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Locus coeruleus neurons are most sensitive to chronic neuroinflammation-induced neurodegeneration

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Abstract

Parkinson's disease (PD) develops over decades through spatiotemporal stages that ascend from the brainstem to the forebrain. The mechanism behind this caudo-rostral neurodegeneration remains largely undefined. In unraveling this phenomenon, we recently developed a lipopolysaccharide (LPS)-elicited chronic neuroinflammatory mouse model that displays sequential losses of neurons in brainstem, substantia nigra, hippocampus and cortex. In this study, we aimed to investigate the mechanisms of caudo-rostral neurodegeneration and focused our efforts on the earliest neurodegeneration of vulnerable noradrenergic locus coeruleus (NE-LC) neurons in the brainstem. We found that compared with neurons in other brain regions, NE-LC neurons in untreated mice displayed high levels of mitochondrial oxidative stress that was severely exacerbated in the presence of LPS-elicited chronic neuroinflammation. In agreement, NE-LC neurons in LPS-treated mice displayed early reduction of complex IV expression and mitochondrial swelling and loss of cristae. Mechanistically, the activation of the superoxide-generating enzyme NADPH oxidase (NOX2) on NE-LC neurons was essential for their heightened vulnerability during chronic neuroinflammation. LPS induced early and high expressions of NOX2 in NE-LC neurons. Genetic or pharmacological inactivation of NOX2 markedly reduced mitochondrial oxidative stress and dysfunction in LPS-treated mice. Furthermore, inhibition of NOX2 significantly ameliorated LPS-induced NE-LC neurodegeneration. More importantly, post-treatment with NOX2 inhibitor diphenyleneiodonium when NE-LC neurodegeneration had already begun, still showed high efficacy in protecting NE-LC neurons from degeneration in LPS-treated mice. This study strongly supports that chronic neuroinflammation and NOX2 expression among vulnerable neuronal populations contribute to caudo-rostral degeneration in PD.

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Keywords

Parkinson's disease; Noradrenergic locus coeruleus neuron; Oxidative stress; Mitochondria; Neuroinflammation; Vulnerability

Introduction

Progressive pathological staging and degeneration in Parkinson's disease (PD) occur along a caudo-rostral axis, especially for patients with young onset and long duration of the disease (Braak et al., 2003; Rietdijk et al., 2017). Noradrenergic locus coeruleus (NE-LC) neurons are among the earliest and most severe sites of degeneration in PD (McKee et al., 2013; Perry et al., 1990; Zarow et al., 2003). Specifically, NE-LC neurons are affected as early as at stage 2 during PD progression, while nigral dopaminergic (DA) neurons and hippocampal/cortical neurons are affected at stage 3 and 5, respectively (Braak et al., 2003). In contrast, neurons in the caudate putamen are entirely spared. Although 'prion-like' hypothesis that neuron-to-neuron transmission of misfolded α -synuclein along caudo-rostral projections has been considered to induce dysfunction and neurodegeneration among selective neuronal populations (Helwig et al., 2016; Holmqvist et al., 2014; Luk et al., 2012; Mason et al., 2016), pathogenic variants of α -synuclein expressed *in vivo* show variable abilities to produce neurodegeneration in rodent models (Gispert et al., 2003; Luk et al., 2012; Martin et al., 2006; Osterberg et al., 2015; Sacino et al., 2014). Therefore, elucidating other potential mechanisms responsible for the spatiotemporal pattern of neurodegeneration in PD is important.

Neuroinflammation and oxidative stress are critical driving forces causing delayed neurodegeneration (Schapira and Gegg, 2011). Excessive production of reactive oxygen species (ROS) generated by both neuroinflammation and damaged mitochondria drives oxidative stress, bioenergetics failure and eventual death among afflicted neurons (Fukui and Moraes, 2008). Chronic neuroinflammation can further exacerbate neuronal oxidative stress through collateral lipid, protein and DNA oxidation caused by cytotoxic factors generated by microglia (Gao et al., 2012). Recent work investigating neurodegeneration in Prion Protein (PrP) model—which spreads using neuron-to-neuron transmission and results in neurodegeneration—found the key factor between distal spreading of PrP and neurodegeneration was the presence of neuroinflammation and pro-inflammatory factor transcripts (Alibhai et al., 2016). Considering that neuroinflammation can spread along neuronal projections (Hunter et al., 2009; Vitkovic et al., 2000) and NE-LC and DA substantia nigra pars compacta (SNpc) neurons have far greater innate susceptibilities to oxidative stress than cortical and hippocampal neurons, we hypothesized that chronic neuroinflammation might differentially exacerbate the oxidative load in these distinct neuronal populations based on their own innate susceptibilities, ultimately resulting in progressive caudo-rostral degeneration.

