Calcium entry induces mitochondrial oxidant stress in vagal neurons at risk in Parkinson’s disease

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Mitochondrial oxidant stress is widely viewed as being critical to pathogenesis in Parkinson’s disease. But the origins of this stress are poorly defined. One possibility is that it arises from the metabolic demands associated with regenerative activity. To test this hypothesis, we characterized neurons in the dorsal motor nucleus of the vagus (DMV), a population of cholinergic neurons that show signs of pathology in the early stages of Parkinson’s disease, in mouse brain slices. DMV neurons were slow, autonomous pacemakers with broad spikes, leading to calcium entry that was weakly buffered. Using a transgenic mouse expressing a redox-sensitive optical probe targeted to the mitochondrial matrix, we found that calcium entry during pacemaking created a basal mitochondrial oxidant stress. Knocking out DJ-1 (also known as PARK7), a gene associated with early-onset Parkinson’s disease, exacerbated this stress. These results point to a common mechanism underlying mitochondrial oxidant stress in Parkinson’s disease and a therapeutic strategy to ameliorate it.

Parkinson’s disease is the second most common neurodegenerative disease. Nothing is known to slow its inexorable progression. As a consequence, there has been a concerted effort to understand its pathogenic mechanisms. Many lines of study, ranging from the functional analysis of genetic mutations in rare familial forms of Parkinson’s disease to the characterization of postmortem tissue from Parkinson’s disease patients, point to mitochondrial oxidant stress as an important step in the cascade of events leading to degeneration.

As compelling as the mitochondrial theory of pathogenesis is, it does not explain the pattern of neuronal pathology and degeneration seen in Parkinson’s disease. The cardinal motor symptoms of Parkinson’s disease are largely attributable to the degeneration of a small group of dopaminergic neurons in the substantia nigra pars compacta (SNpc). However, it has become clear that the pathology in Parkinson’s disease is more widespread, encompassing a variety of small, scattered nuclei in the brainstem and olfactory system in the early stage of the disease.

The most obvious sign of pathology in these nuclei is the presence of proteinaceous intracellular inclusions known as Lewy bodies or Lewy neurites (LBNs). Neurons in the DMV, in the caudal medulla are among the first to show LBN pathology. Moreover, they regulate a variety of parasympathetic functions, including gastrointestinal motility, which is impaired in many Parkinson’s disease patients. We found that cholinergic DMV neurons exhibited a slow autonomous pacemaking, broad spike and robust calcium entry through L-type and other calcium channels. Moreover, calcium entering the cytosol was only weakly buffered by endogenous proteins. Using a transgenic mouse that expressed a mitochondrially targeted, redox-sensitive variant of green fluorescent protein, we found that spike rate, spike duration and calcium entry through L-type channels all factored into elevating mitochondrial oxidant stress in these neurons. Finally, mitochondrial oxidant stress was exacerbated in DMV neurons from DJ-1−/− mice, and L-type channel antagonists effectively ameliorated this stress, suggesting a common phenotype that defines neurons at risk in Parkinson’s disease and a common therapy to protect them.

RESULTS
DMV neurons are autonomous pacemakers with broad spikes
The DMV is situated near the midline, dorsal to the darker and more fibrous hypoglossal nucleus and ventral to the nucleus of the
The DMV is located in the medulla oblongata dorsal to the hypoglossal nucleus (12 nu) and lateral to the central canal (CC). Inset, sagittal view. 5 nu, trigeminal nucleus; Amb, nucleus ambiguous; AP, area postrema; I.O., inferior olive; NST, nucleus of the solitary tract; PYT, pyramidal tract. (b) A confocal image of a transverse slice of the medulla from a Chat-eGFP mouse (approximate location indicated in inset in a). Bilateral DMV can be seen lateral to the central canal and dorsal to the hypoglossal nucleus. The overwhelming majority of neurons in the DMV expressed ChAT. One such cell was filled with biocytin and reacted with streptavidin–fluorescein isothiocyanate (FITC) for visualization. Inset, sagittal view. 5 nu, trigeminal nucleus; Amb, nucleus ambiguous; AP, area postrema; I.O., inferior olive; NST, nucleus of the solitary tract; PYT, pyramidal tract.

Perforated patch recordings from these neurons revealed them to be spontaneously active (Fig. 1c), in agreement with previous work in vivo.20,21 The spontaneous spikes were broad (2.7 ± 0.1 ms, n = 34; Fig. 1d), similar to those of SNpc dopaminergic neurons. The slow spontaneous spiking (Fig. 1e) was autonomously generated, as a cocktail of glutamatergic, GABAergic, cholinergic, serotonergic and adrenergic receptor antagonists failed to significantly alter its rate (1.6 ± 0.2 spikes per s in artificial cerebrospinal fluid (ACSF) versus 1.6 ± 0.2 spikes per s in blockers, P > 0.3, two-tailed Wilcoxon signed-rank test (SRT) for paired samples, n = 40).

As in SNpc dopaminergic neurons, the subthreshold voltage trajectory during this slow spiking was restricted to a relatively depolarized voltage range (−65 to −40 mV). The mechanisms necessary to generate slow autonomous pacemaking in this voltage range are reasonably well-defined.22 The most fundamental requirement is that the cell must not possess a stable membrane potential below spike threshold. In most pacemaking neurons, inward, depolarizing currents carried by voltage-dependent sodium and hyperpolarization/cyclic nucleotide gated (HCN) cation channels destabilize the membrane potential.22,23 In DMV neurons, the Nav1 sodium channel blocker tetrodotoxin (TTX, 1 µM) stopped autonomous spiking and eliminated subthreshold oscillations in voltage, creating a stable resting potential (Fig. 2a). Voltage-clamp measurements of the TTX-sensitive Nav1 channel currents in the soma revealed that these channels had a prominent persistent opening mode that was evident at membrane potentials at and above −60 mV (Fig. 2b), as in other pacemaking neurons.22,23 As reported previously,21 HCN channel currents were also evident in this voltage range. However, HCN channel density varied considerably, and only about half of the neurons tested (33 of 65) responded to negative current steps by initially hyperpolarizing.
and then slowly depolarizing (or sagging); this depolarization is a manifestation of HCN channel activation by hyperpolarization. As further evidence of this variability, the HCN channel antagonist ZD 7288 (20 µM) slowed pacemaking in only three of seven neurons, leaving the rest unaffected (Supplementary Fig. 1).

Another channel that supports pacemaking is the voltage-insensitive cation channel NALCN\(^{23,24}\). Nalcn mRNA was robustly expressed in DMV neurons (Fig. 2c). This mRNA was translated into functional protein, as replacing extracellular sodium with the larger membrane impermeable cation N-methyl-d-glucamine (NMDG) led to a substantial hyperpolarization of the membrane potential in the presence of TTX (median hyperpolarization = 17.7 mV, \(n = 6\), \(P < 0.05\), SRT; Fig. 2d). These results indicate that pacemaking in DMV neurons depends on a combination of Nav1, NALCN and, to a lesser extent, HCN channels.

