Mitochondrial oxidant stress in locus coeruleus is regulated by activity and nitric oxide synthase

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Loss of noradrenergic locus coeruleus (LC) neurons is a prominent feature of aging-related neurodegenerative diseases, such as Parkinson’s disease (PD). The basis of this vulnerability is not understood. To explore possible physiological determinants, we studied LC neurons using electrophysiological and optical approaches in ex vivo mouse brain slices. We found that autonomous activity in LC neurons was accompanied by oscillations in dendritic Ca²⁺ concentration that were attributable to the opening of L-type Ca²⁺ channels. This oscillation elevated mitochondrial oxidant stress and was attenuated by inhibition of nitric oxide synthase. The relationship between activity and stress was malleable, as arousal and carbon dioxide increased the spike rate but differentially affected mitochondrial oxidant stress. Oxidant stress was also increased in an animal model of PD. Thus, our results point to activity-dependent Ca²⁺ entry and a resulting mitochondrial oxidant stress as factors contributing to the vulnerability of LC neurons.

Noradrenergic (NA) LC neurons innervate much of the brain, serving to maintain wakefulness and to modulate neural activity and plasticity during periods of arousal and stress1,2. Despite their importance, this small group of brainstem neurons is vulnerable to aging and aging-related neurodegenerative diseases, such as PD and Alzheimer’s disease. The loss of LC neurons might contribute to many of the non-motor symptoms that accompany these diseases, including memory deficits, depression and daytime hypersomnolence3. Why LC neurons are at risk is not clear. One theory of the aging-related decline in neural function is that it reflects a bioenergetic insufficiency stemming from mitochondrial dysfunction4,5. Neurons require mitochondrial oxidative phosphorylation to meet their bioenergetic needs. In regions such as the substantia nigra pars compacta (SNc), where there is a clear aging-related decline of neuronal number and function, there are substantially more mitochondrial DNA deletions of the type produced by oxidant stress than in unaffected brain regions6. Thus, declining mitochondrial function is likely to be a consequence of cumulative oxidant stress5. Recessive genetic mutations that increase the risk of PD boost this oxidant stress and create deficits in mitochondrial quality control that could amplify the long-term consequences of oxidant damage7.

Mitochondrial oxidant stress can arise from either extrinsic or intrinsic sources. Extrinsic oxidant stress can arise when non-mitochondrial processes, such as lysosomal degradation of proteins, generate reactive oxygen species (ROS) that enter mitochondria8,9. Intrinsic oxidant stress can arise when ROS are generated by electron leakage from the electron transport chain (ETC)7. This oxidant stress can be amplified by genetic or pharmacological perturbations that alter the balance between ROS generation and clearance. In SNc dopaminergic neurons, whose loss is responsible for the cardinal motor symptoms of PD10, intrinsic mitochondrial oxidant stress has been traced to Ca²⁺ entry through L-type channels during autonomous pacemaking11. Ca²⁺ entering through these channels is weakly buffered by cytosolic proteins, allowing it to be taken up by the endoplasmic reticulum (ER) and then passed to mitochondria12. Mitochondrial Ca²⁺ entry de-represses enzymes of the tricarboxylic acid cycle, increasing the production of reducing equivalents for the ETC and respiration13. However, the precise mechanism by which mitochondrial Ca²⁺ augments oxidant generation has not been fully established.

Our results draw strong parallels between the physiological determinants of vulnerability in SNc and LC neurons, showing that activity-dependent opening of L-type Ca²⁺ channels leads to mitochondrial oxidant stress. As in SNc dopaminergic neurons, this stress was exacerbated by deletion of DJ-1 (also known as PARK7), a gene linked to an early onset form of PD, and alleviated by antagonism of L-type Ca²⁺ channels. Our work on LC neurons makes four additional points of importance. First, L-type channels with a Cav1.3 pore-forming subunit make a substantial contribution to Ca²⁺ oscillations. Second, the endoplasmic reticulum is important for the genesis of mitochondrial oxidant stress. Third, the relationship between activity and oxidant stress is not fixed, but is subject to modulation. Fourth, activity-dependent oxidant stress depends on a mitochondrial form of nitric oxide synthase (NOS).

RESULTS

L-type channels are engaged during neuronal spiking in LC
Initially, we mapped the LC region using immunocytochemical techniques in ex vivo brain slices so that we reliably sampled NA...
neurons (Fig. 1a and Supplementary Fig. 1). In addition, neurons were filled with biocytin and subsequently reconstructed to verify their identity (Fig. 1a). As previously reported,14 LC NA neurons in ex vivo brain slices at physiological temperatures were spontaneously active, spiking at 1–6 spikes per s (Fig. 1b). Their spiking rate did not change with the addition of glutamatergic and GABAergic synaptic blockers, suggesting that LC neurons were autonomous pacemakers.14 Another signature feature of LC neurons described previously15–18 was the presence of small spikelets (20–30-mV amplitude) following blockade of Nav1 channels with tetrodotoxin (TTX; Fig. 1b). The spikes of LC neurons were broad, being 2.5 ms in duration at half amplitude (Fig. 1c).

Previous studies using somatic recording have implicated engagement of Cav1 L-type channels during autonomous spiking in LC neurons.14,16,17 To pursue this suggestion, we loaded LC neurons with a low concentration of Ca2+-sensitive dye (Fluo-4, 50 M) and used two-photon laser-scanning microscopy (2PLSM) to monitor changes in intracellular Ca2+ concentration at dendritic locations. Autonomous spiking was accompanied by phase-locked fluctuations in dendritic Ca2+ concentration (Fig. 2a). Moreover, consistent with previous studies, these dendritic Ca2+ oscillations were still present following blockade of Nav1 channels with TTX and were eliminated by bath application of the Cav1 channel antagonist isradipine (1 M; Fig. 2a).15,16

To determine the molecular identity of the channels expressed by LC neurons, we subjected the neurons to quantitative PCR (qPCR) analysis. LC neurons expressed mRNA for both the Cav1.3 pore-forming subunit of the L-type channel and the Cav1.2 subunit (Fig. 2b). We verified functional expression of Cav1 channels by somatic voltage-clamp measurements (Supplementary Fig. 2). Application of the Cav1.3-selective antagonist BPN4689 (50 M) attenuated Ca2+ spikes and intracellular Ca2+ oscillations in the presence of TTX, and the residual oscillatory activity was eliminated by the nonselective L-type channel antagonist isradipine (Fig. 2c).