Lacking an adequate model of PD progression is a great obstacle to interrogate the etiology and mechanism driving caudo-rostral degeneration. The most widely used animal models of PD do not recapitulate the caudo-rostral progression of degeneration. Instead, they either

induce acute and selective dopaminergic neurotoxicity to the substantia nigra or introduce global genetic mutations associated with pathologies and atrophies that are more widely spread than actually observed in PD patients. Previously we have reported on a Parkinsonian-like mouse model generated by chronic neuroinflammation initiated by a single systemic injection of the bacterial endotoxin lipopolysaccharide (LPS) (Qin et al., 2007). In this model, LPS injection elicits robust and transient inflammatory response in the periphery of mice. However, chronic and sustained microglial activation, neuroinflammation and subsequent progressive loss of SN-DA neurons were observed in the brain (Qin et al., 2007). Recently, we extended our initial findings to show that global chronic neuroinflammation results in early and significant neuronal loss that follows a spatiotemporal pattern (Song et al., 2019a). Specifically, the loss of NE-LC neurons preceded the loss DA-SN neurons by 3-4 months and other cortical and hippocampal neurons by 6-7 months, which is consistent with the observation in patients with PD (Song et al., 2019a).

In this study, by taking advantage of this progressive model, we examined the oxidative load in selective neuronal populations known for their heightened susceptibilities along each stage of ascending progression in both naïve and neuroinflammatory conditions elicited by LPS exposure. Results showed that naïve NE-LC neurons displayed the highest levels of oxidative stress among all the examined brain regions, which was significantly exacerbated by chronic neuroinflammation. Mechanistic inquiry revealed that neuroinflammation and innate neuronal NADPH oxidase (NOX2) expression are the key factors contributing to caudo-rostral neurodegeneration, opening new opportunities for therapeutic interventions in patients suffering from the disorder.

Materials and methods

Animal study

C57BL/6J and B6.129S-Cybb^{tm1Din/J} (gp91^{phox-/-} deficient) were obtained from the Jackson Laboratory (Bar Harbor, ME). Housing and breeding of animals were performed humanely with regard to alleviation of suffering following the National Institutes of Health's Guide for the Care and Use of Laboratory Animals (Institute of Laboratory Animal Resources, 1996). Eight-week-old male mice were randomly divided into two groups, i.e., control and lipopolysaccharide (LPS). Mice were exposed to either a single intraperitoneal injection of LPS (Sigma-Aldrich, L3012, 15×10^6 EU/kg) or a saline vehicle (5 ml/kg) (Wang et al., 2015). LD50 of LPS in mice is around 25–30 mg/kg, which equals 7.9×10^7 EU/kg (Li et al., 2018). In this study, no mice are dead. Brain samples were collected at 1, 4, 7, and 10 months following injections ($n = 5$ and 6 for control and LPS group, respectively, per time point). All procedures were approved by the National Institutes of Environmental Health Sciences Animal Care and Use Committee.

Diphenyleneiodonium treatment

Diphenyleneiodonium (DPI) was administered by using Alzet osmotic mini-pumps as described previously (Song et al., 2019a; Wang et al., 2015). Briefly, mice ($n = 6$ each group in each time point) injected with LPS were subjected to subcutaneous implants of Alzet

osmotic mini-pumps that infuse 10 ng/kg/day of the NOX2 inhibitor DPI at 3 or 6 months after LPS injection. Control mice (n = 6 each group in each time point) were received vehicle with Alzet osmotic mini-pumps. After two weeks implantation, the mini-pumps were removed.

In situ visualization of superoxide and superoxide-derived oxidant production

Analysis of ROS levels was performed using dihydroethidium (DHE) or MitoSox according to a previous report (Wang et al., 2015). DHE is a ROS-sensitive dye that can readily cross the blood–brain barrier and exhibits red fluorescence through interactions with superoxide and other free radicals in the brain. MitoSox is a novel fluorogenic dye specifically targeted to mitochondrial ROS (Zhou et al., 2011). Briefly, after 1, 4, 7 and 10 months of LPS or saline injection, mice were administered a single injection (i.p.) of DHE (20 mg/kg) or MitoSox (10 mg/kg). Eighteen hours later, mice were perfused transcardially with 4% paraformaldehyde and coronal SN and LC sections were examined for the DHE or MitoSox oxidation products using fluorescence microscopy (excitation 534 nm; emission 580 nm). The fluorescence density was quantified as described previously (Wang et al., 2015).

