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The Potential of L-Type Calcium Channels as a Drug Target for Neuroprotective Therapy in Parkinson's Disease

Birgit Liss[1] and Jörg Striessnig[2]

[1]Institut für Angewandte Physiologie, Universität Ulm, 89081 Ulm, Germany; email: birgit.liss@uni-ulm.de

[2]Abteilung Pharmakologie und Toxikologie, Institut für Pharmazie, and Center for Molecular Biosciences Innsbruck, Universität Innsbruck, A-6020 Innsbruck, Austria; email: joerg.striessnig@uibk.ac.at

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Abstract

The motor symptoms of Parkinson's disease (PD) mainly arise from degeneration of dopamine neurons within the substantia nigra. As no disease-modifying PD therapies are available, and side effects limit long-term benefits of current symptomatic therapies, novel treatment approaches are needed. The ongoing phase III clinical study STEADY-PD is investigating the potential of the dihydropyridine isradipine, an L-type Ca^{2+} channel (LTCC) blocker, for neuroprotective PD therapy. Here we review the clinical and preclinical rationale for this trial and discuss potential reasons for the ambiguous outcomes of in vivo animal model studies that address PD-protective dihydropyridine effects. We summarize current views about the roles of Cav1.2 and Cav1.3 LTCC isoforms for substantia nigra neuron function, and their high vulnerability to degenerative stressors, and for PD pathophysiology. We discuss different dihydropyridine sensitivities of LTCC isoforms in view of their potential as drug targets for PD neuroprotection, and we conclude by considering how these aspects could guide further drug development.

INTRODUCTION

Parkinson's disease (PD) is the second most common neurodegenerative disorder, with increasing age being the strongest risk factor (with a prevalence of ~3% above age 80) (1–3) for its development. The typical motor symptoms of PD (i.e., rigor, tremor, akinesia/bradykinesia) are caused by a progressive and selective degeneration of dopamine neurons within the substantia nigra (SN) (SN DA neurons) and an increasing dopamine deficit in their striatal axonal projection areas (4). For reasons that remain unclear, neighboring midbrain DA neurons, expressing the Ca^{2+}-binding protein $calbindin_{d28K}$, in the dorsal tier of the SN and in the ventral tegmental area (VTA) remain largely intact in PD (4, 5). On the other hand, nondopamine neuronal populations are also highly affected by the degenerative process in PD (1, 6–9), such as the noradrenergic neurons in the locus coeruleus, which is probably related to the nonmotor symptoms of PD, including psychosis, depression, fatigue, autonomic dysfunction, sexual dysfunction, and dementia (10). With the exception of some monogenetic familial forms (caused by so-called PARK gene mutations), the cause of most PD cases is still unclear. However, various PD stressors and trigger factors have been identified. In particular, α-synuclein aggregates (a major component of intracellular inclusions, so-called Lewy bodies, which are a pathological hallmark of PD), metabolic stress, and altered Ca^{2+} homeostasis seem to converge in triggering the neurodegenerative process (1, 11–17).

Given PD's unclear pathomechanisms, only symptomatic therapies exist, which mainly reduce motor symptoms and improve quality of life (15, 16). However, no therapies are available to prevent or slow disease progression. Symptomatic treatments for PD include pharmacotherapy, stereotaxic neurosurgery (deep brain stimulation), and supportive treatments such as physiotherapy and speech therapy (1, 16, 18). Pharmacotherapies are mainly dopamine mimetic, aiming at replacing dopamine or its function. The blood-brain barrier permeant precursor of dopamine L-3,4-dihydroxyphenylalanine (L-DOPA) is still the gold standard in PD therapy, although dopamine receptor agonists (acting predominantly on D2-type receptors) are administered particularly in early PD stages. Monoamine oxidase (MAO)-B or catechol-*O*-methyltransferase (COMT) inhibitors inhibit dopamine-degrading enzymes and thus prolong dopamine (and L-DOPA) effects (18). As none of the current therapies are able to stop disease progression, novel neuroprotective PD therapies and also biomarkers for early PD diagnosis (prior to manifestation of motor symptoms, when the majority of SN DA neurons are already lost) are unmet medical needs and the focus of ongoing experimental therapy strategies (1, 19). Potentially innovative PD therapies that are currently in clinical trials include α-synuclein-targeted immunotherapy (1, 20) and gene therapy (e.g., clinical trials NCT02418598 and NCT01621581) as well as cell transplantation (e.g., NCT03119636, NCT02538315).

Another appealing approach is to repurpose drugs that are already approved for other indications with good safety and tolerability. Promising examples are the antidiabetic glucagon-like peptide 1 agonist exenatide (21), the broncholytic drug ambroxol (22), the tyrosine-kinase inhibitor nilotinib (23), and the antihypertensive dihydropyridine (DHP) isradipine (24, 25). Isradipine, a Ca^{2+} channel blocker, selectively inhibits voltage-gated L-type Ca^{2+} channels (LTCCs). DHPs appear to be attractive drugs for neuroprotective or preventive PD therapy (26), as epidemiological studies indicate that prescription intake of blood-brain barrier–permeable Ca^{2+} channel blockers of the DHP type is associated with a 20–30% reduced risk of developing PD later in life (for summaries of individual epidemiological studies, see recent meta-analyses in 27–29). DHPs, which have a favorable safety profile, have been used clinically for decades for the treatment of high blood pressure and angina. Although hypertension may be a risk factor for PD (30), risk reduction was not observed for other antihypertensives. PD risk reduction in DHP-treated patients was found in all epidemiological studies (31–34) and meta-analyses (27–29) in which DHPs were

analyzed separately. In vitro and some in vivo mouse model data (discussed below in detail) suggest that activity-related, LTCC-mediated Ca^{2+} entry into SN DA neurons is directly linked to their particularly high metabolic stress levels as well as to their high vulnerability to PD stressors and to cell death. These findings provide a strong rationale for the ongoing phase III STEADY-PD clinical trial with the DHP Ca^{2+} channel blocker isradipine (5 mg, twice daily) in patients with early PD (NCT02168842) (24, 25). Independent of the outcome of this ongoing STEADY-PD trial (with an estimated completion date of late 2018), several questions regarding the potential of LTCC inhibitors as drugs for PD therapy remain: (*a*) What are the functional roles of LTCC isoforms in SN DA neurons? (*b*) What are the molecular mechanism and the relative contribution of LTCC isoforms to neurodegeneration in PD? (*c*) How well are LTCCs inhibited by currently available DHPs in the brain compared to LTCCs in the arterial vasculature, their primary cardiovascular drug target? (*d*) Do other voltage-gated Ca^{2+} channels (VGCCs) or other Ca^{2+} sources also drive neurodegenerative Ca^{2+} signals and/or could they compensate for chronic LTCC inhibition? These aspects of LTCCs are particularly important to consider in an attempt to develop novel therapeutics for PD, as they could guide further drug discovery in distinct directions.

In this context, we discuss current views about the roles of LTCCs for SN DA neuron function, for PD pathophysiology, and as drug targets for neuroprotective PD therapy.

VOLTAGE-GATED L-TYPE CALCIUM CHANNELS IN SUBSTANTIA NIGRA DOPAMINE NEURONS

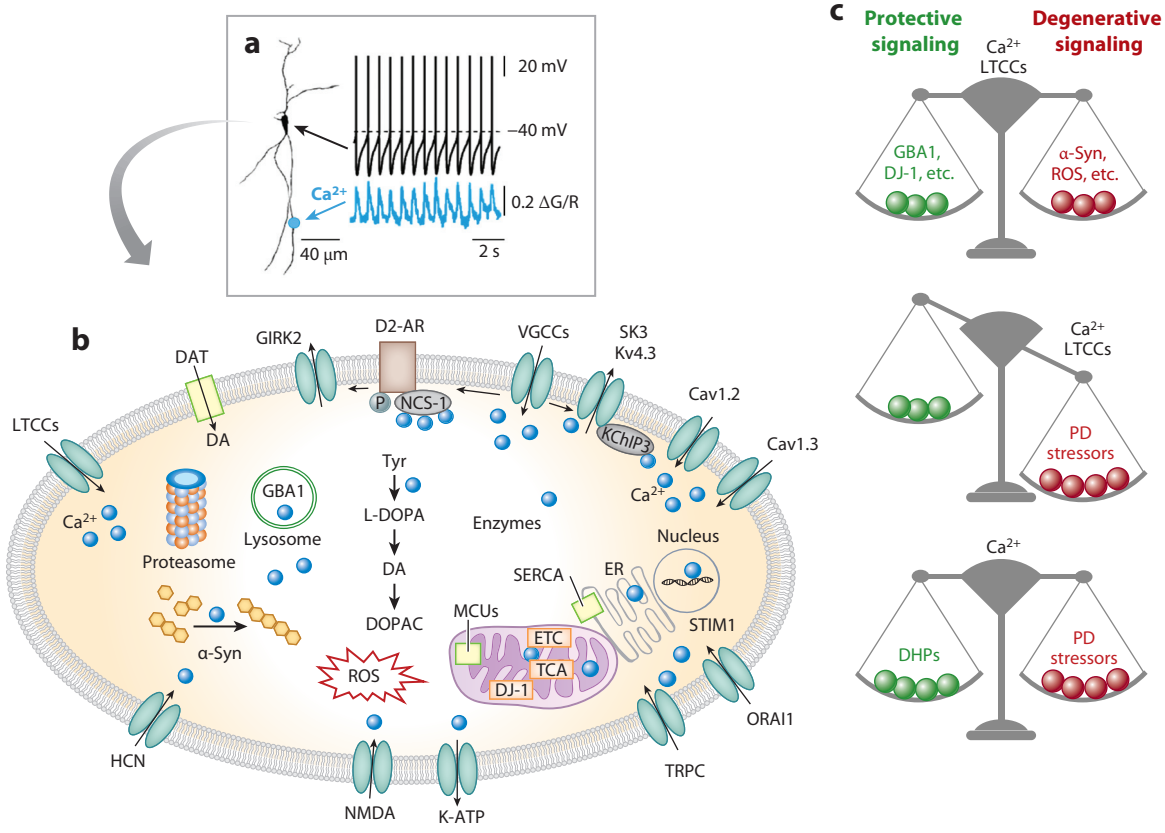
Ten types of VGCCs are distinguished based on their distinct pharmacological and functional properties, each containing a different pore-forming $\alpha 1$ subunit (35, 36). The subcellular expression, activity, gating behavior, and pharmacological properties of VGCCs are tuned by extensive alternative splicing and their association with modulatory accessory subunits ($\beta 1$–$\beta 4$, $\alpha 2\delta 1$–$\alpha 2\delta 4$) (36). Based on their $\alpha 1$ subunit sequence homology as well as their functional and pharmacological properties, three VGCC families have been defined: Cav1, Cav2, and Cav3 (35, 36).

