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Mitochondria and calcium flux as targets of neuroprotection caused by minocycline in cerebellar granule cells.

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Running title: Minocycline modulates mitochondrial functions

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Abstract

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Minocycline, an antibiotic of the tetracycline family, has attracted considerable interest for its theoretical therapeutic applications in neurodegenerative diseases. However, the mechanism of action underlying its effect remains elusive. Here we have studied the effect of minocycline under excitotoxic conditions. Fluorescence and bioluminescence imaging studies in rat cerebellar granular neuron cultures using fura-2/AM and mitochondria-targeted aequorin revealed that minocycline, at concentrations higher than those shown to block inflammation and inflammation-induced neuronal death, inhibited NMDA-induced cytosolic and mitochondrial rises in Ca^{2+} concentrations in a reversible manner. Moreover, minocycline added in the course of NMDA stimulation decreased Ca^{2+} intracellular levels, but not when induced by depolarization with a high K^+ medium. We also found that minocycline, at the same concentrations, partially depolarized mitochondria by about 5-30 mV, prevented mitochondrial Ca^{2+} uptake under conditions of environmental stress, and abrogated NMDA-induced reactive oxygen species (ROS) formation. Consistently, minocycline also abrogated the rise in ROS induced by $75 \mu\text{M}$ Ca^{2+} in isolated brain mitochondria. In search for the mechanism of mitochondrial depolarization, we found that minocycline markedly inhibited state 3 respiration of rat brain mitochondria, although distinctly increased oxygen uptake in state 4. Minocycline inhibited NADH-cytochrome *c* reductase and cytochrome *c* oxidase activities, whereas the activity of succinate-cytochrome *c* reductase was not modified, suggesting selective inhibition of complex I and IV. Finally, minocycline affected activity of voltage-dependent anion channel (VDAC) as determined in the reconstituted system. Taken together, our results indicate that mitochondria are a critical factor in minocycline-mediated neuroprotection.

Key words: anti-oxidant, apoptosis, voltage-dependent anion channel, uncoupler, aequorin, bioluminescence.

Abbreviations: 2,2-diphenyl-1-picrylhydrazyl, DPPH•; 2,4,6-tripyridyl-S-triazine, TPTZ; 5-(and 6-)-chloromethyl-2,7-dichlorodihydrofluorescein diacetate, CM-H₂DCFDA ; adenine nucleotide translocase, ANT; amyotrophic lateral sclerosis, ALS; Carbonylcyanide-4-(trifluoromethoxy)-phenylhydrazone, FCCP; ferric reducing ability of plasma, FRAP; cytosolic Ca²⁺ concentrations, [Ca²⁺]_{cyt} ; fluorescein diacetate, DFCA; Green Fluorescence protein, GFP; Hydroethidine, Het; mitochondrial Ca²⁺ concentrations, [Ca²⁺]_m; mitochondrial inner membrane potential, ΔΨ_m; mitochondrial permeability transition pore, MPTP; N-methyl-D-aspartic acid , NMDA; region of interest, ROI; reactive oxygen species, ROS; superoxide anion radical, •O₂⁻; 2,4,6-tripyridyl-S-triazine, TPTZ; tetramethylrhodamine methyl ester, TMRM; voltage-dependent anion channel, VDAC.

1. Introduction

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Minocycline, a semi-synthetic derivate of tetracycline, is under scientific debate as a potential therapeutic agent in neurological disease processes (for review see [1, 2]. Support for this new application of a time-tested antibiotic comes from observations that minocycline readily crosses the blood-brain barrier to the greatest extent of all of the tetracyclines and is well tolerated. It affords neuroprotection in experimental models of Parkinson's disease, Huntington's disease, multiple sclerosis, amyotrophic lateral sclerosis (ALS), and acute inflammation after brain trauma or cerebral ischemia [1, 3]. Although there are inconsistent reports that show detrimental effects in different models of neurodegeneration [4, 5], numerous studies have focused on the mechanisms underlying minocycline-mediated cell protection. However, the possible mechanisms described so far are poorly understood and need to be clarified.

We and others have postulated that mitochondria could be the pharmacological target affected by minocycline to afford cytoprotection. Proton-translocation by components of the respiratory chain generates the mitochondrial inner membrane potential ($\Delta\Psi_m$). This transmembrane potential can be used to phosphorylate ADP to ATP or for Ca^{2+} uptake into mitochondria [6]. When added to isolated mitochondria, minocycline is able to decrease the $\Delta\Psi_m$, possibly preventing mitochondrial permeability transition pore (MPTP) opening and the subsequent release of cytochrome *c*. Furthermore, $\Delta\Psi_m$ is considered an important factor in the maintenance of mitochondrial homeostasis: both Ca^{2+} uptake [7] and production of reactive oxygen species (ROS) are attenuated due to a decrease in $\Delta\Psi_m$ [8]. Thus, small changes in $\Delta\Psi_m$ may contribute to the cytoprotection mechanisms. Mitochondrial depolarization, caused by low concentrations of mitochondrial uncouplers, has been shown to be protective against NMDA-induced neuronal death [9].

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Mounting evidence indicates that ROS-mediated stress plays a major role in the pathogenesis of many neurodegenerative disorders. Accordingly, it has been shown that excitotoxicity, which plays a critical role in several neurological pathologies, promotes significant increases in cytosolic Ca^{2+} concentrations ($[\text{Ca}^{2+}]_{\text{cyt}}$), ROS production, oxidative damage, and ultimately cell death [10, 11]. In addition, mitochondrial Ca^{2+} overload plays a pivotal role in many cell death models including excitotoxicity [11]. Specifically, it has been reported that glutamate-induced neurotoxicity depends on mitochondrial Ca^{2+} uptake ($[\text{Ca}^{2+}]_{\text{m}}$) [9, 12]. Under stress conditions, there is an early increase in $[\text{Ca}^{2+}]_{\text{m}}$ that may contribute to mitochondrial dysfunction [13, 14] and MPTP opening, a point-of-no-return step in the intrinsic pathway of apoptotic cell death [15, 16].

Nowadays, the molecular composition of the MPTP is still under debate. Numerous independent studies suggest that the MPTP consists of a voltage-dependent anion channel (VDAC) within the outer membrane, and adenine nucleotide translocase (ANT) within the inner membrane. The mechanism of VDAC contribution to MPTP still remains elusive. The available data indicate that pro-apoptotic stimuli can trigger a state of high or low conductance of VDAC. It has also been suggested that anti-apoptotic activity may involve an inhibitory VDAC interaction with MPTP constituents which appears critical for mitochondrial metabolite transport, volume regulation, and apoptosis [for review see, [17-19]. On the other hand, there is also evidence indicating that VDAC participates in signal generation without direct involvement in subsequent processes (e.g. [20]).

Herein, we have designed several experiments to understand the mechanisms responsible for minocycline-mediated neuroprotection under excitotoxic conditions. After NMDA receptor activation, we analyzed minocycline's effect on two of the earliest events which can tip the scales in favour of neurodegeneration. Close attention was paid to the entry of Ca^{2+} through the plasma membrane, mitochondrial Ca^{2+} uptake, and generation of ROS.

1 We also studied different parameters describing mitochondrial functions. Our results suggest
2 that minocycline, although at concentrations higher than those shown to block inflammation
3 and inflammation-induced neuronal death [21], prevents NMDA-induced excitotoxicity by
4 diminishing NMDA-induced Ca^{2+} entry and altering the $\Delta\Psi\text{m}$ via mitochondrial Ca^{2+} uptake.
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6 The latter may be caused by activity changes in VDAC as well as the respiratory chain.
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17 **2. Materials and Methods**

18 *2.1. Chemicals.*

19 Fura-2, hydroethidine, CM-H₂DCFDA, TMRM and celenterazin were obtained from
20 Invitrogen (Barcelona, Spain). Mitochondrial AEQ construct was a gift from Prof. Brulet
21 (Paris, France). Culture media, sera and antibiotics are from Lonza. If not indicated otherwise,
22 all other chemicals were purchased from Sigma or Merck.
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32 *2.2. Animals*

33 For cerebellar granule cell cultures, 5-7-day-old Wistar rats were used. Brain
34 mitochondria were isolated from 3-month-old male Wistar rats. All animals were housed at 22
35 \pm 2°C with 12 h/12 h light/dark cycles and with full access to water and food. Animal
36 experiments were carried out in accordance with the Guiding Principles for Research
37 Involving Animals and Human Beings of the American Physiological Society, the Guidelines
38 of the European Union Council (86/609/CEE), and the Spanish regulations (BOE 67/8509-12,
39 1988) for the use of laboratory animals. Approval was obtained from the Scientific
40 Committee of the Universities of Cádiz, Castilla-La Mancha, and Valladolid.
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58 *2.3. Cell Cultures*

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Cerebellar granule cells were obtained as reported previously [22], plated on poly-L-lysine coated, 12 mm diameter glass coverslips. They were cultured in high-glucose, low K⁺, Dulbecco's modified Eagle's medium (DMEM, Gibco, Spain) plus 10% fetal bovine serum, 5% horse serum, 100 µg/ml penicillin, and 100 µg/ml streptomycin for 2 days. Then the culture medium was replaced by Sato's medium plus 5% horse serum (to avoid excessive proliferation of glia) and cultured for 2-4 days before experiments.

2.4. Cell viability studies

To study NMDA-induced toxicity, neuronal cultures maintained 7 days *in vitro* were incubated for 30 min with 100 µM NMDA, 2 µM Ca²⁺ /Na⁺ and 10 µM glycine in saline solution. Treatments were terminated by washing cells three times with Krebs solution before incubating with regular medium. Minocycline was added 10 min before NMDA at different concentrations and maintained during the whole experiment. Cell viability was analyzed 24 h later using the fluorescein diacetate/ propidium iodide double-staining procedure. Living and dead cells were counted in 3-4 coverslips per condition (a total of 300-450 cells per coverslip) and normalized to corresponding controls. At least three independent experiments were used for quantification

2.5. Fluorescence imaging of cytosolic [Ca²⁺]

Coverslips containing cerebellar granule cells were incubated in standard medium containing (in mM) NaCl 145, KCl 5, CaCl₂ 1, MgCl₂ 1, glucose 10, Hepes 10 (pH 7.42), and loaded with 4 µM fura2/AM for 60 min at room temperature. Coverslips were then placed on the heated stage of an inverted Zeiss microscope (Axiovert S100 TV), and while continuously perfused with the same pre-warmed standard medium, epi-illuminated alternately at 340 and 380 nm. Light emitted above 520 nm was recorded with an OrcaER Hamamatsu camera

(Hamamtsu Photonics, Hamamatsu, Japan). Pixel-by-pixel ratios of consecutive frames were captured, and $[Ca^{2+}]_{cyt}$ was estimated from these ratios as previously reported[23].