Immunohistochemistry

For immunohistochemistry, mice (n = 5-6 each group) treated with LPS or saline were perfused transcardially with 4% paraformaldehyde and coronal SN and LC sections were used for immune-staining as described previously (Wang et al., 2015). We used the following primary antibodies for immunohistochemistry: tyrosine hydroxylase (TH; 1:2,000; EMD Millipore, Temecula, CA) and ionized calcium binding adaptor molecule-1 (Iba-1; 1:5000; Wako Chemicals, Richmond, VA). Immuno-staining was visualized by using 3,3'-diaminobenzidine (DAB) and urea-hydrogen peroxide tablets or nickel-enhanced DAB or Alexa Fluor 488 (green) and 555 (red) dye. Stereological counts of TH⁺ NE-LC neurons were estimated using an optical fractionator method on an Olympus BX50 stereological microscope within userdefined boundaries (Song et al., 2019a). The quantification of immunohistological staining in different brain regions was performed by Image J software as described previously (Song et al., 2019a; Song et al., 2019b). Briefly, the image was first converted into the grayscale picture, and the background was adjusted before the quantifying area was selected for the measurement of the total pixels. The relative density of the staining was compared based on the density of the total pixels of a certain brain region (total pixels/area).

Confocal double-label immunofluorescence

Free-floating sections including substantia nigra and locus coeruleus brain regions prepared from both saline or LPS-injected mice were immunoblocked with 4% goat serum in 0.25% triton/PBS for 2 hours and then incubated with monoclonal mouse anti-mitochondrial complex IV (COX IV, 1:200, Abcam, Cambridge, MA) or p67^{phox} (1:200, Millipore, Billerica, MA) antibodies overnight at 4 °C. COXIV, a component of the electron transport chain required to maintain adequate flow of electrons through the system in mitochondria, and p67^{phox}, a cytosolic regulatory subunit required for activation of NOX2 (Marty et al., 2006), were used to label mitochondria and NOX2, respectively. On the second day, the sections were washed with 1% BSA in 0.25% triton/PBS before the incubation with

polyclonal rabbit anti-TH antibody (1:2,000 dilutions) overnight at 4 °C. The double-label immunofluorescence pictures were taken under the confocal microscope by using Alexa-488 (green) and Alexa-594 (red) conjugated secondary antibodies (1:1,000) to visualize the double TH and COX IV or p67^{phox}-positive neurons.

Electron microscopic study

The electron microscopic study was performed in both substantia nigra and locus coeruleus regions of mice after 4 or 10 months LPS/vehicle injection (n = 4 each group at each time point). Briefly, mice were transcardially perfused with saline followed by 3.5% paraformaldehyde plus 0.5% glutaraldehyde in 0.1 M PBS. Brains were post-fixed in the same fixative overnight at 4°C and then cut coronally into 50-100 µm thick sections with a microtome. (Lancer Co.). Brain sections (n=3-6) from LC/NE and nigral DA areas of each case were then processed immunohistochemically with anti-rabbit TH antibody (1:1000 dilution; Millipore, Temecula, CA) overnight at 4 °C, further linked to PAP the next morning, and then reacted with DAB. A small area/block (~1 mm × 1 mm) containing TH-immunoreactive neurons in the LC and SN was then dissected out with a No. 10 blade. Typically, 3-4 blocks were dissected from each brain, and then processed with standard EM osmication with *en bloc* staining procedures, flat embedded in epon, attached to beam capsules, trimmed and cut into ultrathin sections. Sections were then collected from the grids and further stained with lead citrate and uranyl acetate. TH-immunoreactive cell body and/or dendrites containing mitochondria from LC and SN were randomly selected and photographed with a Jeol JEM1400 Plus electron microscope equipped with camera attachment. Typically, 8-25 photos were taken at higher magnification (× 6000) from either LC/NE or nigral DA neurons. The number of abnormal mitochondria (~ 100-200 from each case) located within the TH immunoreactive positive profiles were then semi-quantitative analyzed.