The Cav1 family is composed of four LTCC members (Cav1.1–Cav1.4), which are characterized by their sensitivity to low nanomolar concentrations of DHPs. Only Cav1.2 and Cav1.3 are expressed in neurons, predominantly at somatodendritic locations, with Cav1.2 composing about 90% of all LTCCs in the central nervous system (37, 38). In contrast, members of the Cav2 family (Cav2.1–Cav2.3), mediating P/Q-, N-, and R-type voltage-gated Ca^{2+} currents, are located presynaptically and are required for fast neurotransmitter release (36). Low voltage–activated Cav3 channels (Cav3.1–Cav3.3) compose the family of T-type Ca^{2+} channels (TTCCs). TTCCs activate and inactivate at more negative potentials than Cav1 and Cav2 channels. This negative operation range allows them to be active at subthreshold voltages and provides them with a prominent role in the control of neuronal firing patterns (35, 36).

In addition to DHP-insensitive T-type Ca^{2+} currents, low voltage–activated, DHP-sensitive, voltage-gated Ca^{2+} currents are also found in neurons (39–41), including SN DA neurons (42–44), which are likely formed by Cav1.3 $\alpha 1$ subunits (36, 44). Cav1.3 activates at about 10–20 mV more negative potentials than Cav1.2 channels, but still about 20 mV more positive than TTCCs (45). Alternative splicing creates C-terminal short Cav1.3 variants, which activate at even more negative potentials than full-length (long) Cav1.3 channels (36, 46). It also affects the activation voltage range of Cav1.2 channels (47); however only a few splice variants have been functionally studied so far. Thus, although Cav1.2 channel variants have not been shown to activate at such negative voltages, this possibility cannot yet be ruled out.

To understand the physiological functions of VGCCs in SN DA neurons in general, and of LTCC isoforms in particular, one needs to consider the primary function of SN DA neurons, which

is somatodendritic and presynaptic dopamine release, crucial for voluntary movement (48–50). This release is Ca^{2+} dependent and is primarily triggered by the electrical activity pattern of SN DA neurons (48, 49). In vivo, SN DA neurons (from rodents, primates, and humans) fire either in a low-frequency single-spike mode or transiently in a high-frequency so-called burst mode. In vitro, as illustrated in **Figure 1*a***, even in full synaptic isolation, SN DA neurons display a slow, regular, and very robust intrinsically generated pacemaker activity around 1.5 Hz (with a maximum of ~10 Hz), with relatively broad action potentials (>2 ms) (51, 52). This is generated and modulated by the orchestrated activity of a variety of different ion channels, transporters, and receptors (**Figure 1*b***) that can complement and/or compensate for each other. For detailed information about DA neuron activity and the complex underlying molecular mechanisms, we refer the readers to recent reviews (50, 53–56). Here we primarily focus—in the context of the particularly high vulnerability of SN DA neurons to PD stressors—on cellular processes that can be directly or indirectly affected by LTCC activity (**Figure 1**). In essence, although it is now commonly assumed that, at least in vitro, LTCCs are not required for pacemaker generation in SN DA neurons per se, they seem to stabilize pacemaker activity, precision, and robustness, and thus dopamine release (12, 57, 58). On the other hand, LTCC function can also inhibit SN DA neuron activity, e.g., by stimulating inhibitory processes. This includes the stimulation of Ca^{2+}-sensitive small conductance K^+ (SK) channels, Ca^{2+}-sensitive A-type K^+ channels [Kv4.3, via potassium channel–interacting protein 3 (KChIP3) β-subunits], or D2 autoreceptor- and G protein–coupled Girk2 K^+ channels (via the stimulation of the D2-sensitizing neuronal Ca^{2+} sensor NCS-1) (12) (**Figure 1*a*,*b***).



(*Caption appears on following page*)

Figure 1 (*Figure appears on preceding page*)

Activity-dependent functions of LTCCs and Ca^{2+} in highly vulnerable SN DA neurons, and possible mechanisms for neuroprotective effects of DHPs in PD. (*a*) Simultaneous whole-cell current clamp recordings of typical low-frequency pacemaker activity (*black trace*) from an SN dopaminergic cell soma (*black arrow*) and corresponding dendritic Ca^{2+} oscillations (*blue trace*) in the same neuron recorded by two-photon laser scanning fluo-4 Ca^{2+} imaging. In the distal dendrites, these Ca^{2+} oscillations are inhibited by about half by the DHP isradipine (1 μM) or by Cav1.3 short hairpin RNA. (*b*) Selected somatodendritic ion channels, receptors, and transporters that generate or modulate electrical activity patterns of SN DA neurons as well as cytosolic Ca^{2+} levels (*blue circles*), and the related Ca^{2+}-modulated pathways, with a focus on voltage-gated LTCCs as Ca^{2+} sources. VGCC indicates that the specific voltage-gated Ca^{2+} channel subtype(s) involved in the respective signaling pathway are not clear or more than one subtype has been shown to be involved (e.g., for Kv4.3 and D2-AR stimulation). LTCC indicates that the dendritic Ca^{2+} oscillations are DHP sensitive, but the contribution of individual LTCC isoforms has not yet been demonstrated. Cav1.2 or Cav1.3 indicates that for some signaling pathways a distinct LTCC isoform has been identified. Note that in principle, all Ca^{2+} sources could affect Ca^{2+}-dependent signaling and thus, e.g., dopamine metabolism and ATP synthesis as well as ER, mitochondrial, lysosomal, and proteasomal function and α-synuclein aggregation and gene expression in healthy individuals and those with PD. In SN DA neurons, ROS contribute to high levels of metabolic stress, which are partially caused by their electrical activity and related Ca^{2+}-dependent signaling. (*c*) Scales depicting a proposed general balance of protective (*green*) and degenerative (*red*) signaling pathways in SN DA neurons, which are affected by LTCCs and Ca^{2+} signaling. This delicate balance (*upper*) is destabilized by PD stressors, amplifying the detrimental effects of LTCC activity (*middle*). The inhibition of LTCCs by DHPs (*lower*) may restore this balance, reduce degenerative signaling in SN DA neurons, and protect them from degeneration in PD. Abbreviations: α-Syn, α-synuclein (*PARK1*, *PARK4*); D2-AR, dopamine D2 autoreceptor; DA, dopamine; DAT, dopamine transporter; DHPs, dihydropyridines; DJ-1, *PARK7* gene product; DOPAC, 3,4-dihydroxyphenylacetic acid; ER, endoplasmic reticulum; ETC, electron transport chain; GBA1, glucocerebrosidase; GIRK2, G protein–coupled inwardly rectifying K^+ channel 2; HCN, hyperpolarization-activated cyclic nucleotide gated cation channel; K-ATP, ATP-sensitive K^+ channel; KChIp3, Kv channel interaction protein (i.e., β-subunit of Kv4); Kv4, voltage and Ca^{2+} regulated A-type K^+ channel; L-DOPA, L-3,4-dihydroxyphenylalanine; LTCC, L-type Ca^{2+} channel (i.e., Cav1.2, Cav1.3); MCUs, mitochondrial Ca^{2+} transporters; NCS-1, neuronal Ca^{2+} sensor 1; NMDA-R, *N*-methyl-D-aspartate glutamate receptor; ORAI1, Ca^{2+} release activated Ca^{2+} channel modulator protein 1; P, phosphate; PD, Parkinson's disease; ROS, reactive oxygen species; SERCA, sarcoplasmic/endoplasmic reticulum Ca^{2+} ATPase; SK, small conductance Ca^{2+} sensitive K^+ channel; STIM1, stromal interaction molecule 1; TCA, tricarboxylic acid cycle; TRPC, transient receptor potential channel; Tyr, tyrosine; VGCCs, voltage-gated Ca^{2+} channels. Panels *a* and *b* adapted from Reference 12.