2.6. Bioluminescence imaging of mitochondrial $[Ca^{2+}]$

Cerebellar granule cells were transfected with plasmids containing GFP-aequorin targeted to mitochondria [24] using a Nucleofector II[®] device and the VPG-1003 transfection kit (Amaya Biosystems, Cologne, Germany). After 24 h, cells were incubated for 1 h with 1 μ M coelenterazine, washed, and placed into a perfusion chamber set at 37°C under a Zeiss Axiovert S100 TV microscope. They were then perfused at 5-10 ml/min with test solutions based on the standard perfusion solution pre-warmed to 37°C as described above. At the end of each experiment, cells were permeabilized with 0.1 mM digitonin in 10 mM $CaCl_2$ in order to release all the residual aequorin counts. Bioluminescence images were taken with a Hamamatsu VIM photon counting camera handled with an Argus-20 image processor. Photonic emissions were integrated for 10 s periods. Photons/cell in each image was quantified using the Hamamatsu Aquacosmos software. Total counts per region of interest (ROI) ranged between $2 \cdot 10^3$ and $2 \cdot 10^5$, and noise was (mean \pm SD) 1 ± 1 counts per second (c.p.s.) per typical cell area (2,000 pixels). Data were first quantified as rates of photoluminescence emission/total c.p.s remaining at each time (% of remaining counts) and divided by the integration period (L/L_{TOTAL} in s^{-1}). Emission values of less than 4 c.p.s were not used for calculations. Calibrations in terms of $[Ca^{2+}]_{mit}$ were performed as previously reported [23]. Briefly, the total luminescence for every ROI was computed by adding up the values of all images. In addition, the following values were registered for every time point (t): L =Luminescence emission at time t; $Sum(L)=\sum L$ values from $t=0$ to t; $L_{TOTAL}(L_{T1})$ =Total luminescence remaining at time t= $Total(L)-Sum(L)$; % Remaining

luminescence=100xLtotal/Total (L); and ratio (R) =L/L_{TI}. Finally, [Ca²⁺] was calculated using the following algorithm:

[Ca²⁺] (in M)=[R+(R*K_{TR})+1] / [(K_R-(R*K_R)), where R=L/L_{TI}*λ)]^(1/n), using the constant values K_R, K_{TR}, n and λ reported previously (see 54 for a detailed description). In some experiments, cells were permeabilized with 20 μM digitonin in “intracellular” medium with the following composition: 130 mM KCl, 10 mM NaCl, 1 mM MgCl₂, 1 mM K₃PO₄, 0,2 mM EGTA, 1 mM ATP, 20 μM ADP, 2 mM succinate, 20 mM HEPES/KOH, pH 6.8. Cells were then incubated for 5 min with the same medium containing 200 nM Ca²⁺ (buffered with EGTA), with or without minocycline. Finally, perfusion was switched to “intracellular” medium containing 5 μM Ca²⁺, with or without minocycline.

2.7. Mitochondrial potential

The effects of treatment on ΔΨ in intact cerebellar granule cells were estimated by fluorescence imaging of cells loaded with the ΔΨ sensitive probe tetramethylrhodamine methyl ester (TMRM), one the most sensitive probes available [25]. Briefly, cerebellar granule cells were loaded with 10 nM TMRM for 10 min, washed with a standard, external medium, and placed on the perfusion chamber of a Zeiss Axiovert S100 TV inverted microscope. Cells were then continuously perfused with pre-warmed (37°C) external medium, with and without treatments. Fluorescence images were taken at 10 s intervals with a Hamamatsu VIM photon counting camera handled with an Argus-20 image processor. Traces from individual cells were normalized relative to the value before the addition of either vehicle or treatment and averaged. Background fluorescence after collapse of the mitochondrial potential induced by 10 μM FCCP was subtracted. Further details have been reported previously [26].

2.8. Superoxide intracellular production

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Superoxide production was monitored using hydroethidine (HEt, Molecular Probes) as described previously [27]. Coverslips containing cerebellar granule cells were incubated in standard medium containing (in mM): NaCl 145, KCl 5, CaCl₂ 1, MgCl₂ 1, glucose 10, and Hepes 10 (pH 7.42) and loaded with 10 μM hydroethidine. Next, coverslips were placed on the heated stage of an inverted Nikon (Eclipse TE2000) microscope. Light emitted above 535 nm was recorded with an emission filter of 635 nm (Omega Optical) with an OrcaER Hamamatsu camera (Hamamatsu Photonics, Hamamatsu, Japan). Background was subtracted. Frames were recorded every 10 s over a 7 min period. Linear regression of fluorescence data was obtained of each condition and the slope of the best fitting line was taken as an index of superoxide anion radical ($\bullet\text{O}_2^-$) production.

2.9. Isolation of mitochondria and preparation of mitochondrial membranes

Brain mitochondria were isolated from the whole rat brain in a small Potter homogenizer with a Teflon pestle. Homogenization was carried out in a medium of 230 mM mannitol, 70 mM sucrose, 1.0 mM EDTA, and 10 mM Tris-HCl, pH 7.40, at a ratio of 9 ml of homogenization medium/g of tissue. The homogenate was centrifuged at 700 g for 10 min and the supernatant at 8,000 g for 10 min to precipitate mitochondria that were washed in the same conditions [28]. The obtained mitochondrial suspensions of about 20 mg protein/ml were used immediately after isolation for the determination of respiration or frozen in liquid N₂ and kept at -80°C. Sub-mitochondrial membranes (disrupted mitochondria) were obtained from brain mitochondria that were twice frozen-and-thawed and homogenized each time by passage through a tuberculin needle. The procedure yielded a preparation of disrupted mitochondria with 0.20-0.24 nmol cytochrome *aa*₃/mg protein, subsequently used for the determination of enzymatic activity of the respiratory complexes and the presence of markers

1 of oxidative damage. The protein content of the samples was determined using Folin reagent
2 and bovine serum albumin as the standard. To study the effects of minocycline, the brain-
3 coupled mitochondria or sub-mitochondrial membranes were added or incubated for 5 min at
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5 4°C, respectively, with 0, 75, 100, 125, or 150 μM minocycline.
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10 11 12 *2.10. Mitochondrial oxygen uptake*

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14 The oxygen uptake of rat brain mitochondria (0.7-0.8 mg/ml) was determined with a
15 Clark electrode in a 1.5 ml chamber at 30°C, in an air-saturated reaction medium consisting
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17 of: 0.23 M mannitol, 0.07 M sucrose, 20 mM Tris-HCl, 1 mM EDTA, 5 mM phosphate, 4
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19 mM MgCl_2 , at pH 7.4. Respiration rates were determined with 5 mM malate-5 mM glutamate
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21 or 6 mM succinate as substrates, and state 3 respiration was established by addition of 0.5
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23 mM ADP. Mitochondria oxygen uptake was assayed in uncoupled mitochondria with 1 μM
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25 FCCP. Three determinations were performed for each value.
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32 33 34 *2.11. Measurement of ROS levels in mitochondrial suspension*

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36 Mitochondrial ROS production was evaluated with the fluorescent probe 5-(and 6-)-
37 chloromethyl-2,7-dichlorodihydrofluorescein diacetate (CM-H₂DCFDA) in isolated
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39 mitochondrial suspension. CM-H₂DCFDA is a reduced form of fluorescein diacetate (DFCA)
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41 that requires oxidation before becoming fluorescent. Therefore, any measurable change in
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43 fluorescence intensity can be directly related to changes in free radical activity, mainly the
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45 generation of hydrogen peroxide and hydroxyl radicals. Mitochondrial suspensions were
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47 incubated with CM-H₂DCFDA (10 $\mu\text{g/ml}$), and fluorescence intensity was measured in a
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49 Spectra Max Gemini XS (Molecular Devices, Sunnyvale, CA). Brain mitochondria were
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51 challenged with 75 μM Ca^{2+} to induce of ROS formation during 30 min with or without
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53 minocycline. Experiments were performed in 96-multiwell plates including four samples for
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1 each experimental point. The average of relative DFCA fluorescence from at least three
2 separate mitochondrial preparations was determined. Results are expressed as mean \pm SEM
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4 values.
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9 *2.12. Mitochondrial electron transfer activity*

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11 The enzymatic activity of Complexes I-III, II-III, and IV were determined
12 spectrophotometrically at 30°C with sub-mitochondrial membranes suspended in 60 mM
13 phosphate buffer (pH 7.4) with corresponding substrates added. For NADH-cytochrome c
14 reductase (the activity of complexes I and III) and succinate-cytochrome c reductase (the
15 activity of complexes II and III), mitochondrial membranes were added with 0.20 mM NADH
16 or 5.0 mM succinate as substrates, 0.10 mM cytochrome c^{3+} , and 1.0 mM KCN. The
17 enzymatic activity determined at 550 nm ($\epsilon = 19 \text{ mM}^{-1}\text{cm}^{-1}$), with the results expressed as
18 nmol cytochrome c reduced / min \times mg protein. Cytochrome oxidase (complex IV) was
19 determined in the same buffer with 0.05 mM cytochrome c^{2+} added, prepared by reduction
20 with ascorbate, and filtration across a Sephadex G25 column. The rate of cytochrome c
21 oxidation was calculated as the first order reaction constant (k') per mg protein, and expressed
22 as nmol cytochrome c oxidized at 10 μM cytochrome c / min \times mg protein. This result
23 determines the electron transfer rates of physiological respiration [29]. Three determinations
24 for each value.
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49 *2.13. DPPH radical-scavenging activity*

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51 The scavenging activity of minocycline was measured according to the method
52 described by Brand-Williams et al. [30] with some modifications. Briefly, 500 μl of
53 minocycline methanolic solutions (100 μM) at different concentrations (12.5 to 150 $\mu\text{l/ml}$)
54 were added to 1 mL of 2,2-diphenyl-1-picrylhydrazyl (DPPH \bullet) methanolic solution (0.1 mM).
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Estimated time of reaction (20 min) was determined considering the reduction of absorbance at 517 nm (monitored every 5 minutes) until the reaction curve reached a plateau. The absorbance was measured at room temperature, in darkness, with a blank (500 μ l of sample plus 1 ml of methanol). The absorbance of the control (500 μ l of methanol in 1 ml of DPPH• solution) was measured daily. All of the assays were conducted in triplicate. The percentage activity for the DPPH• technique was calculated according to % discoloration = $[1 - (\text{Abs sample}/\text{Abs control})] \times 100$.

2.14. Ferric reducing ability assay

The ferric reducing ability of minocycline was measured according to the method developed by Benzie and Strain (1996) [31]. Antioxidant compounds are able to reduce ferric compounds to ferrous-TPTZ, which develops a blue color with a maximum absorption at 593 nm. FRAP reagent was freshly prepared from 300 mM acetate buffer (pH 3.6), 10 mM 2,4,6-tripyridyl-S-triazine (TPTZ) made up in 40 mM HCl, and 20 mM $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$ solution. All three solutions were mixed together in the ratio of 10:1:1(v/v/v). An aliquot of 40 μ l of minocycline was added to 1.2 ml of FRAP reagent. The absorption of the reaction mixture was measured at 593 nm after 2 min of incubation at 37°C. Measurements were performed in triplicate. Fresh working solutions of known Fe (II) concentrations ($\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$, 0-2 mM) were used for calibration. The antioxidant capacity, based upon the ability to reduce ferric ions of each sample, was calculated from the linear calibration curve and expressed as mmol FeSO_4 equivalents.

