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Calcium, mitochondrial dysfunction and slowing the progression of Parkinson's disease

D. James Surmeier[1], **Glenda M. Halliday**[2,3], and **Tanya Simuni**[4]

[1]Department of Physiology and [2]Department of Neurology, Feinberg School of Medicine, Northwestern University, Chicago, IL 60611, USA

[2]Brain and Mind Centre, Sydney Medical School, The University of Sydney, 2006 Australia

[3]School of Medical Sciences, University of New South Wales and Neuroscience Research Australia, Sydney, 2052, Australia

[4]Department of Neurology, Feinberg School of Medicine, Northwestern University, Chicago, IL 60611, USA

Abstract

Parkinson's disease is characterized by progressively distributed Lewy pathology and neurodegeneration. The motor symptoms of cPD are unequivocally linked to the degeneration of dopaminergic neurons in the substantia nigra pars compacta (SNc). Several features of these neurons appear to make them selectively vulnerable to factors thought to cause cPD, like aging, genetic mutations and environmental toxins. Among these features, Ca^{2+} entry through Cav1 channels is particularly amenable to pharmacotherapy in early stage cPD patients. This review outlines the linkage between these channels, mitochondrial oxidant stress and cPD pathogenesis. It also summarizes considerations that went into the design and execution of the ongoing Phase 3 clinical trial with an inhibitor of these channels – isradipine.

Determinants of pathogenesis in Parkinson's disease

Parkinson's disease (PD) is the most common form of a broad class of movement disorders called parkinsonism defined by the the appearance of bradykinesia, rigidity or tremor. The cardinal motor manifestations of clinical cPD (cPD) are attributable to the progressive loss of dopaminergic (DA) neurons in the SNc that innervate the basal ganglia (Berg et al., 2014; Hornykiewicz, 2002). In addition to the loss of SNc DA neurons, a hallmark of cPD is the appearance of Lewy pathology (LP) — proteinaceous inclusions exclusively found in neurons. In cPD, LP is found in a number of brain regions outside the SNc, particularly within the brainstem (Goedert et al., 2012). Braak and others have argued that this

Correspondence to D.J.S. (j-surmeier@northwestern.edu).

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distribution of LP evolves over time (or is staged) from well-defined starting points in the caudal medulla or olfactory bulb (Beach et al., 2009; Del Tredici et al., 2002; Kosaka et al., 1984). Moreover, it was hypothesized that the progressive accumulation of LP led to neuronal loss, including that in the SNc that results in the motor symptoms of cPD.

Recent work showing that misfolded alpha-synuclein (aSYN) fibrils, which are a major component of LP, can spread from a site of brain injection through synaptically coupled networks has garnered the Braak hypothesis new adherents (Luk et al., 2012; Peelaerts et al., 2015; Volpicelli-Daley et al., 2014; 2011). Moreover, because aSYN fibrils appear to be capable of templating the misfolding of endogenous aSYN, the hypothetical spreading of LP has been likened to a prion-like process (Brundin et al., 2010; Olanow and Brundin, 2013). This hypothesis has obvious translational implications. If transynaptic spreading of LP is the driving force in cPD, then the goal of disease modifying therapies should be focused on strategies to slow or stop this spread. Indeed, this effort is underway (NCT02459886).

Although attractive in its simplicity, there are compelling reasons to think that the neuropathology in cPD is not *simply* a consequence of a prion-like spreading of misfolded aSYN in the brain. Many of the issues surrounding this hypothesis have been recently outlined (Uchihara and Giasson, 2016; Walsh and Selkoe, 2016), including one by us (Surmeier et al., 2017). There are four basic problems with the simple prion variant of the Braak model. First, the pattern of LP in cPD brains is variable, with only about half the reliably diagnosed brains conforming to the Braak model, raising questions about the nature of the seeding event (Halliday et al., 2012; Kalaitzakis et al., 2008). Second, the distribution of LP in cPD cases is not predicted by what is known about the brain connectome alone; in particular, the strength of synaptic connections between sites of early LP and the rest of the brain does not predict the distribution of LP later in the disease (Surmeier et al., 2017). The pattern of LP also is not consistent with a spread to nearest neighbors, even within the same nucleus (Kingsbury et al., 2010).. Third, the relationship between LP and neurodegeneration is uncertain. In humans, neuronal loss, which can cleanly be linked to symptoms, evolves with a very different spatiotemporal pattern than does LP. For example, neuronal loss in the SNc precedes any discernible LP. Moreover, some patients with cPD have no discernible LP, whereas others who do, fail to manifest cPD symptoms (Dijkstra et al., 2014). The question is not whether aSYN fibrils or monomers in sufficient quantities can kill neurons; they clearly can. The issue is whether this is what happens in the human brain. And lastly, there is no compelling longitudinal data from cPD patients to support a spreading pathology. Adherents of the spreading hypothesis would argue that this is simply because there aren't good biomarkers for disease progression. While it is true that there aren't validated progression markers, particularly for the early, 'presymptomatic' phases of the disease, the absence of longitudinal data is a shortcoming of the case for the prion hypothesis and should promote a healthy skepticism.

