
# Neuroprotective Effects of Carnitinoid Compounds in Rodent Cellular and *in vivo* Models of Mitochondrial Complex I Dysfunction

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**Abstract:**

Rotenone-mediated mitochondrial complex I inhibition was used to model Parkinson's disease-like syndrome in Lewis rats. Tyrosine hydroxylase immunolabeling demonstrated a decrease in the number of dopaminergic neurons as well as aberrant morphology in surviving neurons. Administration of carnitinoid compounds (synthetic lipoylcarnitine or butyrylcarnitine compounds) reduced dopaminergic neuronal cell loss with characteristic morphology observed in surviving neurons. In a rat primordial hippocampal cell line (H19-7/IGF-IR), rotenone treatment resulted in increased ROS and reduced cellular ATP, while co-treatment with lipoylcarnitine maintained ROS and ATP at control levels. These results illustrate the therapeutic potential of small-molecule carnitinoids in treating neurodegenerative diseases associated with mitochondrial dysfunction.

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## INTRODUCTION

Mitochondria are the primary source of cellular metabolic energy in eukaryotes and are essential for normal cellular function, proliferation and survival. Mitochondrial dysfunction can have severe negative ramifications on cellular function, particularly in tissues with elevated energy requirements. Mitochondria have been implicated in a number of human neurodegenerative diseases affecting both the old and the young [1]. As the entry point of high-energy electrons affording maximal proton-motive force, mitochondrial complex I (CI; NADH-CoQ reductase) of the mitochondrial electron transport chain is a critical initiator of mitochondrial energy transduction. Functional disruption of CI can drastically reduce neuronal energy production while increasing generation of harmful reactive oxygen species (ROS). If the resulting oxidative damage is not somehow alleviated, intrinsic pro-apoptotic pathways may be activated [2], setting the stage for a variety of human neuropathological conditions [3]. In animal models, experimentally-induced CI inhibition results in aberrant neuronal bioenergetics that can manifest as abnormal neurobehavioral phenotypes. This fundamental etiology is representative of many progressive neurodegenerative diseases, including mitochondrial encephalomyopathy; lactic acidosis, and stroke-like episodes (MELAS); Leigh's syndrome; Alzheimer's disease; and Parkinson's disease (PD) [4,5]. While the prevalence of MELAS and Leigh's syndrome is low, Alzheimer's disease affects more than five million people in the United States alone [6], while PD affects at least one million people in the United States and is projected to affect over nine million people worldwide by 2030 [7].

Dysfunctional mitochondria are normally degraded by mitophagy, a specialized form of autophagy in which mitochondria are sequestered into autophagosomes that subsequently fuse with lysosomes for enzymatic degradation of their contents [8]. Mitophagy is critical for maintaining mitochondrial quality, reducing the generation of ROS from damaged mitochondria, and preventing errant triggering of intrinsic apoptotic pathways [9]. Reports suggest that, while moderate oxidative stress and mitochondrial damage might compromise some autophagic processes [10], mild oxidative stress appears to promote mitophagy [11]. In some progressive neurodegenerative diseases, continued production of ROS by dysfunctional mitochondria is believed to overwhelm the capacity for clearance by mitophagy. The overproduction of ROS can contribute to cellular damage and triggering of apoptosis, or, in extreme situations, uncontrolled cell death

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(necrosis). Mitochondrial-targeted antioxidants have the potential to mitigate ROS generation by CI and thereby reduce mitochondrial injury and its consequences.

The pesticide rotenone is a potent and irreversible inhibitor of mitochondrial CI [12]. Rotenone exposure was found to reduce ATP production and increase accumulation of reactive oxygen and nitrogen species, including superoxide and peroxynitrites. These accumulations result in free radical-induced damage to mitochondrial DNA (mtDNA) and mitochondrial proteins and membranes [13] along with nuclear DNA and other cellular components. Impaired mitochondrial function and eventual death of dopaminergic (DA) neurons of the ventral SNpc region of the midbrain are associated with PD [14]. DA neurons are thought to be particularly vulnerable to oxidative stress due to their high basal rate of oxygen metabolism, high iron content, and insufficient cellular antioxidants [15]. Demonstrable neurodegeneration of SNpc neurons in certain rodent models of PD makes these models useful in evaluating potential therapeutic compounds—not only for PD, but for a variety of human diseases in which mitochondrial dysfunction contributes to disease pathology. In general, animal models of PD may be regarded as representative of CI-associated energy metabolism defects and associated neuropathology [16]. As such, the rat-rotenone model is useful in evaluating potential therapies for mitochondrial disease.