Recently, all three VGCC classes, Cav1–Cav3, have been described in the somatodendritic compartments of rodent SN DA and ventral tegmental area dopamine (VTA DA) neurons (59, 60). Presynaptically, P/Q- and N-type currents predominantly contribute to axonal dopamine release from VTA DA neurons into the ventral striatum, while in SN DA neurons LTCCs and TTCCs also contribute to dopamine release into the dorsal striatum (61). The complex physiological functions of distinct VGCC subtypes in SN DA neurons are still unclear. In general, they seem to contribute to pacemaker activity robustness and to the complex subthreshold integration of electrical inputs. Their functional roles in SN DA neurons seem to (*a*) change with age, (*b*) be different between $calbindin_{d28K}$-positive and -negative SN DA neurons, and (*c*) depend on subcompartments and homeostatic states of the neurons (43, 57, 58, 62–71).

LTCCs seem to contribute about 40% of voltage-gated Ca^{2+} currents in SN DA neurons from juvenile rodents [postnatal day (PN) 15–20] (59), but LTCC currents decrease with rodent age by about 80% (62; but see 72). LTCCs are active during the pacemaking of SN DA neurons, not only during an action potential but also during the interspike interval (44, 50, 73, 74). They generate slow voltage oscillations (slow oscillatory potentials) in both somatic and dendritic compartments (75–77). In the distal dendrites of DA neurons and SN DA neurons, a compartment that is important for determining the spontaneous firing rates (78, 79), oscillations in free intracellular Ca^{2+} levels are associated with pacemaker activity, which are partially inhibited by DHPs (44, 58, 80) (**Figure 1*a*,*b***). The more negative activating Cav1.3 L-type channels are ideally suited to contribute to the subthreshold voltage changes that are sufficient to trigger dendritic depolarizations and activity-associated Ca^{2+} spikes (44, 58, 74, 78, 81).

However, both Cav1.3 and Cav1.2 are expressed in rodent and human SN DA neurons (72, 74), and this raises the question about their relative contribution to DHP-sensitive currents and to the potentially neuroprotective effects of DHPs. The direct quantification of the individual contributions of LTCC subtypes (or VGCCs in general) to voltage-gated Ca^{2+} currents in SN DA neurons using patch-clamp current recordings is complicated by the high vulnerability of these (in particular adult) neurons to the highly artificial recording conditions and by the fast rundown that obscures drug effects (42, 64). Dissecting the individual current components and the functional roles of Cav1.2 and Cav1.3 LTCCs in SN DA (and other) neurons is further complicated because no Cav1.3- or Cav1.2-selective drugs are available thus far (36, 83). One compound (compound 8, BPN4689) (84) has been suggested to be Cav1.3-selective, but this could not be confirmed by independent laboratories (reviewed in 83). Interpreting BPN4689-resistant Ca^{2+} transients as Cav1.2- and not Cav1.3-mediated is therefore not based on robust pharmacological evidence (7). The analysis of Cav1.3 knockout mice for distinguishing Cav1.2 and Cav1.3 functions is complicated, as compensation by Cav3.1 TTCCs in SN DA neurons has been described (64). A mouse model that expresses Cav1.2 channels that are insensitive to DHPs (Cav1.2 $DHP^{-/-}$) allows discrimination between Cav1.2- and Cav1.3-mediated DHP effects in SN DA neurons using available DHPs (67), but it has not been used so far to separate Cav1.2 from Cav1.3 Ca^{2+} currents in SN DA neurons. However, Cav1.3 channels likely contribute to activity-dependent dendritic Ca^{2+} inward current, because the Ca^{2+} transient amplitudes decrease by half in SN DA neurons expressing Cav1.3 short hairpin RNA (shRNA) (44).

Immunohistochemical reports about the subcellular distribution of Cav1.3 α1-subunit protein in SN DA neurons should be interpreted with caution, since the specificity of antibody staining for Cav1.3 α1-subunits on SN DA neurons has thus far not been unequivocally demonstrated by reporting proper control staining in Cav1.3 knockout brains (see, e.g., 59, 85–88).

THE ROLES OF NEURONAL L-TYPE CALCIUM CHANNELS IN PARKINSON'S DISEASE PATHOLOGY

Excellent reviews have summarized environmental and cell-autonomous risk factors as well as pathophysiological events implicated in SN DA neuron degeneration and PD (1, 6, 12–14, 89, 90). Here we briefly summarize the main PD stressors and pathomechanisms and focus on the role of LTCCs in these pathways and for Ca^{2+} homeostasis in SN DA neurons (see also **Figure 1*c***) (17). The modulation of neuroinflammatory responses due to LTCCs in activated microglia and astrocytes and the anti-inflammatory effects of LTCC blockers through glial inhibition are additional important aspects that are not discussed here (but see 1, 13).

The preferential death of SN DA neurons in the course of PD (and during physiological aging) is considered to be the result of a synergistic network of toxicity pathways, which are initiated by multiple intrinsic and extrinsic disease-triggering factors. Such a multiple-hit hypothesis (91–93) integrates well-known PD risk factors (e.g., age, toxins, inflammation, and *PARK* gene mutations) as well as pathogenic mechanisms implicated in PD, such as iron content, dopamine metabolism itself, elevated metabolic stress, reduced mitochondrial quality control, and dysregulated Ca^{2+} homeostasis. The identification of gene mutations underlying familial forms of PD (e.g., *PARK* genes) (11, 94–97) has helped to identify pathogenic signaling pathways, in particular proteasomal, lysosomal, and mitochondrial (especially complex I) dysfunction (96, 98). For instance, heterozygous mutations in the *GBA1* gene, coding for a lysosomal glucocerebrosidase, emerged as an important risk factor for PD, accounting for at least 5% of all PD cases (14, 22). iPSC-derived differentiated DA neurons from patients with *GBA1* mutations show, besides lysosomal dysfunction and elevated metabolic stress, a dysregulation of Ca^{2+} homeostasis and increased vulnerability to metabolic stress (99).

The specific intrinsic physiology of SN DA neurons renders them particularly vulnerable to identified PD stressors. In particular, their large axonal arborization as well as the distinct molecular mechanisms underlying their autonomous electrical activity lead to elevated metabolic stress levels already under physiological conditions. Additional PD stressors are thus more likely to tip the neurons over the edge into degeneration (6, 50, 100, 101).

The metabolism of dopamine itself is strongly linked to elevated oxidative stress, as its degradation generates reactive oxygen species (ROS), and dopamine oxidation can lead to the formation of endogenous neurotoxins (102). Furthermore, SN DA neurons are some of the most highly arborized neurons in the nervous system with billions of transmitter release sites (103), and the ATP requirement for propagation of action potentials grows exponentially with the level of axonal branching (104). In addition, intracellular Ca^{2+} buffering is particularly low in SN DA neurons as they do not express Ca^{2+} binding proteins, such as calbindin$_{d28k}$, for reasons that remain unclear and in contrast to more resistant DA neurons (105, 106).

On top of this already metabolically challenging situation, each action potential is accompanied by large Ca^{2+} transients that are associated with elevated mitochondrial stress in highly vulnerable SN DA neurons, but not in VTA DA neurons (6, 43, 44, 58, 77, 78, 107, 108). Elevated metabolic stress could thus easily lead to a vicious cycle of further impairment to mitochondrial functions, resulting in even more metabolic stress. One consequence is a reduced bioenergetic reserve capacity of DA neurons, which in turn makes them particularly vulnerable in conditions of increased metabolic demand (109), i.e., when continuous dopamine release into the dorsal striatum might be essential for movement and survival (12). Additional scenarios by which elevated Ca^{2+} levels may become deleterious for SN DA neurons are the activation of Ca^{2+}-dependent apoptotic enzymes, the Ca^{2+}-dependent stimulation of enzymes for dopamine and ATP synthesis, Ca^{2+}-dependent modifications of α-synuclein, or alterations in gene expression (reviewed in 6, 12–14, 17, 93, 107, 108, 110). Together, these intrinsic causes might explain why an LTCC-involving pacemaker mode is particularly stressful for SN DA neurons (but facilitates their physiological functions), and why DHPs might protect them from degeneration.

The activity- and Ca^{2+}-related mitochondrial stress levels in vitro are elevated in SN DA neurons from *dj-1* (i.e., *park7*) knockout mice (brain slices, PN 21–30), indicating a direct link to PD pathology. This stress is attenuated by the DHP isradipine (5 μM) (80), which also reduces activity-dependent mitochondrial oxidative stress and oxygen consumption in cultured mouse SN DA neurons and slices (1–5 μM) (PN 0–5) (86, 101).

A recent study also showed that mitochondrial mass is lower in SN DA neurons than in VTA neurons due to enhanced mitophagy (44). More importantly, chronic in vivo isradipine treatment of adult mice (7–10 days), resulting in isradipine plasma concentrations of about 5 nM (44), reduced metabolic stress and reversibly increased mitochondrial mass in SN DA neurons relative to that of VTA DA neurons.