![Figure 1](https://example.com/figure1.png)

**Figure 1** LC neurons were autonomous pacemakers with broad action potential spikes. (a) Biocytin-labeled neuron (red, stained with streptavidin-conjugated to alexa594) colocalized with neurons immunoreactive for the LC biomarker tyrosine hydroxylase (TH). Right, a representative reconstruction of a biocytin-filled LC neuron. (b) Representative LC recording displaying autonomous spiking activity, recordings performed in the presence of glutamate and GABA receptor synaptic blockers. Bottom, a representative trace of LC recording with spikelet activity following blockade of Nav1 channels with TTX (1 M). Under our recording conditions, more than 90% of the LC neurons recorded displayed spikelet activity (n = 20 neurons, from 10 mice). (c) Representative traces showing spike width in LC neurons (n = 5 neurons, from 3 mice, median = 2.4 s) compared with globus pallidus (GP) neurons (n = 6 neurons, from 3 mice, median = 0.28 s). Right, box-plot quantification with spike widths in LC neurons significantly wider than GP neurons (P = 0.004). Data are presented as whisker box plots displaying median, lower and upper quartiles, and whiskers representing minimum and maximum of the data, and analyzed using Mann-Whitney test. **P < 0.01 compared with control.

![Figure 2](https://example.com/figure2.png)

**Figure 2** Engagement of L-type channels mediated dendritic Ca2+ oscillations during spiking activity in LC. (a) Left, a schematic of an LC neuron and the whole-cell current-clamp recording configuration of spiking activity synchronized to 2PLSM dendritic Ca2+ line-scan imaging. Spiking activity was accompanied with phase-locked Ca2+ oscillations in distal dendrites (80–100 µm away from soma, asterisk), detected in full-spike mode (control) or in the presence of TTX. Antagonism of L-type Ca2+ channels with 1 M isradipine eliminated Ca2+ oscillations in the presence of TTX (n = 4 neurons from 4 mice, P < 0.0037).

(b) qPCR analysis revealed mRNA expression of both Cav1.2 (also known as Cacna1c) and Cav1.3 (also known as Cacna1d) L-type channel subunits in LC neurons (n = 6 mice). (c) Representative spiking activity traces in the presence of TTX before and after selective antagonism of Cav1.3 channels with 50 M BPN4689, and nonselective antagonism of Cav1.2 and Cav1.3 with 1 M isradipine. Antagonism of Cav1.3 channels with BPN4689 significantly decreased spike amplitude (P = 0.017), frequency (P = 0.018) and amplitude of dendritic Ca2+ oscillations (P = 0.023) in LC neurons (n = 5 neurons from 5 mice). Residual spiking activity and Ca2+ oscillations were attenuated completely by 1 M isradipine, suggesting that Cav1.2 channels mediated the residual activity (P < 0.001). Data are presented as whisker box plots displaying median, lower and upper quartiles, and whiskers representing minimum and maximum of the data, and analyzed using Mann-Whitney test.
Although antagonism of Cav1 channels eliminated the dendritic fluctuations in Ca^{2+} concentration, it did not alter pacemaking rate or regularity (Fig. 3a), arguing that, as in SNc DA neurons, Cav1 channels contribute to pacemaking, but are not essential for its generation. In agreement with this inference, in the presence of TTX (1 μM), autonomous spiking continued, but at a slower rate (Fig. 3b), suggesting that proximally generated Na^{+} spikes were pacing dendritic Ca^{2+} spikes. To test this proposition, we initiated ectopic spikes in the somatic region by injection of brief current pulses through the patch electrode while monitoring dendritic Ca^{2+} fluctuations. These ectopic somatic spikes reset the dendritic Ca^{2+} oscillations, as predicted (Supplementary Fig. 3).

We used qPCR analysis of LC neurons to identify channels that help support pacemaking in addition to Cav1 channels and found robust expression of mRNA for voltage-dependent Na^{+} channels (Nav1.1, Nav1.2 and Nav1.6) and Na^{+} 'leak' channels (NALCN). Both types of Na^{+} channels are inwardly rectifying, causing depolarization at subthreshold membrane potentials. The expression of mRNA for NALCN was greater in LC neurons than in SNc DA neurons, suggesting a more central role in pacemaking. To verify functional expression of NALCN channels, we replaced extracellular Na^{+} with channel impermeable N-methyl-D-glucamine in the presence of TTX and isradipine (to eliminate the contribution of Nav1 and Cav1 channels, respectively); this intervention hyperpolarized LC neurons by ~20 mV, supporting the proposition that NALCN channels make an important contribution to the membrane potential near spike threshold (Supplementary Fig. 4).

Although L-type channels were not required for pacemaking, they might enhance the ability of the cell to sustain spiking when driven by extrinsic inputs. This robustness could be manifested by the ability to sustain an elevated spike rate, as this elevation would increase slow inactivation of Nav1 Na^{+} channels that normally drive pacemaking. To test this idea, we increased the spike rate by elevating the extracellular [K^{+}] to 17 mM (from 2.5 mM) led to a sustained elevation in spike rate (Fig. 3c). Antagonizing L-type Ca^{2+} channels had no effect on basal spiking rate either before or immediately after elevating extracellular K^{+}; however, after approximately 20 min, the spike rate fell in the presence of isradipine (Fig. 3c), suggesting that L-type channels help to maintain spiking in this condition.

Spiking triggers Ca^{2+}-induced mitochondrial oxidant stress
In SNc DA neurons, Ca^{2+} is less efficiently buffered by cytosolic proteins than in neighboring ventral tegmental area DA neurons, allowing it to diffuse in the cytoplasm more readily. In LC neurons, the situation was similar. Intrinsic Ca^{2+} buffering was estimated using the added extrinsic buffer method. The amplitude and decay time constant of the fluorescence signal evoked by a spike was monitored...
Figure 5 L-type channels triggered mitochondrial oxidant stress in LC. (a) Mito-roGFP–positive LC neuron immunoreactive to TH, labeled by white asterisk. (b) Mito-roGFP calibration with relative oxidation of 0.4. Isradipine attenuated oxidation in a dose-dependent manner (control, \(n = 5\) neurons, 5 mice; 200 nM isradipine, \(n = 4\) neurons, 4 mice, \(P = 0.028\); 1 µM isradipine, \(n = 5\) neurons, 4 mice, \(P = 0.002\)). Ryanodine (\(n = 6\) neurons, 5 mice, \(P = 0.001\)) and Ru360 (\(n = 4\) neurons, 4 mice, \(P = 0.007\)) also attenuated mitochondrial oxidation. (c) Ca\(^{2+}\) entry through L-type channels was pumped into ER via the sarcoplasmic reticulum Ca\(^{2+}\)-induced Ca\(^{2+}\) release (CICR)\(^{12}\). To test this possibility, we antagonized ryanodine receptors (RYRs) with isradipine (green) for 1 h attenuated oxidant stress in DJ-1\(^{-/-}\) (\(P = 0.032\), \(n = 5\) DJ-1\(^{-/-}\) neurons with isradipine, 3 mice). Data presented as whisker box plots with median, lower and upper quartiles, and whiskers representing minimum and maximum of the data, and analyzed using Mann-Whitney test. *\(P < 0.05\), **\(P < 0.01\), compared with control.