Laser Capture Microdissection and Real-time PCR

Laser capture microdissection was performed in sections prepared from saline or LPS-injected mice (n = 5 per group) using anPixCellIIIe (Life Technologies, CA). TH antibody stained cells in substantia nigral and LC regions were identified under the FITC filter of fluorescent microscope of Pixcell instrument and captured using a Capsure™ HS LCM Cap (Life Technologies). 2500-3000 cells were captured and pooled. Total RNA was extracted from the cap after 5 minutes incubation at room temperature using the RNeasy Micro Kit. Total RNA was isolated and eluted in 14 µl of elution buffer following the manufacturer's instructions. The yield and quality of total RNA were determined using 2100 Bioanalyzer 6000 Pico assay. Real-time PCR amplification was performed using SYBR Green PCR Master Mix (Applied Biosystems) and Applied Biosystems 7900HT Fast Real-Time PCR System according to the manufacturer's protocols. The primers were designed by Vector NTI Version: Advance 11 software. The PCR conditions were 95°C for 10 seconds, 55°C for 30 seconds, and 72°C for 30 seconds for 40 cycles. All of the data were normalized to GAPDH.

Statistical analysis

All values were expressed as mean $\pm$ SEM. Differences among means were analyzed using one-way ANOVA with treatment as the independent factors. When ANOVA showed significant differences, pair-wise comparisons between means were tested by Newman-Keuls post-hoc testing. In all analyses, the null hypothesis was rejected at the 0.05 level.

Results

LC neurons exhibit high oxidative stress levels under naïve and chronic neuroinflammation conditions along the caudo-rostral axis

To investigate the reasons why neurons in various brain regions are differentially affected by LPS-induced damage, we first evaluated if oxidative load in naïve mice coincided with this delayed and progressive degenerative profile along the caudo-rostral axis. We used the sensitive intracellular reactive oxygen species (ROS) dye DHE that crosses the blood-brain barrier and emits red fluorescence with variable intensities that correspond proportionally to the oxidized DHE metabolites generated by ROS, such as superoxide (Wang et al., 2015). DHE fluorescence was the most intense at LC in naïve mice and displayed very little fluorescence in the SN (Fig. 1A, B). Confocal analysis showed that DHE fluorescence in the LC was mainly concentrated to TH⁺ noradrenergic neurons (Fig. 1C).

We next evaluated DHE fluorescence in NE-LC and DA-SNpc neurons in mice after 1, 4, 7 and 10 months following LPS exposure. Though aging is associated with increases in ROS in both NE-LC and DA-SNpc neurons of naïve mice (Fig. 1D and Supplementary Fig.1), LPS exposure significantly increased the production of ROS in the LC by 4 months and the SNpc by 7 months (Fig. 1D and Supplementary Fig.1). Consistent with the prolonged resilience of cortical and hippocampal neurons along the caudo-rostral axis of neurodegeneration, DHE fluorescence intensity was only slightly increased by 10 months after LPS exposure (Supplementary Fig. 2). In contrast, no significant increases in ROS production were observed in the VTA (Supplementary Fig. 2).

Chronic neuroinflammation damages mitochondria in NE-LC neurons before DA-SNpc neurons on the caudo-rostral axis

High magnification analysis of DHE fluorescence in NE-LC neurons shows cytosolic punctate resembling mitochondria (Fig. 1C). To determine if the increased oxidative burden in NE-LC neurons compared to DA-SNpc neurons is attributed to mitochondrial dysfunction, we stained sections along the caudo-rostral axis in mice at 4 and 10 months after LPS exposure with MitoSox, a mitochondrial dye that primarily detects superoxide production. During chronic neuroinflammation MitoSox fluorescence displayed a similar caudo-rostral pattern of progression between NE-LC and DA-SNpc neurons at (Fig. 2A, B and Supplementary Fig. 3).

The concordance between the levels of mitochondrial ROS and the spatiotemporal order of neurodegeneration along the caudo-rostral axis suggests that mitochondrial health and function may dictate regional neuronal vulnerability to LPS-elicited damage. To test this hypothesis, we analyzed surrogate markers of mitochondrial function in NE-LC and DA-

SNpc neurons at 4 and 10 months after LPS exposure. We co-immunostained against complex IV (COXIV), a component of the electron transport chain required to maintain adequate flow of electrons through the system, and TH to assess mitochondrial dysfunction in both NE-LC and DA-SNpc neurons. Fluorescent pixel counts showed that mitochondrial COXIV expression was significantly reduced in NE-LC neurons by 40.6% at 4 months and 55.7% at 10 months after LPS exposure compared to their respective age-matched naïve controls (Fig. 2C, D). By contrast, DA-SNpc neurons did not show changes in the expression of mitochondrial COXIV until 10 months after LPS exposure (Fig. 2C, D). Electron micrographs confirm that NE-LC and DA-SNpc neurons show swelling and loss of cristae in 36.3% and 11.4% of their mitochondria, respectively, at 4 months after LPS exposure. Ten months following LPS exposure, abnormal mitochondria accounted for 45.1% of the mitochondrial evaluated in NE-LC neurons and 13.3% in DA-SNpc neurons (Fig. 2E, F).