**Pacemaking elevates intracellular calcium concentration**

In SNpc dopaminergic neurons, low-threshold L-type calcium channels with a Cav1.3 pore-forming subunit contribute to the inward currents driving pacemaking\(^{12,14,25}\). When Nav1 channels are blocked in these cells to eliminate spikes, the Cav1.3 channels generate slow oscillatory potentials (SOPs)\(^{13}\). SOPs were not evident in DMV neurons (Fig. 2a), suggesting that Cav1.3 channels have a less substantial role. To explore this issue, we voltage-clamped the somatic membrane and measured the cobalt-sensitive calcium channel currents evoked by the normal pacemaking voltage trajectory\(^{22}\). We observed a calcium channel current during the subthreshold portion of the trajectory in most neurons, but this current was relatively small, amounting to a few tens of picoamperes (Fig. 3a). Comparing this calcium current to the TTX-sensitive sodium currents over the same subthreshold

**Figure 3** Calcium currents during the pacemaking cycle in DMV neurons. (a) Cobalt-sensitive calcium currents recorded (bottom) in response to a voltage-clamped spike waveform recorded from a DMV neuron (top). Inset, distribution of the cobalt-sensitive subthreshold calcium current measured before the spike. The thick bar indicates the median, the box limits represent the 25th and 75th percentiles, and the whiskers indicate the range. (b) Population average (mean ± s.e.m.) of the persistent cobalt-sensitive calcium currents as a function of voltage. Solid red line is a fit as described in Figure 2c, but with a calcium Nernst potential of +120 mV. The fit for the TTX-sensitive current from Figure 2c is shown in black for comparison. (c) Single-cell RT-PCR revealed the presence of mRNA for Cav1.2 and Cav1.3 channels in DMV cholinergic neurons (in 7 of 8 and 3 of 8 cells, respectively). (d) Cobalt-sensitive calcium action currents generated by a physiological spike (red) and one expanded to twice the duration (blue). The vertical dashed line is the time of spike threshold, as determined by maximal dV/dt. Inset, calcium charge (shaded area) as a function of spike duration.

**Figure 4** 2PLSM calcium imaging reveals that calcium dynamics are dominated by spike-associated influx. (a) Left, somatic voltage recording and 2PLSM Fluo-4 imaging from a distal dendritic location (140 μm from soma) of a DMV cell revealed spontaneous discharge that is accompanied by spike-associated calcium transients. Right, application of TTX revealed a stable resting potential without any subthreshold calcium oscillations. (b) Spike-triggered average of calcium transients in a DMV neuron revealed a rapid increase in calcium that is followed by an exponential decay. (c) Left, 2PLSM calcium imaging from the soma in response to a sequence of 10-s current injections from −60 pA to +60 pA to either hyperpolarize and silence discharge or to depolarize and increase discharge. The value of fluorescence for 0 pA is defined as the ambient fluorescence. Bottom right, plot of fluorescence as a function of current before (filled circles) and after (empty circles) TTX treatment. The majority of calcium entry was spike dependent. At the population level, the rise in free calcium as a function of applied current, estimated from the slope of this function from −20 to +60 pA, decreased significantly in TTX (data not shown, \(n = 6\) cells, \(P < 0.05\), SRT). Top right, frequency-intensity (f-I) curve of this neuron in response to the same current steps. The dashed line in the fluorescence plot indicates 0 ΔF/F₀.
limits represent the 25th and 75th percentiles, and the whiskers indicate
the range.

The expression of mRNA for the Cav1.3 subunit in DMV neurons was verified by single-cell reverse transcription PCR (RT-PCR). This analysis also revealed robust expression of the high voltage-activated Cav1.2 subunit (Fig. 3c). These channels, along with other high voltage-activated channels, contribute to the calcium currents evoked by the spike itself. These currents in DMV neurons were large, constituting 0.5–2.9 pC per spike (n = 14; data not shown and Fig. 3d).

To complement these experiments, we used two-photon laser-scanning microscopy (2PLSM) to monitor intracellular calcium concentration in DMV neurons dialedyzed with calcium dye Fluo-4 (100 μM). During normal pacemaking, intracellular calcium concentration rose rapidly following the spike and then fell exponentially during the interspike interval (Fig. 4a,b). Abolishing spikes with TTX eliminated the fluctuations in intracellular calcium concentration (Fig. 4a), providing additional evidence against a subthreshold, calcium channel-dependent oscillation. Manipulating spike rate by intracellular current injection revealed that spike rate was the primary determinant of free cytosolic calcium concentration in DMV neurons (Fig. 4c).

Precisely which channels are responsible for the high-threshold, spike-evoked calcium currents in DMV neurons? Previous work has implicated both Cav1 and Cav2 channels. Cav2.2 channel currents

**Figure 5** Contribution of Cav1 channels to discharge patterns and ambient calcium levels in DMV neurons. (a) Pre-incubation in 200 nM of either isradipine or calciseptine, which antagonize Cav1 channels, did not alter the distribution of DMV neurons firing rates. (b) Application of 5 μM nifedipine, a Cav1 channel antagonist, significantly reduced the depth of the sAHP, measured from spike threshold that follows long depolarizing pulses (P < 0.05, SRT). Inset, distribution of changes in sAHP amplitude (ΔsAHP) in response to this drug. (c) Left, recording of spontaneous discharge followed by a hyperpolarizing pulse to silence the cell (bottom) can be used to measure the ambient fluorescence during autonomous discharge (top). Treatment with 5 μM isradipine consistently reduced the baseline level of fluorescence in cholinergic DMV neurons. The dashed line in the fluorescence plot indicates ∆F/∆F_0. Right, distribution of ambient levels of fluorescence before and after treatment with 5 μM isradipine (**P < 0.01, SRT). The thick bar indicates the median, the box limits represent the 25th and 75th percentiles, and the whiskers indicate the range.

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**Figure 6** DMV neurons have a low endogenous buffering capacity relative to dopaminergic VTA neurons. (a) Raw fluorescence as a function of time (green). Brief depolarizing pulses (2 nA, 2 ms) were delivered every 10 s for 2 min to induce a spike as the cell was filled with the Fluo-4 indicator. These spikes were accompanied by brief steps in raw fluorescence (ΔF). Prior to these pulses, and between them, the cell was silenced with a constant hyperpolarizing current. Fitting an exponential curve to the temporal profile of basal fluorescence (F_0) can be used to estimate the cytosolic concentration of the Fluo-4 dye as a function of time (black). A.U., arbitrary units. (b) Spike-induced transients in free cytosolic calcium concentration, measured with 0.2 F/∆F_0, decreased and were prolonged as the concentration of the added buffer increased (data excised from around the points marked with vertical arrows in a). (c) Fitting a linear regression line to the scatter plot of the reciprocal of ΔF as a function of the reciprocal of the incremental buffering capacity k' (see Online Methods) for one DMV neuron (black) and one dopaminergic neuron of the VTA (red) yielded estimates of the endogenous buffering capacity k_0 in these cells. (d) The distribution of k_0 for both populations is shown on the right. The thick bar indicates the median, the box limits represent the 25th and 75th percentiles, and the whiskers indicate the range. The buffering capacity of DMV neurons was significantly smaller than that of dopaminergic VTA neurons (**P < 0.005, RST).
have been reported to exert a strong effect on the medium duration after-hyperpolarization (mAHP) mediated by the small-conductance, calcium-activated (SK) potassium channel<sup>29</sup>. As predicted, the Cav2.2 antagonist ω-conotoxin GVIA (1 μM) reduced the mAHP, resulting in a significant acceleration in pacemaking rate (n = 7, P < 0.05, SRT; Supplementary Fig. 2a). Blocking SK channels directly with the bee venom apamin (100 nM) or calcineurine (200 nM) had no effect on basal pacemaking rate (Kruskal-Wallis one-way ANOVA, degrees of freedom = 77, χ² = 0.86, P > 0.6; Fig. 5a), but did reduce the slow AHP (sAHP) evoked by a burst of spikes (median decrease = 6.9 mV, n = 7, P < 0.05, SRT; Fig. 5b), as reported previously<sup>29</sup>. Although they had little effect on the rate of regular spiking, L-type channels did contribute to intracellular calcium entry such as Fluo-4 (equation (5)). To estimate the endogenous incremental buffering capacity of the cell (κ<sub>e</sub>; Fig. 6c). The median κ<sub>e</sub> of DMV neurons was 49 (n = 8); this means that roughly 2 of every 100 calcium ions entering the cell are unbound. This κ<sub>e</sub> value is very low for neurons and close to that of other neurons that are prone to degeneration (for example, spinal motoneurons<sup>32</sup> and SNpc dopaminergic neurons<sup>15</sup>).