Continuously after rupturing the membrane at the tip of a cell-attached patch electrode filled with Fluo-4 (200 µM). As the dye filled the cell, the relative oxidation of the fluorescence signal fell and the time constant rose (Fig. 4a and Supplementary Fig. 5). These parameters allowed the intrinsic buffering (\(K_{b}\)) to be estimated (Fig. 4a). The median \(K_{b}\) for LC neurons was just greater than 50 (the smaller the number the lower the intrinsic buffering). This \(K_{b}\) estimate was similar to that for SNc DA neurons and was substantially lower than that for neurons with strong Ca\(^{2+}\) buffering (for example, cerebellar Purkinje neuron \(K_{b} = 2,000\))\(^{23}\).

Given the relatively low intrinsic buffering capacity of LC neurons, Ca\(^{2+}\) entering the cytoplasm could reach the ER, potentially evoking Ca\(^{2+}\)-induced Ca\(^{2+}\) release (CICR)\(^{12}\). To test this possibility, we antagonized ryanodine receptors (RYRs) by application of ryanodine (10 µM). Doing so decreased the dendritic Ca\(^{2+}\) transients evoked by pacemaking, indicating that RYR-dependent CICR contributes to the observed Ca\(^{2+}\) oscillation (Fig. 4b).

In SNc DA neurons, Ca\(^{2+}\) entry through plasma membrane L-type channels leads to increased mitochondrial oxidant stress. To determine whether the situation was the same in LC neurons, we prepared ex vivo slices from transgenic mice expressing a mitochondrially targeted ratiometric redox probe, mito-GFP\(^{11,23}\), under the control of the cytomegalovirus (CMV) promoter (Fig. 5a). LC neurons exhibited a basal mitochondrial oxidant stress that was typically 40–50% of the dynamic range of the probe (Fig. 5a). Antagonizing plasma membrane L-type channels with isradipine dose-dependently diminished this stress without altering pacemaking (Fig. 5b,c). Blocking Ca\(^{2+}\) entry into mitochondria with the mitochondrial unipporter (MCU) blocker RU360 (10 µM) also virtually eliminated oxidant stress (Fig. 5b,c) without changing the pacemaking rate (data not shown).

Mitochondrial Ca\(^{2+}\) entry is widely thought to occur at junctions between the ER and mitochondrion, where the local Ca\(^{2+}\) concentration can rise into the micromolar range\(^{25}\). Both RYRs and inositol triphosphate receptors (IP\(_{3}\)Rs) populate these junctions (referred to as mitochondria-associated membrane or MAMs)\(^{36}\). Consistent with the idea that mitochondrial Ca\(^{2+}\) entry occurs at MAMs, antagonizing RYRs markedly lowered mitochondrial oxidant stress, diminishing it by roughly the same amount as blocking the MCU with RU360 (Fig. 5b,c). These results suggest that plasma membrane Ca\(^{2+}\) entry through L-type channels is sequestered by the ER and then passed through RYRs to mitochondria, leading to oxidant stress (Fig. 5b,c).

Mitochondrial oxidant stress was accompanied by fluctuations in the mitochondrial membrane potential, as measured with the cationic dye tetramethyl rhodamine methylester (TMRM). These potential fluctuations had a frequency in the range of 10–30 mHz and amplitudes of about 10% of the TMRRM dynamic range (Fig. 5d), suggesting that they were attributable to the opening and closing of an ion channel in the inner mitochondrial membrane. The frequency and amplitude of fluctuations were diminished by genipin (100 µM), an inhibitor of uncoupling proteins (UCPs), which are ion channels that are capable of depolarizing the inner mitochondrial membrane\(^{27}\) (Fig. 5e). The potential fluctuations also were attenuated in frequency and amplitude by diminishing Ca\(^{2+}\) entry through plasma membrane L-type channels (Fig. 5f).

Oxidant stress is elevated in LC neurons lacking DJ-1

Loss of function of DJ-1 protein is associated with autosomal recessive early-onset forms of PD in humans\(^2\) and has been described as a redox–sensitive protein that participates in orchestrating oxidant defense\(^{28}\). Previous studies have shown that deletion of DJ-1 exacerbates mitochondrial oxidant stress in SNc and dorsal motor nucleus of the vagus (DMV) neurons\(^{11,12,29}\). If mitochondrial oxidant stress is a driver of LC degeneration, it should be increased in mice lacking functional DJ-1. To test this hypothesis, we used stereotoxic injection of an AAV viral vector to deliver a CMV–mito-roGFP expression construct to LC neurons in DJ-1 knockout (DJ-1\(^{-/-}\)) and wild-type mice. Brain slices were prepared and oxidant stress assayed 10–14 d later as described above. These experiments were done at 23–25 °C,
as previous studies have shown that probe oxidation was nearly complete at physiological temperatures. In LC neurons from DJ-1−/− mice, mitochondrial oxidant stress was substantially greater than in wild-type mice. Moreover, antagonism of L-type channels attenuated mitochondrial oxidant stress in DJ-1−/− neurons.