Over-activation of NOX2 contributes to early mitochondrial dysfunction in response to inflammation in NE-LC neurons

Activation of NOX2 expressed on neurons catalyzes the production of superoxide during neuroinflammation generate intracellular ROS that likely cause oxidant damage to mitochondria to produce greater levels of ROS. We hypothesized that the differences in mitochondrial dysfunction susceptibilities along neurons of the caudo-rostral axis could be attributed to elevated levels of NOX2 activation. To test this possibility, we evaluated if the expression levels of p67^{phox}, a cytosolic regulatory subunit required for activation of NOX2 (Marty et al., 2006), differed between NE-LC and DA-SNpc neurons. Dual immunofluorescence staining against TH and p67^{phox} revealed that 4 months after LPS injection NE-LC neurons had elevated p67^{phox} expression, but not DA-SNpc neurons. (Fig. 3A, B). Significant elevation in p67^{phox} expression in DA-SNpc neurons was observed at 10 months after LPS exposure, but the increase was smaller than that of NE-LC (Fig. 3A, B). Consistent with these findings, qPCR analysis of p67^{phox} and gp91^{phox}, the membrane-bound catalytic subunit of NOX2, revealed LC neurons had nearly three-times higher mRNA expression of both subunits compared to SNpc neurons (Fig. 3C).

To test whether NOX2 contributed to mitochondrial dysfunction along the caudo-rostral axis, we used gp91^{phox}-deficient mice. Mice deficient in gp91^{phox}, the catalytic subunit of NOX2, display no functional NOX2. The levels of mitochondrial ROS were compared between gp91^{phox}-deficient and aged-matched naïve mice (8-mon old). As seen in Fig. 3D and E, gp91^{phox}-deficient mice had significantly reduced MitoSox staining in NE-LC neurons compared to wild type controls with no obvious differences between strains in DA-SNpc neurons.

Pharmacological inhibition of NOX2 attenuates caudo-rostral neurodegeneration *in vivo*

We previously reported that DPI administered at ultra-low doses could specifically inhibits NOX2 to protect DA-SNpc neurons from degeneration in several *in vivo* models of PD even when administered after the onset of neurodegeneration (Wang et al., 2015). Thus, we sought to test whether inhibiting the activation of neuronal NOX2 using DPI could mitigate mitochondrial dysfunction and the caudal-rostral neurodegeneration in our *in vivo* model of chronic neuroinflammation. Mice exposed to a single injection of LPS. Three months later,

they were infused with low dose DPI for two weeks (10 ng/kg/day; s.c. via minipump) and permitted to age for an additional 7 months prior to evaluating immunohistological markers of mitochondrial function (Fig. 4A). DPI significantly inhibited MitoSox localization on NE-LC neurons (Fig. 4B, D), increased the pixel counts of COXIV expression (Fig. 4C, D), and significantly protected neurons against degeneration (Fig. 4E, F) when compared to LPS alone group. DPI administered 6 months after the initial LPS injection showed similar levels of protection, even though at this time point NE-LC neurodegeneration had already begun (Fig. 4G, H). Together, this data provides strong evidence that NOX2 serves as a mediator of mitochondrial dysfunction during conditions of chronic neuroinflammation and its differential expression contribute to the ascending caudo-rostral neurodegeneration phenomenon associated with many neurodegenerative disorders.

Discussion

Three significant findings can be drawn from this study. First, chronic neuroinflammation exacerbates oxidative stress with increased severity towards the most vulnerable NE-LC neurons that are the first to degenerate along the caudo-rostral axis. Second, the degree of vulnerability of these neuronal populations was proportionally corresponded to the number of dysfunctional mitochondria they possess. Third, the activation of NOX2 on degenerating neurons along the caudo-rostral axis is both necessary and sufficient to dictate the severity of the mitochondrial dysfunction since its inhibition can mitigate mitochondrial damage and neurodegeneration. Collectively, these findings imply that extinguishing chronic neuroinflammation may delay the caudo-rostral progression of PD, providing new therapeutic opportunities to ameliorate the course and symptoms of neurodegenerative disorders.