In contrast, the endogenous buffering capacity of ventral tegmental area (VTA) dopaminergic neurons (n = 6), which are known to express calbindin and to be relatively resistant to degeneration, was greater by a factor of 3 (P < 0.005, two-tailed Wilcoxon rank-sum test (RST) for independent samples; Fig. 6d).

Activity-dependent calcium entry oxidizes mitochondria
The combination of autonomous pacemaking, depolarized voltage trajectory, broad spikes, robust calcium entry and low calcium-buffering capacity could create a metabolic stress on DMV neurons similar to that found in SNpc dopaminergic neurons. To test this prediction, we generated transgenic mice expressing mito-roGFP under the control of the cytomegalovirus (CMV) promoter. Previous work with the mito-roGFP construct driven by the tyrosine hydroxylase promoter demonstrated the efficacy of the mitochondrial matrix targeting of this construct<sup>12</sup>. In these CMV–mito-roGFP mice, DMV neurons robustly expressed mito-roGFP and the construct had a subcellular distribution similar to that of the tyrosine hydroxylase–mito-roGFP mice<sup>12</sup> (Fig. 7a). This provided a means of measuring oxidation of mitochondrial matrix thiol proteins in pacemaking DMV neurons. Using 2PLSM approaches in brain slices, we measured the fluorescence of mito-roGFP; for every neuron, the fluorescence was calibrated at the end of the experiment with strong oxidizing and reducing agents to allow correction for variation in expression level of the probe or optics<sup>12</sup>. As hypothesized, the matrix of mitochondria in most DMV neurons

![Figure 7](image_url)

**Figure 7** Mitochondria in DMV neurons are normally oxidized and are reduced by pre-incubation in isradipine. (a) A confocal image of the soma and proximal dendrite of a DMV neuron from a CMV–mito-roGFP mouse. (b) The relative oxidation was estimated by measuring the resting fluorescence of the mitochondria relative to post-treatment with strong reducing (dithiothreitol, DTT) and oxidizing (aldrithiol) agents. Pre-incubation in 200 nM isradipine reduced the cells (green). (c) DMV neurons exhibited a broad range of relative oxidation. Pre-incubation in 200 nM isradipine or 1 μM TTX reduced the mitochondria matrix proteins, as did lowering the bath temperature to 22 °C. (d) Bath application of 0.5 mM TEA significantly and reversibly (data not shown) broadened the spike in every DMV neuron recorded. (e) Pre-incubation in 0.5 mM TEA significantly oxidized the mitochondria (red). (f) Autonomous discharge of a DMV neuron from CMV–mito-roGFP mice lacking the DJ-1 gene (DJ-1<sup>−/−</sup>). (g) The basal oxidation of mitochondrial matrix proteins was significantly higher in DJ-1<sup>−/−</sup> mice.

Pre-incubation in 200 nM isradipine significantly reduced the mitochondria of DMV cells in these animals. *P < 0.05, **P < 0.01, RST. The thick bar indicates the median, the box limits represent the 25th and 75th percentiles, and the whiskers indicate the range.
was relatively oxidized (Fig. 7b). Isradipine (200 nM) diminished mitochondrial oxidant stress \((n = 9, P < 0.005, \text{RST})\) without altering spiking or pacemaking frequency, pointing to the importance of calcium entry in creating it (Fig. 7b,c).

Because spike rate and spike duration were major determinants of cytosolic calcium levels in DMV neurons, we hypothesized that manipulating them would affect the oxidation of mitochondrial oxidant stress. This hypothesis was confirmed by experiment. First, antagonizing Nav1 channels with TTX, which eliminates spiking and calcium entry into DMV neurons, significantly diminished mitochondrial oxidant stress \((n = 10, P < 0.005, \text{SRT})\). Second, lowering the temperature of the bath significantly reduced the firing of DMV neurons \((32 °C, 2.39 \pm 0.37 \text{ spikes per s}, n = 58; 22 °C, 1.28 \pm 0.15 \text{ spikes per s}, n = 58; P < 0.05, \text{RST})\) and diminished mitochondrial oxidant stress \((n = 5, P < 0.05, \text{RST}; \text{Fig. 7c})\). Third, increasing spike duration by 50% \((n = 6, P < 0.05, \text{RST}; \text{Fig. 7d})\) with 0.5 mM tetraethylammonium (TEA, an antagonist of the large conductance voltage- and calcium-activated (BK) potassium channel)\(^3\) resulted in a significant increase in mitochondrial oxidant stress \((n = 19, P < 0.05, \text{RST}; \text{Fig. 7e})\). Although TEA broadened the spike, it did not change spike rate (data not shown). Thus, spike duration and rate, the primary determinants of calcium entry into DMV neurons, determine the level of mitochondrial oxidant stress in DMV neurons.

**Mitochondrial oxidant stress is exacerbated in DJ-1\(^{-/-}\) mice**

Mutations in DJ-1 are linked to an autosomal recessive early-onset form of Parkinson’s disease\(^3\). DJ-1 helps to orchestrate mitochondrial oxidant defenses\(^3\), making its loss potentially important to metabolically challenged neurons. For example, SNpc dopaminergic neurons lacking the DJ-1 gene have a higher basal level of mitochondrial oxidant stress\(^12\). Relieving the metabolic burden on these cells by antagonizing L-type channels normalizes mitochondrial oxidant stress levels.

To determine whether the metabolic burden on DMV neurons created a similar dependence on DJ-1, we crossed DJ-1\(^{-/-}\) mice with CMV–mito-roGFP mice. In DMV neurons from these mice, basal pacemaking was normal in rate (Fig. 7f). Spike width was also normal (data not shown). However, basal mitochondrial oxidant stress levels were elevated in DMV neurons \((n = 7, P < 0.05, \text{RST})\). Furthermore, as in SNpc dopaminergic neurons, this basal mitochondrial stress was ameliorated by pre-incubation with isradipine \((200 \text{ nM}, n = 9, P < 0.05, \text{RST}; \text{Fig. 7g})\).

**DISCUSSION**

Our findings indicate that DMV cholinergic neurons have a physiological phenotype that results in basal mitochondrial oxidant stress. The core elements of this physiological phenotype are slow autonomouslypacemaking, broad spikes, calcium entry through voltage-dependent ion channels and modest intrinsic calcium-buffering capacity. Antagonizing L-type calcium channels, one of the primary contributors to calcium entry during pacemaking, led to a substantial reduction in mitochondrial oxidant stress without altering ongoing activity of DMV cholinergic neurons. Antagonizing L-type channels also ameliorated the elevation in mitochondrial oxidant stress created by deletion of DJ-1, a gene associated with familial forms of Parkinson’s disease. The similarities between DMV cholinergic and SNpc dopaminergic neurons suggest that there is a common neuronal phenotype underlying neurodegenerative risk in Parkinson’s disease. Moreover, because L-type channel antagonists with good brain bioavailability are well tolerated by humans, our results suggest that their use in early stages of Parkinson’s disease could slow the evolution of both motor and non-motor symptoms.