2.15. Isolation of VDAC from *Saccharomyces cerevisiae* mitochondria

1 The yeast *Saccharomyces cerevisiae* M3 cells (MAT α , lys2, his4, trp1, ade2, leu2,
2 ura3) [32] were grown at 28°C in YPG medium (1% yeast extract, 2% peptone, 3% glycerol)
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4 at pH 5.5 to OD₅₄₆ of 2.2. Mitochondria and the mitochondrial outer membrane were isolated
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6 according to the published procedure [33]. The obtained outer membrane pellet was
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8 suspended in the solubilization buffer containing 3% Triton X-100, 10 mM Tris-HCl (pH
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10 7.0), and 1 mM EGTA, incubated for 30 min at 0°C. The suspension was then loaded onto a
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12 dry hydroxyapatite/celite column [34] and the first two fractions were collected. Before
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14 reconstitution, the preparations of VDAC were checked by Western blotting with anti-yeast
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16 anti-serum for the presence of marker proteins of mitochondrial compartments as well as for
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18 VDAC (not shown).
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27 *2.16. Conductance measurements in planar phospholipid membranes*

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29 Membranes were formed from soybean asolectin suspended at concentration of 25
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31 mg/mL in n-decane, across a circular hole (aperture diameter about 250 μ m) in the thin wall
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33 of a Delrin chamber (Warner Instruments) separating two compartments (*cis-trans*) filled
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35 with unbuffered 1 M KCl, pH 7.0. The chamber was connected to the recording equipment
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37 through a matched pair of Ag-AgCl electrodes. Preparations of VDAC were added in small
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39 aliquots (2-3 μ l) to the *cis* compartment. *Cis* also refers to the compartment where the voltage
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41 was held. Signals were amplified by a BLM-120 bilayer amplifier (Bio-LOGIC Science
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43 Instruments) and computer software was used for data collection. The average amount of
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45 analyzed insertion events for a given set of conditions was 70.
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58 *2.17. Statistics*

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When only two means were compared, Student's *t* test was used. For more than two groups, statistical significance of the data was assessed by analysis of the variance (ANOVA) and compared using Turkey's test. Differences were considered significant at $p < 0.05$. For the conductance measurements in planar phospholipid membranes, the differences between calculated values of average conductance were tested by *t*-test (at $\alpha = 0.01$) for statistical significance.

3. Results

3.1. Minocycline and cell viability

Consistent with previous data from our laboratory and elsewhere, minocycline afforded cytoprotection to neuronal cultures when exposed to excitotoxic conditions. Cerebellar granular cells were treated with minocycline at different concentrations (1-150 μM) and challenged with NMDA (100 μM) during 30 min. Twenty four hours later, neuronal viability was evaluated by using fluorescein diacetate and the propidium iodide staining method. As shown in Fig. 1, NMDA treatment decreased cell viability by about 35% ($p < 0.05$). Pre-treatment with minocycline, which did not affect cell viability (Figure 1, black bars), afforded neuroprotection in a concentration-dependent manner. Concentrations lower than 50 μM did not significantly modify NMDA-induced cell death (Fig. 1).

3.2. Minocycline and calcium signals

It has been clearly established that NMDA-induced cell death depends on Ca^{2+} entry through the plasma membrane and mitochondrial Ca^{2+} uptake [12]. We therefore studied the effects of minocycline on NMDA induced increases in cytosolic ($[\text{Ca}^{2+}]_{\text{cyt}}$) and mitochondrial Ca^{2+} ($[\text{Ca}^{2+}]_{\text{mit}}$) concentrations in cerebellar granule cells. Fig. 2A shows pseudocolor images of a representative microscopic field corresponding to $[\text{Ca}^{2+}]_{\text{cyt}}$ levels during perfusion with

1 the glutamate receptor agonist NMDA (100 μM) in the presence of minocycline (100 μM)
2 and after its removal. We found that in the presence of 100 μM minocycline, NMDA induced
3 a rather modest increase in $[\text{Ca}^{2+}]_{\text{cyt}}$. Five min after minocycline withdrawal, cells were
4 stimulated again with NMDA and $[\text{Ca}^{2+}]_{\text{cyt}}$ was monitored. Under these experimental
5 conditions, NMDA increased $[\text{Ca}^{2+}]_{\text{cyt}}$ to a much larger extent ($p < 0.001$, $n=4$) indicating that
6 minocycline inhibits Ca^{2+} entry to cells mediated by activation of NMDA receptor channel
7 (**Fig. 2A**). This result is consistent with our previous findings that minocycline decreases the
8 amplitude and the number of excitatory postsynaptic currents with EC_{50} between 42 and 64
9 μM , indicating that glutamatergic synaptic transmission is drastically affected by
10 neuroprotective concentrations of minocycline (30 μM or higher [35]).
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25 To study the effects of minocycline on $[\text{Ca}^{2+}]_{\text{mit}}$ cerebellar granule cells were
26 transfected with mitochondria-targeted aequorin, a bioluminescent probe widely used for
27 subcellular Ca^{2+} measurements [22, 23]. This probe also contains GFP to help selecting
28 transfected cells for bioluminescence imaging. Cerebellar granule cells were transfected with
29 the GA plasmid and incubated for 24 h to allow for the expression of the Ca^{2+} probe. **Fig. 2B**
30 shows the bioluminescence (AEQ) and fluorescence (GFP) images of transfected cerebellar
31 granule cells. We found that NMDA, in the presence of 100 μM minocycline, failed to induce
32 any rise in $[\text{Ca}^{2+}]_{\text{mit}}$. However, after removal of minocycline, NMDA promoted the increase in
33 $[\text{Ca}^{2+}]_{\text{mit}}$ ($n=2$). Thus, these results indicate that minocycline inhibits both the cytosolic and the
34 mitochondrial rises in Ca^{2+} concentrations induced by NMDA in a reversible manner.
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49 To address whether minocycline inhibits the increase in $[\text{Ca}^{2+}]_{\text{cyt}}$ in a specific manner,
50 we studied the effects of minocycline on $[\text{Ca}^{2+}]_{\text{cyt}}$ induced by either NMDA or high K^+
51 medium, added here to promote opening of voltage-gated Ca^{2+} channels. **Fig. 3** shows that
52 minocycline blocked NMDA-induced $[\text{Ca}^{2+}]_{\text{cyt}}$ rise but failed to reduce the increased $[\text{Ca}^{2+}]_{\text{cyt}}$
53 induced by a high- K^+ medium. Traces are Mean \pm SEM values of all cells present in
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representative experiments. These data indicate that minocycline does not inhibit voltage-gated Ca^{2+} channels that can be activated by NMDA-induced plasma membrane depolarization.

In the next set of experiments we studied whether minocycline may affect the mitochondrial inner membrane potential and mitochondrial Ca^{2+} uptake acting on intact cells. Cerebellar granule cells were loaded with the mitochondrial inner membrane potential probe TMRM and subjected to fluorescence microscopy. **Fig. 4A** shows TMRM staining of cerebellar granule cells before and after the addition of 100 μM minocycline. An image of the same cells after adding the mitochondrial uncoupler FCCP is also shown. Perfusion of TMRM-loaded cells with minocycline induced a dose-dependent decrease in TMRM fluorescence consistent with a mitochondrial depolarization. This effect was absent at 1 μM minocycline (not shown), slightly apparent at 10 μM and quite strong at 100 μM . As expected, the mitochondria uncoupler FCCP (10 μM) caused organelle depolarization (**Fig. 4A**). Therefore, these results indicate that minocycline depolarizes mitochondria in intact cells in a dose-dependent manner. Minocycline decreases TMRM fluorescence at 10 and 100 μM by 17 ± 4 and 70 ± 6 % respectively, relative to the effect of the total uncoupler FCCP at 10 μM . We have shown previously that decreases in TMRM can be used to estimate mitochondrial potential decrements [12]. According to the algorithm proposed [12], we estimate that minocycline, at 10-100 μM , depolarizes mitochondria in intact cerebellar granule cells by 5-30 mV.

We have reported recently that a weak depolarization of mitochondria may largely influence mitochondrial Ca^{2+} uptake [23]. Accordingly, we studied whether minocycline inhibited mitochondrial Ca^{2+} uptake in permeabilized cells exposed to Ca^{2+} concentrations similar to those achieved in Ca^{2+} microdomains (10 μM). Cerebellar granule cells were transfected with the GA plasmid and incubated for 24 h. Then cells were incubated with

1 digitonin and subjected to bioluminescence imaging. **Fig. 4B** shows that addition of Ca^{2+} to
2 permeabilized cells in the presence of 100 μM minocycline failed to rise $[\text{Ca}^{2+}]_{\text{mit}}$. After
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4 removal of minocycline, addition of Ca^{2+} induced release of photonic emissions
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6 corresponding to a increase in $[\text{Ca}^{2+}]_{\text{mit}}$ (n=36 cells, 6 independent experiments). Therefore,
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8 these results indicate that minocycline induces mitochondrial depolarization to such an extent
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10 that inhibits mitochondrial Ca^{2+} uptake.
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17 3.3. Minocycline and ROS production

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19 Increasing amount of data indicates that ROS play a major role in the pathogenesis of
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21 neurodegenerative disorders [16] and also in excitotoxic cell death models [36]. As shown in
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23 Fig. 5A, cerebellar granular neurons challenged with NMDA (100 μM) showed a higher rate
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25 of oxidation of dihydroethidine, an indicator of superoxide anion production. Specifically,
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27 cerebellar granular neurons presented a basal superoxide production rate of 0.019 ± 0.0023
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29 A.F.U./min (n=78). The addition of NMDA (100 μM) induced an increase in the rate of
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31 superoxide production, to a value of 4.18 ± 0.9645 A.F.U./min (n=186). The effect that was
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33 abrogated by minocycline in a concentration-dependent manner.
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40 In order to ascertain whether minocycline blocked ROS production at the
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42 mitochondrial level, we used CM- H_2DCFDA to measure peroxide-like formation in brain
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44 isolated mitochondria challenged with high Ca^{2+} . First, minocycline decreased basal
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46 mitochondrial ROS production (Fig. 5B). The addition of 75 μM Ca^{2+} to the mitochondrial
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48 suspension resulted in a significant increase in the production of ROS (2.8 fold increase).
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50 Minocycline inhibited this increase (Fig. 5B). In addition, the mitochondrial uncoupler FCCP
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52 (10 μM) was also able to inhibit the increase in ROS production induced by the applied
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54 concentration of Ca^{2+} (Fig. 5B).
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2 Taking into account these results, we went further to explore whether minocycline
3 might function as a superoxide dismutase (SOD) or a catalase mimetic in a cell free system.
4 Our results revealed that minocycline does not show any of the above mentioned enzymatic
5 activities (data not shown). Interestingly, and consistent with previous data, minocycline
6 showed a diphenylpicrylhydrazyl (DPPH) radical scavenger activity [37], an effect that
7 reached statistical significance at a concentration of 25 μM and above (Fig. 5C). Furthermore,
8 our data also confirmed that minocycline mediated the reduction of Fe^{3+} to Fe^{2+} , at
9 concentrations higher than 10 μM (Fig. 5D).
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22 *3.4. Minocycline-induced effects on mitochondrial function*

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24 The O_2 uptake of isolated rat brain mitochondria was determined in resting state, in
25 phosphorylating state and in uncoupled state triggered by FCCP. Malate-glutamate and
26 succinate were used as respiratory substrates in the presence of minocycline in the range of
27 75-125 μM . **Fig. 6** and **7** present typical recordings of the oxygen uptake obtained in the
28 presence of 125 μM minocycline for phosphorylating state (Fig. 6) and resting and uncoupled
29 states (Fig. 7). Minocycline increased the oxygen uptake in resting state, by about 80% in the
30 case of malate-glutamate as respiratory substrate and by about 60% with succinate as
31 respiratory substrate but simultaneously decreased the uncoupling capacity of FCCP by about
32 50% for the both respiratory substrates. However, in phosphorylating state minocycline
33 caused decrease in oxygen uptake, by about 60% in the case of malate-glutamate as
34 respiratory substrate and by about 50% with succinate as respiratory substrate. The effect of
35 minocycline on the oxygen uptake by brain mitochondria in uncoupled state in the presence of
36 malate-glutamate and succinate as respiratory substrates were similar to that observed for
37 phosphorylating state and the calculated levels of inhibition were about 50 and 45%
38 respectively. The inhibition was not caused by minocycline-mediated rupture of the
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mitochondrial outer membrane as minocycline did not cause increase in cytochrome c release (not shown). **Fig. 8** summarizes the effect of different concentrations of minocycline on the oxygen uptake during the studied respiratory states. Independently of the studied respiratory substrate we observed minocycline-concentration dependent inhibition of phosphorylating and uncoupled states and stimulation of resting state. However, the obtained calibration curves differed in shape between malate-glutamate and succinate that suggests differences of the applied mechanisms. Therefore we analyzed the influence of minocycline on enzymatic activities of the respiratory chain complexes I+III (NADH-cytochrome c reductase), II+III (succinate-cytochrome c reductase), and IV (cytochrome c oxidase). As shown in **Fig. 9**, minocycline in the range of 75-150 μ M inhibited markedly complexes I+III and IV. Thus minocycline appeared to affect the oxygen uptake supported by malate-glutamate at the level of complexes I and IV and the oxygen uptake supported by succinate only at the level of complex IV.