The rationale for a Cav1 Ca^{2+} channel inhibitor

What other factors might contribute to cPD pathology? One approach to this question is to look at the properties of vulnerable neurons to determine if they have common features that might be affected by risk-factors associated with cPD: age, exposure to environmental toxins

and a collection of genetic mutations (Surmeier et al., 2017). Indeed, many of the neurons that are most profoundly affected in cPD have a loosely connected functional role in the brain. They are principal neurons in neuromodulatory control networks, contrasting them with neurons in brain networks responsible for epicritic sensation and precise motor control. The SNc, locus ceruleus (LC), raphe nuclei (RN), pedunculopontine nucleus (PPN), basal forebrain nuclei (BFN), gigantocellularis nucleus (GCN), lateral hypothalamus (LH) and thalamic intralaminar nuclei (ILN) are involved in arousal or mobilization of sensorimotor networks necessary for rapid and effective action, which is critical to vigilance, escape and attack (Alexandre et al., 2013; Aston-Jones and Waterhouse, 2016; Palmiter, 2011; Pfaff et al., 2012; Saper et al., 2005; Sara and Bouret, 2012). The dorsal motor nuclei (DMV) and nucleus tractus solitarius (NTS) – two caudal brainstem nuclei manifesting LP in cPD patients – share a similar role through their control of the autonomic nervous system (Saper, 2003; Silvani et al., 2016). In the forebrain, the amygdala and limbic cortices, which have LP early in cPD, are part of a default activity network in the forebrain that regulates fear and affective behaviors – again, key parts of coordinating appropriate action in response to salient events in a threatening environment (Miskovic and Schmidt, 2012).

Although much remains to be done, it appears that these neurons share a number of traits that might put them at-risk. The most notable and best characterized of these is a long and highly branched axon with a large number of transmitter release sites. This diffuse axonal arbor helps these neurons coordinate the activity in large networks, like the basal ganglia or the spinal cord. For example, SNc DA neurons in the rodent have axons that branch profusely in the striatum and possess as many as 200,000 vesicular release sites (Matsuda et al., 2009). Although less well characterized, neurons in the DMV, GCN, RN, LC, PPN, BFN, LH and ILN all share this feature (large, diffuse axonal projections) to varying degrees, distinguishing them from the vast majority of sensory or motor neurons in the brain, which typically have spatially focused, modestly branched axons that conform to topographic maps (Aston-Jones and Waterhouse, 2016; Baufreton et al., 2009; Hornung, 2003; Hu et al., 2015; Liu et al., 2015; Martinez-Gonzalez et al., 2011; Pfaff et al., 2012; Ratcliffe et al., 2011). Why might a long and highly branched axon increase vulnerability? There are several theories that have been proposed (Bolam and Pissadaki, 2012; Hunn et al., 2015; Pacelli et al., 2015). But, not all neurons with long, branched axons are vulnerable in cPD (e.g., striatal cholinergic interneurons (Zhou et al., 2002)), suggesting that some other factor is in play.