PMX-500FI (lipoyl-L-carnitine methyl ester iodide) is a novel, synthetic small-molecule compound that contains both L-carnitine (LC) and α-lipoic acid (ALA) moieties. In normal metabolism, cells utilize LC to shuttle long-chain fatty acids into mitochondria where they are burned for energy. LC also displays antioxidant activity. ALA is an essential cofactor in aerobic metabolism and one of the most potent antioxidants in nature. Although ALA is present in a variety of foods, its bioavailability from dietary intake is limited. Both ALA and a therapeutic combination of LC and ALA were shown to have beneficial effects in rodent models of PD [17,18]. ALA and LC are also common components in various formulations of the "Mito Cocktail", a patient-specific combination of vitamins, co-factors, antioxidants and other supplements intended to alleviate some of the symptoms of mitochondrial disease.

PMX-550DBr (butyryl-L-carnitine ethyl ester bromide) was engineered to leverage the mitochondrial targeting ability of carnitine with the histone deacetylase activity of butyrate [19]. Butyric acid was shown to stimulate neuroprotective cellular pathways in response to oxidative damage [20,21] and has shown promise in treating a variety of neuropathologies including certain types of cancer [22].

Previously, our group demonstrated that PMX-500FI protected against rotenone-induced impairment of neuronal function and bioenergetics in the mouse hippocampus [23]. Oxidative stress and neuromotor deficits were also reduced by PMX-500FI in rotenone-treated mice [24,25]. The present study evaluated the neuroprotective potential of PMX-500FI and PMX-550DBr in rotenone-treated Lewis rats, a common animal model of mitochondrial dysfunction-induced PD.

We modified the protocol of Cannon et al. [26] to produce a PD phenotype in Lewis rats via rotenone administration and utilized this model for in vivo testing of synthetic small-molecule carnitinoid compounds that were developed specifically for restoring normal mitochondrial function in mitochondrial disease and other human pathologies associated with mitochondrial dysfunction [21,27]. These compounds were designed to be actively taken up by mitochondria and to neutralize harmful oxidants, while potentially modulating the expression of genes involved in recycling of defective mitochondria. Our experiments demonstrated that both PMX-500FI and PMX-550DBr co-administration in rotenone-treated rats had a neuroprotective effect on SNpc DA neurons; however, there was no discernable neuromotor/behavioral improvement. In addition, we include further evidence for a protective effect of PMX-500FI on mitochondrial bioenergetics and ROS clearance in rotenone-induced mitochondrial dysfunction in the rat H19-7/IGF-IR primordial hippocampal cell line ("H19-7 cells").

## RESULTS

### Neither PMX Compound Improved Performance in Neuromotor Testing in Rotenone-Treated Rats.

In our modeling of mitochondrial dysfunction in rats, daily injections of rotenone for 14 days produced a characteristic phenotype in Lewis rats, and resulted in degeneration of TH-ir DA neurons of the SNpc (Figure **1b**). A variety of tests were performed to assess the CI inhibition-mediated phenotype in this model and to determine if PMX co-treatment had a beneficial effect on various neuromotor parameters. The rotenone-induced phenotype included bradykinesia, hind limb rigidity and weight loss, along with reduced performance in balance beam, rotarod, and most facets of the open field test ($p<0.05$). In initial trials, testing two days after treatment showed no beneficial effect for either PMX-500FI or PMX-550DBr, prompting modification of the protocol to introduce a one-week recovery period so that the animals could recuperate from possible systemic effects of acute rotenone toxicity. Even with an extended (one-week) recovery period, no performance differences were detectable in any neuromotor or behavioral tests in PMX-treated rats. Co-administration of rotenone treated rats with either PMX-550DBr or ALA + LC had a positive effect on weight gain during the recovery period ($p<0.05$).

### PMX-500FI and PMX-550DBr Protected DA Neurons in Rotenone-Treated Rats.

Following neuromotor/behavioral testing, post-mortem samples of brain were collected for immunohistochemical analyses [28]. DA neurons in sections of midbrain were labeled with an antibody to tyrosine hydroxylase, the rate-limiting enzyme involved in dopamine synthesis. Neuroprotection was visually discernable in midbrain sections from rats co-treated with PMX compounds (Figure **1**). Quantitative analysis confirmed that PMX-500FI and PMX-550DBr co-treatment protected DA neurons from rotenone-induced degeneration, and that PMX-500FI, PMX-550DBr and ALA+LC co-