The DMV neurons have features common to pacemakers

Pacemaking in DMV neurons relies on ionic mechanisms that are shared with other pacemaking neurons that have been characterized in depth. For example, in DMV neurons, TTX-sensitive, voltage-dependent sodium channels were critical to the generation of rhythmic membrane potential oscillations and pacemaking, as in many other neurons\(^22,23\). Voltage-dependent HCN and voltage-independent NALCN cation channels contributed to the inward currents driving pacemaking in DMV neurons, as in a number of other slow pacemaking neurons\(^23,38\).

Voltage-dependent, L-type calcium channels also contributed to the inward currents driving pacemaking in DMV cholinergic neurons. However, as in the situation in VTA dopaminergic neurons, these currents were substantially smaller than those of voltage-dependent sodium channels\(^23\). The channels underlying these subthreshold currents are highly likely to have a Cav1.3 pore-forming subunit\(^25,26\). The mRNA coding for this subunit, as well as the mRNA for the high threshold Cav1.2 subunit, were readily detected in single DMV neurons.

However, the calcium currents evoked by spikes were clearly larger than the subthreshold currents estimated with somatic point-clamp experiments. The broad spikes of DMV neurons enhanced calcium entry through both Cav1 channels and Cav2 channels, leading to the monotonic rise in intracellular calcium concentration with spike rate. Although the voltage-dependence of dihydropyridine binding prevented an accurate estimate of the contribution of L-type channels to the calcium influx during a single spike\(^3\), their contribution to basal intracellular calcium concentration during pacemaking could be estimated by silencing neurons in the presence and absence dihydropyridine; our results suggest that L-type channels are responsible for roughly half of the free calcium in the somatic cytoplasm during pacemaking.

Notably, antagonism of L-type channels generically did not have a substantial effect on the overall pacemaking rate. In part, this is attributable to compensatory ionic mechanisms. In SNpc dopaminergic neurons, where Cav1.3 channel currents are relatively larger, their antagonism does not substantially affect pacemaking either, largely because their antagonism also reduces the opening of SK channels, leading to little net change in transmembrane current\(^26\).

**Calcium entry promotes mitochondrial oxidant stress**

As in SNpc dopaminergic neurons\(^12\), mitochondrial matrix proteins in DMV neurons exhibited signs of oxidant stress during pacemaking. This measurement was made possible by expression of the reversible, ratiometric redox probe roGFP with a mitochondrial matrix targeting sequence\(^40\). The expression of the probe was driven by the cytomegalovirus (CMV) promoter, yielding widespread and robust neuronal expression in the brain. Although our experiments did not take advantage of the ratiometric capabilities of roGFP, the optical signal was calibrated at the end of every experiment by washing on strong oxidizing and reducing agents. This allowed us to eliminate the effects of expression level or optics and compare the relative redox status of neurons in different conditions.

The mitochondrial oxidant stress in DMV neurons was activity dependent. Spikes and the concomitant dissipation of ionic gradients increase ATP use in neurons, diminishing their respiratory reserve capacity\(^41\). Calcium entry poses an additional metabolic burden because of the steep electrochemical gradient against which it must be pumped by ATP-dependent mechanisms\(^9\). The combination of pacemaking, broad spikes and robust calcium channel expression appears to create a substantial metabolic challenge for DMV neurons.
That being said, VTA dopaminergic neurons showed no signs of mitochondrial oxidant stress, in spite of their similar pacemaking phenotype\textsuperscript{12}. What distinguishes DMV and VTA neurons is the expression of Ca\textsuperscript{2+}P. VTA neurons express high levels of the buffering protein calbindin, leading to estimates of intrinsic calcium-buffering capacity in excess of 150. In contrast, DMV cholinergic neurons had much lower intrinsic buffering capacity. Why might this be important? In the absence of fixed calcium buffering proteins, such as calbindin and parvalbumin, the burden of maintaining cytosolic calcium homeostasis falls more definitively on ATP-dependent pumps in the plasma membrane and intracellular organelles, such as the endoplasmic reticulum. The ability of calcium to diffuse away from the plasma membrane means that much of the calcium is likely to be pumped into intermediate organelle storage sites and then released back into the cytoplasm for eventual extrusion out of the cell\textsuperscript{30}. Although this allows calcium to perform signaling functions, it inescapably adds to its metabolic cost.

What is less clear is why this increases mitochondrial oxidant stress. Neurons depend on mitochondria and oxidative phosphorylation to meet their considerable metabolic needs\textsuperscript{41}. Neurons cannot survive on glycolysis alone. However, the relationship between oxidative phosphorylation and oxidant stress in the mitochondrion is incompletely understood. It also is not clear how neurons balance mitochondrial mass and regulation of respiratory rate in individual mitochondria to meet their metabolic demands. Calcium appears to be an important regulator of this bioenergetic decision making process, as it controls the Cav1.3 L-type channels need to be antagonized to achieve any antagonism is necessary to achieve protection. In toxin models of Parkinson's disease (where inflammation\textsuperscript{50}, begin to drive pathogenesis, diminishing the neuroprotective value of dihydropyridines. It could also be that, because neurons are compromised at this stage in the disease, a greater degree of L-type channel antagonism is necessary to achieve protection. In toxin models of Parkinson's disease (where inflammation is a factor), roughly half of the Cav1.3 L-type channels need to be antagonized to achieve any substantial level of protection in the SNpc\textsuperscript{16}; this level of antagonism is greater than that achievable with dihydropyridines because of their preference for Cav1.2 channels in the cardiovascular system, pointing to the need for Cav1.3 channel selective antagonists.

Implications for the treatment of Parkinson's disease

Impaired gastrointestinal motility is common in Parkinson's disease patients and this could be a result of diminished outflow from DMV neurons\textsuperscript{17,18}. This inference is consistent with the prominent LBN pathology and neuronal loss in the DMV of Parkinson's disease patients\textsuperscript{7}. As outlined above, the origins of this vulnerability have been unclear. An idea advanced early on was that vulnerable neurons have long unmyelinated axons that create a metabolic burden, ultimately resulting in bioenergetic deficits, proteostatic dysfunction and LBN pathology\textsuperscript{7,44}. Indeed, many of the neurons that are vulnerable in Parkinson's disease have long, richly branching axons (for example, SNpc, locus ceruleus and raphe neurons). Our results and the available physiological literature\textsuperscript{9} suggest that this anatomical trait is correlated with a physiological phenotype that adds to the metabolic burden these neurons have to carry. This metabolic hypothesis also links mitochondria to pathogenesis. As stated at the outset, many lines of evidence implicate mitochondrial dysfunction in Parkinson's disease\textsuperscript{4}. As neurons depend on mitochondria to meet their bioenergetic needs, it is not surprising that deficits in their ability to generate ATP could lead to neurodegeneration\textsuperscript{41}. What has been lacking is an explanation of why some neurons and not others should be susceptible to modest impairments in mitochondrial function. Our finding that the physiological phenotype of vulnerable neurons results in sustained mitochondrial oxidant stress fills this gap. Over decades, this oxidant stress could lead to mitochondrial decline, bioenergetic deficits, compromised ATP-dependent proteostatic function (resulting in LBN pathology) and, ultimately, death. Genetic mutations that further compromise mitochondria viability (for example, Dj-1, Pink1 mutations) or add to the proteostatic workload (for example, alpha synuclein overexpression) accelerate this decline.