Another shared feature of at-risk neurons appears to be their distinctive physiology. *In vivo*, at-risk neurons that have been studied have slow tonic activity (Surmeier et al., 2017). The best studied member of this class is the SNc DA neuron. The action potential of these neurons is slow and broad, which maximizes Ca^{2+} entry and promotes slow rhythmic activity (Bean, 2007). The slow, rhythmic activity (2–10 Hz) in these neurons is autonomously generated and accompanied by slow oscillations in intracellular Ca^{2+} concentration that are triggered by the opening of plasma membrane Cav1 (Cav1.2, Cav1.3) Ca^{2+} channels and release of Ca^{2+} from intracellular, endoplasmic reticulum (ER) stores (Guzman et al., 2010; Morikawa and Paladini, 2011; Nedergaard et al., 1993; Puopolo et al., 2007). Once in the cytoplasm, Ca^{2+} is relatively free to interact with other proteins as the abundance of Ca^{2+} buffering proteins, like calbindin, is low (Anderegg et al., 2015; Brichta

et al., 2015; Poulin et al., 2014; Sulzer and Surmeier, 2013; Surmeier et al., 2016). This combination of features – broad spikes, pacemaking, low intrinsic Ca^{2+} buffering and cytosolic Ca^{2+} oscillations – (not any one) is what distinguishes SNc DA neurons. For example, VTA DA neurons, which are significantly less vulnerable than SNc DA neurons (see above), are autonomous pacemakers with broad spikes, but have smaller Cav1 channel currents and strong intrinsic Ca^{2+} buffering (Guzman et al., 2009; Khaliq et al., 2010). Although there have been very few studies that have examined these features in other at-risk neurons, those that have (LC, DMV, PPN) show that this phenotype is largely shared (Goldberg et al., 2012; Kang and Kitai, 1990; Sánchez-Padilla et al., 2014).

The slow Ca^{2+} oscillations in at-risk neurons sub-serve two complementary functions. First, because they are electrogenic, the oscillations help maintain the slow tonic spiking in these neurons (Nedergaard et al., 1993; Puopolo et al., 2007; Putzier et al., 2009a). Ca1.3 channels, because they activate at sub-threshold membrane potentials, are critical to this function (Guzman et al., 2009; Helton et al., 2005; Puopolo et al., 2007). Second, although less well established, they promote Ca^{2+} entry into mitochondria, oxidative phosphorylation (OXPHOS) and the production of ATP (Guzman et al., 2010; Llorente-Folch et al., 2015; Sánchez-Padilla et al., 2014). In principle, this feed-forward control of OXPHOS helps to ensure that bioenergetic needs are met (Balaban, 2009; Nicholls, 1998) and that intracellular ATP levels do not fall into a range that would trigger protective activation of K-ATP channels and cessation of on-going activity (Dragicevic et al., 2015). Even temporary cessation of activity in neuronal networks necessary to mobilize sensory and motor systems directing escape or attack behavior would lessen the chances of survival in an unpredictable environment. As a consequence, there should have been strong evolutionary pressure to design neurons in these ‘too important to fail’ networks with this type of feed-forward control mechanism.

There are two obvious downsides of this design. First, stimulating OXPHOS when mitochondria are hyperpolarized in the absence of strong ATP demand increases the production of reactive oxygen species (ROS) and reactive nitrogen species (RNS) (Goldberg et al., 2012; Guzman et al., 2010; Sánchez-Padilla et al., 2014; Votyakova and Reynolds, 2001). ROS and RNS damage proteins, lipids and DNA, particularly in mitochondria. Sustained oxidant stress could be a major factor underlying declining mitochondrial function in at-risk neurons with age (Reeve et al., 2014). ROS and RNS also can exacerbate the impact of genetic mutations and environmental toxins affecting mitochondria (Gegg and Schapira, 2016), as well as increase the propensity of aSYN to aggregate (Gupta et al., 2009). The second downside is that this mechanism results in sustained elevations in cytosolic Ca^{2+} concentration. Ca^{2+} promotes aSYN aggregation both directly (Rcom-H'cheo-Gauthier et al., 2014) and indirectly through activation of calpain and calcineurin (Caraveo et al., 2014; Diepenbroek et al., 2014; Dufty et al., 2007). Elevated cytosolic Ca^{2+} also impairs lysosomal function and turnover of misfolded proteins (Gómez-Sintes et al., 2016; Medina and Ballabio, 2015), potentially synergizing with other defects in proteasomal/autophagic function to increase the likelihood of LP (Wong and Cuervo, 2010). Thus, these vulnerable neurons appear to reside close to mitochondrial and degradative ‘tipping points’.