Extrinsic signals differentially regulate oxidant stress

The activity of LC neurons varies with behavioral state, raising the possibility that oxidant stress varies in parallel. For example, during sleep, the activity of LC NA neurons is suppressed by GABAergic input, which could lessen mitochondrial stress. Indeed, application of the GABA receptor agonist muscimol hyperpolarized LC neurons, stopped spiking and markedly diminished mitochondrial oxidant stress (Fig. 6a,b). Conversely, during arousal, the discharge rate of LC NA neurons increases, which could augment mitochondrial oxidant stress. The increase in LC discharge rate during arousal is attributed to the release of orexin in the LC by hypothalamic neurons. Bath application of orexin increased the discharge rate of LC neurons in our preparation (as predicted; Fig. 6a). However, orexin attenuated, rather than increased, mitochondrial oxidant stress (Fig. 6b). One possible explanation of this change is that orexin altered the pacemaking mechanism to rely more on Na+ channels and less on the opening of L-type Ca2+ channels. To test this hypothesis, we carried out two experiments. First, if orexin inhibited L-type channels, then spiking in the presence of TTX (which depends on L-type channels) should be impaired. Indeed, orexin virtually eliminated spiking in the presence of TTX (Fig. 6c). Second, inhibition of L-type channels should decrease dendritic Ca2+ transients during normal pacemaking. As predicted, orexin diminished these transients (Fig. 6d). Thus, mitochondrial oxidant stress in LC neurons is state dependent, falling both during GABAergic suppression of activity and during orexin-mediated acceleration of activity.

Hypercapnia also increases the spiking rate of LC neurons, which may contribute to the modulation of breathing. Moreover, hypercapnia-induced acceleration in the spiking of LC neurons is dependent on L-type Ca2+ channels, suggesting that hypercapnia might increase mitochondrial oxidant stress. To test this hypothesis, we bathed LC neurons in brain slices in a solution bubbled with gas containing 15% carbon dioxide (CO2) to induce hypercapnia. As shown previously, hypercapnia increased the spiking rate of LC neurons monitored in cell-attached recording mode (Fig. 6e).

To determine whether mitochondrial oxidant stress was affected, we bathed slices in the hypercapnic solution for 1 h and then assessed their mitochondrial redox status using 2PLSM. As predicted, hypercapnia increased mitochondrial oxidant stress relative to slices maintained in the normal 5% CO2 environment (Fig. 6f).

Oxidant stress was dependent on NOS

How does Ca2+ entry into mitochondria elevate oxidant stress? There is no consensus on this point. Mitochondrial Ca2+ influx de-represses matrix dehydrogenases of the tricarboxylic acid cycle, leading to increased generation of electron donors for the ETC. In physiological circumstances, increasing the proximal flux of electrons into the ETC could increase the production of superoxide and other ROS. However, there are other ways in which mitochondrial Ca2+ entry might increase ROS production. For example, sustained Ca2+ influx into mitochondria could stimulate a mitochondrial form of NOS. NO produced by NOS activation competes with O2 at cytochrome oxidase, thereby changing the redox status at earlier points along the ETC and increasing the production of superoxide. To determine whether these mechanisms were at work in LC neurons, we loaded...
the cells with the NO-sensitive probe 4-amino-5-methylamino-2,7-difluorescein (DAF-FM) with a local perfusion pipette and then imaged the neurons using 2PLSM. In unperturbed LC neurons, DAF-FM fluorescence was readily detectable (Fig. 7a and Supplementary Fig. 6). The NO signal was decreased by pre-incubation with the NOS inhibitor L-N^6-nitroarginine methyl ester (L-NAME, 100 µM; Fig. 7a). Antagonizing plasma membrane L-type channels also decreased the DAF-FM signal (Fig. 7b). Although the source of NO could not be determined directly, as the dye was largely cytosolic, blocking Ca^{2+} entry into mitochondria with RU-360 decreased the cytosolic NO signal (Fig. 7b), arguing that mitochondria are a site of origin. Conversely, blocking Ca^{2+} efflux from the mitochondria by antagonizing the mitochondrial Na^{+}-Ca^{2+} exchange (NCX) with 5 µM CGP37157 (refs. 26,38) led to an increased cytosolic NO signal (Fig. 7b), arguing that increasing intramitochondrial [Ca^{2+}] stimulates NO production.

To determine whether NO production increased mitochondrial oxidant stress, we incubated slices with L-NAME (100 µM) before determining the redox status of mitochondria. Previous work has shown that NO does not directly alter mito-roGFP fluorescence, so any change in these experiments should be attributable to altered dithiol formation\textsuperscript{23}. Pre-incubation with L-NAME diminished mitochondrial oxidant stress in LC neurons (Fig. 7c). L-N^6-nitroarginine (L-NNA, 10 µM), a different NOS inhibitor, also decreased mitochondrial oxidant stress (Fig. 7c). Exposure of SNc DA neurons to L-NAME had a very similar effect (Fig. 7c), suggesting that NOS activity is a major source of oxidant stress in both cell types. To provide an additional test of the inference that NOS activity was increasing mitochondrial oxidant stress, we used TMRM to monitor mitochondrial membrane potential. As predicted, L-NAME diminished the amplitude and frequency of fluctuations in mitochondrial potential (Fig. 7d).

**DISCUSSION**

Three conclusions can be drawn from our results. First, LC NA neurons have a robust pacemaking phenotype that leads to basal mitochondrial oxidant stress that is exacerbated in a genetic model of PD. Second, although mitochondrial stress generally tracked spike activity and Ca^{2+} entry through L-type channels, this relationship was subject to modulation. Third, the activity-dependent oxidant stress stems in large part from the stimulation of a mitochondrial form of NOS. These conclusions provide a framework for understanding how age and disease might lead to the loss of LC NA neurons, as well as how this loss might be averted.

**Spiking elevates oxidant stress in LC neurons**

Previous work on LC neurons has not provided a mechanistic foundation for understanding their sensitivity to aging and aging-related neurodegenerative diseases, such as PD and Alzheimer’s disease. A number of studies have shown that LC neurons are spontaneously active and that this activity is autonomously generated\textsuperscript{14,15}. Our results were consistent with these studies and extend them in several important respects.

Using 2PLSM Ca^{2+} imaging approaches, we found that autonomous pacemaking was accompanied by large fluctuations in dendritic Ca^{2+} concentration that were attributable to opening of a mixture of L-type channels with Cav1.3 and Cav1.2 pore-forming subunits. As in SNc DA neurons, the low-threshold Cav1.3 L-type channels in LC neurons were active throughout the interspike interval, leading to a sustained Ca^{2+} influx. Although Ca^{2+} current through L-type channels contributed to the inward currents driving LC pacemaking, they were not necessary to maintain it, as low micromolar DHP concentrations virtually eliminated dendritic fluctuations in intracellular Ca^{2+} concentration, but had no effect on pacemaking rate and regularity.