3.5. Minocycline affects VDAC conductance and voltage dependence

There is a growing amount of data showing that the voltage dependent anion channel (VDAC) is a dynamic regulator, or governor, of mitochondrial functions (for review see [17, 18]). Therefore we have studied the effects of minocycline on channel-forming activity of VDAC reconstituted into lipid bilayers. It was achieved by adding VDAC preparation to the aqueous phase of one side (so called *cis* side) of a black membrane bilayer made of asolectin. It is well known that VDAC behavior in planar phospholipid membranes made of asolectin is voltage dependent and symmetrical [38-40], meaning that the channel closes at about the same rate and to about the same extent depending on the applied potential value but regardless its sign. Thus, VDAC reconstituted into planar phospholipid membranes displays the ability to adopt a fully open state and multiple closed states of significantly smaller

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conductance that differ in permeability. The VDAC protein was isolated from mitochondria of the yeast *Saccharomyces cerevisiae* that contain only one channel-forming VDAC isoform [41] and are commonly used as a model to study mitochondrial biology. Since 100 μM minocycline did not change formation and stability of the planar phospholipid membrane, we perform comparative experiments at different values of the applied positive and negative potential and in the presence of different concentrations of minocycline. The obtained distributions of conductances in 1 M KCl were used to build histograms and to calculate values of average conductance of VDAC under a given experimental conditions.

The effect imposed by minocycline on VDAC conductance did not depend on the sign of the applied potential and was clearly visible under conditions of the dominance of VDAC fully open state, i.e. at a membrane potential of 10 mV. Examples of single channel insertions at a membrane potential of +10mV shown in **Fig. 10A** indicate that unexpectedly minocycline caused flickering of VDAC conductance in the second or even millisecond time scale dependently of minocycline concentration. Single channel insertions were then used to create histograms. **Fig. 10B** presents histograms obtained at +10 mV and in the presence of increasing concentrations of minocycline. Addition of minocycline distinctly changed distribution of VDAC conductance and consequently caused decrease in calculated values of average conductance dependently of its concentration (Fig. 10C). The calculated values of average conductance at +10 mV were as follows (in nS): 3.9 ± 0.1 nS (in the absence of minocycline), 3.4 ± 0.3 (10 μM minocycline), 3.2 ± 0.4 (50 μM minocycline) and 2.9 ± 0.4 (100 μM minocycline). This denotes a serious change in a functional state of VDAC towards lower conductance states as the dominance of VDAC closure observed at +70 mV corresponded to an average conductance of 1.9 ± 0.2 nS. The observed effect imposed by minocycline on VDAC conductance could result from changes in voltage dependence of the channel. Voltage dependence can be observed in reconstitution systems and is defined as the

1 probability of a channel occurrence in a given conductance state depending on the value of the
2 applied potential [32, 42]. As shown in **Fig. 10D**, in the absence as well as in the presence of
3 minocycline an increase in the value of the applied potential facilitated closure of the studied
4 channels that resulted in a decrease of the value of an average conductance calculated for a
5 given potential (G). This, in turn, caused a decrease in the value of the ratio G/G_0 , where G_0
6 denotes an average conductance at the lowest applied potential (10 mV). The sign of the
7 applied potential did not influence these changes. However, in the presence of minocycline
8 the VDAC voltage dependence was less pronounced and the weakening was concentration-
9 dependent.
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24 **4. Discussion**

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26 The present data, built upon our group's previous work [22, 35, 43], posits the intricate
27 involvement of mitochondria in minocycline-mediated cytoprotection for neuronal cells.
28 Following excitotoxic NMDA receptor activation, the accumulation of Ca^{2+} and the
29 generation of ROS are modulated as minocycline targets mitochondria. Intervention with
30 minocycline may prove useful as these are two events that occur early in neurodegenerative
31 processes.
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41 Consistent with previous observations [44, 45], minocycline afforded cytoprotection to
42 neuronal cultures under conditions of NMDA receptor-mediated excitotoxicity, although at a
43 concentration range higher than that needed to block inflammation and inflammation-induced
44 neuronal death [21]. In cultures pre-treated with minocycline, the NMDA-induced $[Ca^{2+}]_{cyt}$
45 rise was dramatically inhibited. We can exclude this effect as a consequence of the well
46 established association between tetracyclines and Ca^{2+} buffer capacity [43]. Minocycline
47 failed to modify the intracellular levels of Ca^{2+} in resting conditions, and did not attenuate a
48 Ca^{2+} increase following cellular depolarization with 50 mM KCl. Our data also revealed that
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1 this effect was reversible, demonstrated when minocycline removal allowed neurons to
2 respond to NMDA in a similar extent as control cell cultures. Furthermore, minocycline
3 actually decreased $[Ca^{2+}]_{cyt}$ during the course of NMDA receptor stimulation. This later result
4 might explain recent studies showing minocycline blocking centrally mediated hyperalgesia
5 induced by intrathecal NMDA [46], or shed some light on the favourable effects of
6 minocycline in a therapeutic window between 6 and 24 hours after a stroke [47].
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14 Minocycline does more than just preventing Ca^{2+} fluxes at the plasma membrane. It
15 also reduced $[Ca^{2+}]_{mit}$ overload, a pivotal role in excitotoxicity and other models of cell death
16 [9, 12, 15]. By using bioluminescence imaging with mitochondria-targeted aequorin, we
17 directly observed the effects of minocycline on NMDA-induced mitochondrial Ca^{2+} overload.
18 This effect remained in permeabilized cells as minocycline abrogated mitochondrial Ca^{2+}
19 uptake induced by Ca^{2+} concentrations that surpass the activation of the mitochondrial Ca^{2+}
20 uniporter. Supporting the hypothesis that small changes in mitochondrial potential may
21 dramatically influence mitochondrial Ca^{2+} uptake, minocycline, at 100 μ M, decreased by
22 about 5-30 mV the $\Delta\Psi_m$ in cerebellar granular cells. Therefore, minocycline reduces the
23 electrochemical driving force required for mitochondrial Ca^{2+} uptake, resulting in the
24 prevention of $[Ca^{2+}]_{mit}$ overload in excitotoxicity. Accordingly, prevention of mitochondrial
25 Ca^{2+} uptake by the mitochondrial Ca^{2+} uncoupler FCCP largely abrogated NMDA-induced
26 cell death [9]. Therefore, minocycline inhibit mitochondrial Ca^{2+} overload induced by NMDA
27 acting both at the plasma membrane level and at the mitochondrial level. These two actions
28 seem independent as FCCP does not prevent NMDA-induced Ca^{2+} influx (data not shown).
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51 Minocycline also weakened ROS production, both in cultured neurons and in isolated
52 mitochondria. Minocycline reduced the rate of increase of hydroethidine oxidation in neurons
53 exposed to NMDA. Minocycline also reduced peroxide-like generation in isolated
54 mitochondria suspensions when challenged with 75 μ M Ca^{2+} . Similar to minocycline's effect
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1 on $\Delta\Psi_m$, this anti-oxidant action was mimicked by the mitochondrial uncoupler FCCP. We
2 can not exclude a structure-dependent anti-oxidant effect of minocycline, since previous
3 findings [48] have shown a direct DPPH radical scavenging property and the capacity to
4 reduce Fe^{3+} . However, such properties failed to afford SOD or catalase activity of
5 minocycline. We have previously shown that minocycline does not prevent malonate-induced
6 peroxide formation in cerebellar granular cell [22], and is unable to prevent ROS-mediated
7 MPTP opening that supporting the above statement [43].
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10 Consistent with a role of mitochondria as a target of minocycline-mediated
11 neuroprotection, we found that minocycline affects distinctly activity of the respiratory chain
12 although the observed effect is rather complicated.. First, minocycline inhibits complex I and
13 complex IV of the respiratory chain. Interestingly, only these two complexes are true proton
14 pumps within the respiratory chain able to physically move protons across a membrane [49].
15 Nevertheless, the observed inhibition occurs in phosphorylating and uncoupled states
16 supported by malate-glutamate or by succinate. On the other hand, the inhibition limits
17 uncoupling capacity of uncoupler (FCCP) although minocycline added during resting state
18 stimulate the oxygen uptake that might be caused by uncoupling as minocycline is able to
19 decrease the inner membrane potential. However, the uncoupling effect is rather week when
20 compared to that of FCCP. The apparent contradictory effects of minocycline on
21 mitochondrial respiration (i.e., stimulation vs. inhibition) have been, in part, previously
22 reported for others drugs such as local anaesthetics [50]. Since activation of NMDA receptors
23 responsible for excitotoxicity imposes a high energetic demand on the *in situ* mitochondria
24 [51] one could hypothesize that minocycline exerts its neuroprotective effect by affecting
25 bioenergetic functions of mitochondria. Indeed, this effect could be responsible for the recent
26 report showing that minocycline has a harmful effect on patients with ALS [52]. Accordingly,
27 it was proposed by Kupsch et al. [53] that minocycline impairs mitochondria functions
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1 causing $\Delta\psi$ dissipation. Subsequently, deenergized mitochondria lose the ability to undergo
2 Ca^{2+} -triggered MPTP resulting in cell death.
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5 Minocycline might also act as a modulator of the voltage dependent anion channel
6 (VDAC). Minocycline added to a reconstituted system caused VDAC transition into lower-
7 conductance states and decreased the voltage dependence in a concentration-dependent way.
8 Thus, in the presence of minocycline the voltage dependence of reconstituted VDAC is
9 weaker although simultaneously the channel is modified to assume lower conductance states
10 that suggests an effect of minocycline on VDAC gating mechanism [39]. Furthermore
11 flickering of VDAC conductance observed in the presence of minocycline might result from
12 VDAC transient blockade/closure [54]. This, in turn, could have anti-apoptotic effects [19].
13 Moreover, VDAC closing is accompanied by a change of its selectivity toward cations (e.g.
14 [38, 40]). However, it has been recently proven that VDAC also has a cation selective open
15 state [55]. Therefore, it was proposed by Mannella and Kinnally [18] that VDAC modulators
16 triggering a transition to lower conductance states might not be acting to close but to open.
17 Nevertheless in the presence of minocycline, the permeability of the mitochondrial outer
18 membrane that is mediated by VDAC might be affected, thus contributing to neuroprotection.
19 In support of this view is the neuroprotective activity of rasagiline, a known anti-Parkinsonian
20 drug, related to its ability to modulate VDAC [56]. Noteworthy, minocycline does not change
21 formation or stability of the planar phospholipid membrane as measured by reconstitution
22 experiments. This implies the absence of nonspecific perturbations of lipid membranes by
23 minocycline. However, it can not be excluded that minocycline acts at the protein-lipid
24 interface causing redistribution of membrane lateral pressure sensed by specific membrane
25 proteins [57].
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55 In conclusion, the results obtained support a pleiotropic mechanism of minocycline
56 neuroprotective activity that involves direct interaction of minocycline with proteins (e.g.
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with VDAC), minocycline-mediated changes in the interface between lipids and membrane proteins (e.g. VDAC, NMDA receptor, complexes of the respiratory chain) and antioxidant effects of minocycline that control protein activity (e.g. MPTP opening). Although further investigations are required to clarify these mechanisms based in our results (Fig. 11) we can propose that minocycline affects VDAC activity, as well as the mitochondrial respiratory chain at the level of complex I and IV, inhibiting NMDA-induced entry of Ca^{2+} . As a result, minocycline may contribute to change the mitochondrial inner membrane potential, to ROS formation, and to mitochondrial Ca^{2+} uptake. The effects of minocycline on mitochondria complement its influence on NMDA-mediated Ca^{2+} influx into the cytosol, as changes in cytosolic Ca^{2+} concentration may in turn affect mitochondrial respiration, potentially leading to the prevention of NMDA-mediated excitotoxicity that is also conveyed by mitochondria; as lowering of cytosolic Ca^{2+} concentration inhibits formation and opening of MPTP crucial for apoptotic cell death. Simultaneously, formation and opening of MPTP may be inhibited by minocycline directly and/or indirectly due to its intricate effect on mitochondria. Therefore, under pathological conditions, minocycline may prove beneficial in preventing apoptotic signalling via stabilization of mitochondria-mediated signal cascades.