Do other at-risk neurons conform to this model? In-depth analysis has only been performed in SNc, LC and DMV neurons. While much of the brainstem data is consistent with a shared phenotype, more in-depth phenotyping needs to be done. However, healthy, young telencephalic neurons are not phenocopies of SNc dopaminergic neurons. That said, many of the telencephalic regions at-risk in cPD (and AD) are part of a 'default' network that manifests high resting activity, albeit of synaptic origin (Andrews-Hanna et al., 2007). It is possible that in aged, late stage cPD patients, network dysfunction (Hammond et al., 2007; Ko et al., 2013) triggers adaptations that bring these neurons and networks phenotypically closer to other at-risk neurons. Cav1 Ca^{2+} channels, which are key determinants of the SNc phenotype, could be a major factor in this process. Sustained Ca^{2+} entry through Cav1 channels in forebrain neurons has long been associated with aging-related cognitive decline and AD (Disterhoft et al., 1994; Thibault et al., 2007). Moreover, in cPD patients, Cav1 Ca^{2+} channels are up-regulated in limbic and motor cortices (Hurley et al., 2013; 2014).

Can the phenotype of at-risk neurons account for LP staging? The simple answer is no. From what we currently know about cell autonomous risk factors, LP should appear in the SNc before it does in the DMV. Barring the emergence of some other cell autonomous factor that drives LP, the most parsimonious explanation of the LP pattern in cPD is that there is spreading of aSYN pathology – as posited by Braak et al. and the proponents of the prion model – but that spreading is limited to a subset of neurons whose phenotype renders them susceptible to spreading – a proposition that is very consistent with the phenotype outlined above.

What is better explained by cell autonomous factors is the sequence of cell death in cPD. The earliest known loss of neurons in cPD is the SNc (Surmeier et al., 2017). These neurons are at one extreme of the anatomical, physiological and molecular spectrum of vulnerable neurons as we currently understand it (Anderegg et al., 2015; Brichta et al., 2015; Poulin et al., 2014; Sulzer and Surmeier, 2013; Surmeier et al., 2016), exhibiting the highest basal levels of mitochondrial oxidant stress and free cytosolic Ca^{2+} of any cell examined. Mitochondria and intracellular Ca^{2+} are linchpins of all three major death cascades (apoptotic, autophagic and necrotic) (Nagley et al., 2010). In human SNc, there are telltale signs of sustained mitochondrial oxidant stress with aging and cPD, such as mitochondrial DNA deletions (Bender et al., 2006; 2008). Against this backdrop, it makes sense that genetic mutations that compromise mitochondrial oxidant defenses, biogenesis or quality control cause the preferential loss of SNc dopaminergic neurons and early onset forms of cPD (Kumaran and Cookson, 2015; Lin and Farrer, 2014; Mullin and Schapira, 2015). The tipping point for these neurons also could be reached by other genetic mutations that indirectly compromise mitochondrial function (Brini et al., 2014; Gegg and Schapira, 2016; Guardia-Laguarta et al., 2015; McCoy and Cookson, 2012; Mullin and Schapira, 2013).

Elevated cytosolic Ca^{2+}, aSYN and DA in SNc DA neurons could be a particularly toxic combination, especially in axon terminals and dendrites (Brimblecombe et al., 2015; Caraveo et al., 2014; Dryanovski et al., 2013; Mosharov et al., 2009). Indeed, striatal DA axon terminals appear to be lost early in the development of cPD, preceding the loss of DA cell bodies (Kordower et al., 2013). In this regard, the inference that levodopa therapy does not accelerate disease progression (Fahnand the Parkinson Study Group, 2005) might be

wrong if the primary site of DA toxicity is the axon terminal – terminals that are largely gone by the time levodopa therapy is usually started.

If cell autonomous factors are critical to the evolution of cPD, then 'normalizing' one or more of these factors should slow disease progression. As outlined above, Ca^{2+} entry through Cav1 – particularly Cav1.3 – Ca^{2+} channels appears to be a major driver of mitochondrial oxidant stress in the at-risk neurons examined to date. Moreover, these channels can be targeted. Dihydropyridines (DHPs) are FDA-approved, selective negative allosteric modulators (NAMs) of Cav1 channels that have good brain bioavailability (Anekonda et al., 2011a; Striessnig et al., 1998; Surmeier et al., 2016). Epidemiological studies have consistently found that the use of DHPs is associated with a decreased risk of developing cPD (Becker et al., 2008; Gudala et al., 2015; Lee et al., 2014; Pasternak et al., 2012; Ritz et al., 2010); their use even seems to slow progression after diagnosis (Marras et al., 2012). The combination of preclinical and clinical data implicating Cav1 channels in cPD pathogenesis provide the rationale for testing DHPs as potential diseas modifying agents in cPD.