Mouse SN DA neurons in culture are also more susceptible than VTA DA neurons to the neurotoxic effects of 1-methyl-4-phenyl-pyridine (MPP^+), the toxic metabolite of 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) (101, 111), which inhibits complex I of the mitochondrial respiratory chain (112, 113). As detailed below, chronic MPTP treatment is the gold standard neurotoxin-based in vivo PD model. In cultured SN DA neurons, MPP^+ leads to a delayed rise in cytosolic Ca^{2+} levels followed by an elevation of mitochondrial Ca^{2+} concentrations and mitochondrial oxidation. This rise in Ca^{2+} is exacerbated by human wild-type α-synuclein (*PARK4*), and it is prevented by isradipine (5 μM) (111).

In mouse midbrain slices (PN 21–30), the activity-related Ca^{2+} transients are partially inhibited in distal and proximal dendrites by isradipine (44, 58, 74, 86). This suggests that, besides LTCCs, additional Ca^{2+} sources could contribute to SN DA neuron vulnerability, including a variety of

other Ca^{2+} channels and Ca^{2+} transporters in the plasma membrane and in intracellular organelles (12, 13).

Indeed, a perturbed mitochondrial endoplasmic reticulum (ER) network, due to the altered activity of sarcoplasmic/endoplasmic reticulum Ca^{2+} ATPase (SERCA) or mitochondrial Ca^{2+} transporters, and thus mitochondrial Ca^{2+} overload, has been implicated in PD pathology (114, 115). In cultured SN DA neurons, LTCC-triggered, Ca^{2+}-induced Ca^{2+} release (CICR) contributes to MPP^+-induced Ca^{2+} surges (111). CICR is modulated in SN DA neurons by the phospholipase Pla2g6 (*PARK14* gene) (116). Noradrenergic locus coeruleus neurons (mouse brain slices PN 21–32), which show a similar stressful electrical activity as SN DA neurons, are also highly vulnerable to PD stressors. In these neurons, ryanodine-sensitive CICR from the ER mediates most of the DHP-sensitive, activity-related Ca^{2+} transients (7); a similar coupling to intracellular Ca^{2+} stores may also exist in SN DA neurons (44).

Notably, the activity of SN DA (and also of locus coeruleus and other) neurons is generated and modulated by a complex network of various ion channels in which LTCCs are embedded (**Figure 1*b***) (12, 13, 50, 55). One example is the transient receptor potential canonical channel TRPC1 that can suppress Cav1.3 activity in SN DA neurons in mouse brain slices (PN 14–35) via stromal interacting molecule-1 (STIM1). This is associated with protection from MPP^+-induced death in cell culture (117). A variety of other voltage-gated ion channels can functionally interact with LTCCs and modulate not only SN DA neuron function but also their vulnerability, including hyperpolarization-activated cyclic nucleotide gated (HCN) cation channels (81, 118), Ca^{2+}-sensitive A-type channels (119), SK- or BK-K^+ channels (65, 68, 120), and metabolic stress–activated ATP-sensitive K^+ channels (K_{ATP}) (82, 121). As an illustration of this complexity, metabolically gated K_{ATP} channels activate in SN DA neurons (in adult mice) in response to PD stressors (e.g., MPP^+, rotenone), thereby reducing their electrical activity (121). This hyperpolarization could in turn activate TTCCs and HCNs (63). However, under physiological conditions, K_{ATP} activity in SN DA neurons in vivo can do the opposite and trigger glutamate- and NMDA-mediated burst activity (82), which may favor enhanced Cav1.2 LTCC activity (74). Although it has been demonstrated that the loss of K_{ATP} channels in vivo protects SN DA neurons in the chronic MPTP PD model and in a genetic PD model (82, 121), it is not clear if this protective effect is caused by reducing (burst) activity or by reducing the chronic activity inhibition and hyperpolarization of SN DA neurons.

These signaling networks are also important when considering LTCC inhibition as a neuroprotective strategy. There is equally strong evidence showing that not only elevated Ca^{2+} concentrations but also a drop in intracellular Ca^{2+} concentrations below threshold levels can compromise the survival of SN DA neurons (reviewed in 12, 13). In this view, SN DA neurons might display a flexible homeostatic bandwidth of activity patterns and Ca^{2+} levels that allows them to adapt dopamine release and voluntary movement to metabolic needs to ensure, e.g., ongoing activity and dopamine release, particularly during metabolic duress when movement is essential for survival (like starving or being chased by a predator) (12). Further PD stressors could perturb this delicate balance (**Figure 1*c***). Hirsch and colleagues (13) suggested a similar scenario where SN DA neurons might cycle through hypo- and hyperactive phases, during which they could endure Ca^{2+} deficiency and Ca^{2+} overload, prior to degeneration in PD.

In conclusion, although they are still incompletely understood, LTCCs appear to serve a delicate and perhaps age-dependent physiological role for SN DA neuron fine-tuning within a complex signaling network. PD stressors affect this network, thereby allowing LTCCs to contribute to stressful Ca^{2+} signaling (**Figure 1*c***). This complex network and the bidirectional functions of

LTCCs and Ca^{2+} signaling in SN DA neurons should be considered in view of LTCC inhibition as a novel therapy, as it could affect the neuroprotective potential of these drugs.

In view of the critical transition from animal model findings to human PD, it is important to note that almost all studies summarized above or below analyzed SN DA neurons from rodents—in in vivo neurotoxin models, in brain slices (juveniles or adults), or in cell culture. A recent study provided strong evidence that dopamine metabolism, Ca^{2+} homeostasis, and the response to PD stressors actually differ between SN DA neurons from mice and humans (122). Burbulla et al. (122) compared adult wild-type and *dj-1* knockout mice, or iPSC-derived DA-like neurons from these mice, to human iPSC-derived SN DA-like neurons from idiopathic or DJ-1 (*PARK7*) PD patients and from healthy controls. Only the iPSC-derived DA neurons of PD patients displayed decreased basal respiration as well as increased oxidized dopamine accumulation and oxidant stress levels, accompanied by reduced lysosomal *GBA1* function and elevated α-synuclein aggregation due to oxidative modifications. Isradipine exposure (500 nM for 30 days) significantly diminished the accumulation of oxidized dopamine and its consequences (122).

Considering the PD mouse model studies that addressed the protective effects of DHPs and LTCC inhibition on SN DA neurons, it is important to note that in the same study a pathophysiological cascade similar to that described in human SN DA neurons could be triggered in mouse SN DA neurons when dopamine levels were elevated by L-DOPA application (122). These findings indicate quantitative differences between human and mouse SN DA neurons, but qualitative similarity in disease mechanisms, and they justify the in vivo mouse model studies for assessing the neuroprotective effects of LTCC inhibition.

MOLECULAR AND CLINICAL PHARMACOLOGY OF L-TYPE CALCIUM CHANNEL BLOCKERS

In this section, we discuss the pharmacology and functional properties of currently available LTCC blockers in view of the desired characteristics for their use as novel drugs for neuroprotective PD therapy. Such a blocker should ideally inhibit, with high affinity and selectivity, only those LTCC isoforms that are contributing to neurodegeneration in PD; it should reach sufficiently high brain concentrations for efficient channel inhibition; and it should be easy to administer, i.e., orally once a day.

DHPs belong to the class of Ca^{2+} channel blockers (Ca^{2+} antagonists) that have mainly vascular effects (123). At therapeutic doses, DHPs preferentially relax arterial vascular smooth muscle and lower peripheral vascular resistance. Effects on cardiac function (in particular, negative inotropic effects) are only present at high doses. This makes DHPs widely used first-line antihypertensive drugs (123). Notably, Cav1.2 predominantly mediates the cardiovascular effects of Ca^{2+} channel blockers, since Cav1.3 channels are absent in arterial and ventricular myocytes (36, 37, 124).

DHPs like isradipine inhibit Cav1.2 channels in vascular smooth muscle more effectively than in the heart because their channel-blocking activity in intact cells is strongly determined by membrane voltage (modulated receptor hypothesis) (125, 126). Consequently, depolarized membrane voltages that favor inactivated channel states also facilitate channel blocking by DHPs (126). Since membrane voltage is, on average, more depolarized in arterial smooth muscle than in the working myocardium, this offers one possible explanation for their vascular selectivity. Shifting the holding potential from negative to more depolarized voltages (e.g., −50 mV) decreases the half maximal inhibitory concentration for DHP (IC_{50}) values for Cav1.2 by about fivefold (47). In addition, alternative splicing also affects the biophysical and pharmacological properties of Cav1.2, rendering smooth muscle (and brain) splice variants about ten times more sensitive than cardiac muscle variants (47). Therefore, membrane potential and alternative splicing seem to explain DHP

tissue selectivity, although a contribution from additional factors (such as protein interactions and subunit composition) cannot be ruled out.