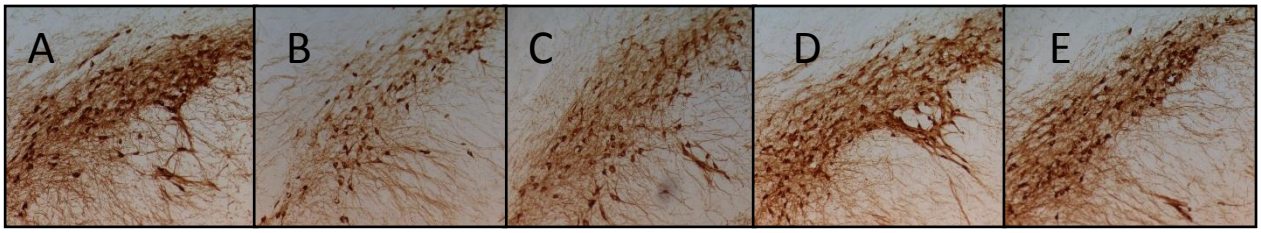


**Fig. (1).**Immunohistochemistry of rat brain SNpc labeled with a primary antibody specific for tyrosine hydroxylase. Control untreated (A), Rotenone treated (B), Rotenone co-treated with ALA+LC (C), Rotenone co-treated with PMX-500FI (D), Rotenone co-treated with PMX-550DBr (E).

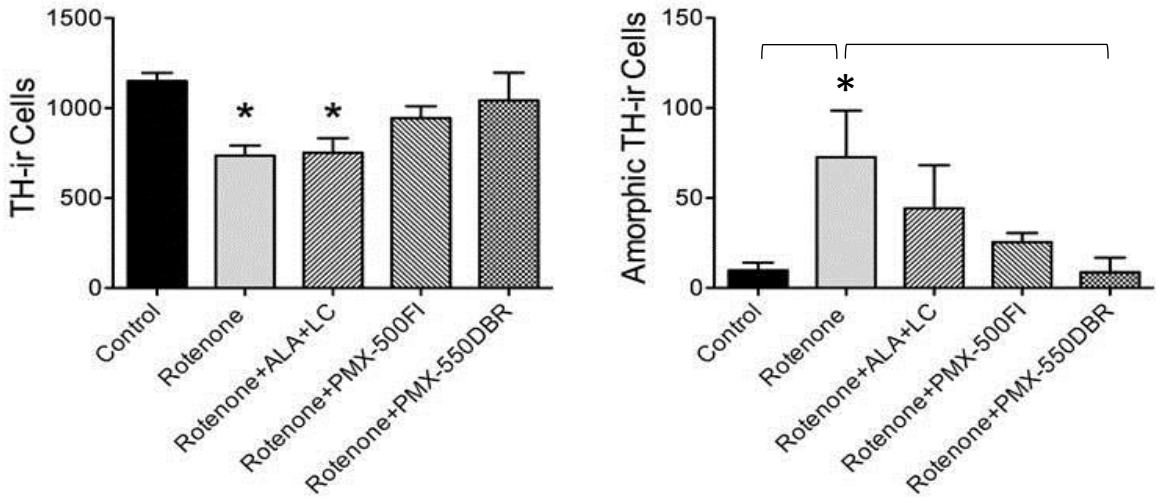


**Fig. (2).**Quantitative immunohistochemical analysis of TH-ir neurons in SNpc of rat brain striatum in rats treated with rotenone only and in rotenone-treated rats that were co-treated with the lipoylcarnitine PMX-500FI or the butyrylcarnitine PMX-550DBr. TH-ir (DA neurons) cell counts and necrotic (amorphic) TH-ir cell counts were similar in samples from control rats and rotenone-treated rats that were co-treated with either PMX-500FI or PMX-550DBr, while rotenone-only treated rats showed decreased TH-ir cell counts and increased amorphic TH-ir cell counts. TH-ir cell counts from rotenone-treated rats that were co-treated with a combination of ALA and LC showed no improvement compared to rotenone only. [*$p < 0.05$; control untreated, n= 5; rotenone only, n=6; rotenone + co-treatments (ALA + LC, PMX-500FI, or PMX-550BDr), n=5].

treatments also resulted in fewer surviving TH-ir cells displaying abnormal morphology (Figure **2**).