One of the shortcomings of DHPs as a disease modifying therapy is their 'off-target' inhibition of Cav1 channels in vascular smooth muscle (and elsewhere). These off-target effects, which are primarily mediated by Cav1.2 channels, limit dosing. Because DHPs are voltage-dependent NAMs that bind preferentially to channels that are relatively depolarized (Bean, 1984), some of these off-target effects will be diminished by the fact that most neurons reside primarily at relatively hyperpolarized membrane potentials, where DHPs have a lower affinity; this should limit DHP action to cells that are depolarized for prolonged periods of time, like pacemaking neurons at-risk in cPD. Another translational consideration is that Cav1.3 Ca^{2+} channels, rather than the more common Cav1.2 channels, are likely to be the most important drivers of risk in PD. Most DHPs preferentially inhibit Cav1.2 channels; isradipine differs from most DHP in that it has nearly the same affinity for Cav1.2 and Cav1.3 channels in membrane binding assays (Sinnegger-Brauns et al., 2009). This consideration motivated the use of isradipine in preclinical studies and subsequent clinical trials (see below).

A fundamental question is whether isradipine can be given to humans at doses high enough to produce a clinically significant inhibition of Cav1.3 channels in SNc DA neurons and other neurons at-risk. There are several reasons to think this is achievable. First, peripheral administration of isradipine, at doses that were well tolerated in mice, protected SNc DA neurons against a mild, distributed striatal injection of the toxin 6-OHDA (Ilijic et al., 2011). Second, unpublished work by our group has shown that systemic administration of isradipine suppresses cytosolic Ca^{2+} transients, elevates mitochondrial mass and lowers mitophagy in SNc DA neurons (Guzman et al., unpublished results). Third, epidemiological data have consistently found that DHPs lower PD risk (see above).

A recent study has challenged this conclusion (Ortner et al., 2017). The authors claim that systemic administration of isradipine does not protect SNc DA neurons against striatal injection of 6-OHDA. As pointed out by our earlier work (Ilijic et al., 2011), the ability of isradipine to protect against a 6-OHDA challenge requires that the insult be modest and

spatially distributed. Nevertheless, in their attempt to test isradipine, Ortner et al. used a 6-OHDA concentration that Ilijic et al. (2011) had shown to be refractory to systemic isradipine treatment; this choice makes their negative result problematic. Also, it is worth noting that other groups have reported that systemic administration of DHPs affords protection in both PD and Alzheimer's disease models (Anekonda et al., 2011b; Kupsch et al., 1996). In an attempt to explain their result, the authors estimate the isradipine IC50s of Cav1.2 and Cav1.3 Ca^{2+} channels using a voltage trajectory recorded from SNc DA neurons; this interesting set of expeirments showed that in this circumstance Cav1.2 channels and Cav1.3 channels had IC50 values that were less than 10 nM, with the IC50 value of Cav1.2 channels being somewhat lower than that of Cav1.3 channels (cf., Sinnegger-Brauns et al., 2009). The authors take this information and then use imaging to assess the ability of isradipine – at more than three times the IC50 value for both Cav1.2 and Cav1.3 channels (30 nM) – to reduce the intracellular Ca^{2+} transient associated with pacemaking in SNc DA neurons. Paradoxically, the authors found no inhibition, contradicting a large literature showing the rich expression of Cav1.2 and Cav1.3 Ca^{2+} channels in these neurons throughout their somatodendritic membrane (e.g., Guzman et al., 2009; Nedergaard et al., 1993; Puopolo et al., 2007; Putzier et al., 2009b). The authors provide no plausible explanation for this apparent contradiction. On the basis of these two negative results, the authors assert that isradipine is unlikely to produce a significant inhibition of Cav1.3 channels in patients. Not only do we disagree with this conclusion because of the shortcomings in the work, their IC50 estimates suggest the opposite conclusion; namely, that at clinically relevant doses of isradipine where brain levels should reach or exceed 5–10 nM, there should be significant (~50%) inhibition of both Cav1.2 and Cav1.3 channels in SNc DA neurons. That said, it is unclear whether this will be enough to slow PD pathogenesis. Nevertheless, the caveats of using non-selective DHPs are real and underscores the need to develop a more selective Cav1.3 channel inhibitor.