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Figure legends

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5 **Figure 1. Minocycline protects neuronal cultures against excitotoxic stimulus in a**
6 **concentration-dependent manner.** After 7 days in vitro, cultured cerebellar granule cells
7 were exposed to 100 μ M NMDA for 30 min. Minocycline (10-150 μ M) was added 10 min
8 before exposure to NMDA and maintained until the end of experiment. Cell viability was
9 assayed 24 h later. Black bars in this figure represent treatment with the indicated
10 minocycline concentrations in the absence of NMDA. The data represent mean \pm SD of at
11 least 9 coverslips (three independent cell cultures). * $p < 0.01$ different from 0 minocycline and
12 NMDA (analysis of the variance and Tukey test).
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27 **Figure 2. Minocycline inhibits the increases in cytosolic and mitochondrial $[Ca^{2+}]$**
28 **induced by NMDA.** A. Cerebellar granule cells were loaded with fura2/AM and $[Ca^{2+}]_{cyt}$
29 levels were monitored by fluorescence microscopy. Pictures show $[Ca^{2+}]_{cyt}$ levels (coded in
30 pseudocolor, color scale, 0 to 2500 nM) during perfusion with NMDA (100 μ M) with and
31 without minocycline (100 μ M). Traces show recordings of $[Ca^{2+}]_{cyt}$ in individual,
32 representative cerebellar granule cells. (n=193 cells, 4 independent experiments). B.
33 Cerebellar granule cells were transfected with GA plasmid. 24 h later were incubated with
34 coelenterazine for 2 h at room temperature and subjected to $[Ca^{2+}]_{mit}$ measurements by photon
35 counting imaging. Pictures show the bioluminescence (AEQ) and fluorescence (GFP) images
36 of transfected cells. Color scale 0 to 100 photonic emissions per pixel. Traces show
37 recordings of $[Ca^{2+}]_{mit}$ in individual, representative cerebellar granule cells (n=11 cells, 2
38 independent experiments).
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Figure 3. Minocycline decreases cytosolic Ca^{2+} levels when added in the presence of NMDA but not in the presence of high K^+ medium. Cerebellar granule cells were loaded with fura2/AM and $[\text{Ca}^{2+}]_{\text{cyt}}$ levels were monitored by fluorescence microscopy. Traces show the effect of minocycline (100 μM) over the increased $[\text{Ca}^{2+}]_{\text{cyt}}$ induced by NMDA (100 μM) (A) or high K^+ (B) medium. Traces are mean \pm SEM values of $[\text{Ca}^{2+}]_{\text{cyt}}$ on all cells present in the same microscopic field and are representative of at least 3 independent experiments.

Figure 4. Minocycline depolarizes mitochondria and inhibits mitochondrial Ca^{2+} uptake. A. Cerebellar granule cells were loaded with 10 nM TMRM for monitoring the effects of minocycline on the mitochondrial inner membrane potential. Pictures show TMRM staining of the same cells before and after treatment with 10 and 100 μM minocycline μM and 10 μM FCCP. Traces show average recordings of TMRM fluorescence in representative experiments. Fluorescence values were normalized to the value before treatment and averaged (n=50 cells, 3 experiments). B. Cerebellar granule cells were transfected with GA plasmid, cultured for 24 h and incubated with coelenterazine for 2 h at room temperature. Cells were permeabilized by digitonin in intracellular medium (see methods) and subjected to bioluminescence imaging of mitochondrial $[\text{Ca}^{2+}]$. Permeabilized cells were stimulated with intracellular medium containing 10 μM $[\text{Ca}^{2+}]$ in the presence of 100 μM minocycline and after its removal. Traces shown are recordings from representative, individual cells (n=36 cells, 6 independent experiments).

Figure 5. Minocycline presents anti-oxidant functions. A. Minocycline declines intracellular superoxide production rate induced by NDMA (100 μM) in cerebellar granular cells. The slopes of the lines fitting the fluorescence intensity changes (an index of the rate of superoxide production) were individually measured and averaged. At least 84 individual cells

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were averaged to calculate the slope. The mean slope values were used to generate a theoretical line using the following equation: $y=ax$. The figure represents generated lines of O_2^- production in both basal conditions and during 100 μ M NMDA with and without minocycline (1, 10, 50, 100 μ M). B. Minocycline abrogates ROS production caused by 75 μ M Ca^{2+} in brain isolated mitochondria. DFCA fluorescence was measured 30 min after the addition of Ca^{2+} to mitochondrial suspensions. Data are mean \pm SD, of at least 4 different preparations. Columns marked with the same small letters denotes values not significantly different ($p>0.05$). C-D. Minocycline showed a DPPH radical scavenger activity (C) and mediates the reduction of Fe^{3+} to Fe^{2+} (FRAP) (D). The data represent mean \pm SD of at least 4 or 3 independent experiments. * $p<0.05$ ** $p < 0.001$ vs control conditions (without minocycline).

Figure 6. Mitochondrial oxygen uptake of rat brain mitochondria in phosphorylating state. Respiratory rates were determined at 30 °C. Additions: mitochondria (0.7-0.8 mg/ml), 5 mM malate-5 mM glutamate, 6 mM succinate, 0.5 mM ADP and 125 μ M minocycline. The numbers near the traces indicates oxygen uptake in ng-at O/min x mg protein.

Figure 7. Mitochondrial oxygen uptake of rat brain mitochondria in resting state and in uncoupled state induced by FCCP. Respiratory rates were determined at 30 °C. Additions: mitochondria (0.7-0.8 mg/ml) 5 mM malate-5 mM glutamate, 6 mM succinate, 0.1 μ M FCCP and 125 μ M minocycline. The numbers near the traces indicates oxygen uptake in ng-at O/min x mg protein (n=3).

Figure 8. Effects of minocycline on the mitochondrial oxygen uptake of rat brain mitochondria in state 4, state 3 (0.5 mM ADP) and state 3u (0.1 μ M FCCP) (n=3). Respiratory rates are determined with mitochondria (0.7-0.8 mg/ml) at 30 °C. Panel A: 5 mM malate, 5 mM glutamate as respiratory substrate; panel B: 10 mM succinate as substrate.

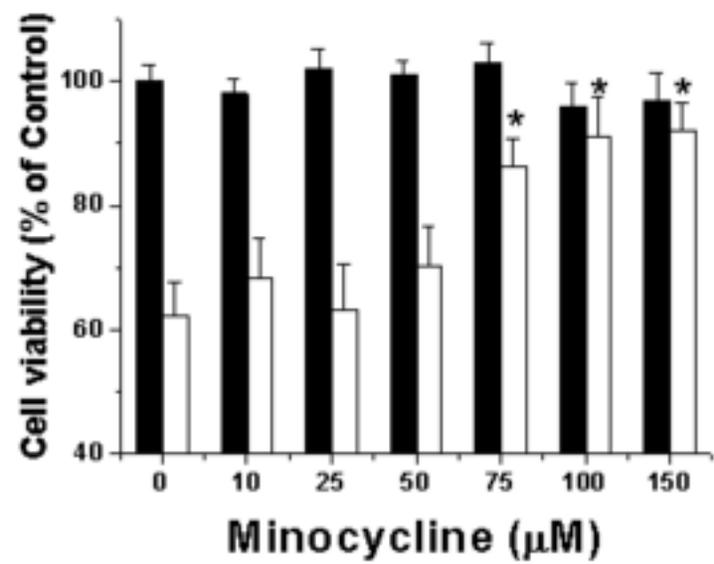
Figure 9. Effects of minocycline on the electron transfer activities of rat brain mitochondrial membranes. NADH-cytochrome c reductase activity (Complexes I-III) and succinate-cytochrome c reductase activity (Complexes II + III) expressed as nmol cytochrome c reduced/min \times mg protein; and cytochrome oxidase activity (Complex IV) expressed as nmol cytochrome c oxidized at 10 μ M cytochrome c/ min \times mg protein. (n=3).