### Rotenone-Induced Intracellular ROS Generation in H19-7 Cells was Reduced by PMX-500FI

Dysfunctional mitochondria, such as those with diminished CI function, are major sources of cellular ROS generation. In pilot experiments, different concentrations and durations of rotenone exposure were tested for ROS production. Initially, we determined that 10 μM rotenone induced high levels of ROS generation, without causing cell loss ($p<0.05$), in H19-7 primordial hippocampal neurons. Neurons were subsequently exposed to 10 μM rotenone for different durations; results from these experiments showed that 4 h exposure was optimal for inducing ROS production (Figure **3A**; n=6, $p<0.01$), whereas longer exposures resulted in excessive cell death (data not shown). We then assessed the effectiveness of PMX-500FI in reducing ROS using 0.2, 0.5 or 1.0 mM PMX-500FI. After 3.5 h of exposure to rotenone and PMX-500FI, 2',7'–dichlorofluorescein diacetate (DCFDA, a fluorogenic dye used to measure hydroxyl, peroxyl and other ROS) was added to a final concentration of 20 μM and incubated for an additional 30 min. Results illustrated that rotenone exposure increased ROS generation (514 ± 11.1 arbitrary units (AU) after rotenone exposure and 415 ± 19.0 AU in controls [mean ± SEM]) and PMX-500FI reduced ROS levels (347 ± 29.7, 334 ± 7.7 and 297 ± 6.2 AUs for PMX-500FI doses 0.2, 0.5, and 1 mM, respectively) generated by 4 h rotenone exposure (Figure 3B; n=6, $p<0.001$).

### Reduction of ATP Levels by Rotenone in H19-7 Cells was Reversed by PMX-500FI

Oxidative stress from excessive ROS production results in mitochondrial damage and reduction in mitochondrial abundance. Since PMX-500FI reduced intracellular ROS, we tested the ability of PMX-500FI to preserve mitochondrial energy production in H19-7 cells. Results of these experiments illustrate that rotenone reduced ATP production (476 ± 6.2 nM in rotenone treated cells when compared to 520 ± 19.5 nM in controls) and PMX-500FI prevented the reduction in ATP (529 ± 9.1 and 519 ± 5.5 nM for 0.5 mM and 1 mM PMX-500FI doses, respectively) in rotenone-exposed H19-7 cells

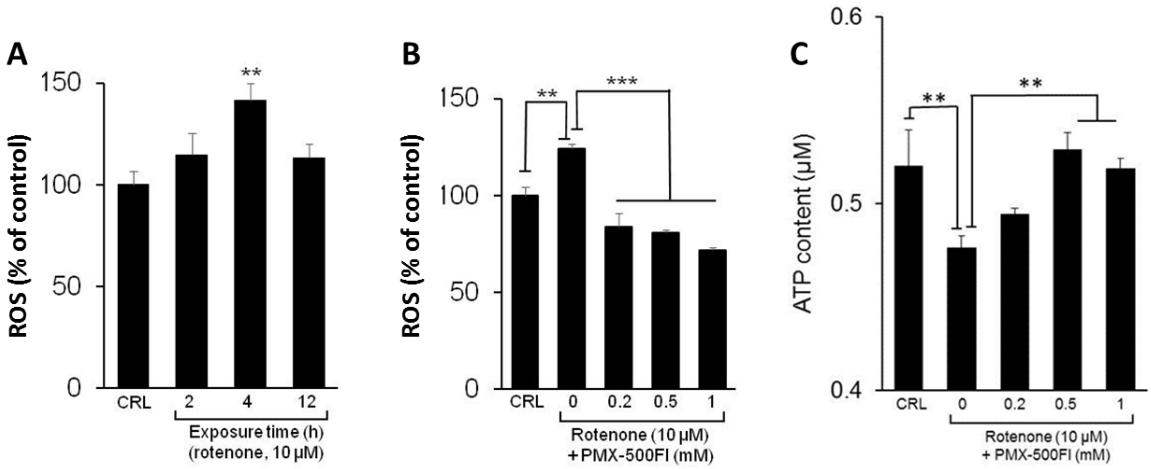


**Fig. (3).** ROS production and ATP content in H19-7 rat primordial hippocampal neurons after selective CI inhibition by rotenone and protective effects of PMX-500FI. (A) Four hour duration of rotenone (10 μM) exposure increased ROS (n=6; **p<0.01). (B) Co-exposure of PMX-500FI with rotenone (4 h) resulted in reduced ROS (n=6; **p<0.01, ***p<0.001). (C) Rotenone exposure (10 μM, 4 h) reduced cellular ATP levels. Treatment with PMX-500FI resulted in preservation of ATP levels (n=4; **p<0.01).

(Figure 3C; n=4, p<0.01). Hence, PMX-500FI mitigated ROS production and improved bioenergetics in cells with rotenone-induced impairment of CI function.