From the bench to clinical trial

The section below outlines the pathway for clinical translation of the preclinical data, summarizes the design of the on-going Phase III clinical trial of isradipine and highlights the challenges of development of cPD disease modifying inetrventions in absence of validated biomarkers of disease progression.

As outlined above, DHPs are selective NAMs of Cav1 Ca^{2+} channels. They are Food and Drug Administration (FDA) approved for use as anti-hypertensive agents (Zhang et al., 2007). Isradipine has been FDA approved since 1990 and has extensive safety data in the hypertensive patient population. Isradipine was selected for clinical testing because it has a high affinity for both types of Cav1 channels expressed in SNc DA neurons, having roughly the same binding affinity for Cav1.2 and Cav1.3 channels (Sinnegger-Brauns et al., 2009) (see above). In contrast, nifedipine has a roughly five times higher affinity for Cav1.2 channels. Isradipine also has excellent brain penetration (Anekonda et al., 2011a; Ilijic et al., 2011).

As a first step toward a disease modification trial, the safety of isradipine was tested in Phase Ib and Phase II studies in early stage cPD patients. These studies found that isradipine had

acceptable safety and tolerability at doses of 10mg/day or less (Parkinson Study Group, 2013; Simuni et al., 2010). The combination of the preclinical rationale, epidemiological data linking DHPs to reduced risk of cPD and the Phase Ib/II safety data motivated NIH to mount a 5 year, Phase III, disease modification clinical trial of isradipine in early stage cPD (STEADY-PD III, clintrials.gov NCT02168842). The study is being conducted at 57 Parkinson Study Group (PSG) sites in North America and is funded by the National Institute of Neurological Disorders and Stroke (NINDS) and the Michael J. Fox Foundation.

Design and execution of the Phase III trial

STEADY-PD III (NCT02168842) is an ongoing 36-month double-blind randomized, placebo-controlled study of isradipine in 336 participants with early stage cPD, who at enrollment were not receiving or requiring symptomatic therapy (ST). The study is testing the hypothesis that individuals given isradipine will have slower progression of cPD disability over the 36 month trial period, as determined by the change in the total Unified Parkinson Disease Rating Scale (UPDRS) score. Isradipine (5 mg) is being given twice daily for a total daily dose of 10 mg.

Trial participants had to fulfill several **inclusion and exclusion criteria**. Inclusion criteria were: 1) they had to be diagnosed with early stage, idiopathic cPD based upon the presence of two out of three cardinal manifestations of cPD (Hughes et al., 1992); 2) they had to be older than 29 years of age at the time of diagnosis; 3) they had to be Hoehn and Yahr stage less than or equal to 2 (Hoehn and Yahr 1967); 4) they had to have been diagnosed less than 3 years prior to enrollment; 5) they were not to have received symptomatic medication (levodopa, dopamine agonist or MAO-B inhibitors) and not projected to require medication for at least 3 months from enrollment. Use of amantadine and/or anticholinergics was allowed prior to enrollment, as was use of anti-hypertensives other than Ca^{2+} channel inhibitors. The key exclusion criteria were: 1) a diagnosis of atypical parkinsonism; 2) prior exposure to symptomatic medication; 3) a history of orthostatic hypotension, bradycardia, congestive heart failure or other cardiac and other systemic diseases; 4) abnormalities on the screening labs or ECG that might preclude safe participation in the study; 5) the presence of cognitive dysfunction defined by a Montreal Cognitive assessment (MOCA) score of less than 26 (Nasreddine et al., 2005); 6) clinically significant depression as determined by a Beck Depression Inventory II (BDI-II) score greater than 15 (Beck and Beamesderfer, 1974).

The **primary outcome** measure of the trial is the change in total UPDRS score during the 36 month trial period. Majority of participants were expected to start taking symptomatic medications during the trial and as such the study was designed and powered to test isradipine efficacy by comparing the baseline UPDRS with the end-of-trial UPDRS, measured in the medication on-state, accounting for the effect of the symptomatic treatment. The UPDRS is a valid and reliable measure of cPD disability that has been effectively used in a number of cPD trials. The study is powered to detect a 25% slowing of functional decline with isradipine above the benefit from symptomatic therapy, a difference that would be sufficient to influence clinical practice and may suggest the likelihood of longer term benefit.