Chronic neuroinflammation drives caudo-rostral degeneration

Chronic neuroinflammation is associated with many neurodegenerative diseases, including PD (Ouchi et al., 2009). Unbiased genome-wide association studies (GWAS) have shown strong associations between mutations to genes that impair anti-inflammatory functions in microglia and alter leukocyte recruitment (*TREM2*, *CLU*, *CD31*, *EPHA1*, *CR1* and *HLA*) to the pathogenesis of AD and PD (Carter, 2011; Neumann and Daly, 2013). We recently reported that chronic neuroinflammation elicited by a single systemic injection of LPS in mice is sufficient to produce not only degeneration of DA-SNpc neurons, but also vulnerable neuronal populations outside the basal ganglia, in a progressive temporal order (Song et al., 2019a). Specifically, chronic neuroinflammation significantly and selectively degenerates NE-LC neurons within 4 months of initiation to precede significant DA-SNpc neurodegeneration by 3 months (Song et al., 2019a). Cortical and hippocampal neurodegeneration is observed at later stages (Song et al., 2019a), which is overall consistent with the progression of neurodegeneration that occurs along the caudo-rostral axis in PD patients.

Since this model of chronic neuroinflammation is global (Supplementary Fig. 4), we suspected that innate vulnerabilities among the neuronal populations along the caudo-rostral axis may be essential in determining the order at which they degenerate. DA-SNpc and NE-LC

are vulnerable neuronal populations to irreversible oxidative damage which accounts for their respective degeneration of 20-46% (Lohr and Jeste, 1988; Rudow et al., 2008; Stark and Pakkenberg, 2004) and 31-46% (Lohr and Jeste, 1988) during aging in elderly control mice. DA-SNpc neurons have elevated ROS damage from high neuromelanin concentrations in the presence of transition metals, reactive dopamine metabolites, Fenton reactions from elevated heavy metal accumulation and an inability to restore oxidant: antioxidant balance due to intrinsic tonic firing [26]. NE-LC neurons share many features that cause high levels of oxidative damage in DA-SNpc neurons, yet NE-LC neurons have greater levels of oxidative and nitrosylative stress and mitochondrial injury compared to DA-SNpc neurons as shown in this study and previous reports [27, 28], suggesting that other innate factors may account for the heightened vulnerability of NE-LC neurons.

Chronic neuroinflammation triggers mitochondrial oxidative stress on vulnerable neurons along the caudo-rostral axis

To better understand the mechanism by which global chronic neuroinflammation selectively drives neurodegeneration along caudo-rostral axis, we focused on understanding the differences in susceptibilities between NE-LC and DA-SNpc neurons. The death of neurons in neurodegenerative disorders is thought to be attributed primarily to apoptosis resulting from oxidative stress, mitochondrial dysfunction, and perturbed calcium homeostasis (Mattson, 2000). Thus, we investigated whether chronic neuroinflammation enhanced oxidative damage in neurons along the caudo-rostral axis and whether these changes proportionally preceded their respective sites of neurodegeneration. The most vulnerable population of neurons (i.e. NE-LC neurons) along the caudo-rostral axis had heightened mitochondrial dysfunction including higher levels of mitochondrial ROS, decreased complex IV expression and ultrastructural abnormalities.

Though the link between chronic neuroinflammation, neuronal ROS and mitochondrial dysfunction has been speculated by many to be involved in neurodegenerative diseases (Witte et al., 2010), our findings reveal new insights into this relationship. For instance, our findings showed that mitochondrial ROS not only preceded neurodegeneration but also predict it along the caudo-rostral axis in accordance to the unique susceptibility of distinct neuronal populations along this axis. NE-LC neurons were far more susceptible to neuroinflammation-induced mitochondrial damage than DA-SNpc neurons. One possible explanation for this susceptibility is that there are 10-20 fold more DA-SNpc neurons than NE-LC neurons in rodents (Sara, 2009), yet NE-LC neurons have ~11-times more varicosities per neuron than DA-SNpc neurons due to their significantly wider projections (Berridge and Waterhouse, 2003). Varicosities are rich in mitochondria, which is required to provide adequate energy for neurotransmission, and thus replacing damaged mitochondria is rather energetically demanding given the cost to transport new mitochondria from the cell body using the axoplasmic transport system (Aiello and Bach-y-Rita, 2000). Thus, we speculated that the energy demands required to maintain normal physiological functions in NE-LC neurons, particularly during inflammation-mediated oxidative stress, is likely far greater than that of DA-SNpc neurons and renders them more susceptible to bioenergetic failure attributed to exogenous insults that alter their function.