## DISCUSSION

The central nervous system (CNS) is highly susceptible to oxidative injury due to its high oxygen consumption combined with a relatively high basal production of ROS and the loss of regenerative capacity of the majority of neurons after differentiation. This implies that an effective first line of therapeutic defense against oxidative injury could be the use of potent exogenous antioxidant compounds that supplement the actions of endogenous antioxidants, increasing the net antioxidant defense capacity. Unfortunately, synthetic antioxidants have generally proven severely limited in their ability to provide neuroprotection, necessitating more effective alternatives [29]. We recently demonstrated that a novel lipoylcarnitine antioxidant compound, PMX-500FI, protected mouse hippocampi from rotenone-induced deficits in synaptic plasticity and cellular stress signaling [25]. Here we demonstrate that PMX-500FI reduced ROS and improved energy (ATP) production in a neuronal cell line challenged with rotenone.

Although the mechanisms of CI inhibition by rotenone have not been fully elucidated, there is an established correlation between CI inhibition and apoptosis, triggered by excess generation of ROS [30]. In non-proliferating mature neurons, mitochondrial dysfunction and apoptosis are associated with increased ROS production [31]. We found a substantial increase in ROS production with concentrations of rotenone higher than 0.1 μM, in agreement with reports that showed a similar increase in ROS in HL-60 cells [30] and HeLa cells [32]. Interestingly human neural stem cells required higher concentrations of rotenone for ROS production [33]. Results of this study show a relatively high dose but short duration (4 h) of rotenone exposure produced high levels of ROS. However, a longer (12 h) exposure time resulted in markedly reduced ROS levels likely due to rapid onset of death and clearance of cells. Results of this and other studies confirm that CI inhibition by rotenone results in enhanced generation of ROS in neurons. A major protective mechanism of natural cellular antioxidants is to scavenge ROS and thereby reduce cellular injury. Several natural and synthetic compounds were reported to effectively scavenge ROS resulting in neuroprotection [34,35]. Results of this study clearly demonstrate that PMX-500FI treatment reduced ROS levels, indicating a direct ROS scavenging capability and/or prevention of ROS generation by this antioxidant.

There is an intricate relationship among mitochondrial dysfunction, ROS production and decreased ATP production. Increased cellular ROS production is a potent factor that can reduce mitochondrial ATP output [36] and even a modest 30% decrease in ATP is capable of precipitating apoptosis [37]. Results of this study also show that rotenone-induced ROS generation was associated with reduced ATP levels. Some antioxidants have been shown to preserve ATP levels in cells and tissues under oxidative stress [38]; and, indeed, results of this study indicate that PMX-500FI decreased ROS levels while also preventing reduced generation of ATP.

Various genetic approaches have been used to model human neurodegenerative diseases in rodents, from fusion of cytoplasts with ES cells (resulting in "cybrid" cells [39]) to produce transmitochondrial mice harboring mtDNA mutations [39,40] to conventional transgenic models of PD [41], Alzheimer's [42], Huntington's [43] and other CNS neurodegenerative diseases. Although the technology for manipulating nuclear genes in a directed fashion is well established and straightforward (particularly for mice), production of animal models that faithfully mimic human diseases associated with mtDNA mutations has proven elusive [40]. However, considering the fact that seven of the thirteen polypeptides encoded by mtDNA are CI subunits, it would logically follow that CI function is particularly vulnerable to random mutations of mtDNA. Therefore, specific inhibition of CI (e.g., with chemicals such as rotenone) might prove useful

for modelling some of the general phenotypic effects of mtDNA mutation, including neurodegenerative diseases in which a decline in CI function is often associated with CNS pathology.

Rotenone neurotoxicity in rodent models of PD is believed to be mediated principally by CI dysfunction and the resulting concomitant production of excess ROS and oxidative damage. While rotenone is clearly a specific and irreversible inhibitor of CI, it also displays microtubule depolymerization activity, which should not be discounted in terms of its potential to affect neuronal function [44]. In addition, there is evidence that at least part of the rotenone-induced phenotype in rodent models is due to general systemic toxicity and bears no relevance to PD-like symptoms [45]. Although rodent models of PD are useful, the complexity of environmental and multigenic interactions that come into play in spontaneous idiopathic cases of PD should serve to dissuade one from making overarching inferences regarding the utility of any particular animal or cellular model for the study of this disease [46].