The **secondary outcomes** of clinical importance include: 1) time to initiation of symptomatic therapy, which has been used as a primary outcome measure in several previous studies of putative disease modifying agents and reflects progression early in disease not obscured by symptomatic therapy (Parkinson Study Group, 1996; Parkinson Study Group PRECEPT Investigators, 2007); 2) time to and severity of motor complications, which is a another measure of disease progression (Rascol et al., 2000) ; 3) reduced need for symptomatic therapy (as measured by the levodopa equivalent dosages between treatment groups (Tomlinson et al., 2010); 4) the incidence and severity of non-motor symptoms, as these contribute disproportionately to quality of life and reflect clinically relevant outcomes in cPD.

Finally, there are **exploratory outcome measures** that include global measures of functional disability, quality of life, ambulatory capacity and cognitive function as measured by MOCA. The trajectory of UPDRS change before and after initiation of symptomatic therapy also will be modeled.

Biosamples are obtained from patients. Blood is obtained at enrollment and at 3 and 6 month follow-up visits to confirm isradipine pharmokinetic profiles. In addition, blood samples obtained at enrollment will be used to extract DNA for genetic testing and samples obtained at the conclusion of the study will be stored for future reserach.

The rationale for the study design

PD is a slowly progressing neurodegenerative disease. Previously conducted disease-modification studies enrolled participants with newly diagnosed cPD not yet requiring symptomatic treatment and followed them for a relatively short period of time (12–24 months) and data were censored at the time of initiation of symptomatic treatment (Hart et al., 2009). These previous trial designs were driven by lack of objective biomarkers of cPD progression and the significant impact of symptomatic treatment on standard clinical outcome measures. However, they do not address the “real life scenario” in which the disease modification strategy might slow but not stop disease progression, requiring that all patients ultimately be treated for cPD symptoms. On average 50% of *de novo* cPD patients initiate symptomatic treatment within one year of diagnosis and nearly all patients require therapy within three years (Parashos et al., 2009)(Ravina et al., 2009). If isradipine slows this progression, it would be an important advance. With our trial design, this can be assessed by the secondary outcome measures (time to initiation of symptomatic treatment and differential use of symptomatic therapy). It also is unclear whether an intervention that is effective early in the course of the disease will affect progression later in the disease; that is, will the effects of treatment persist? Previous trial designs don’t allow this question to be answered.

STEADY-PDIII attempts to address these limitations by extending the drug treatment period to 36 months and allowing symptomatic medication to be initiated early in the trial. At 36 months post diagnosis, nearly all participants are expected to be treated with symptomatic therapies. The primary outcome measure is the change in UPDRS score from the time of enrollement to that obtained in the “on-state” at the end of the trial 36 months later. This will allow us to identify the benefit of isradipine “on top” of the benefit conferred by

symptomatic therapy – an outcome with “real world” relevance to patients and clinicians. The UPDRS “off-state” was considered as an alternative primary outcome. While it may be argued that this assessment is a better representation of dopaminergic deficit, this is not supported by the clinical data. Both levodopa and dopamine agonists have shown long duration effects on UPDRS, lasting for days and even weeks. So, the traditional 12 hours off medication for “off-state” assessment would not reflect the true dopaminergic deficit (Fahn et al., 2004; Stocchi et al., 2001). Nevertheless, this assessment is of value and the “off-state” motor UPDRS will be used as a secondary outcome measure once symptomatic treatment has been initiated.

In regard to the study duration, 36 months was chosen as a compromise between the attempt to assess long-term efficacy of isradipine (if it exists) and feasibility. While 36 months is still a short period for a slowly progressing disease like cPD, and long-term cPD complications are not expected (e.g., like postural instability and dementia) a longer study was fiscally problematic and would have been compromised by retention issues. Nevertheless, this is the longest duration ever proposed for a study in baseline *de-novo* untreated cPD population and is likely long enough to provide insight into the effects of isradipine on relevant motor and nonmotor outcomes. It is also short enough to maximize participant retention. Thus, the study design is novel in that it allows us to use a relatively small cohort of patients to better test the hypothesis that isradipine will slow disease progression and add to the benefit derived from symptomatic treatment.