Although not necessary for pacemaking, L-type channels increased the robustness of pacemaking. In fact, dendritic L-type Ca^{2+} channels sustained pacemaking after blocking Nav1 channels with TTX, albeit at a lower rate. This contrasts with the situation in SNc DA neurons in which L-type channels are capable of only sustaining a subthreshold...
dendritic oscillation after block of Nav1 channels\textsuperscript{40}. For this type of Ca\textsuperscript{2+}-dependent dendritic pacemaking to work, Ca\textsuperscript{2+} must be weakly buffered by cytosolic proteins so as to allow free [Ca\textsuperscript{2+}] to control the onset of repolarization by Ca\textsuperscript{2+}-activated K\textsuperscript{+} channels\textsuperscript{41}. As a consequence, much of the entering Ca\textsuperscript{2+} must be slowly sequestered by intracellular organelles. The ER is widely viewed as a key mediator of intracellular Ca\textsuperscript{2+} buffering on this timescale\textsuperscript{32}. Although ER Ca\textsuperscript{2+} content was not monitored, we found that blocking ER RYRs diminished cytosolic Ca\textsuperscript{2+} transients during pacemaking, suggesting that the ER is important for intracellular Ca\textsuperscript{2+} dynamics. In many cell types, ER buffering leads to Ca\textsuperscript{2+} being shuttled to mitochondria at specialized junctions, so-called MAMs\textsuperscript{26}. RYRs are localized on the ER side of MAMs and MCU are found on the mitochondrial side, creating a mechanism by which Ca\textsuperscript{2+} can move from the ER to mitochondria. This same arrangement appeared to be present in LC neurons, where Ca\textsuperscript{2+} shuttle into mitochondrial was sufficient to create an oxidant stress as measured with a matrix-targeted redox-sensitive variant of GFP.

Given the negative consequences of sustained Ca\textsuperscript{2+} entry, why do LC NA neurons express such high levels of the Cav1 channel? One possibility is that Ca\textsuperscript{2+} drives the synthetic machinery necessary for proper functioning of LC neurons\textsuperscript{43}. Another possibility is that the pacemaking function of LC NA neurons is essential for organismal fitness and survival. During waking states, even a transient loss of spiking in LC NA neurons results in impaired brain function, which could be catastrophic (if, for example, one were being pursued by a predator). This would create evolutionary pressure to put in place redundant pacemaking mechanisms that minimize the likelihood of failure, even with sustained excitation. In slices, LC NA neurons were able to continue spiking at elevated rates for as long as the recording could be sustained. However, in the presence of L-type channel antagonists, spiking was less robust, failing in ~20 min after increasing extracellular K\textsuperscript{+}. This difference is attributable to the fact that, unlike Cav1 Ca\textsuperscript{2+} channels, Nav1 Na\textsuperscript{+} channels are subject to slow, voltage-dependent inactivation with sustained depolarization. Thus, it is easy to imagine that there would be strong evolutionary pressure to creating a ‘backup’ dendritic oscillator that was not going to fail under the same conditions, an oscillator such as that involving Cav1 Ca\textsuperscript{2+} channels. The cost of this design choice is the sustained elevation in mitochondrial oxidant stress. However, this cost is one that could be tolerated, as there are biochemical systems in place to mitigate oxidant stress and delay its negative consequences. Indeed, the effect on the function of the LC NA system takes five or six decades to become manifest, which is well beyond the life expectancy of animals with this design, including humans until recently.

Mitochondrial stress was state dependent

The engagement of the dendritic oscillator was modulated by extrinsic inputs. During sleep, the autonomous activity of LC NA neurons is actively suppressed by GABA\textsubscript{A}ergic input\textsuperscript{40,44}. This suppression could be mimicked in ex vivo brain slices by application of the GABA\textsubscript{A} receptor agonist muscimol. As expected, suppression of pacemaking diminished Ca\textsuperscript{2+} entry and mitochondrial oxidant stress. In contrast, arousal leads to an elevation in the spiking rate of LC neurons, increasing the release of NA in brain regions controlling behavioral activation\textsuperscript{45}. One of the drivers of this type of arousal is orexin, which is released by hypothalamic neurons innervating the LC\textsuperscript{31}. As anticipated, exogenous orexin increased the discharge rate of LC neurons in brain slices. What was not anticipated was that this increase in discharge rate would lead to lowered mitochondrial oxidant stress. This happened because orexin negatively modulated L-type channel opening while increasing Na\textsuperscript{+} currents, presumably those through NALCN channels. This observation is consistent with early work showing that the effects of orexin on LC spiking are independent of Ca\textsuperscript{2+} (ref. 34). It is not clear at this point how this modulation was brought about, but L-type Ca\textsuperscript{2+} channels are well known to be regulated by G protein–coupled receptors, such as those mediating the effects of orexin.

Hypercapnia also increased the spiking rate of LC neurons. Augmented LC spiking is thought to modulate brainstem respiratory circuits, promoting increased respiratory rate\textsuperscript{46}. However, unlike the response to orexin, the excitatory effects of hypercapnia depend on L-type Ca\textsuperscript{2+} channels in LC neurons\textsuperscript{15,16}. Accelerating the discharge rate of LC neurons without suppressing the opening of L-type Ca\textsuperscript{2+} channels should lead to increased Ca\textsuperscript{2+} entry and mitochondrial Ca\textsuperscript{2+} loading. Indeed, hypercapnia increased mitochondrial oxidant stress in LC neurons. These results suggest that persistent respiratory insufficiency could have a negative effect on LC and contribute to neurodegenerative decline.

Mitochondrial Ca\textsuperscript{2+} entry stimulates NOS and oxidant stress

Ca\textsuperscript{2+} that enters the mitochondrial matrix stimulates oxidative phosphorylation by de-repressing dehydrogenases, thereby helping to meet bioenergetic needs\textsuperscript{37}. Our results suggest that another consequence of mitochondrial Ca\textsuperscript{2+} entry is the stimulation of NOS. NO production, as measured with cytosolic DAF-FM, was robust in pacemaking LC neurons. Not only was NO production sensitive to Ca\textsuperscript{2+} entry through plasma membrane L-type channels, it was sensitive to antagonism of the mitochondrial uniporter with RU360. Moreover, although blocking entry decreased NO production, disrupting the removal of Ca\textsuperscript{2+} from the matrix by NCX increased NO levels. The ability of L-NAME to block NO production suggests the involvement of an enzymatic process, again implicating NOS. Studies in other tissues have shown that a Ca\textsuperscript{2+}-dependent form of NOS is localized to mitochondria\textsuperscript{36}, providing a clear precedent for the observations in LC neurons.