In this study, we found that two synthetic carnitinoid compounds, a lipoylcarnitine (PMX-500FI) and a butyrylcarnitine (PMX-550DBr), conferred neuroprotection from rotenone-induced depletion of TH-ir neurons in the SNpc of Lewis rats, and that surviving TH-ir neurons appeared more normal by morphometric analysis. The mechanisms of neuroprotection in these models likely relate in large part to improved mitochondrial function, given that rotenone acts primarily on CI function. However, epigenetic histone deacetylase inhibitory (HDACi) activity of PMX-500FI and PMX-550DBr [47], might also provide neuroprotective effects via induction of antioxidant response elements which upregulate transcription of cytoprotective genes, including components of the cellular antioxidant system that functions to balance high ROS levels.

Rotenone-induced CI inhibition in Lewis rats is a well-established animal model of PD [12,26]; however, most studies to date administered rotenone over longer periods (typically 28 days) and used very narrowly defined, single-method measures for assessing neuromotor impairment. In this study, we sought to develop a treatment regimen that produced a less debilitating phenotype that would allow for more subtle and more diverse assessment of neuromotor function, and would thus lend itself to more informative preclinical evaluation of potential therapeutic compounds, such as the PMX compounds investigated in this study. With our modified protocol using Lewis rats, neuromotor testing showed a clear rotenone-induced deficit; however, although PMX-treated rats subjectively appeared to recover somewhat from rotenone-induced bradykinesia and hind limb rigidity, improved performance in neuromotor testing was not documented.

In this study, a clear neuroprotective effect of PMX-500FI or PMX-550DBr co-treatment by quantitative analysis of tyrosine hydroxylase immunohistochemical labeling of DA neurons in the SNpc was demonstrated. As it was our aim to correlate neuromotor performance gains with neuroprotection of SNpc DA neurons, we modified the protocol to include a longer (one-week) recovery period after rotenone/PMX treatments (prior to neuromotor testing). Although we found a beneficial effect on recovery from rotenone-induced weight loss for PMX-550DBr with the one-week recovery period, no improvements in neuromotor or behavioral performance were noted.

Previous studies from our laboratory on rotenone-induced CI dysfunction in normal mice showed beneficial effects from PMX-500FI in a variety of neuronal functions, including protection against ROS production in brain and neuromotor decline [25], increased long term potentiation in hippocampal neurons and stabilization of synaptic physiology and transmission [23]. PMX-500FI co-treatment revealed a beneficial effect on rotenone-mediated activation of oxidative stress-related proteins SAPK/JNK and pERK1/2. PMX-500FI showed presumptive antiapoptotic activity via phosphorylation of BAD (pBAD) and decreased BAX translocation to mitochondria [23]. PMX-500FI and PMX-550DBr also demonstrated HDACi activity via hyperacetylation of histone H3 in assays using the H19-7 cell line [47].

Impaired mitochondrial function is associated with most progressive neurodegenerative diseases as well as with the normal aging process itself [48], and is central to the pathological changes in mitochondrial disease proper. Increased production of ROS along with insufficient ATP production by dysfunctional mitochondria could potentially overwhelm the cellular capacity for clearance by mitophagy and accelerate oxidative damage and triggering of intrinsic apoptotic pathways. Mitochondrial-targeted antioxidants that also possess HDACi activity have the potential to mitigate ROS generation and potentially stimulate recycling of dysfunctional mitochondria, thereby reducing mitochondrial injury and its consequences in these devastating diseases. As such, they represent a class of compounds with possible clinical relevance that warrant continued study.

## METHODS

### Reagents

Rotenone used in cell culture and rat experiments was obtained from MP Biomedicals (Solon, OH, USA). Miglyol 812N (Sasol Germany GmbH), used as a vehicle for rotenone injection [26], became unavailable early on in the study and was replaced with a similar product, Neobee M-5 (a caprylic/capric triglyceride; Spectrum Chemical Mfg., New Brunswick, NJ, USA). PMX-500FI, PMX-550DBr, ALA and LC were supplied by PhenoMatriX, Inc. (Natick, MA, USA). Each was dissolved in dimethyl sulfoxide (DMSO) at 40-80 mg/ml, except for LC, which was dissolved in PBS at 40 mg/ml. Rotenone was dissolved in DMSO at 5 mg/ml and was prepared fresh twice a week during the course of the experiments. All solutions were sterile filtered prior to use. Treatment compounds were vortex-mixed with Neobee M-5 vehicle immediately prior to intraperitoneal (IP) injection. All other reagents were of the highest grade commercially available from Sigma-Aldrich (St. Louis, MO, USA) or Cayman Chemical (Ann Arbor, MI, USA).