From the clinical standpoint, the question is how can this process be slowed or stopped without compromising neuronal function? Certainly, reducing the axonal projections of vulnerable neurons is not an option. Attempts to directly target mitochondrial function have not been successful to date\textsuperscript{49}. Our results point to another therapeutic path. Diminishing activity-dependent calcium entry through L-type channels with dihydropyridines reduced signs of mitochondrial oxidant stress in DMV cholinergic neurons without altering their autonomously generated activity. These compounds have been used safely in humans for decades to treat hypertension and their use is associated with a reduced risk of developing Parkinson's disease\textsuperscript{46–48}. Our results suggest that this reduction in risk is attributable to not just an effect on SNpc neurons\textsuperscript{12}, but to an effect on DMV neurons as well, raising the possibility that a single therapeutic agent might delay the onset of both motor and non-motor symptoms in Parkinson's disease. Whether antagonism of L-type channels can slow the progression of the disease once patients have become symptomatic is uncertain. Recent epidemiological work failed to find a substantial effect of dihydropyridine use on time to reach late stage milestones in Parkinson's disease patients\textsuperscript{49}. It could be that at this stage other factors, such as inflammation\textsuperscript{50}, begin to drive pathogenesis, diminishing the neuroprotective value of dihydropyridines. It could also be that, because neurons are compromised at this stage in the disease, a greater degree of L-type channel antagonism is necessary to achieve protection. In toxin models of Parkinson's disease (where inflammation is a factor), roughly half of the Cav1.3 L-type channels need to be antagonized to achieve any substantial level of protection in the SNpc\textsuperscript{16}; this level of antagonism is greater than that achievable with dihydropyridines because of their preference for Cav1.2 channels in the cardiovascular system, pointing to the need for Cav1.3 channel selective antagonists.

METHODS

Methods and any associated references are available in the online version of the paper.

Note: Supplementary information is available in the online version of the paper.

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AUTHOR CONTRIBUTIONS

J.A.G. designed and conducted the experiments and analyzed the data. J.N.G. conducted some of the control roGFP experiments. C.M.E. and E.I. conducted the histological experiments. J.K. and J.S.-P. generated the CMV–mito-roGFP and Dj-1\textsuperscript{1420}mice. D.J.S. was responsible for the overall direction of the experiments. J.A.G. and D.J.S. prepared the manuscript and illustrations.

COMPETING FINANCIAL INTERESTS

The authors declare no competing financial interests.