What was more unexpected was the observation that inhibition of NOS decreased the oxidation status of matrix proteins and attenuated the fluctuations in mitochondrial potential driven by that stress. This suggests that NOS contributes to oxidant stress. How? NO generated in proximity to the ETC can compete with O\textsubscript{2} at the binuclear center of cytochrome oxidase, leading to an increase in the reduction state of more proximal electron carriers and to an increase in the generation of superoxide\textsuperscript{37}.

But what end does this signaling pathway serve? One possibility is that NO generated in response to mitochondrial matrix Ca\textsuperscript{2+} entry helps to prevent Ca\textsuperscript{2+} overload; that is, it serves as a negative feedback regulator of mitochondrial calcium uptake. By inhibiting cytochrome oxidase, NO can slow electron transport, leading to a decrease in mitochondrial membrane potential and a slowing of mitochondrial Ca\textsuperscript{2+} influx. By regulating matrix [Ca\textsuperscript{2+}], this mechanism would prevent activation of the mitochondrial permeability transition pore, which could inflict lethal injury on the cell\textsuperscript{7}. This model is consistent with the demonstration that tricarboxylic acid enzymes are more sensitive to Ca\textsuperscript{2+} than is mitochondrial NOS\textsuperscript{13}. Thus, modest mitochondrial Ca\textsuperscript{2+} entry can maintain oxidative phosphorylation necessary for neuronal survival\textsuperscript{47}, without activating NOS. However, when mitochondrial Ca\textsuperscript{2+} influx increases, NOS-mediated NO production would lead to an inhibition cytochrome oxidase, a slowing of the ETC and a diminished driving force for Ca\textsuperscript{2+} entry\textsuperscript{37}. This braking mechanism also provides a compelling rationale for the expression of NOS in the mitochondria, as this localization confers sensitivity to matrix
Ca\textsuperscript{2+} that would not be detected by NOS expressed in the cytosol. An unfortunate byproduct of this mechanism is the production of superoxide by the ETC\textsuperscript{C,47}. However, in most neurons, the engagement of the NOS brake (and the production of superoxide) should be infrequent. LC NA neurons (and SNc DA neurons) are exceptions, where large and sustained mitochondrial Ca\textsuperscript{2+} influx leads to the continuous activation of this protective brake. During a typical human lifespan, the cumulative oxidant damage produced by this mechanism could contribute to mitochondrial DNA defects that impair the ability to generate ATP, leading to bioenergetic failure and cell death.\textsuperscript{5,6}

As appealing as the model is, there is an alternative explanation for our DAF-FM data that needs to be considered. For DAF-FM fluorescence to increase with NO exposure, its amide group must have been linked to reduced risk of developing PD \textsuperscript{49} and to slowing the progression of Alzheimer’s disease\textsuperscript{50} and the nonmotor symptoms of PD, of progression. These drugs also might have utility in slowing the progression and not from mitochondrial localization of NOS? This seems highly unlikely, as an extra-mitochondrial NOS localization would not explain the ability of NOS inhibitors to diminish mitochondrial matrix oxidant stress or oscillations in the inner mitochondrial membrane potential.

**Therapeutic strategy in aging-related neurodegeneration**

Many of the neurons that succumb to aging and aging-related degenerative diseases have essential physiological functions that require the ability to maintain autonomous spiking under normal and stressful states. In at least three of these cell types, DMV cholinergic neurons, SNc DA neurons and LC NA neurons, the sustained engagement of L-type Ca\textsuperscript{2+} channels enhances the reliability of autonomous spiking at the cost of increased mitochondrial oxidant stress.\textsuperscript{1,2,9} We found that, in a mouse model of PD, LC neurons also displayed exacerbated mitochondrial stress, which was attenuated in this mouse model by using Cav1 channel antagonists. The engagement of L-type channels during autonomous spiking might be considerably more widespread than previously recognized. Use of Cav1 channel antagonists to lower blood pressure that cross the blood brain barrier have no obvious deleterious neurological consequences for humans and have been linked to reduced risk of developing PT\textsuperscript{49} and to slowing of progression. These drugs also might have utility in slowing the progression of Alzheimer’s disease\textsuperscript{50} and the nonmotor symptoms of PD, particularly those potentially dependent on the loss of LC neurons.

**METHODS**

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

*Note: Any Supplementary Information and Source Data files are available in the online version of the paper.*

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**AUTHOR CONTRIBUTIONS**

D.J.S., J.S.-P. and J.N.G. were responsible for the design and execution of experiments, as well as the analysis of results and overall direction of the experiments. Analysis of data, construction of figures and communication of the results, W.O. participated in the design and the communication of the results. S.S. and D.J.G. contributed to collecting electrophysiological and relative oxidation data, respectively. B.Y. performed stereotaxic viral injections. D.W. provided expertise in optical approaches. J.S.-P. and E.I. conducted the immunocytochemical experiments. J.K. generated the AAV virus for the generation of the CMV–mito-roGFP mice and participated in the design, analysis and communication of the results. D.J.S. and J.S.-P. prepared the manuscript and the illustrations.

**COMPETING FINANCIAL INTERESTS**

The authors declare no competing financial interests.

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ONLINE METHODS

Brain slice preparation. Acute pontine horizontal slices (220 μm thick) were prepared from wild-type male C57BL/6 mice (Charles River) or DJ-1−/− mice (backcrossed to C57BL/6) between postnatal days 21 and 32, unless specified otherwise. The handling of mice and all procedures performed on them were approved by the Institutional Animal Care and Use Committee at Northwestern University and were in accordance with the US National Institutes of Health Guide to the Care and Use of Laboratory Animals and Society for Neuroscience guidelines. Mice were anesthetized with a ketamine/xylazine mixture, followed by a transcardial perfusion with ice-cold, oxygenated ACSF containing 125 mM NaCl, 2.5 mM KCl, 25 mM NaHCO3, 1.25 mM NaH2PO4, 2 mM CaCl2, 1 mM MgCl2, and 25 mM dextrose, pH 7.3 (osmolality 315–320 mOsm L−1). After perfusion, mice were decapitated and brains removed rapidly, followed by sectioning in ice-cold oxygenated ACSF using a vibratome (VT1000S, Leica Microsystems). Pontine slices were incubated in ACSF at 32–34 °C for 30 min before electrophysiological recordings. Slices were then transferred to a small-volume (<0.5 mL) recording chamber that mounted on a fixed-stage, upright microscope (BX51, Olympus America) equipped with infrared differential interference contrast (0.9 numerical aperture (NA)) with de Sénarmont compensation (Olympus). Electrophysiological recordings were performed at 33–34 °C, unless specified otherwise. The recording chamber was superfused with carbogen-saturated ACSF with a flow rate of 2–3 mL min−1 running through an in-line heater (SH-27B with TC-324B controller, Warner Instruments). Neuronal somata and proximal dendrites were visualized by video microscopy at high magnification (60×, 0.9 NA water-immersion objective, Olympus). Brain slices were used within 3 h of slice preparation.

