeat kinase 2
- LB: Lewy bodies
- L-DOPA: levodopa or L-3,4-dihydroxyphenylalanine
- LC: locus coeruleus
- MPTP: 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine neurotoxin
- mGluR: metabotropic glutamate receptors
- Na-K ATPase: sodium/potassium ATPase
- NMDA: N-methyl-D-aspartate
- PD: Parkinson’s Disease
- PPN: pedunculopontine nucleus
- PINK-1: PTEN-induced putative kinase 1
- RN: raphe nuclei
- ROS: reactive oxygen species
- SK: small conductance calcium activated-potassium channel
- SNC: substantia nigra pars compacta
- SNCA: gene name for alpha-synuclein
- TTX: tetrodotoxin
- UCP: uncoupling proteins
- VTA: ventral tegmental area

**References**


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