1. de Lau, L.M. & Breteler, M.M. Epidemiology of Parkinson's disease. Lancet Neurol. 5, 525–535 (2006).
2. Biskup, S. & Moore, D.J. Detrimental deletions: mitochondria, aging and Parkinson's disease. Bioessays 28, 963–967 (2006).
3. Lees, A.J., Hardy, J. & Revész, T. Parkinson's disease. Lancet 373, 2055–2066 (2009).
4. Schapira, A.H. Mitochondria in the aetiology and pathogenesis of Parkinson's disease. Lancet Neurol. 7, 97–109 (2008).
5. Albin, R.L., Young, A.B. & Penney, J.B. The functional anatomy of disorders of the basal ganglia. Trends Neurosci. 18, 63–64 (1995).
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6. Jellinger, K.A. A critical reappraisal of current staging of Lewy-related pathology in human brain. Acta Neuropathol. 116, 1–16 (2008).
7. Braak, H., Ghebremedhin, E., Rub, U., Brat, D.O. & Del Tredici, K. Stages in the development of Parkinson's disease-related pathology. Cell Tissue Res. 318, 121–134 (2004).
8. Dickson, D.W. et al. Evidence that incidental Lewy body disease is pre-symptomatic Parkinson's disease. Acta Neuropathol. 115, 437–444 (2008).
9. Surmeier, D.J., Guzman, J.N., Sanchez-Padilla, J. & Goldberg, J.A. The origins of oxidant stress in Parkinson's disease and therapeutic strategies. Antioxid. Redox Signal. 14, 1289–1301 (2011).
10. Sulzer, D. Multiple hit hypotheses for dopamine neuron loss in Parkinson's disease. Trends Neurosci. 30, 240–250 (2007).
11. Grace, A.A. & Bunney, B.S. The control of firing pattern in nigral dopamine neurons: single spike firing. J. Neurosci. 4, 2866–2876 (1984).
12. Guzman, J.N. et al. Oxidant stress evoked by pacemaking in dopaminergic neurones is attenuated by DJ-1. Nature 468, 696–700 (2010).
13. Nedergaard, S., Flatman, J.A. & Engberg, I. Nifedipine- and omega-conotoxin-sensitive Ca2+ conductances in guinea-pig substantia nigra pars compacta neurones. J. Physiol. (Lond.) 466, 727–747 (1993).
14. Puopolo, M., Raviola, E. & Bean, B.P. Roles of subthreshold calcium current and sodium current in spontaneous firing of mouse midbrain dopamine neurones. J. Neurosci. 27, 645–656 (2007).
15. Foehring, R.C., Zhang, X.F., Lee, J.C. & Callaway, J.C. Endogenous calcium buffering capacity of substantia nigral dopamine neurones. J. Neurophysiol. 102, 2326–2333 (2009).
16. Hijic, E., Guzman, J.N. & Surmeier, D.J. The L-type channel antagonist isradipine is neuroprotective in a mouse model of Parkinson's disease. Neurobiol. Dis. 43, 364–371 (2011).
17. Natale, G., Pasquali, L., Ruggieri, S., Paparelli, A. & Fornai, F. Parkinson's disease and the gut: a well known clinical association in need of an effective cure and explanation. Neurogastroenterol. Motil. 20, 741–749 (2008).
18. Miller, V.M. et al. Dorsal motor nucleus of vagus protein aggregates in Lewy body disease with autonomic dysfunction. Brain Res. 1286, 165–173 (2009).
19. Gao, H. et al. Morphological and electrophysiological features of motor neurons and putative interneurons in the dorsal vagal complex of rats and mice. Brain Res. 1291, 40–52 (2009).
20. Mo, Z.L., Katafuchi, T., Muratani, H. & Horii, T. Effects of vasopressin and angiotensin II on neurones in the rat dorsal motor nucleus of the vagus in vitro. J. Physiol. (Lond.) 458, 561–577 (1992).
21. Tawagl, R.A. & Gillis, R.A. Hyperpolarization-activated currents, IH and IK1, in rat dorsal motor nucleus of the vagus neurones in vitro. J. Neurophysiol. 71, 1308–1317 (1994).
22. Bean, B.P. The action potential in mammalian central neurons. Annu. Rev. Physiol. 66, 727–747 (1993).
23. Klahi, Z.M. & Bean, B.P. Pacemaking in dopaminergic ventral tegmental area neurones: depolarizing drive from background and voltage-dependent sodium conductances. J. Neurosci. 30, 7401–7413 (2010).
24. Lu, B. et al. The neuronal channel NALCN contributes resisting sodium permeability and is required for normal respiratory rhythm. Cell 129, 371–383 (2007).
25. Putzier, I., Kulmann, P.H., Horn, J.P. & Levitan, E.S. Cav1.3 channel voltage dependence, not Ca2+ selectivity, drives pacemaker activity and amplifies bursts in nigral dopamine neurones. J. Neurosci. 29, 15414–15419 (2009).
26. Guzman, J.N., Sanchez-Padilla, J., Chan, C.S. & Surmeier, D.J. Robust pacemaking in substantia nigra dopaminergic neurones. J. Neurosci. 29, 11011–11019 (2009).
27. Koschak, A. et al. alpha 1D (Cav1.3) subunits can form L-type Ca2+ channels activating at negative voltages. J. Biol. Chem. 276, 22100–22106 (2001).
28. Hocherman, S.D., Werman, R. & Yarom, Y. An analysis of the long-lasting after-hyperpolarization of guinea-pig vagal motoneurones. J. Physiol. (Lond.) 456, 325–349 (1992).
29. Sah, P. & McLachlan, E.M. Ca2+-activated K+ currents underlying the afterhyperpolarization in guinea pig vagal neurones: a role for Ca2+-activated Ca2+ release. Neuron 7, 257–264 (1991).
30. von Lewinski, F. & Keller, B.U. Ca2+, mitochondria and selective motoneuron vulnerability: implications for ALS. Trends Neurosci. 28, 494–500 (2005).
31. de León, M., Covenas, R., Narvaez, J.A., Aguirre, J.A. & Gonzalez-Baron, S. Distribution of parvalbumin immunoactivity in the cat brain stem. Brain Res. Bull. 23, 639–646 (1993).
32. Marwali, M., Mainen, Z.F., Sabatini, B.L. & Svoboda, K. Estimating intracellular calcium concentrations and buffering without wavelength ratioing. Biophys. J. 78, 2655–2667 (2000).
33. Neher, E. & Augustine, G.J. Calcium gradients and buffers in bovine chromaffin cells. J. Physiol. (Lond.) 450, 273–301 (1992).
34. Helmchen, F., Imoto, K. & Sakmann, B. Ca2+ buffering and action potential-evoked Ca2+ signaling in dendrites of pyramidal neurones. Biophys. J. 70, 1069–1081 (1996).
35. Lips, M.B. & Keller, B.U. Activity-related calcium dynamics in motoneurones of the nucleus hypoglossus from mouse. J. Neurophysiol. 82, 2936–2946 (1999).
36. Lang, D.G. & Ritchie, A.K. Tetraethylammonium blockade of apamin-sensitive and insensitive Ca2+-activated K+ channels in a pituitary cell line. J. Physiol. (Lond.) 425, 117–132 (1990).
37. Kahle, P.J., Waak, J. & Gasser, T. DJ-1 and prevention of oxidative stress in Parkinson's disease and other age-related disorders. Free Radic. Biol. Med. 47, 1354–1361 (2009).
38. Mercuri, N.B., Bonci, A., Calabresi, P., Stefani, A. & Bernardi, G. Properties of the hyperpolarization-activated cation current i_h in rat midbrain dopaminergic neurones. Eur. J. Neurosci. 7, 462–469 (1995).
39. Helton, T.D., Xu, W. & Lipscombe, D. Neuronal L-type calcium channels open quickly and are inhibited slowly. J. Neurosci. 25, 10247–10251 (2005).
40. Dooley, C.T. et al. Imaging dynamic redox changes in mammalian cells with green fluorescent protein indicators. J. Biol. Chem. 279, 22284–22293 (2004).
41. Nicholls, D.G. & Budd, S.L. Mitochondria and neuronal survival. Physiol. Rev. 80, 315–360 (2000).
42. Yi, M., Weaver, D. & Hajnoczky, G. Control of mitochondrial motility and distribution by the calcium signal: a homeostatic circuit. J. Cell Biol. 167, 661–672 (2004).
43. Brown, G.C. Control of respiration and ATP synthesis in mammalian mitochondria and cells. Biochem. J. 284, 1–13 (1992).
44. Bohnen, N.J. & Albin, R.L. The cholinergic system and Parkinson disease. Behav. Brain Res. 221, 564–573 (2011).
45. Meissner, W.G. et al. Priorities in Parkinson's disease research. Nat. Rev. Drug Discov. 10, 377–393 (2011).
46. Becker, C., Jick, S.S. & Meier, C.R. Use of antihypertensives and the risk of Parkinson disease. Neurology 70, 1438–1444 (2008).
47. Ritz, B. et al. L-type calcium channel blockers and Parkinson disease in Denmark. Ann. Neurol. 67, 600–606 (2010).
48. Pasternak, B. et al. Use of calcium channel blockers and Parkinson's disease. Am. J. Epidemiol. 175, 627–635 (2012).
49. Marras, C. et al. Dihydropyridine calcium channel blockers and the progression of parkinsonism. Ann. Neurol. 71, 362–369 (2012).
50. Hirsch, E.C. & Hunot, S. Neuroinflammation in Parkinson's disease: a target for neuroprotection? Lancet Neurol. 8, 382–397 (2009).
ONLINE METHODS

Slice preparation. Experimental procedures adhered to the Northwestern University Animal Care and Use Committee. We deeply anesthetized 3–7-week-old choline acetyltransferase–enhanced green fluorescent protein (ChAT-eGFP) BAC transgenic mice, transgenic mice with mitochondrial matrix–targeted redox-sensitive GFP (Mito-MitoGFP) driven by a CMV promoter (CMV–mito-roGFP mice)12 or the latter mice crossed with DsRed m1, m2, both of sexes, with ketamine–xylazine and transcardially perfused with iced-modified ACSF, bubbled with 95% O2/5% CO2 containing 2.5 mM KCl, 26 mM NaHCO3, 1.25 mM Na3HPO4, 0.5 mM CaCl2, 10 mM MgSO4, 0.4 mM ascorbic acid, 10 mM glucose and 210 mM sucrose. The cerebellum, pons and medulla were rapidly removed, blocked in the coronal plane and sectioned at a thickness of 240 µm in ice-cold modified ACSF. Slices were then submerged in ACSF, bubbled with 95% O2/5% CO2 containing 2.5 mM KCl, 126 mM NaCl, 26 mM NaHCO3, 1.25 mM Na3HPO4, 2 mM CaCl2, 2 mM MgSO4, and 10 mM glucose, and stored at 20–22 °C for at least 1 h before recording and/or imaging. VTA was blocked and sectioned as described previously12.