Such state-dependent determinants of DHP potency can also explain the different DHP sensitivities of Cav1.2 and Cav1.3. In electrophysiological recordings applying square pulse protocols, isradipine inhibits Cav1.3 with about five- to tenfold-higher IC_{50} values than Cav1.2 (127). Similar Cav1.2 selectivity is also observed for nimodipine (128). Statements that isradipine is a Cav1.3-selective blocker (13) are incorrect, misleading, and in contrast to solid experimental evidence. As discussed below, isradipine selectively inhibits Cav1.2 channels even under experimental conditions in which SN DA neuron pacemaking is simulated (74). This also rules out the possibility that Cav1.3 channels are preferentially inhibited by isradipine because more Cav1.3 channels inactivate (and thus facilitate isradipine inhibition) at more hyperpolarized membrane potentials compared to Cav1.2 (44). Similar to Cav1.2, Cav1.3 alternative splicing also affects the channels' sensitivity to DHPs. C-terminal alternative splicing can produce several short splice variants, which all share an even more negative activation voltage range and faster inactivation during prolonged depolarizations (reviewed in 46). Notably, C-terminal short variants show lower sensitivity to nimodipine (three- to fourfold) (129) and to isradipine (**Figure 2**) (74).

The state-dependent action of DHPs raises the important question of the extent to which Cav1.2 and Cav1.3 channels are inhibited in SN DA neurons at therapeutic, well-tolerated DHP plasma concentrations. For isradipine, 10 mg has been identified as the maximum daily dose of DHPs that can be chronically administered to PD patients once daily as a slow-onset, controlled-release formulation (Dynacirc CR) (130), resulting in mean serum concentrations of 1.53 ng/mL (~4 nM) (NCT00753636). In the ongoing phase III clinical PD trial, this daily dose of 10 mg is administered as 5 mg immediate-release tablets twice daily. This regimen is expected to lead to peak plasma concentrations of about 5 ng/mL (13.5 nM) with fast decline to one-tenth before the next dose (131, 132) and less than 2 ng/mL during most of the dosing interval.

In contrast to heart and smooth muscle, neither Cav1.2 and Cav1.3 current components nor their DHP sensitivities have been quantified in rodent SN DA neurons due to the methodological reasons discussed above. DHP sensitivity in these neurons must also be affected by the expressed splice variants and by the electrical activity pattern. The action potentials of SN DA neurons are significantly broader (~2.5 ms) compared to, e.g., those of Purkinje cells (below the millisecond range) (73). Furthermore, due to their pacemaker activity, SN DA neurons have no stable membrane potential, but their average membrane potential during single-spike activity is within about −40 mV rather depolarized, and they are even more depolarized during burst activity. These electrical properties of SN DA neurons should generally favor LTCC blocking by DHPs. One study has addressed this important question by quantifying isradipine effects on Cav1.2 and Cav1.3 LTCCs in stably transfected HEK293 cells (74). Brain Cav1.2 (corresponding to a smooth muscle splice variant) and Cav1.3 long or short α1-subunits were each coexpressed with human β3 and $\alpha2\delta1$ subunits (**Figure 2**). In vitro mouse SN DA neuron action potential–like waveforms or bursts were used as command voltages to mimic SN dopaminergic electrical activity (74). As shown in **Figure 2**, these recordings confirmed that more negatively activated Cav1.3, but not Cav1.2, LTCCs carry a substantial Ca^{2+} current during the interspike intervals of SN DA neuron pacemaker activity, while Cav1.2 was particularly important during action potentials and during burst activity (74). In steady-state SN DA neuron pacemaker mode, both human Cav1.3 and Cav1.2 currents were inhibited by low nanomolar concentrations of isradipine (74); however, Cav1.3 channels were significantly less sensitive to the drug. In particular, a C-terminal short Cav1.3 splice variant (short variants constitute half of the Cav1.3 channel transcripts in SN DA neurons) (74) was about six times less sensitive ($IC_{50} \sim 17$ nM) than Cav1.2 ($IC_{50} \sim 3$ nM) under these recording conditions (**Figure 2**). When Cav1.2 channels

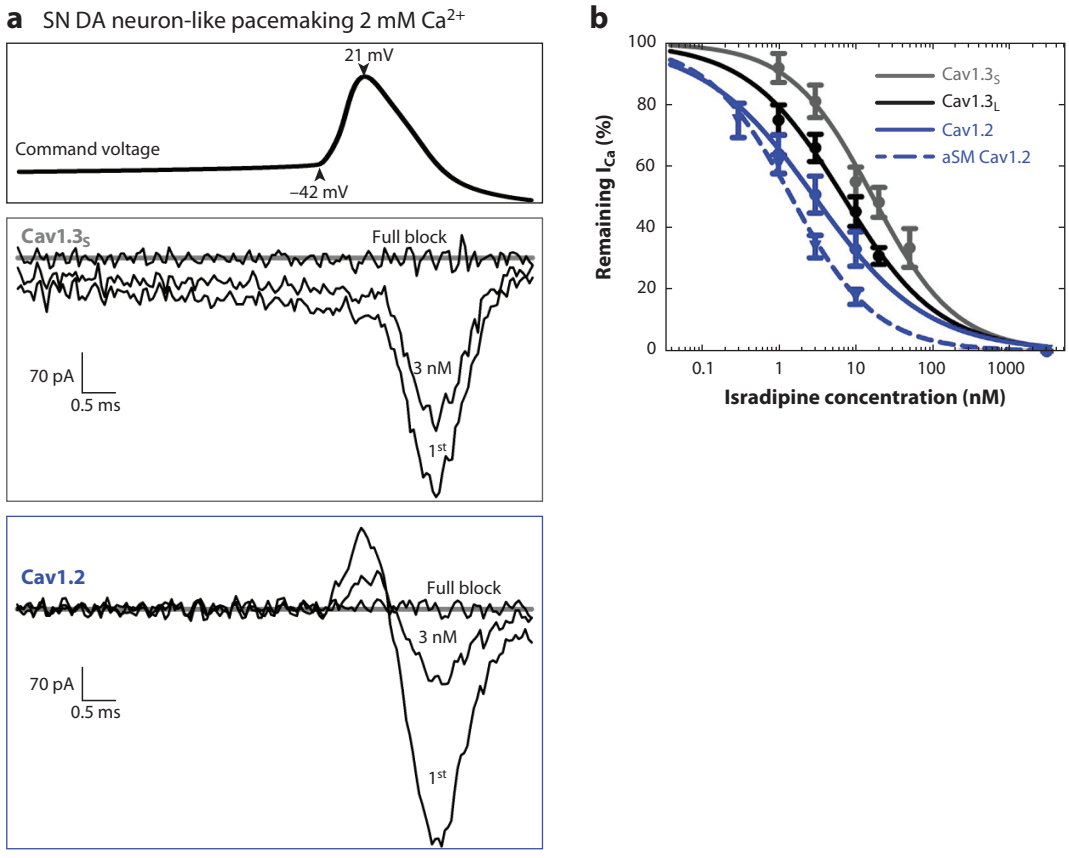


Figure 2

Steady-state sensitivity of stably expressed LTCC isoforms to isradipine during SN DA neuron-like activity. (*a*) SN DA neuron action potential waveforms were used as command voltage to mimic regular pacemaking (2.5 Hz) to elicit Ca^{2+} currents through human Cav1.3 (Cav1.3$_S$) and human Cav1.2 LTCCs that were stably expressed in HEK293 cells (together with $\alpha 2\delta 1$ and $\beta 3$ subunits). Starting from −89 mV, simulated SN DA neuron-like pacemaking resulted in an ∼80% current decay in both channels (not shown). After reaching steady-state I_{Ca} (sweep 1), 3 nM isradipine was applied followed by complete channel inhibition with 3 μM isradipine. The remaining isradipine-insensitive current components were then subtracted to obtain pure LTCC-mediated I_{Ca} and drug effects were corrected for linear I_{Ca} decay quantified in cells perfused with the vehicle only (see 74 for details). Notice that unlike Cav1.2, Cav1.3 conducted I_{Ca} already during the interspike interval. (*b*) Concentration-response curves for LTCC steady-state I_{Ca} inhibition by isradipine (n = 4–10 per data point) during simulated SN DA neuron-like pacemaking (*solid lines*) and aSM-like activity (*dashed line*, Cav1.2 only). Data were fitted to a sigmoidal dose-response equation with variable slope, resulting in the following IC_{50} values given as the mean (95% confidence interval): Cav1.3$_S$, 16.8 nM (14.1–19.9); Cav1.3$_L$, 6.9 nM (5.8–8.3); Cav1.2 SN dopaminergic, 2.9 nM (2.2–3.9); Cav1.2 aSM, 1.5 nM (1.2–1.7). IC_{50} values differed significantly from each other (extra sum-of-squares F-test; Hill slopes, $p = 0.797$; IC_{50} values, $p < 0.0001$). Abbreviations: aSM, arterial smooth muscle; Cav1.3$_L$, human Cav1.3 long variant; Cav1.3$_S$, human Cav1.3 short splice variant; DA, dopamine; I_{Ca}, Ca^{2+} inward current; IC_{50}, half maximal inhibitory concentration; LTCC, L-type Ca^{2+} channel (i.e., Cav1.2, Cav1.3). Figure adapted from Reference 74.

were stimulated using command voltages resembling arterial smooth muscle electrical activity, the IC_{50} for isradipine dropped twofold to ∼1.5 nM, as expected from state-dependent DHP action (see above). These experiments, although controversially discussed (74, 133), indicate that brain LTCCs, and in particular Cav1.3 channels, will be more difficult to engage therapeutically than vascular Cav1.2 channels, which are responsible for therapy-limiting hypotensive side effects

and leg edema. Thus, the development of novel Cav1.3-selective LTCC inhibitors with high brain exposure would be desired for PD therapy. However, no such drug is currently available (83, 134).