Electrophysiology. Cell-attached and whole-cell patch-clamp recordings were performed in LC neurons located near the floor of the fourth ventricle. LC neurons were recognized as a cluster of neurons with large somatic compartments. Recording patch electrodes (resistance of 3–6 MΩ) were prepared with a Sutter Instruments horizontal puller using borosilicate glass with filament. For current-clamp recordings, patch electrodes were filled with internal solution containing 135 mM KMeSO4, 5 mM KCl, 5 mM HEPES, 0.05 mM EGTA, 10 mM phosphocreatine-di(tris), 2 mM ATP-Mg and 0.5 mM GTP-Na, the pH adjusted to 7.3 (osmolality adjusted to 290–300 mOsm L−1). The liquid junction potential in our recording ACSF using this internal solution is 7 mV and not corrected for. Conventional tight-seal (>2 GΩ) whole-cell patch-clamp and cell-attached recordings were made on visually identified LC neurons based on size, somatodendritic morphology and regular spiking between 1 and 6 Hz. Signals were filtered at 1–4 kHz and digitized at 5–20 kHz with a Digidata 1400 (Molecular Devices). For current-clamp recordings, the amplifier bridge circuit was adjusted to compensate for electrode resistance and monitored. For perforated-patch current-clamp experiments, internal solution was supplemented with amphotericin-B (Sigma), prepared fresh and added in the internal solution to a final concentration of 180 μg ml−1. Alexa658 was added in the internal solution to confirm recordings were done under perforated-patch mode. Electrode capacitance was compensated and if series resistance increased >20% during recording, the data were discarded.

2PLSM Ca2+ imaging. For dendritic Ca2+ measurements, LC neurons were loaded with internal solutions supplemented with Alexa Fluor 594 (20 μM) and Fluor-4 (50 μM) through the patch pipette. All experiments were performed at 32–34 °C. Imaging took place after 15–20 min of dye loading. Images were acquired with an Olympus LUMPLFL 60×/1.0 NA water-dipping objective lens. The two-photon excitation source was a Chameleon–ultra1 laser system (Molecular Devices). For current-clamp recordings, the amplifier bridge circuit was adapted to 2PLSM based on protocols described previously by other groups. Images were captured using 200 μM Fluor-4 (Kd = 300 nM, Rf = 85–100) and Alexa568, which was added in the internal solution to confirm recordings were done under perforated-patch mode. Somatic fluorescence was each detected by a multi–alkali-cathode PMT. The system digitized the current from detected photons to 12 bits. The laser light transmitted through the sample was collected by the condenser lens and sent through a Dود contrast tube (Ludwig and Neumann) to another PMT (R3896, Hamamatsu). Somatic fluorescence was saturated as a function of time before breaking into the cells while inducing spikes to evoke Ca2+ transients. Ca2+ signal was detected using 2PLSM as described above. For data analysis, we used previously described equations22. To measure changes in intracellular Ca2+ ([Ca2+]i) ([Ca2+]ipeak − [Ca2+]i Rest) was first calculated and then calculated added buffering capacity (Kf) using equation (3):

\[ \Delta [Ca^{2+}]_0 = \frac{(1 - R_f)}{(R_f - 1)} \frac{\delta f}{\delta f_{\text{max}}} \]  

The value of \( \delta f_{\text{max}} \) was calculated by a depolarizing current step that achieves a Ca2+ plateau to saturate Fluor-4 dye. From this value, we calculated resting [Ca2+]i from equation (2):

\[ [Ca^{2+}]_0 = \frac{R_f - 1}{1 - R_f} \frac{\delta f}{\delta f_{\text{max}}} \]  

The estimated value of Rf = 85–100 for Fluor-4 was obtained from ref. 22, making Rf = 1–value small; we therefore disregarded it from equations (1) and (2). The dynamic range of the dye (Δfmax/Δf) was estimated as 1 + δfmax. After determining [Ca2+]i and related parameters, we then calculated added buffering capacity (Kf) using equation (3):

\[ K_f = \frac{[Fluo-4]}{[K_d] + [Ca^{2+}]_0 ([K_d] + [Ca^{2+}]_{\text{peak}})} \]  

The inverse of \( [Ca^{2+}]_0 \) was then plotted as a function of added buffering capacity and with a linear regression, the estimated endogenous buffering capacity (Kd) was determined from the linear fit intersecting the negative x axis.
pixel size between 0.18–0.21 μm and a 10-μs pixel dwell time. This wavelength was chosen by determining the two-photon excitation spectrum of mito-roGFP in cultured dopaminergic neurons from TH-mito-roGFP mice. Laser was a Chameleon-Ultra, 690–1,040 nm, 80-MHz ~250-fs sample pulse duration, Coherent Laser Group. The roGFP fluorescence was detected (490–560 nm) by a non–de-scanned GaAs PMT (H7422PA, Hamamatsu) and a laser-scanned Dodt contrast transmission PMT (R3896, Hamamatsu) detector system (Prairie Technologies) that provided a bright–field transmission image. 60 frames of the roGFP signal were collected in one optical plane at a rate of 3–4 frames per s. Records with drifting baseline (resulting from photo-bleaching or photo-oxidation of roGFP) were discarded. At the end of all experiments, the maximum and minimum fluorescence of mito-roGFP was determined by application of 2 mM dithiothreitol (DTT) to fully reduce the mitochondria, and then 200 μM aldrithol (ald) to fully oxidize mitochondria. Relative oxidation was calculated with the equation 1− (F−Fald)/FDTT−Fald).