Slice visualization, electrophysiology and histology. The slices were transferred to the recording chamber mounted on an Olympus BX51 upright, fixed-stage microscope and perfused with oxygenated ACSF at 32 °C. A 60×, 0.9 NA water–immersion objective was used to examine the slice using standard infrared differential interference contrast video microscopy. For whole-cell current cur-
rent recordings, we used HEPES-based solutions14. The first solution contained 137 mM NaCl, 1.8 mM CaCl2, 1 mM MgCl2, 5.4 mM TEA-Cl, 10 mM 4-AP, 0.001 mM TTX, 5 mM HEPES and 10 mM glucose (pH = 7.3 with NaOH), and the second solution was identical except for an equimolar substitution of CaCl2 with CoCl2 (pH = 7.3 with NaOH). For recording of voltage-insensitive Na+ currents, we used solutions23: the first contained 151 mM NaCl, 2 mM CaCl2, 2.5 mM KCl, 5 mM MgCl2, 0.001 mM TTX, 5 mM HEPES and 10 mM glucose (pH = 7.3 with NaOH), and the second was identical except for an equimolar substitution of NaCl with NMDG (pH = 7.3 with HCl). Patch pipette resistance was typically 3–4.5 MΩ when filled with recording solution. For whole-cell current clamp recordings the pipette contained 135.5 mM potassium methylsulfate, 5 mM KCl, 2.5 mM NaCl, 5 mM sodium phosphocreatine, 10 mM HEPES, 0.2 mM EGTA, 0.2 mM Na2GTP, and 2 mM Mg2+(ATP)3 (pH = 7.3 with KOH, 280–290 mM osm kg−1). In some experiments, we added biocytin (5%, wt/vol) to this internal solution. At the end of these experiments, slices were fixed by immersion into 4% paraformaldehyde (wt/vol) in 0.15 M phosphate buffer, refrigerated for a period of 2 d, reacted with streptavidin–Alexa Fluor 594 and plated for laser-scanning confocal imaging on the Olympus Fluoview FV1000 system.

For perforated patch recordings, we front-filled the pipettes with the K+-based internal solution and back-filled with the same solution that was sonicated after addition of 1.5 µg ml−1 gramicidin B. For whole-cell voltage–clamp recordings of calcium currents, the pipette contained 111 mM cesium methylsulfate, 12.5 mM CsCl, 1 mM MgCl2, 0.1 mM CaCl2, 10 mM HEPES, 1 mM EGTA, 0.21 mM Na2GTP, and 2 mM Mg2+(ATP)3 (pH = 7.3 with CsOH, 280–290 mM osm kg−1). For calcium imaging experiments, the pipette contained 135 mM K2SO4, 5 mM KCl, 5 mM Na3HPO4, 5 mM Tris-phosphocreatine, 10 mM HEPES, 0.1 mM Fluo-4, 0.05 mM Alexa Fluor 568, 0.21 mM Na2GTP, and 2 mM Mg2+(ATP)3 (pH = 7.3 with KOH, 280–290 mM osm kg−1).

Electrophysiological recordings were obtained with a Multiclamp 700B amplifier (Molecular Devices). Junction potential, which was 7–8 mV, was not corrected. Signals were digitized at 20–100 kHz and logged onto a personal computer with the Clampex 9.2 software (Molecular Devices) or, in the imaging experiments, using the custom-written shareware package WinFluor (J. Dempster, Strathclyde University), which automates and synchronizes the 2PLSM imaging and electrophysiological protocols.

2PLSM imaging. The 2PLSM system was described previously12. Briefly, the two-photon excitation source was a Chameleon Ultra 2 tunable laser system (706–1,000 nm). The 2PLSM fluorescence signal was detected with non-descanned photomultiplier tubes (Prairie Technologies). A Dott contrast detector system was used to provide a bright-field transmission image in registration with the fluorescent images. For the calcium-buffering experiments, regions of interest were chosen to cover a small rectangular patch of soma, and 20–50 Hz scans were conducted, using 0.183-µm pixels and 12-µs dwell time.

Optical imaging of roGFP signals was performed by using a 920-nm excitation beam. The two-photon excitation source was a Chameleon Ultra 2 tunable laser system (706–1,000 nm). The 2PLSM fluorescence signal (490–560 nm) was detected by a photomultiplier tube. We collected 60 frames of the roGFP signal in one optical plane at a rate of 3–5 frames per s to determine the baseline fluorescence (Fo) of the cell. At the end of all experiments, 2 mM DTt was applied for 10 min to reduce the mitochondria fully so as to measure the maximal fluorescence (Fmax), followed by 100 µM Ald for 0.75 to 1.5 h to oxidize the mitochondria fully, so as to determine the minimal fluorescence (Fmin). The relative oxidation was then calculated as (Fmax − F)/Fmax. Drugs and reagents. For recording of autonomous discharge, we used a cocktail of synaptic receptor blockers that included 50 µM m(-)-(2-amino-5- phosphonovaleric acid, 5 µM 6-nitro-2,3-dioxo-1,4-dihydropyridine[8]quinoline-7-sulfonamide, 10 µM SR 95531, 1 µM CGP 58845, 10 µM mecamylamine, 10 µM ketanserin and, in many experiments, 10 µM phenolamine. Voltage-sensitive Na+ channels were blocked with 1 µM TTX. Cav2.2 channels were blocked with 1 µM ω-conotoxin GIVA. Cav1 channels were antagonized acutely with 5 µM of either nifedipine or isradipine, or pre-incubated in 200 nM isradipine. Alternatively, 200 nM of calcineurin was used for this purpose (either acutely or with pre-incubation). Small-conductance calcium-activated (SK) K+ channels were blocked with 100 nM amapid. Large-conductance voltage- and calcium-activated (BK) K+ channels were blocked with 0.5 mM TEA. HCN channels were blocked with 20 µM ZD 7288. The acute effects of solution exchanges or drug applications were measured at least 5 min after wash on, except for dihydropyridine, where 10 min were given. Fluo-4 pentapotassium and Alexa Fluor 568/594 hydrazide Na+ salts were obtained from Invitrogen. TTX, ω-conotoxin and calcineurin were obtained from Alomone Labs. The rest of the drugs and reagents were obtained from Tocris or Sigma.

Single-cell RT-PCR. In some experiments, the contents of DMV neurons in the slice were aspirated into an electrode. The electrode tip was broken in a 0.6-m1 presiliconized tube (Midwest Scientific) containing 1.9 µl of DEPC-treated water, 0.7 µl of RNaseOUT (20 U µl−1), 0.7 µl of oligo-dT (50 µM), 1.0 µl random hexamers (50 ng µl−1), 0.7 µl of BSA (143 µg µl−1), 1.0 µl of dNTPs (10 mM), and the contents were ejected into the presiliconized tube, and frozen at –80 °C for reverse transcription. The single-cell cDNA generated from the reverse transcription step was subjected to conventional PCR. Primers for Cav1.2, Cav1.3 and ChAT were designed in-house. Primer Ndkt mRNA (GenBank, NM177393) was designed with a pair of primers, 5′-GGTGCACTCCTCCTGTCGCCGA (position 479) and 5′-GCGACATGCCCCACAGGTGA (position 796). The predicted PCR product length was 317 bp.