**Cell Culture, Chemicals and Treatment**

Rat H19-7/IGF-IR primordial hippocampal cells (H19-7 cells; CRL-2526, ATCC) were seeded onto sterile 10 cm culture dishes and 6- or 24-well culture plates pre-coated with poly-L-lysine (Gibco/ThermoFisher, Waltham, MA, USA) and were allowed to proliferate at 34 °C in 10% CO2 in air until they reached ~80% confluence. The culture medium contained high-glucose Dulbecco's modified Eagle's medium supplemented with 10% Fetal Clone IIITM (Gibco/ ThermoFisher), 50 U/ml penicillin, 50 U/ml streptomycin, 1X GlutaMAXTM (Gibco/ThermoFisher), 1 mM sodium pyruvate and 200 μg/ml G418 sulfate (Mediatech/Corning, Tewksbury, MA, USA). Differentiation was induced by increasing incubation temperature to 39 °C and including N2 supplement (Gibco) and basic fibroblast growth factor (10 ng/ml; Invitrogen/Thermo-Fisher) to the culture medium. Rotenone and PMX-500FI were dissolved in DMSO and diluted in complete medium. DMSO final concentration was kept at or below 0.1% to avoid cell toxicity issues.

**Intracellular ROS and ATP Content**

H19-7 cells were cultured in 24-well plates until reaching 80% confluence and were then differentiated as described above. Generation of intracellular ROS in response to different doses of rotenone, exposure time and co-treatment with PMX-500FI was measured by loading culture medium with 20 μM 2',7'-dichlorofluorescin diacetate (DCFDA) for 30 min. Cellular ATP content among different groups was determined using an ENLITEN® ATP Assay System Bioluminescence Detection Kit (Promega, Madison, WI, USA) according to the manufacturer's instructions. Fluorescence (at Ex485nm/ Em535nm) and luminescence were measured using a Tecan Infinite M200 multi-function microplate reader (Tecan, Männedorf, Switzerland) equipped with Magellan 7.x software (Tecan).

**Animals**

Male Lewis rats (9-10 weeks old, 200-250 g) were purchased from Envigo (Prattville, AL, USA) and were handled exclusively by female technicians in all procedures [49]. Animals underwent a one-week acclimation period in which they were subjected to one-handed dorsal recumbency positioning by the handler. Rats were weighed daily to determine treatment dosages. After two weeks of daily IP rotenone injections (3.0 mg/kg body weight), rats displayed the characteristic hind limb rigidity and bradykinesia that are hallmarks of the rat-rotenone model [18]. Separate groups of rotenone-treated rats were co-treated daily with PMX compounds, while others were treated with a mixture of ALA and LC [18]. Animals received daily IP injections of experimental compounds (PMX or ALA+LC) in the morning and rotenone in the early afternoon.

All animal procedures, including housing, neuromotor testing and euthanasia, conformed to Institutional Animal Care and Use Committee (IACUC) guidelines and the Guide for the Care and Use of Laboratory Animals, under Office for Laboratory Animal Welfare (OLAW) assurance #A3152-01. Specifically, all animal procedures used in this study were reviewed and approved by the Auburn University IACUC (reference protocol number 2015-2646).

**Immunohistochemistry**

Immunolabeling of brain slices [28] was performed on rats from experiments in which neuromotor testing had commenced two days post-treatment (i.e., euthanasia at four days post-treatment). Animals were anesthetized with a combination of ketamine (100 mg/kg) and xylazine (20 mg/kg) and fixation was accomplished by transcardial perfusion with 4% paraformaldehyde in phosphate buffered saline (PBS), pH 7.2. Whole fixed brains were removed and stored in fixative at 4 °C for two days, followed by 30% sucrose with daily changes for 3-4 days before sectioning and immunohistochemical analysis. Coronal brain slices (30μm thick) were prepared using a cryostat. Immunohistochemical analysis was performed on every fourth free-floating section. All procedures prior to antibody incubation were carried out at room temperature. Sections were washed in PBS with 0.1% Triton X-100 (PBST) for several hours to remove cryoprotectant, and were incubated in PBST containing 3% normal goat serum (ICN Biochemical, Costa Mesa, CA, USA) for 1 h. Sections were then incubated in a 1:1000 dilution of a rabbit polyclonal antibody against tyrosine hydroxylase (AB152 from Merck Millipore, Billerica, MA, USA) in PBST for 2 days at 4 °C. Following incubation, sections were washed and then placed in biotinylated goat anti-rabbit IgG (Jackson Immunoresearch, West Grove, PA, USA), 1:000 dilution in PBST for 1 h. The sections were washed in PBST and then incubated in PBST containing ABC-elite (1:1000; Vector Laboratories, Burlingame, CA, USA) for 1 h. After rinsing in PBS, sections were incubated in 0.05% 3,3'-*Diaminobenzidine* (*DAB*) and 0.015% H2O2 in PBS for 10 min. Sections were rinsed in PBS, mounted on positively charged glass slides and allowed to dry. Slides were dehydrated in ethanol and cover-slipped with Permount (Electron Microscopy Sciences, Hatfield, PA, USA). Controls included omission of the primary antibody from the immunohistochemical analysis protocol, the absence of which completely eliminated DAB staining for the antigen. Tyrosine hydroxylase immunoreactive (TH-ir) cells were counted unilaterally in four consecutive sections of the dorsal tier of SNpc (SNpc-A9d) by a blinded observer. Imaging was performed using a Nikon Eclipse E800M microscope and whole-section mosaics of high-magnification images were assembled using Nikon Elements 5.1 software, which was also used for quantitative analysis of TH-ir cells.