Several alternative study designs were considered. For example, a ”simple long duration study” design (LS-1) was considered, but it would have required in excess of 1500 participants and 7–8 years to complete (Elm,NINDS NET-PD Investigators, 2012). Another design utilized in cPD disease modification trials is the delayed-start design (Olanow et al., 2009). The arguments against a delayed start design in our case are 1) the lack of demonstrable symptomatic benefit of isradipine, 2) the requirement of > 1000 participants for sufficient power, and 3) controversy on its ability to demonstrate disease modification in cPD. Another design to consider would enroll individuals at the time of initiation of symptomatic therapy (like CALM-PD) (Parkinson Study Group CALM Cohort Investigators, 2009). However, this would not allow to test the impact of isradipine on progression early in disease (before symptomatic treatment). In addition, enrolling subjects as early as possible in the disease process would allow the neuroprotective benefit of isradipine (if such an effect exists) to be maximized. A prolonged wash out at the end of study or at the time of initiation of symptomatic treatment was considered to reassess for the evidence of symptomatic benefit, but there are strong arguments against such design, including lack of obvious symptomatic effect of isradipine in our Phase II STEADY-PDII study; the participant burden is also a drawback. Moreover, there is no consensus on the necessary duration of the washout that would be required for isradipine (Holford and Nutt, 2011). Therefore, although not without shortcomings, our design appears to be the most valid currently available approach to study the effect of isradipine on progression of disability in cPD. It should be highlighted that as of today based on expert concensus and discussions with the FDA, there is no single “preferred” study design to test disease modification in cPD.

There are a number of study limitations to be considered. The major limitation of our trial – and all others at this point – is lack of validated biomarkers of disease progression. This has two consequences. One is that our study is limited to patients who have already progressed to the point that symptoms are manifest corresponding to more advanced stages of neurodegeneration. Disease modification with isradipine or other drugs may only be feasible in the premotor phases of the disease. The other is that we use a clinical scale, UPDRS, as the primary outcome measure. UPDRS, while validated and a widely used scale in cPD clinical trials, does not directly assess biological disease progression. DNA and plasma samples are being collected for future analyses of novel biomarkers that could address these limitations.

Another limitation of our study is that it is not clear that the dose of isradipine chosen is high enough. At present, there is no good way to assess the relevant pharmacokinetic features of isradipine in humans, particularly target (Cav1 channel) engagement in the SNc. The tested dose (10 mg/day) was chosen based on the tolerability in the Phase II study and while that dose achieves serum concentrations that were neuroprotective in animal models, it is not clear that it is sufficient in humans. Again, pharmacokinetic samples will help determine whether isradipine concentrations that were effective in animal models were achieved and whether variability in clinical response was related to variations in serum concentrations.

Our study uses a novel primary outcomer measure, UPDRS in the medications ON state. While such approach has a number of advantages as discussed above, the study may be criticized for lack of definitive way to exclude symptomatic effect of isradipine which could account for the benefit compared to placebo (if the study was positive). Our rationale for such a design are data from the phase II study that demonstrated lack of symptomatic effect of isradipine based on the short term change in UPDRS after initiation of treatment (assessed every 2 weeks up to 3 months) and after 2 weeks taper between active treatment arms and placebo. In addition, the mechanism of action of isradipine preclinical data do not point to a potential symptomativc effect of the compound. However, such effects can’t be completely excluded short of conducitng a study with a prolonged wash out which is also subject to pitfalls as discussed above. Even if a symptomatic effect of isradipine existed and persisted long term ON TOP of exisiting symptomatic therapy, that in conjunction with the compelling preclinical data of neuroprotecitve effect of NAMs, would be a significant contribution to the existing armamentarium of cPD therapeutics.

Lastly, it should be noted that STEADY cPD III is conducted under the FDA Investigational New Drug (IND) path with no plans to license isradipine for cPD use. Such a path was chosen based on the fact that isradipine is an FDA approved drug that is generic and provided the study showed positive results, it wil be readily available to the cPD community.

Current status of the study

Enrollment of the 336 participants began in November 2014 and was completed in October 2015 at 55 of the 57 active PSG sites. Enrollment was completed 6 months ahead of the expected timeline. The final subject is expected to complete the study in November 2018. As of May 2017, 330 participants remain active in the study.

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