NOX2 activation during neuroinflammation differs along caudo-rostral neurons to dictate mitochondrial dysfunction severity

Nitrosylation from increased nitric oxide synthase (NOS) activity is implicated in mitochondrial damage in NE-LC and DA-SNpc neurons (Chinta and Andersen, 2011; Nakamura and Lipton, 2011). It was shown that pharmacological inhibition of NOS in naïve neuronal cultures is sufficient to decrease mitochondrial oxidation and reduce fluctuations in the mitochondrial membrane potential (Sanchez-Padilla et al., 2014). Nitrosylation increases superoxide byproducts from the electron transport chain (ETC) and nitric oxide (NO) can react with superoxide anions to generate peroxynitrite, which impairs mitochondrial respiration to ultimately inducing cell death (Szabo et al., 2007). Superoxide generated by NOX2 was originally thought to be restricted to immune cells that sequester and destroy microbial pathogens via oxidative bursts within their phagosomes (Block et al., 2007). Recently, NOX2 has been identified in neurons and verified to generate intracellular ROS upon activation, resulting in self-oxidation and cell death if sustained (Infanger et al., 2006). We found NE-LC neurons expressed the highest levels of NOX2 in comparison to other neuronal populations along the caudo-rostral axis of mice with chronic neuroinflammation. More importantly, pharmacological or genetic inhibition of NOX2 can mitigate mitochondrial oxidative stress and protected NE-LC neurons from degeneration. Thus, we concluded that NOX2-generated superoxide is important in driving mitochondrial dysfunction in the vulnerable neuronal populations along the caudo-rostral axis.

How neuronal NOX2 activation during chronic neuroinflammation impacts mitochondrial function is not fully understood. In angiotensin II (AngII)-induced models of hypertension and vascular dysfunction, Dikalov *et al* (Dikalov et al., 2014) and Doughan *et al* (Doughan et al., 2008) reported that depletion of NOX2 using siRNA or genetic ablation of gp91^{phox}/p22^{phox} inhibits mitochondrial superoxide production. Subsequent studies revealed that activating PKC ϵ and the mitochondrial ATP-sensitive potassium channel (mitoK_{ATP}) as well as reverse electron transfer is the potential mechanism underlying the regulation of mitochondria by NOX2 (Dikalov et al., 2014; Doughan et al., 2008). Opening of mitoK_{ATP} channel by NOX2-generated superoxide has been proposed to increase potassium influx that results in matrix alkalinization, swelling, mild mitochondrial uncoupling, and increased ROS production (Costa et al., 2006). Since proinflammatory cytokines, such as TNF α , are capable of stimulating activation of mitoK_{ATP} (El-Ani and Zimlichman, 2003), we speculated that the same mechanism could also be applied to the regulation of mitochondrial function by NOX2 under neuroinflammatory conditions. It is interesting to point out that mitochondria are not only a passive target for superoxide generated by NOX2 but also a significant source of ROS, which under certain conditions can stimulate activation of NOX2 to produce more intracellular ROS, resulting in additional oxidative damage (Schulz et al., 2014). Therefore, a self-propelling vicious cycle is formed between NOX2 and damaged mitochondria within neurons that could be served as a driving force for persistent oxidative stress and mitochondrial injury. Interrupting this self-propelling cycle by inhibiting NOX2 could be a novel therapeutic strategy to halt the progressive degeneration along the caudo-rostral axis in neurodegenerative disorders.

NOX2 inhibition as a therapeutic strategy for caudo-rostral neurodegeneration

Under the presumption that NOX2 activation is indispensable for neurodegeneration, we identified several NOX2 inhibitors capable of protecting dopaminergic neurons from PD-related degeneration (Block et al., 2007; Gao et al., 2012). Unlike traditional NSAID therapies that suppress production of prostaglandins or human monoclonal antibody therapies that target cytokines and their receptors, we have shown that infusion of ultralow levels of NOX2 inhibitors can extinguish chronic neuroinflammation (Wang et al., 2015) and directly rescue the vulnerable neuronal populations along the caudal-rostral axis by suppressing the accumulation of intracellular ROS and mitochondrial dysfunction. NOX2 inhibitors are therefore potent targets for the development of novel drugs for the treatment of neurodegenerative disorders.

Conclusion

In summary, our findings reveal innate differences in susceptibility of distinct neuronal populations along the caudo-rostral axis. We found that the expression of NOX2 dictates the severity of mitochondrial dysfunction and vulnerability to neuroinflammation-driven degeneration in these populations, thus providing a target to prevent the ascending pattern of neurodegeneration observed in PD.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1.