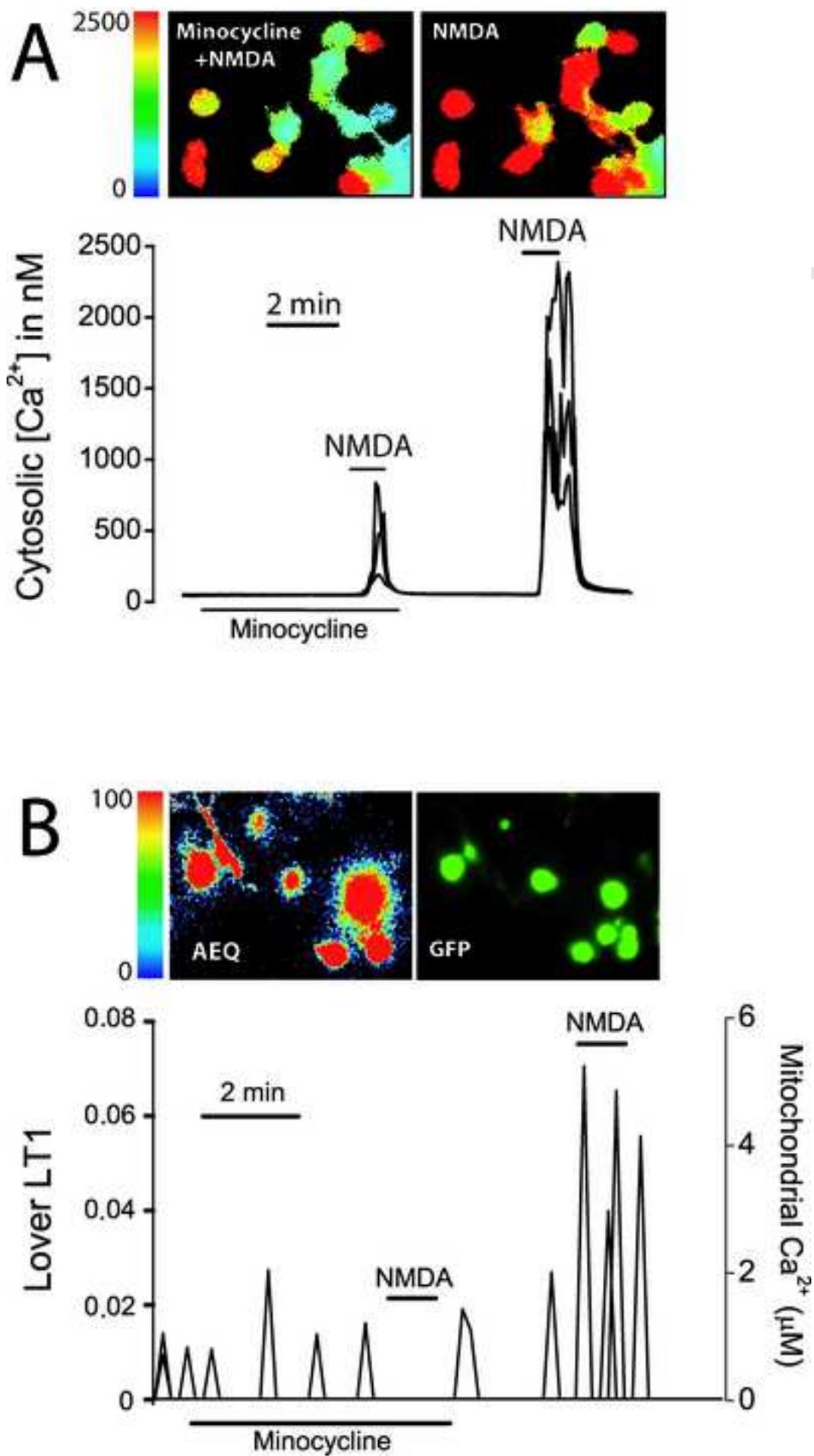
Figure 10. The effect of minocycline on reconstituted VDAC. (A) Examples of single channel insertions in the presence of increasing minocycline concentrations at a membrane potential of +10 mV. (B) Histograms of VDAC conductances obtained in the presence of increasing minocycline concentrations at a membrane potential of +10 mV. P (G) is the probability that a given conductance increment G is observed. (C) VDAC average conductance as a function of minocycline concentration. Values of average conductance were calculated at a membrane potential of +10 mV. The dominance of VDAC closure at a membrane potential of + 70 mV corresponded to an average conductance of 1.9 ± 0.2 nS. (D) The dependence of VDAC on the applied potential in the presence of minocycline increasing concentrations. G/G_0 is the ratio of the conductance (G) at a given voltage and the conductance (G_0) at the lowest applied potential (10 mV). The average amount of analysed insertion events for a given conditions used to calculate average conductance was 70. The obtained values of average conductance were statistically different.

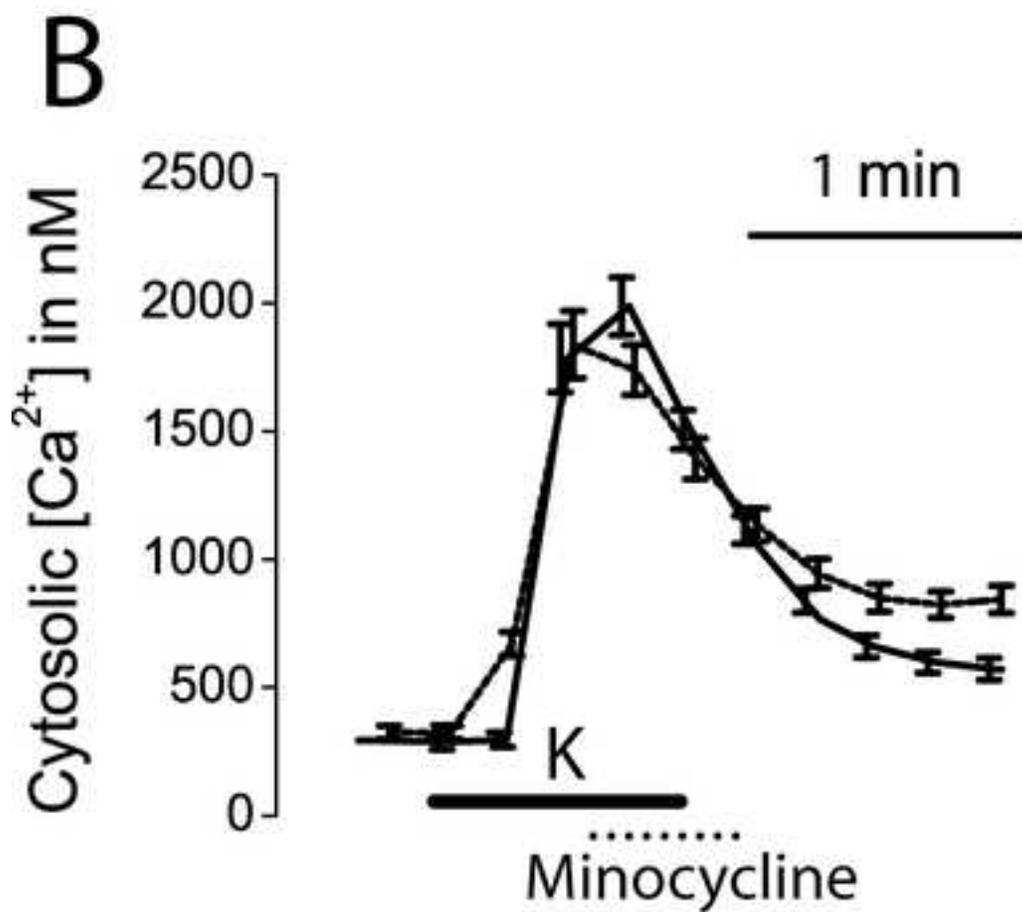
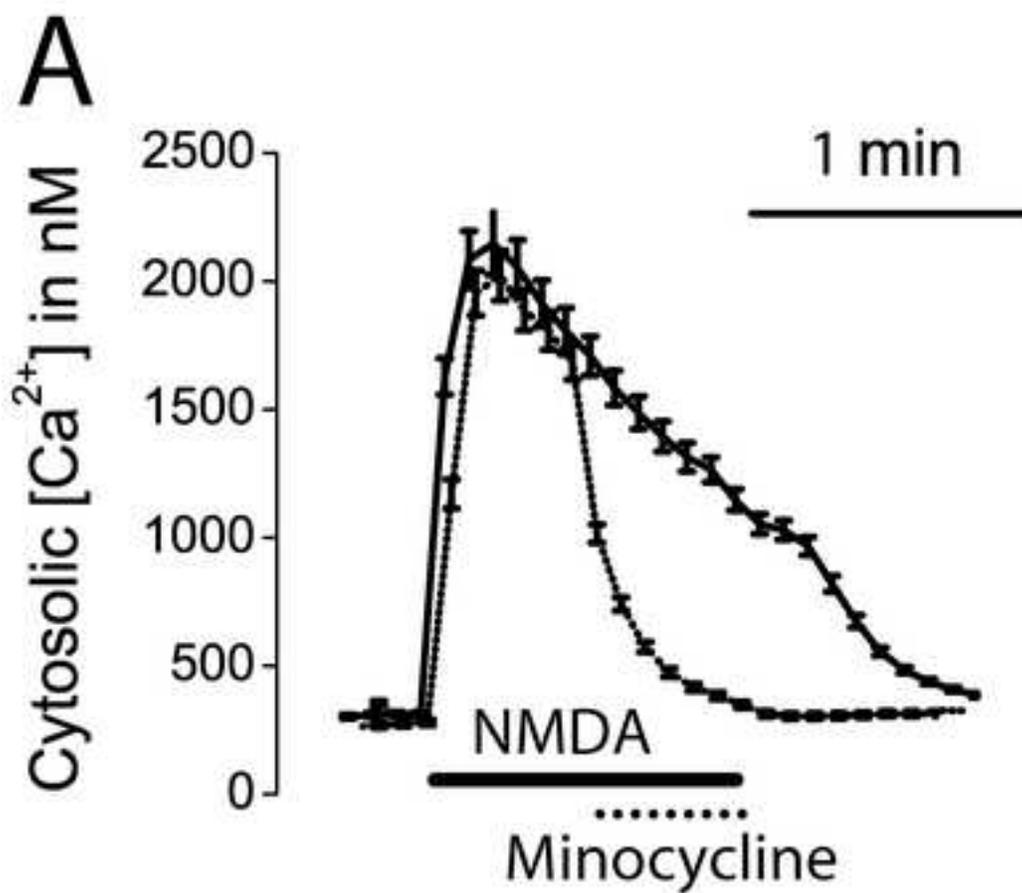
Figure 11. A model of minocycline-mediated neuroprotection. Minocycline inhibits Ca^{2+} entry supported by NMDA receptor being a hallmark of excitotoxicity. In addition, minocycline modulates both VDAC and the respiratory chain (RC) leading to mitochondrial depolarization. Mitochondrial depolarization diminishes the driving force for mitochondrial Ca^{2+} uptake through the mitochondrial Ca^{2+} uniporter (MCU), which prevents mitochondrial Ca^{2+} overload, ROS production and apoptosis execution. These pleiotropic effects may contribute to explain the neuroprotection afforded by minocycline.