Additional factors must be considered when comparing DHP concentrations for LTCC inhibition in vitro with therapeutic steady-state DHP plasma concentrations in humans: The drug concentrations used in in vitro studies are unopposed by DHP plasma protein binding, which is >90% for isradipine (131). Therefore, in in vitro studies, the total drug concentration corresponds to the free concentration available for equilibration into the membranes of the tissues or cells under investigation, complicating direct comparisons of in vitro IC_{50} values with plasma concentrations. In contrast, in in vivo animal studies, comparing the total steady-state DHP plasma concentrations with the respective human therapeutic plasma levels is valid (assuming that plasma protein binding and the fundamental mechanisms of distribution into the brain in particular are similar). Despite the very high plasma protein binding, lipophilic DHPs easily unbind from plasma proteins (135) and are quickly enriched in lipophilic plasma membrane compartments at 37°C (136). This also allows for fast first-pass brain extraction of these highly lipophilic drugs (135). Since free drug concentrations in the cerebrospinal fluid and plasma are very similar (137, 138), the equilibrium with central and peripheral lipophilic plasma membrane compartments of LTCCs must also be similar. Consequently, distribution into the LTCC membrane compartments in the brain should parallel that of the cardiovascular system. Based on the predicted relative sensitivities of vascular and SN dopaminergic LTCCs to isradipine discussed above (74), this suggests that the inhibition of SN dopaminergic Cav1.3 channels may be less complete at standard blood pressure–lowering doses of the drug.

A recent study provided evidence that Cav1.3 channels are inhibited in vivo by therapeutic plasma concentrations of isradipine in mice (44). After 7–10 days of chronic dosing [subcutaneous (SC) release of 3 mg/kg body weight per day], median plasma concentrations of ∼5 nM were reached. In in vitro slices isolated from isradipine-treated mice, cytosolic Ca^{2+} oscillations were reduced by about half (to similar levels as for shRNA treatment in drug-naive mice) in dendrites of SN DA neurons. This inhibition was observed in in vitro slice recordings despite perfusion of mouse brains with artificial cerebrospinal fluid (containing no isradipine) and extensive washing of the slices before recordings (44). This raises the question of whether this persistent effect in brain slices indeed reflects direct inhibition of Cav1.3 channels in the isolated slices or if chronic isradipine treatment reduced dendritic Ca^{2+} oscillations in a more complex manner that outlasts the inhibition of LTCCs by the drug. Moreover, 10 nM of isradipine (corresponding to 10 nM free drug in plasma) added directly to the slices caused less inhibition than 5 nM plasma concentrations (corresponding to less than 0.5 nM free drug in plasma; see above). This also could indicate that responses of SN DA neurons to chronic isradipine exposure differ from those of acute exposure. For instance, Cav1.2 (and also Cav1.3) is important for the regulation of activity- and Ca^{2+}-dependent gene transcription (12, 139–141). Thus, their chronic inhibition could reduce Ca^{2+} oscillations by affecting other regulatory mechanisms [e.g., alternative splicing, phospholipid-mediated pathways (36), or activity-related Ca^{2+} release from intracellular stores]. In this scenario, inhibition of Cav1.2 or Cav1.3 by isradipine could alter gene expression, and this change could lead to smaller Ca^{2+} oscillations, which may also include downregulation of Cav1.3 activity. Note that a comparison of cytosolic Ca^{2+} oscillations in chronically treated mice with placebo controls, which received the same surgical procedures, was not reported in this study but only with an isradipine-treated group where the minipumps were explanted 5 days prior to recordings.

In summary, DHPs like isradipine (or nimodipine) should currently be regarded as primary candidates for targeting brain LTCCs as a neuroprotective PD therapy for a number of reasons,

including their history of safe use in humans as prescription drugs and their brain permeability (74, 132, 142–148). Isradipine has a short half-life (132); therefore, twice-daily dosing of immediate-release tablets is required. From a safety standpoint, slow-onset, extended-release formulations are preferred to avoid fast absorption, which has the potential to cause reactive sympathetic activation, with reflex tachycardia and increased cardiac oxygen consumption, and other side effects such as hypotension, flush, discomfort, and headache (149, 150). Extended-release formulations are currently available in some countries for isradipine and were used for the phase II clinical trial in early PD patients (130).

IN VIVO NEUROPROTECTION STUDIES WITH DIHYDROPYRIDINE CALCIUM CHANNEL BLOCKERS IN ANIMAL MODELS OF PARKINSON'S DISEASE

In Vivo Animal Models of Parkinson's Disease

Studies in animal models were carried out to directly demonstrate the SN DA neuron protective effect of DHPs in PD paradigms. One important factor for such in vivo neuroprotective studies in general is the choice of a suitable PD animal model. Various neurotoxic and genetic PD models have been established. However, given the multifactorial nature of PD, none of the current animal models can fully recapitulate its complexity, and all established and newly described in vivo PD models have individual advantages and disadvantages (151–153). As a comprehensive discussion of all these models is beyond the scope of this review, we focus on those PD models that have thus far been utilized to study neuroprotective DHP effects in vivo.

In all these studies, neurotoxin-based PD animal models were used—either the neurotoxin MPTP or hydroxylated dopamine (6-OHDA) was administered, and in different paradigms (for more details, see **Table 1** and **Supplemental Table 1**). MPTP can pass the blood-brain barrier, and in the brain, MPTP is converted by glial MAO-B into the active toxin MPP^+. MPP^+ is transported via the dopamine transporter into DA neurons where it inhibits the complex I of the mitochondrial respiratory chain and introduces a neurodegenerative process that affects SN DA neurons in particular. 6-OHDA is injected directly into the SN, the striatum, or the medial forebrain bundle where it autooxidizes, and the oxidation products (ROS) are assumed to mediate 6-OHDA neurotoxicity (153–157).

Supplemental Material >

The chronic low-dose MPTP PD model (i.e., 20 mg/kg MPTP, administered twice a week over a month, together with 250 mg/kg probenecid to reduce biological variabilities and potentiate MPTP effects) is currently regarded as the gold standard for studying neuroprotective mechanisms in PD. It introduces a selective, progressive, and persistent degeneration of SN DA neurons and their striatal axonal projections, similar to PD (112, 153–155, 158). However, compared to 6-OHDA experiments, MPTP experiments are more strictly regulated and require special permissions and a laboratory equipped with specific safety features to protect the experimenter from toxin exposure (155). The disadvantages of 6-OHDA models are the necessity for brain injection, a less chronic time course (similar to acute MPTP models), no Lewy body production (as is present in the chronic MPTP model and in PD), and higher variability in the outcome depending on, e.g., injection site, 6-OHDA dose, and infusion rate. These factors make standardization between laboratories more difficult (153, 155).

Given these methodological considerations, we discuss in vivo animal model studies separately for those using MPTP (seven studies) and 6-OHDA models (five studies). It is important to note that, even within these two models, experimental variabilities exist due to differences in toxin and drug application regimens (**Table 1**; **Supplemental Table 1**).

Table 1 Short summary of preclinical studies investigating DHP-mediated protective effects in animal models of PD

Publication	Protection[a]	Species (strain), age	PD model, dosage	DHP (dosage)	DHP plasma:brain[b]	Sample size
Kupsch et al. (161)	N: yes	Mice (C57Bl/6N), 10–12 weeks	MPTP (acute), SC, 40 mg/kg per injection, 2×, 16 h apart	NIM (10 mg pellet, SC)	ND	$n = 4–14$
Singh et al. (162)	N, DA, B: yes	Mice (Balb/c), adult	MPTP (acute), IP, 30 mg/kg per injection, 2×, 16 h apart	NIM (5, 10, or 15 mg/kg, IP, once)	ND	$n = 5–18$
Gerlach et al. (159)	DA: no	Mice (C57Bl/6), 6 weeks	MPTP (subacute), IP, 30 mg/kg per injection, 1 per day for 3 days	NIM (5 or 20 mg/kg, PO, daily)	ND	$n = 7–10$
Kupsch et al. (148)	N: yes DA, B: no	Common marmosets, 43–50 months	MPTP (subacute), SC, 2 mg/kg per injection, 4×, 24 h apart	NIM (80 + 120 mg pellet, SC)	Day 18: 1:2	$n = 4–8$
Chan et al. (72)	N, B: yes	Mice (C57Bl/6N), 8–10 weeks	MPTP (chronic), SC, 25 mg/kg per injection, 10×, 3.5 days apart, plus 250 mg/kg probenecid IP	ISR (3 mg/kg per day pellet, SC)	ND	ND
Price et al. (160)	N, DA: no	Mice (C57Bl/6N), 9–10 weeks	MPTP (chronic), SC, 25 mg/kg per injection, 10×, 3.5 days apart, plus 250 mg/kg probenecid IP 30 min before	ISR (3 mg/kg per day pellet, SC)	Day 7: 2:1	$n = 4–12$
Wang et al. (163)	N, DA, B: yes	Mice (C57Bl/6), 8–10 weeks	MPTP (chronic), IP, 30 mg/kg per injection, 2× per week for 1–4 weeks	ISR (3 mg/kg per injection, SC, daily)	ND	$n = 5–10$
Sautter et al. (165)	N, DA, B: no	Rats (Sprague-Dawley), adult	6-OHDA, 20 μg, 0.5 μL/min	NIM (80 mg pellet, SC)	Day 35: 2:1	$n = 9–12$
Chan et al. (72)	N: yes	Mice (C57Bl/6N), Postnatal day 28–31	6-OHDA, 1 μg, 0.05 μL/min	ISR (3 mg/kg per day pellet, SC)	ND	$n = 9$
Ilijic et al. (164)	N: yes	Mice (C57Bl/6J), 6–7 weeks	6-OHDA, 2.5 μg, 0.1 μL/min	ISR (3 mg/kg per day pump, SC)	Plasma only	$n = 7–9$
Wang et al. (166)	DA, B: yes	Rats (Wistar), adult	6-OHDA, 19.8 μg, 1.0 μL/min	Nifedipine (3.5 mg/kg per day, SC)	ND	ND
Ortner et al. (74)	N: no	Mice (C57Bl/6N), 12–15 weeks	6-OHDA, 4.1 μg, 0.1 μL/min	ISR (3, 6, or 9 mg/kg per day pellet or pump, SC)	Plasma only	$n = 18–28$
	N: no	Mice ($Cav1.3^{-/-}$), 12–15 weeks		No DHP, global constitutive Cav1.3 knockout	No DHP	$n = 23–27$