Estimation of endogenous buffering capacity. To estimate the endogenous buffering capacity Kc, we use the added buffer method. In this method, a competition is created between endogenous buffers and the added Fluo-4 dye, whose buffering capacity is denoted Kc, in binding to the free calcium as the dye diffuses into the cellular compartment, which in our case is the soma34. Because the time course of this diffusion (measured in minutes) is much longer than the timescale of dissociation (measured in milliseconds), the concentration of bound exogenous buffer, [CaB], can be considered to be at equilibrium with [Ca], the concentration of free calcium, while the cell fills. This assumption is particularly true when the cells is quiescent and hence truly at steady state. In this case, the law of mass action yields

\[
\frac{d [CaB]}{dt} = \frac{\partial [CaB]}{\partial [Ca]} \frac{d [Ca]}{dt} + \frac{[Ca]}{K_c} \frac{d [B]}{dt}
\]

where [B] and Kc are the concentration and the dissociation constant of the exogenous buffer Fluo-4, respectively. Differentiation of this equation with respect to time yields

\[
\frac{d}{dt} [CaB] = \frac{\partial [CaB]}{\partial [Ca]} \frac{d [Ca]}{dt} + \frac{[Ca]}{K_c} + [Ca] \frac{d [B]}{dt}
\]
The pre-factor of the first term on the right side is the definition of the binding capacity of the dye and is denoted as

\[ \kappa_B = \frac{\partial [CaB]}{\partial [Ca]} = \frac{[B]K_D}{K_D + [Ca]} \]

and measures the number of calcium ions bound to the buffer per each free ion. The time course of the rise in \([B]\) in the soma is quite generally exponential shaped because the driving force of the diffusion is the difference between the concentration of the dye in the pipette \([B]_1\) and \([B]\) so that \([B]\) is expected to behave more or less like

\[ [B] = [B]_1 \left(1 - e^{-\tau/\tau} \right) \]

with some decay time constant \(\tau\) (which in our experiment was on the order of a minute or so)\(^{33}\). Thus, equation (1) can be rewritten as

\[ \frac{d}{dt} [CaB] = \kappa_B \frac{d}{dt} [Ca] + \frac{[Ca][B]_1}{K_D + [Ca]} \frac{1}{\tau} e^{-t/\tau} \]  

(2)

In the added buffer method, we take advantage of the first term in the right side of equation (2) and generate well-controlled perturbation in \([Ca]\), namely by silencing the cell with hyperpolarizing currents and evoking well-spaced single spikes in the cell that are assumed to introduce a reproducible amount of calcium ions [in moles], denoted \(u\) \(\Delta [Ca]_{AP}\) into the soma, whose volume is denoted \(V\). We can therefore write\(^{33,34}\)

\[ (1 + \kappa_S + \kappa_B) \frac{d}{dt} [Ca] = \frac{u}{V} \Delta [Ca]_{AP}^2 + \sum_{\text{AP}} \delta(t - t_{\text{AP}}) + \text{other terms} \]  

(3)

where \(\delta(t - t_{\text{AP}})\) is a Dirac delta function representing an impulse when the spikes occur. Substituting equation (3) in (1) and solving equation (1) reveals that \([CaB]\) will undergo a stepwise increase when spikes occur superimposed on the slow dynamics described by the first term on the right side of equation (2). Namely

\[ [CaB] = \frac{u}{V} \Delta [Ca]_{AP}^2 + \sum_{\text{AP}} \frac{\kappa_B(t_{\text{AP}})}{1 + \kappa_S + \kappa_B(t_{\text{AP}})} H(t - t_{\text{AP}}) + \text{other terms} \]  

(4)

where \(\kappa_B\) is the incremental binding ratio, defined below in equation (6), and \(H(t - t_{\text{AP}})\) is the Heaviside step function added with each spike.

Because Fluo-4 has a very high dynamic range (that is, \(R = F_{\text{max}}/F_{\text{min}}\) is on the order of 100)\(^{32}\), the brightness of the Fluo-4 dye \(F\) can be regarded as proportional to the concentration of the bound dye, that is, \(F = S_{\text{calc}}[CaB]\). Thus, equation (4) implies that step-like increases in the raw value of \(F\) should be observed with each spike. Notably, because \([B]\) rises slowly as the soma fills, the sequence of spikes will produce montonically increasing values \(\kappa_B(t_{\text{AP}})\). If we denote the resting concentration of free calcium as \([Ca]_0\), and the fluorescence corresponding to it as \(F_0\), then the amplitude of the steps in the raw value of \(F\) induced by each spike \(\Delta F_{\text{AP}} = F_{\text{AP}} - F_0\) that corresponds to an increment in free calcium denoted \(\Delta [Ca]_{AP}\) should also increase montonically, like a ‘binding isotherm’ and generate the following relationship between \(\Delta F_{\text{AP}}\) and \(\kappa_B\)

\[ \Delta F_{\text{AP}} = \Delta F_{\text{AP}}^{\text{max}} \left( \frac{\kappa_B^*}{1 + \kappa_S + \kappa_B^*} \right) \]  

(5)

Equation (5) can be used to extract \(\kappa_B\) (equation (25) in ref. 33). We estimated \(\kappa_B^*\) by substituting the expressions they derived for \([Ca]_0\) and \(\Delta [Ca]_{AP}\) into the definition of the incremental binding ratio of the buffer\(^{32}\) to yield

\[ \kappa_B^* = \frac{[B]K_D}{(K_D + [Ca]_{0})(K_D + [Ca]_{0} + \Delta [Ca]_{AP})} \left(1 + \frac{df_{\text{AP}}}{df_{\text{max}}^*} \right) \]  

(6)

where \(df_{\text{AP}} = \Delta F_{\text{AP}}^{\text{max}}/F_0\).

\(F_0\) is the value of fluorescence when the cells are quiescent\(^{34}\) (in the case of pacemaking neurons such as the DMV or VTA neurons, this required a constant hyperpolarizing currents). \(df_{\text{max}}\) is defined as \((F_{\text{max}} - F_0)/F_0\) and \(F_{\text{max}}\) is measured as explained below: \([B]\) is estimated at the time \(F_0\) is measured from the baseline fluorescence normalized so that it approaches a steady state of the pipette concentration of Fluo-4 (that is, 100 or 200 \(\mu\)M). The \(K_D\) of Fluo-4 was taken to be 345 nM. Because all buffering capacity estimates are uniformly scaled by this value, it may introduce a systematic error, but will not affect comparisons among different populations\(^{32}\). Notably, in deriving equation (6), the 1/R term that appears in ref. 32 is neglected because of the very high dynamic range of Fluo-4.

The experiment was conducted as follows. A cell was patched and the 2PLSM imaging of a small rectangular patch of the soma was begun. The seal was ruptured and a hyperpolarizing current was then injected to silence the cell. On the background of this hyperpolarizing current, a series of 2-nA, 2-ms pulses were injected every 10 s for 2.5–3 min to generate nominally identical and instantaneous calcium increments. The measurements of the resulting increments in raw fluorescence \(\Delta F_{\text{AP}}\) in conjunction with the estimated \([B]\) and \(df_{\text{max}}\) enabled the estimation of \(\kappa_B^*\) (equation (6)). Using the fact that the a double reciprocal plot of equation (5) yields a linear relation, we generated a scatter plot of \((1/\kappa_B^*)/\Delta F_{\text{AP}}\) to estimate \(\kappa_B\) (which can be extracted from fitting a linear regression to the scatter plot)\(^{33}\). At the end of the experiments, \(df_{\text{max}}\) was estimated as follows: a sequence of 7-s-long current injections into the soma of increasing intensity, in 100-pA increments, were used to saturate the brightness of the fluorescence and the dye\(^{34}\).

**Data and statistical analysis.** Data were analyzed and curve fitting was done using custom–made code on custom–made (Winfluor; NU Pver, N. Schwarz, Northwestern University; Oscilloscope, C. Wilson, University of Texas at San Antonio) and commercial (Mathematica 8, Wolfram Research; Matlab 7, Mathworks) software. Spike threshold was identified as the maximal \(dF/dt\) of the voltage trajectory, and the width of the spike as measured as the time from threshold crossing to repolarization back to threshold. Nonparametric statistical tests for changes in medians were used. For independent samples, the two-tailed Wilcoxon RST and Kruskal Wallis one-way ANOVA were used, and, for matched pairs, the two-tailed Wilcoxon SRT was used. Null hypotheses were rejected if the \(p\) value was below 0.05.