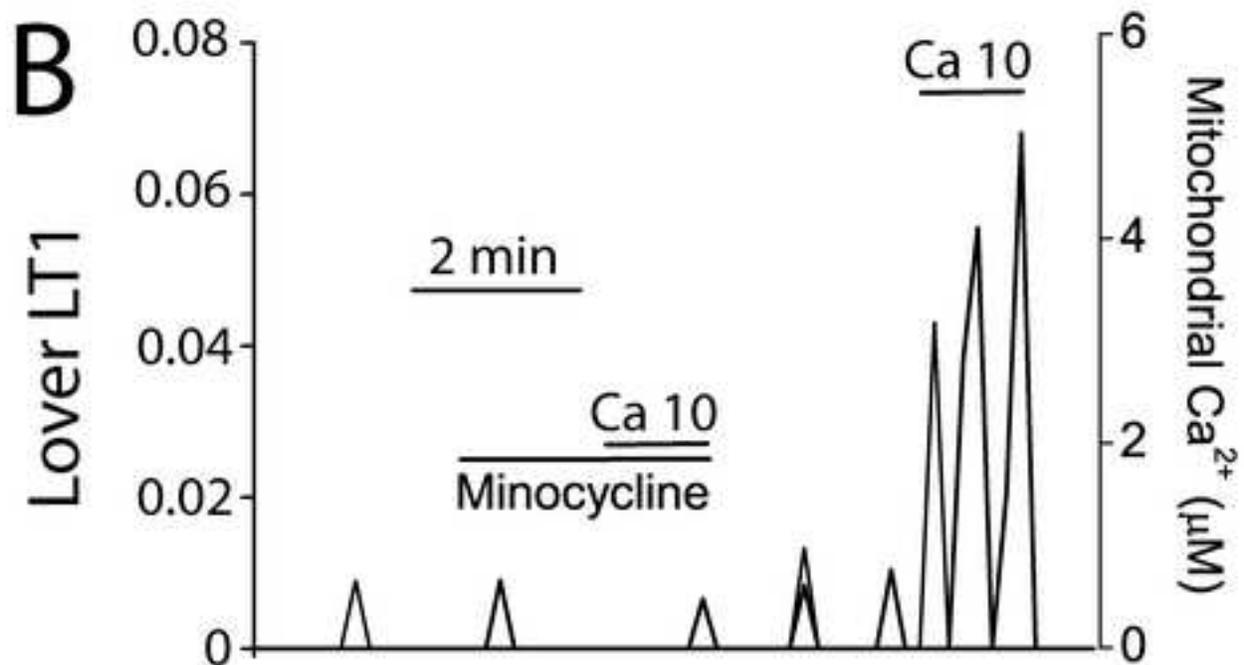
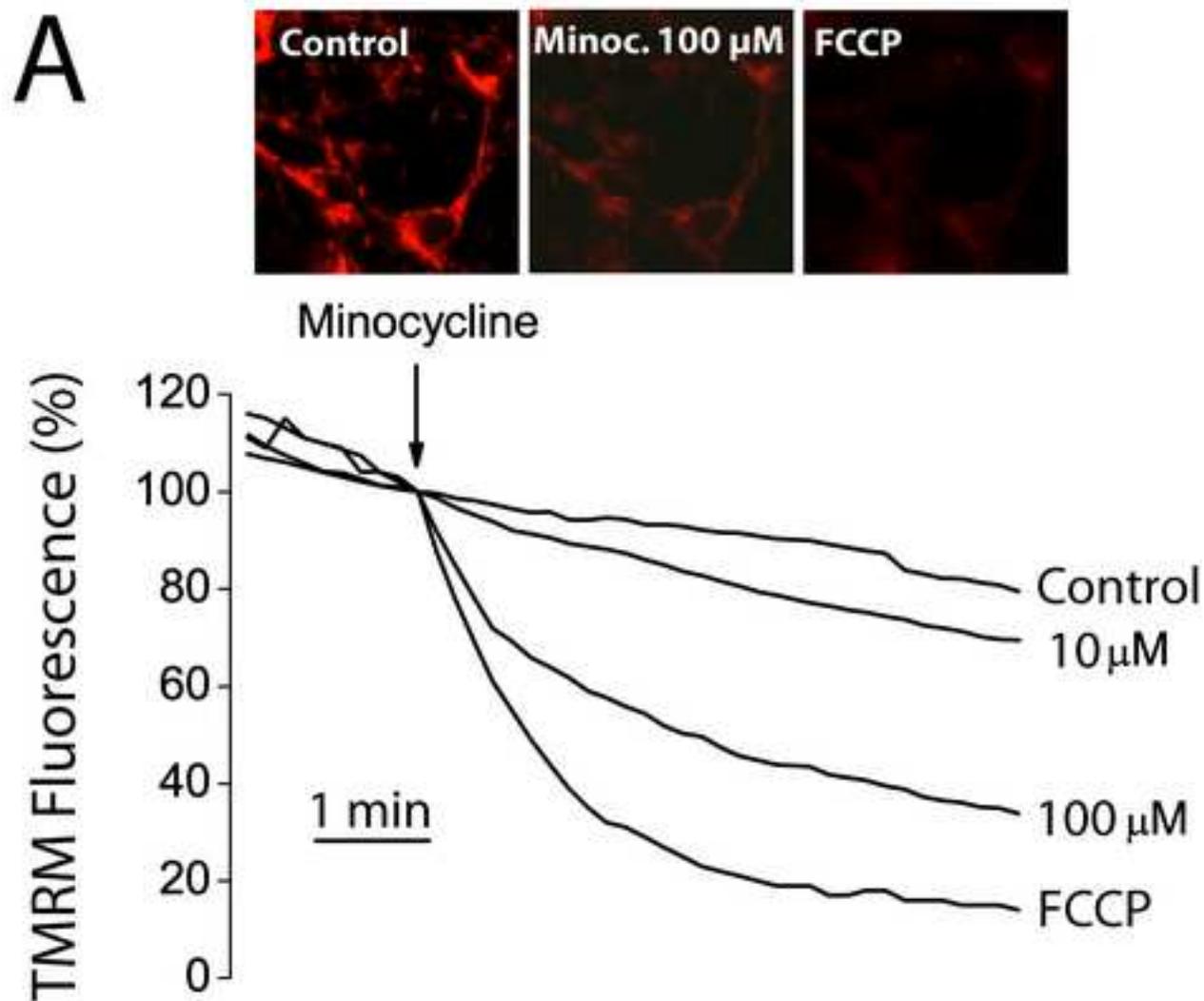
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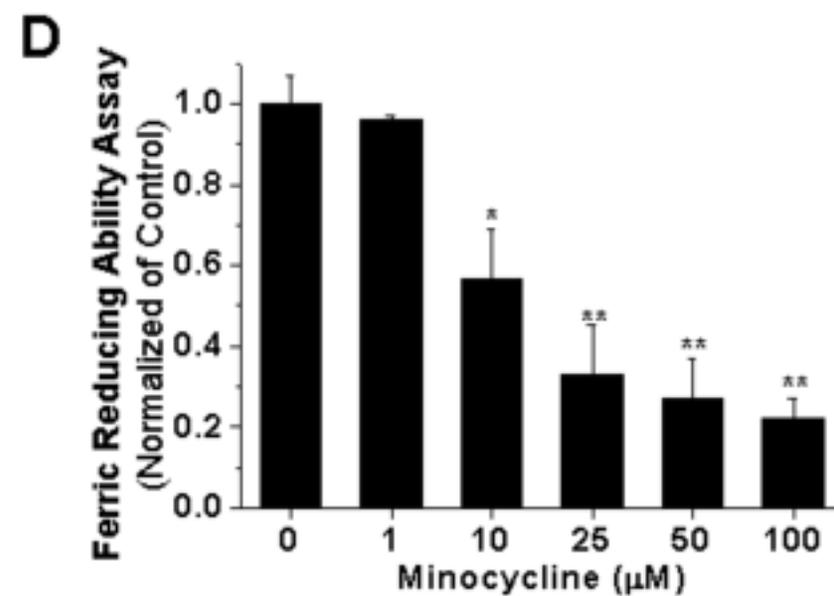
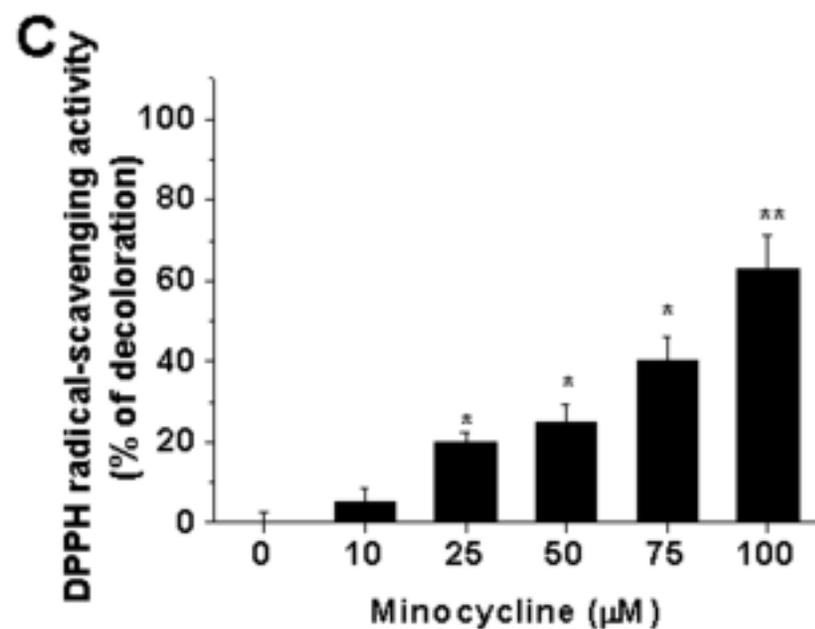
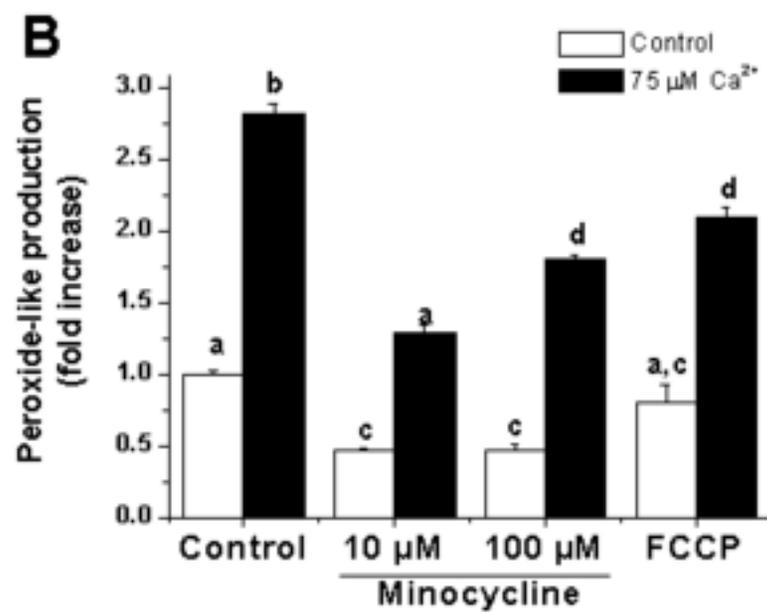
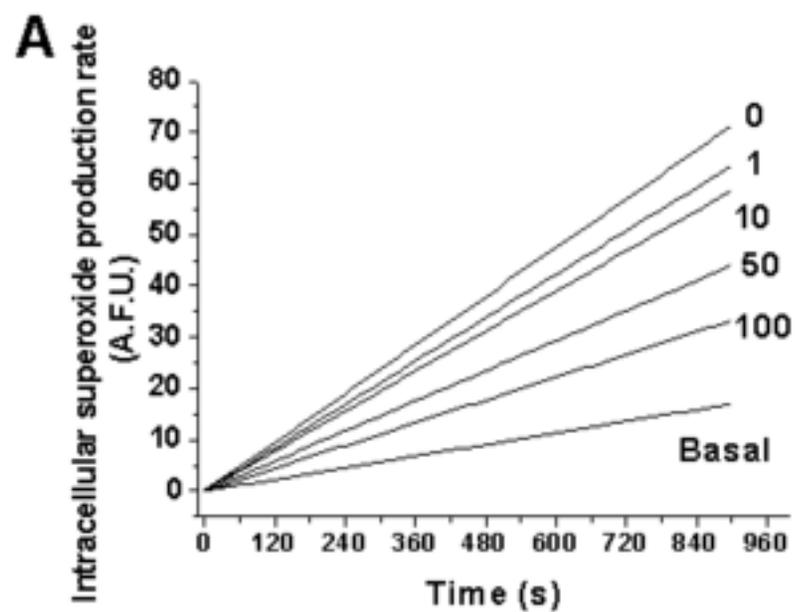