Only studies that investigated striatal DA levels, SN TH^+ cell count or behavioral outcomes were included. A study using an experimental formulation of NIM was not included (179).
Abbreviations: 6-OHDA, 6-hydroxydopamine; B, behavior; DA, dopamine; DHP, dihydropyridine; IP, intraperitoneal; ISR, isradipine; MPTP, 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine; N, SN TH^+ neuron count; NIM, nimodipine; ND, not determined/not reported; PD, Parkinson's disease; PO., per os; SC, subcutaneous; SN, substantia nigra; TH, tyrosine hydroxylase.
[a]Protection is defined as a significant positive outcome in SN TH^+ neurons, striatal DA content, and/or behavior analysis.
[b]Plasma or brain DHP concentration was determined on day X after pellet/pump implantation and is given as the ratio plasma:brain.

Studies in MPTP Parkinson's Disease Models

From the seven reported studies in MPTP models (**Table 1**), only one pioneering study has investigated nonhuman primates. Kupsch et al. (148) analyzed male and female adult common marmosets to study the effects of nimodipine (80 or 120 mg, $n = 4$ each) and placebo pellets ($n = 8$) in an acute MPTP model 1 week after MPTP or saline treatment. The chosen MPTP protocol led to selective degeneration of about 50% of SN DA neurons. Neither dose of nimodipine had a significant effect on toxin-induced behavioral deficits or the depletion of dopamine and its metabolites in the dorsal striatum. However, 120 mg of nimodipine led to the almost complete protection of SN DA neurons, while 80 mg resulted in a nonsignificant trend towards rescue. No unbiased stereological analysis was used to determine SN DA neuron numbers. From all eight nimodipine-treated animals, four showed complete protection by the drug, whereas four were nonresponsive to it. No significant correlation was found between striatal dopamine levels, SN DA neuron numbers, and nimodipine brain concentrations. This, and the observation that nimodipine plasma (~60 ng/mL) and brain concentrations (~115 ng/g of tissue) did not differ between the 80 mg and 120 mg groups despite different outcomes, weakens the validity of the findings. However, this early study provides some evidence for a neuroprotective effect of the DHP nimodipine at therapeutic plasma concentrations.

Another early study reported no neuroprotective effects of nimodipine on striatal dopamine levels 13 days after the last MPTP dose in a subacute MPTP mouse model (6-week-old C57Bl/6 mice, gender and substrain not reported) (159). Nimodipine was administered at a dose of 5 or 20 mg/kg per os once a day for 9 days, starting 2 days before MPTP.

In contrast, the laboratory of Surmeier (72) found neuroprotective effects of DHPs in the gold standard chronic MPTP mouse model (8–10-week-old C57Bl/6N mice from Charles River). Isradipine (3 mg/kg per day extended-release pellets) prevented MPTP-induced motor deficits and partially rescued SN DA neurons (~50%). The number of animals in each group, isradipine plasma levels, and detailed statistical analysis were not reported. In this study, the authors mentioned additional experiments, indicating that Cav1.3 deficiency in mice confers neuroprotection comparable to isradipine ($n = 3$), but data have not been published.

However, this protective effect of isradipine in the chronic MPTP mouse model was not reproduced by Price et al. (160), who also used a C57Bl/6N strain and very similar MPTP and isradipine administration protocols to those of Chan et al. (72). A lower general mortality in the DHP group (determined at day 44) in MPTP-treated mice (3/12 placebo versus 1/12 isradipine) was observed, but there was no effect on MPTP-induced SN DA neuronal loss. Plasma isradipine levels were above human therapeutic levels (12–19 ng/mL), and MPTP-induced increases of astrocyte and microglia markers were not attenuated by isradipine treatment. It is important to note that although a detailed experimental workup was presented in abstract and poster format, a full report of the data has not yet been published in a peer-reviewed journal. Therefore, these data cannot yet be taken as strong experimental evidence.

Several laboratories (using C57BL/6 or Balb/c mice) also tested either nimodipine in acute MPTP mouse models (161, 162) or isradipine in a subacute MPTP model (163). All three studies reported the significant neuroprotection of SN DA neurons (no unbiased stereological analysis was performed). Two of these studies also found improvements in motor performance and striatal dopamine contents (162, 163). Interestingly, in these studies, treatment with the DHPs was not continuous (as in all other MPTP studies) but was given by once-daily subcutaneous injections. Although plasma levels were not reported, the very short half-life of both DHPs in mice (elimination half-life in mice of about 8 min) (146) predicts brief exposure to very high drug concentrations followed by long drug-free periods. Singh et al. (162) even administered only a single dose of

nimodipine before the start of MPTP injections. If reproducible, these findings would challenge the current view that neuroprotection requires constant steady-state plasma concentrations.

Studies in 6-OHDA Parkinson's Disease Models

The Surmeier laboratory (72) also reported that isradipine treatment (3 mg/kg per day, SC pellets) starting 7 days before 6-OHDA injections partially protected SN DA neurons from neurodegeneration in mice (~PN 30 male C57BL/6J, Charles River). Unilateral striatal injections of 1 μg 6-OHDA induced an SN DA neuronal loss of ~70% 3 weeks after lesioning that was reduced to ~25% by isradipine. This finding was confirmed in a second study by the same laboratory (164) in young (6–7 weeks) male C57Bl/6J mice (Jackson Laboratory). For unilateral striatal injection, a higher 6-OHDA concentration was used (2.5 μg), which resulted in a similar loss of about 70% of SN DA neurons. The same dose of isradipine was used as by Chan et al. (72), but it was applied via osmotic minipumps and started only 3 days prior to 6-OHDA. Isradipine rescued SN DA neurons and their striatal axons, and the degree of protection was positively correlated with the plasma isradipine concentrations reached in the individual animals (2.6–46.7 ng/mL, $n = 8$–9). The resulting plasma isradipine IC_{50} values were ~5 ng/mL (~13 nM) for protection of SN DA neurons and ~7.2 ng/mL (~19 nM) for striatal fiber protection. Using a theoretical model of channel inhibition based on the modulated receptor hypothesis (see above), the authors estimated that these concentrations should inhibit about 60–80% of Cav1.3 LTCCs, and they concluded that at least 3 ng/mL of isradipine is required for significant protection of SN DA neurons against 6-OHDA. Although these calculations are valid in principle, their interpretation should take into account that at steady-state plasma concentrations in vivo, less than 10% of isradipine is freely available for channel inhibition (131). Nevertheless, their data indicate a dose-dependent beneficial effect of isradipine on SN DA neurons, with 7 of 9 animals showing protection. It is not reported if such a dose-dependent protection could be reproduced in an independent cohort of animals and/or in the MPTP model.

Ortner et al. (74) evaluated the neuroprotective potential of isradipine and of the genetic deletion of Cav1.3 in male C57BL/6N (Charles River) mice in a similar 6-OHDA model. Similar protocols were used as those reported by Chan et al. (72) and Ilijic et al. (164), however, the mice were older (age 3–4 months). Dose-response experiments were carried out to optimize unilateral intrastriatal 6-OHDA injections to produce a partial lesion of about 50% of SN DA neurons. This was achieved with a dose of 4.1 μg 6-OHDA (**Table 1**). Isradipine was delivered via either extended-release pellets (6 or 9 mg/kg per day) or osmotic minipumps (3 mg/kg per day), resulting in mean plasma levels of ~6 ng/mL (~16 nM) 5 weeks after pellet or pump implantation (range of 3–10 ng/mL, $n = 18$). Stereological analysis indicated that neither isradipine treatment ($n = 18$–28) nor constitutive Cav1.3 knockout ($n = 23$–27) reduced relative 6-OHDA-induced loss of SN DA neurons. In contrast, in adult Cav1.3 knockout mice, the number of SN DA neurons even without 6-OHDA treatment was already significantly lower than in wild-type C57BL/6N (74).