2PLSM imaging of mitochondrial membrane potential. Brain slices from wild-type were incubated in 2–4 μM TMRM for 30–60 min at 32–34 °C; excess dye was washed out with a TMRF-free ACSF solution. Imaging experiments were performed with TMRM-free ACSF solution at 32–34 °C. Fluorescence (580–630 nm) was collected by a GaAs PMT (H7422PA, Hamamatsu) as part of the Prairie Technologies laser-scanning microscope. Fluorescence measurements in regions of interest (ROI) were monitored to ensure the stationarity of the signal; samples with a drifting baseline (resulting from photo-bleaching of dye and/or wash-out) were discarded. Time-series scanning (1,000 frames) in a fixed plane was performed with a 10-μs dwell time at a rate of 2.5–3 frames per s using the 2PLSM system described above. Four to five ROIs in the cell body and one ROI in the nucleus were monitored and changes in TMRM fluorescence were plotted as a function of time. Flickering frequency is defined as the number of transitions in 100-s epochs. The change in mitochondrial membrane potential (V) during flickering was estimated from the fluorescence in an ROI using a Nernst equation: V = (RT/zFln(Fm/Fn)) where R is the gas constant, T is temperature, F is Faraday’s constant, z = 1, Fm is the fluorescence in the mitochondrial ROI, Fn is the fluorescence of the nucleus in the same optical plane and β is a scaling factor. The scaling factor was calculated by assuming that the mitochondrial membrane potential was −150 mV when Fmmax and Fm were determined. For each flickering event, the percent change in V was calculated by taking the difference in the estimates before and then during the drop in fluorescence; the fluorescence during the drops was averaged.

2PLSM NO imaging. The NO probe 4-aminoo-5-methylamino-2,7-dihydrosecurin (DAF-FM, Life Technologies) stock was dissolved in DMSO as instructed by the manufacturer. Stock solution was always prepared fresh on the day of the experiment. Following brain slice preparation, DAF-FM was perfused directly into the LC region via a patch pipette filled with 10 μM of the probe, at 32–34 °C and waited 5–10 min before 2PLSM imaging. Optical imaging of DAF-FM signals acquired using a 920-nm excitation beam, in a fixed plane of focus with a pixel size between 0.18–0.21 μm and a 10-μs pixel dwell time. DAF-FM fluorescence (490–560 nm) was detected by a GaAs PMT (H7422PA, Hamamatsu) and a Dodt contrast detector system that provided a brightfield transmission image (Prairie Technologies). For experiments using NO signaling inhibitor, brain slices were hemi-dissected one sister slice containing LC and the other sister slice corresponding to respective pharmacological manipulation, using same laser power and PMT settings. DAF-FM fluorescence was normalized by Dodt contrast signal to control for variations in laser power (as detected in the condenser path), and termed relative DAF-FM fluorescence in the box plots.

qPCR. SNc and LC brain regions were micro-dissected from wild-type and total RNA was extracted using TriZol reagent (Life Technologies); cDNA was generated by reverse transcription (Quanta Biosciences) from both knockout and wild-type samples. cDNA from each sample was analyzed using qPCR. qPCR experiments were performed using Sybr-Green and sense and antisense primers (Integrated DNA Technologies); primers specific to Cav1, Nav1 and NALCN channels were designed to generate PCR products between 120–200 base pairs. qPCR cycling parameters were: 1 cycle at 95 °C for 3 min, followed by 40 cycles each consisting of 15 s at 94 °C, 1 min at 60 °C, 30 s at 72 °C, followed Sybr Green fluorescence recording. After the completion of 40 cycles, each sample was subjected to a melting temperature (Tm) curve analysis. Expression level was estimated using the comparative Ct, approximation method to calculate relative abundance of mRNA. Samples were run in triplicate for accuracy and normalized to expression levels of seven housekeeping genes to obtain the ΔCt. Relative ion channel mRNA abundance was calculated by equation (2)−DCT.

Immunostaining. Wild types were perfused transcardially with 4% paraformaldehyde (wt/vol) in 0.1 M phosphate buffer, pH 7.3. Brains were removed and thin sections (30 μm thick) were obtained using a Leica vibratome (VTI200S). Sections were blocked in 5% normal goat serum (vol/vol) followed by incubation with a monoclonal antibody to tyrosine hydroxylase (TH, 16–18 h at 4 °C, working dilution of 1:1,000, Immunostar, catalog number 22941) and a polyclonal antibody to dopamine-β-hydroxylase (DBH, 16–18 h at 4 °C, working dilution 1:1,000, Immunostar, catalog number 22806). Staining was visualized with the respective secondary antibody conjugated to Alexa 594 or 405 (2 h incubation at 23–25°C, working dilution 1:1,000, Life Technologies, catalog numbers A31553 and A11012). For biocytin experiments, LC neurons were filled with biocytin and stained with streptavidin–conjugated with Alexa 594. Images were acquired with a Fluoview confocal microscope (Olympus) and stored at 12-bit image depth at a resolution of 1,024 × 1,024 pixels.

Pharmacological reagents and channel ligands. Reagents were purchased from Sigma except for isradipine, L-NNA, DTT, ryanodine, orexin, muscimol and CGP37157 were purchased from R&D Systems; TMRM, Alexa638, Alexa594 and Fluo-4 obtained from Life Technologies; genipin was obtained from Wako Reagents, and Ru360 was obtained from EMD Biosciences. L-NAMe was purchased from Sigma-Aldrich. Drugs stock solutions were prepared in deionized water, DMSO, ethanol, or methanol as instructed by manufacturer on the day of experiment. Stock solutions were diluted to final concentrations in ACSF to achieve a final solvent concentration of <0.01% (vol/vol).

Statistical analysis. Imaging data collected was analyzed with Igor Pro 6.0 (WaveMetrics), Matlab (Mathworks), or GraphPad Prism Version 5.0 (GraphPad Software). The stimulation, display and analysis software for the two-photon imaging data was analyzed using a custom-written shareware package, WinFluor, PicViewer and PowerCAL kindly provided by J. Dempster (Strathclyde University). Data were summarized using box–plots showing median values for small sample sizes and the interquartile range to describe the distribution of the data. Sample n represents the number of neurons collected from brain slices from at least three mice, one brain slice per mouse. Sample size was determined on the basis of initial effect size, and as reported in previous publications. No randomization of samples was employed. Statistical analysis was performed with GraphPad Prism Version 5.0 (GraphPad Software) using non–parametric tests. Probability threshold for statistical significance was P < 0.05.