Figure 6

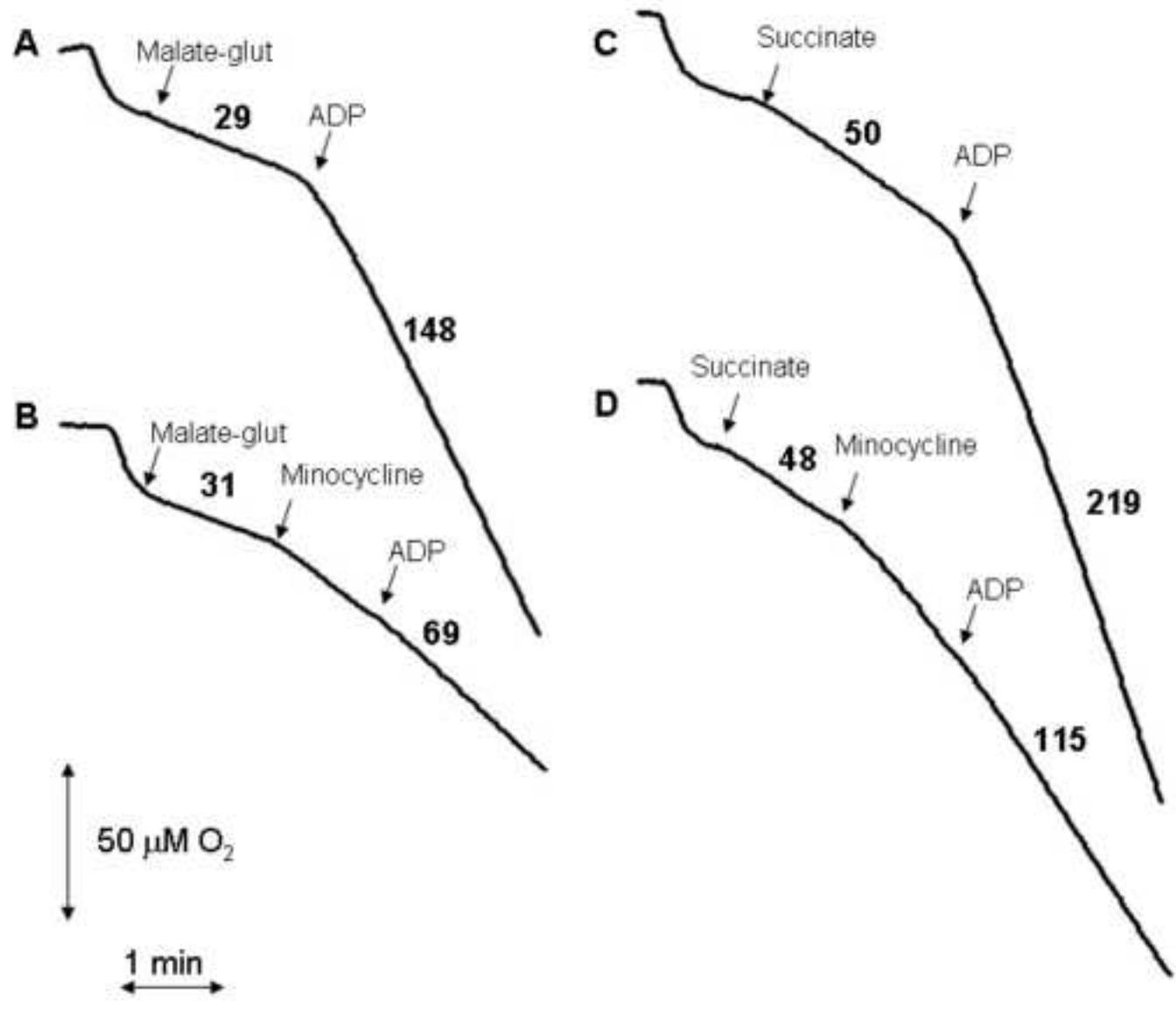


Figure 7

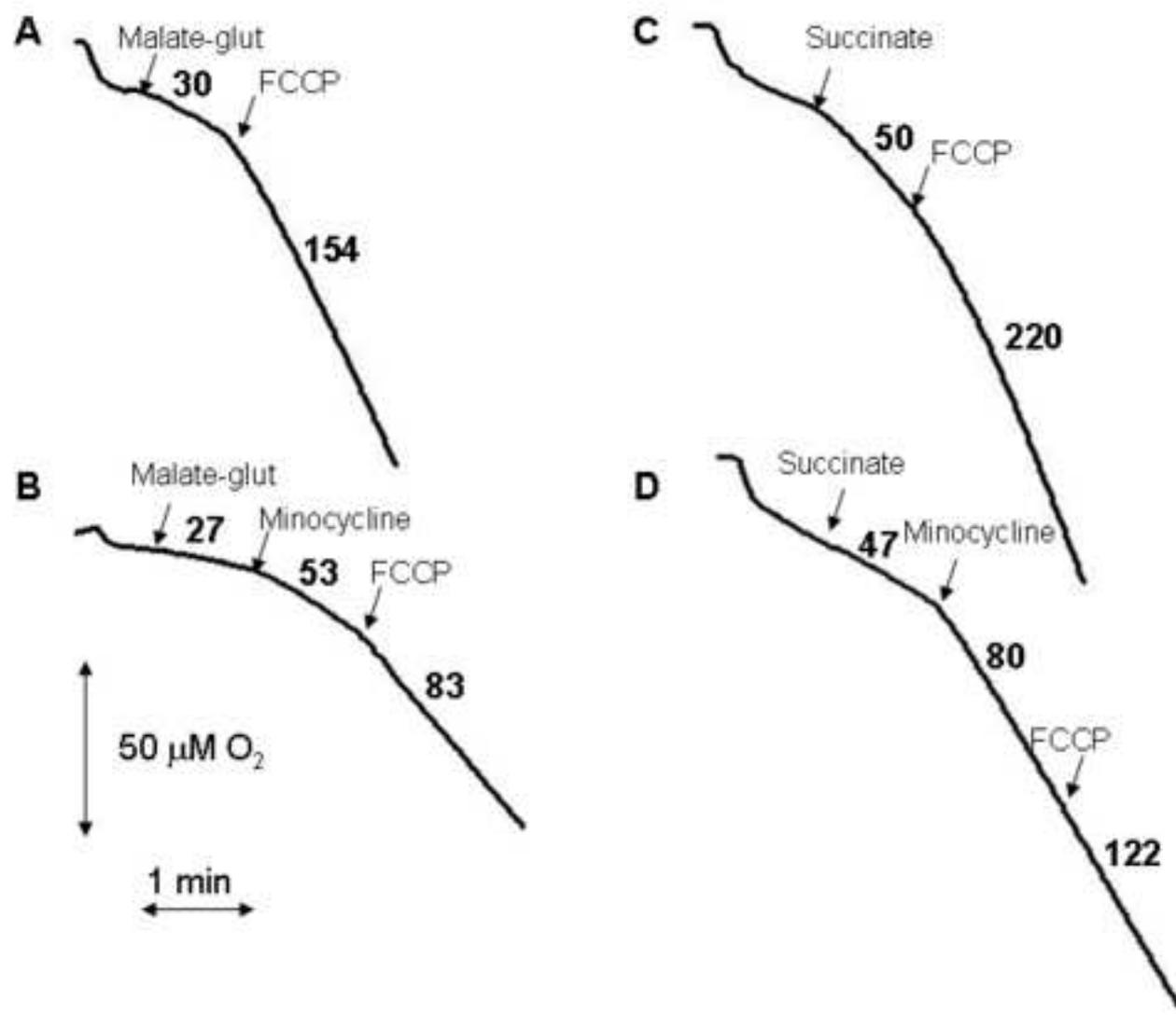


Figure 8

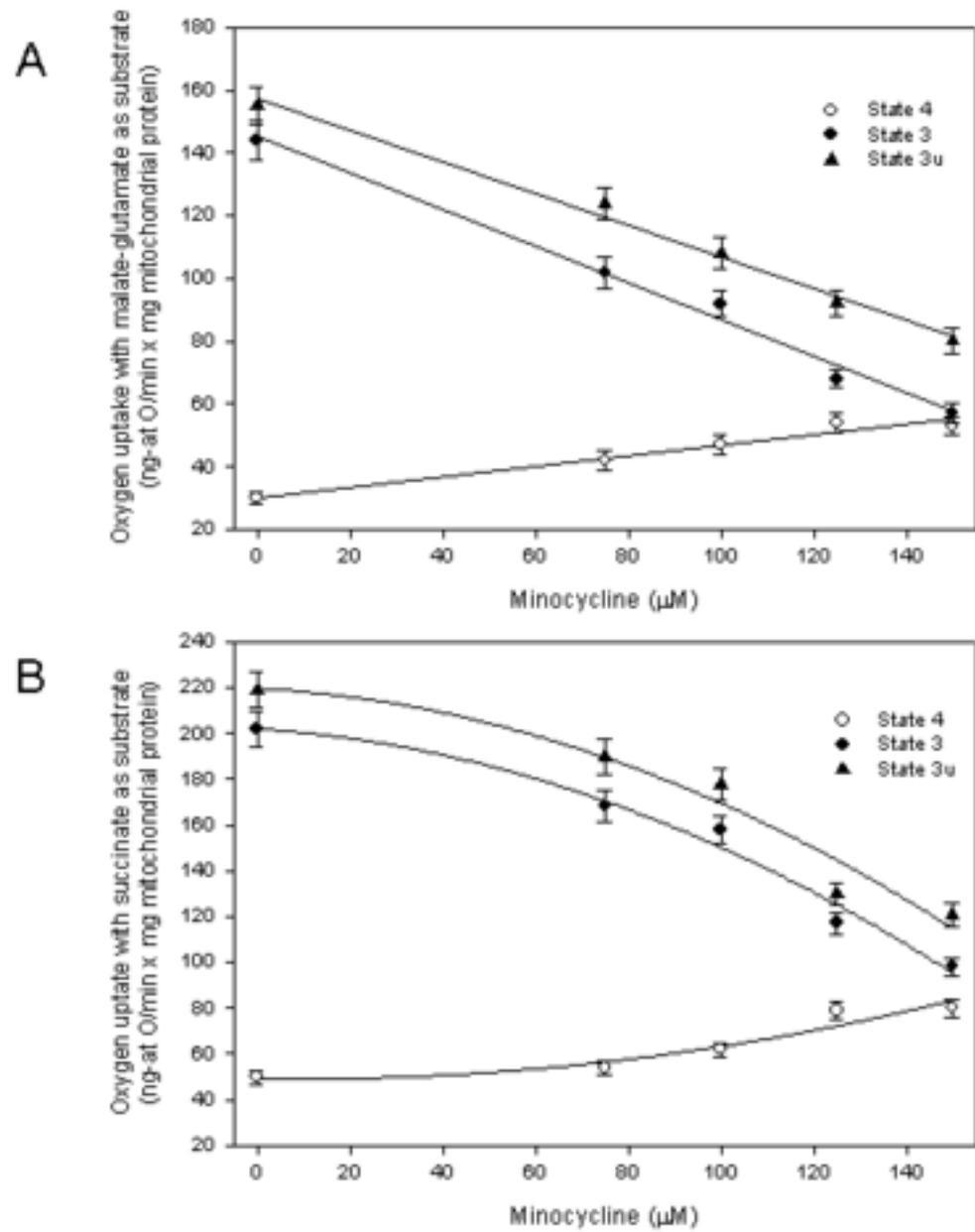


Figure 9