Two other studies addressed DHP effects in different 6-OHDA application paradigms in adult rats, and they also reported inconsistent results (**Table 1**). Sautter et al. (165) found no evidence for a neuroprotective effect on SN DA neurons or striatal projections by chronic nimodipine pretreatment (80 mg pellets, SC) starting 7 days prior to 6-OHDA treatment (20 μg, unilateral intrastriatal injections, 4 weeks) of female adult (250–300 g) Sprague-Dawley rats. Nimodipine treatment resulted in therapeutic serum concentrations of ~30 ng/mL. Wang et al. (166) examined the effects of nifedipine in female adult (180–220 g) Wistar rats treated with unilateral 6-OHDA injections (19.8 μg) into the medial forebrain bundle. Nifedipine was injected subcutaneously once daily (3.5 mg/kg), starting 1 day before lesioning and continuing for 4 weeks. In this

study, a partial rescue of 6-OHDA-induced motor behavior and striatal dopamine depletion was detected. However, the numbers of SN DA neurons were not quantified, and nifedipine plasma concentrations were not reported.

Factors That Could Affect Outcome of Neuroprotection Studies in Parkinson's Disease Models

In 8 of the 13 reports that investigated the neuroprotective effects of DHPs in PD animal models in vivo, a significant protection of SN DA neurons by LTCC inhibition was reported for the brain-permeable DHPs isradipine, nimodipine, and nifedipine. Neuroprotective drug effects have been demonstrated in neurotoxin PD models for mice, rats, and primates (in five of seven MPTP studies, and in three of five 6-OHDA studies). The following considerations might explain why not all studies reported a similar benefit.

Various confounding factors exist that could influence study outcomes, including not only the chosen PD animal model, as discussed above, but also the age, sex, strain, and species of the animals employed. For mice, it is crucial to note that for some C57BL/6 strains from Harlan (Envigo since 2015), a sporadic null mutation in the *Snca* gene has been described (167–170). Hence, they are technically α-synuclein knockouts (described for C57BL/6J-OlaHsd, and possibly other strains) (171, 172). This point is essential, as SN DA neurons from α-synuclein knockout mice are less vulnerable to PD stressors (173–175); thus, this mutation would most likely affect the outcome of studies addressing protective effects in PD mouse models. Also, animal care conditions and related factors could profoundly affect, for example, immune state and stress levels of the individual animals and affect study outcomes (176). This point is of particular importance, as there is accumulating evidence that LTCC functions in SN DA neurons are complex and seemingly depend on a number of factors, including the environmental or metabolic state of an animal, as summarized above.

Moreover, the chosen DHP, the timing and route of administration, the dosing intervals, and other determinants of the plasma concentration time course could affect its neuroprotective effects. This important aspect is apparent from recent studies in mice (MPTP) (162, 163) and rats (6-OHDA) (166) in which only once-daily SC or intraperitoneal injections with DHPs provided neuroprotective effects. This should prompt future animal studies to test if intermittent drug exposure could be equivalent or even superior to continuous treatment regimens.

An additional critical factor to consider is the choice of approaches and protocols that reliably quantify a possible neuroprotective effect in a study (e.g., SN DA neuron number, striatal dopamine content/release, motor behavior). For instance, when using unbiased stereology, for a more accurate SN DA neuron number estimate (necessary to detect smaller changes in neuronal numbers), using a so-called oversampling approach where ideally each section is analyzed is highly recommended (177). This is particularly important in the 6-OHDA model, where the regional loss of SN DA neurons depends largely on the injection site and its size. Every third, fourth, and fifth section was analyzed in the studies conducted by Ilijic et al. (164), Chan et al. (72), and Ortner et al. (74), respectively. For a full quantitative description of possible DHP protection in 6-OHDA models, it is also important to document the individual injection sites and also the 6-OHDA-induced lesions for correlations with numbers of SN DA neurons over the full caudo-rostral axis. This has not been reported in any of the above studies.

These methodological issues should be considered in future experiments investigating neuroprotective strategies in PD models, as they likely contribute to the heterogeneous outcomes. Ideally, all these experimental conditions and the description of all relevant methodological procedures should be standardized to allow better comparisons of findings from different laboratories.

We suggest that studies systematically provide sufficient information about age, sex, animal species and (sub)strains, animal provider, and animal care conditions as well as the chosen PD model. Methods for quantifying neuroprotection should be described in detail. Furthermore, given the high variability of drug and neurotoxin effects, power calculation–based higher numbers of animals might be necessary in order to generate statistically more valid, biologically meaningful data sets (especially important for the interpretation of negative findings). Furthermore, drug plasma concentrations should be measured to ensure sufficient exposure and to allow correlation with neuroprotective outcome measures.

Finally, it would be desirable to confirm neuroprotective outcomes in separate independent cohorts. In this context, it should be noted that, with one exception (Reference 74 reported *n* numbers from four independent cohorts), the studies discussed here analyzed only 10 or fewer animals per group with no independent repeats, and the number of independent cohorts were not reported (**Table 1**).

In conclusion, none of the above animal studies addressing DHP neuroprotective effects fully reproduced the experimental protocol of another study. In other words, all studies used different PD models, different strains or species, and/or different DHP application protocols, and the plasma concentrations that were reached in individual animals were highly variable (or were not reported). Furthermore, different readout measures and analysis methods for determining and quantifying the possible neuroprotective effects of DHPs were chosen. Thus, a direct comparison of the reported outcomes in view of independent repeats is not valid, and findings need to be interpreted with care. An exception would be the studies of Chan et al. (72) and Price et al. (160), who had very similar study design. However, the latter study is not yet published, and in Chan et al.'s study, a more detailed description and analysis of the data are missing.

CONCLUSIONS AND IMPLICATIONS FOR FURTHER DRUG DISCOVERY

Currently, no PD therapy is available that protects SN DA neurons from degeneration. Human epidemiological and preclinical in vitro studies provide a strong rationale for targeting LTCCs as a novel therapeutic PD approach in the ongoing phase III clinical trial for isradipine. Although in vitro data demonstrate LTCC involvement in PD-relevant signaling events (such as dendritic Ca^{2+} transients in SN DA neurons and mitochondrial stress), robust, direct in vivo proof for a causal involvement of Cav1.3 LTCCs in defining the high vulnerability of SN DA neurons to degenerative triggers in PD is still missing. However, this question is highly relevant from a drug discovery point of view.

Both Cav1.2 and Cav1.3 are expressed in SN DA neurons, but their relative contributions to DHP-sensitive currents and their physiological roles are not yet clear. Current data indicate that the functions of distinct LTCC subtypes in SN DA neurons are complex and depend on the (patho)physiological context, which likely might also influence the PD-protective effect of DHPs. Although in vitro and in vivo studies point to a role for Cav1.3 in the high vulnerability of SN DA neurons, a contribution by Cav1.2 to DHP-mediated neuroprotection cannot be ruled out. Particularly during their burst activity, Cav1.2 channels should have a major impact on SN DA neurons, either directly or indirectly and more permanently via Ca^{2+}-dependent regulation of gene expression. In vivo, burst activity appears to contribute to the particularly high vulnerability of SN DA neurons to PD stressors (82) and likely involves the strong activation of Cav1.2 channels (74). Accordingly, the stabilization of single-spike modes and the reduction of energy-consuming burst activity in SN DA neurons might offer an additional explanation as to how DHPs confer a potential PD-protective effect, besides the inhibition of stressful Ca^{2+} oscillations. A possible

contribution of Cav1.2 to the neuroprotective effects of DHPs is of particular interest because of its high sensitivity to currently available DHPs.

If the results of the ongoing clinical trial further support a protective role of DHPs in PD, the unequivocal identification of the contributions of distinct Cav1.2 and Cav1.3 LTCC channel complexes for the neuroprotective effect in SN DA neurons is essential. If a significant role for Cav1.2 channels can be ruled out, then Cav1.3-selective blockers would provide a suitable strategy to optimize PD-protective effects while minimizing cardiovascular side effects. However, if Cav1.2 channel inhibition does significantly contribute to neuroprotection, then strategies for the optimized brain delivery of nonselective (178, 179) or even Cav1.2-selective blockers remains a choice. Finally, given the complex PD pathophysiology and the intricate flexibility of Ca^{2+} signaling in SN DA neurons, combination therapies of LTCC inhibitors with other potentially neuroprotective drugs (e.g., MAO-B inhibitors or novel repurposed substances like ambroxol) could be considered.

Regardless of all the considerations and open issues, the results of the ongoing STEADY-PD phase III trial will soon provide definite empirical proof of whether isradipine (at 10 mg/day) can slow down the progression of the motor symptoms in PD patients. Hopefully, 200 years after the first description of PD by James Parkinson and 50 years after their first clinical use as antihypertensives, DHPs will become the first class of drugs for disease modification in PD.

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