NE-LC neurons exhibit high levels of oxidative stress under baseline and conditions of chronic neuroinflammation. (A) C57BL/6J mice received an injection of DHE and 18 hours later their brains were analyzed for oxidation products (stained in red) by DHE using fluorescence microscopy. Representative images of different brain regions along the caudal-rostral axis are shown. NE-LC, DA-SNpc and DA-VTA neurons were identified by using anti-TH antibody (green) and their respective (B) fluorescence densitometry for DHE oxidation was calculated. (C) The cellular origins of DHE oxidation near TH⁺ NE-LC neurons were also examined by co-labeling for Iba-1⁺ microglia and GFAP⁺ astrocytes. (D) DHE expression in the SNpc and LC of mice were assessed at 1, 4, 7 and 10 months after LPS or saline exposure and their respective fluorescence densitometry was quantified. $n = 5$ or 6 for saline or LPS group, respectively. * $p < 0.05$, ** $p < 0.01$. Scale Bar = 50 μ m.

Figure 2.

Chronic neuroinflammation damages mitochondria in NE-LC neurons prior to afflicting DA-SNpc neurons. (A) Representative images of oxidant damage from mitochondria-derived superoxide was assessed in the SN and LC of mice at 4 or 10 months following LPS or saline exposure using the selective dye MitoSox (red) and their respective (B) fluorescence densitometry was quantified. (C) TH⁺ NE-LC and TH⁺ DA-SNpc neurons were co-immunostained against mitochondrial COX IV at 4 and 10 months following exposure to LPS or a vehicle control and their respective (D) fluorescence densitometry was quantified. $n = 5$ or 6 for saline or LPS group, respectively. (E) Ultrastructural analysis of mitochondria in TH⁺ NE-LC and TH⁺ DA-SNpc neurons were performed 4 and 10 months after LPS or saline injection using immuno-electron microscopy and their respective (F) frequency of structural abnormalities were quantified. $n = 4$ for each group at each time point. * $p < 0.05$, ** $p < 0.01$; Scale Bar = 100 μm in a and c, 200 nm in e(a) and e(b) and 800 nm in e(c) and e(d).

Figure 3.

NE-LC neurons exhibit high levels of NOX2 under baseline and conditions of chronic neuroinflammation. (A) TH⁺ NE-LC and TH⁺ DA-SNpc neurons were immunostained against p67^{phox} at 4 and 10 months following exposure to LPS or a vehicle control and their respective (B) fluorescence densitometry quantified. *n* = 5 or 6 for saline or LPS group, respectively. (C) Laser microdissection of NE-LC and DA-SNpc neurons 10 months after LPS exposure underwent RT-PCR to quantify mRNA transcript levels of p67^{phox} and gp91^{phox}. *n* = 5. (D) Seven to eight-month old WT and NOX2^{-/-} mice (8-mon old) received a single injection of MitoSox. Eighteen hours later, the brains were perfused and the production of superoxide from mitochondria in SN and LC was determined by measuring the oxidation products (red) of MitoSox, a selective mitochondria ROS dye, by fluorescence

microscopy and representative images were shown. (E) The fluorescence densitometry was quantified. $n = 5$ for each group. $*p < 0.05$, $**p < 0.01$; Bar = 100 μm .

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Figure 4.

NOX2 inhibition attenuates mitochondrial dysfunction and protects NE-LC neurons from inflammation-induced degeneration *in vivo*. (A) Mice received a single injection of LPS (15×10^6 EU/kg; i.p.) or saline. Three months post-injection, mice were infused with ultra-low dose DPI via osmotic mini-pump (10 ng/kg/day, s.c) for 2 weeks. Seven months after DPI infusion, the protective effects of DPI against LPS-induced damage were determined. (B) Mitochondria-generated superoxide in NE-LC neurons of LPS-injected mice with or without DPI treatment was detected using MitoSox and representative images were shown. (C) Mitochondrial COXIV in NE-LC neurons of LPS-injected mice with or without DPI treatment was detected by double-immunofluorescence staining with anti-COXIV and TH antibodies and representative images were shown. (D) The density of MitoSox oxidation and COX IV immunostaining was quantified. (E) NE-LC neurons were stained with anti-TH antibody and the representative images were shown. (F) The protective effects of DPI administered 3 months after LPS injection against LC neurodegeneration were determined

by counts THir cells. (G, H) The protective effects of DPI administrated 6 months after LPS injection against LC neurodegeneration were determined by counts THir cells. n = 6 for each group; * $p < 0.05$, ** $p < 0.01$; Bar = 100 μm .

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