**Neuromotor Analyses**

At the conclusion of the two-week treatment period, rats were subjected to neuromotor testing after either a two-day or a one-week recovery. Rat neuromotor and behavioral tests included rotarod, open field test and balance beam, and were conducted over a two day period.

**Rotarod**

Motor coordination and balance in rats were tested using a rotarod (Rotamex, Columbus Instruments, Columbus, OH, USA). Prior to performance testing, rats were trained to balance on the rotarod. Two variations of training were used,

each with different timing of performance testing. In the first variation, rats were introduced to the rotarod apparatus one day after experimental treatments with rotenone and PMX compounds ended, and training lasted for just one day, with a series of short training sessions throughout the day.

In performance trials, the rotarod was programmed to begin at 4 rpm and increase by 1 rpm every 8 sec until a maximum speed of 40 rpm was reached. Latency to fall (measured as time passed since rod rotation began) was recorded by the rotarod apparatus. Three trials were run with the results averaged and rats were given a 30 min rest period between trials.

### Open Field Test

The open field test is a quantitative measure of general locomotor activity and exploratory behavior. Rats were placed in the center of the floor of an empty clear acrylic box (100cm x 100cm x 100cm) and allowed to explore for 10 min. Sessions were video recorded by a centrally-aligned overhead camera, and animal movement was tracked using SMART software (v3.0; Panlab, Harvard Apparatus, Holliston, MA, USA). The full dataset for each rodent was analyzed using the SMART software package. Among the variables analyzed were total distance traveled, percent time spent resting versus moving, and mean speed. Following completion of the software analysis, video footage was reviewed by a human observer and scored for the number of rears.

### Balance Beam

To test balance and coordination, rats were trained to cross a wooden balance beam elevated approximately 3 feet off the floor. The starting end of the beam apparatus consisted of an open platform with a light source directed towards the beam to prompt the rodent to traverse the beam into the darkened "safe" box at the beam terminus. Wooden beams for these experiments were 1.5" square and round dowels. Initial training of animals was performed using the square beam, placing the rodent on the center of the beam with its head facing the box. Once inside the box, the animals were allowed 30 sec of rest and then repositioned at the start of the beam. Following training, each animal was placed at the start of the balance beam a total of 6 times and allowed to cross (3 crossings on the square beam followed by 3 crossings on the round beam after a 30 min break). Each trial was video recorded, with the field of view directly down the beam lengthwise, which allowed for a clear view of hind foot faults. Videos were later manually scored for foot faults/slips (the number of times the animal's foot slipped off the balance beam). After tallying of all foot faults was completed, averages were compared for square beam, round beam, and total foot faults.

## STATISTICAL ANALYSIS

Statistical analyses were performed using R-studio software with the 'asbio' package. Data were analyzed using one-way ANOVA with Tukey HSD post hoc test. Statistical significance was accepted at $p<0.05$.

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## AUTHOR CONTRIBUTIONS

MEH and SH performed neuromotor/ behavioral testing and processing of tissues, and participated in experimental design. MEH and BNA performed statistical analyses of neuromotor/behavioral data. KP participated in the experimental design and performance of ROS and ATP assays with H19-7 cells. CDF designed and conducted immunohistochemical analyses of brain slices. BNA and MHI were responsible for experimental design and directed all technical procedures. JGM participated in experimental design and protocol development. CAP and KS participated in experimental design and interpretation of results. MEH, BNA, CAP and MHI wrote the manuscript.

## COMPETING INTEREST STATEMENT

KS is CEO and a stockholder of PhenoMatriX, Inc. All other authors declare no competing interests.

## DATA AVAILABILITY

The data that support the findings of this study are available on request.

## CONFLICT OF INTEREST

There is no conflict of interest.

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