

Research Article

Heterogeneous Responses to Antioxidants in Noradrenergic Neurons of the Locus Coeruleus Indicate Differing Susceptibility to Free Radical Content

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The present study investigated the effects of the antioxidants trolox and dithiothreitol (DTT) on mouse Locus coeruleus (LC) neurons. Electrophysiological measurement of action potential discharge and whole cell current responses in the presence of each antioxidant suggested that there are three neuronal subpopulations within the LC. In current clamp experiments, most neurons (55%; 6/11) did not respond to the antioxidants. The remaining neurons exhibited either hyperpolarization and decreased firing rate (27%; 3/11) or depolarization and increased firing rate (18%; 2/11). Calcium and JC-1 imaging demonstrated that these effects did not change intracellular Ca^{2+} concentration but may influence mitochondrial function as both antioxidant treatments modulated mitochondrial membrane potential. These suggest that the antioxidant-sensitive subpopulations of LC neurons may be more susceptible to oxidative stress (e.g., due to ATP depletion and/or overactivation of Ca^{2+} -dependent pathways). Indeed it may be that this subpopulation of LC neurons is preferentially destroyed in neurological pathologies such as Parkinson's disease. If this is the case, there may be a protective role for antioxidant therapies.

1. Introduction

Oxidative stress in neurons arises because of an imbalance between free radical production and antioxidant control. This process leads to cell damage and, when severe, can trigger apoptosis or necrosis [1]. Indeed, such oxidative stress may initiate certain neuropathologies such as Alzheimer's and Parkinson's disease whereby oxidative damage to biomolecules causes cellular dysfunction and neuronal death [1, 2].

The exact mechanisms whereby healthy neurons become sensitive to oxidative stress are unknown, and it is also unclear whether antioxidant treatments limit the spread of oxidative damage. For example, it has been reported that varying levels of oxidants can modulate ion channels and consequently effect important neuronal functions such as

pacemaking [3–5]. Thus, antioxidant treatments have been developed and used and have indeed had beneficial effects in preventing or slowing the onset of neurological disease [6].

Antioxidants may modulate transcription factors that ultimately lead to oxidative stress [7]. On the other hand, it has been demonstrated that molecules that were thought to be antioxidants due to their antioxidant capacity *in vitro* (such as vitamin A and retinoids) have different effects in living organisms (*in vivo*) as they can increase oxidative damage to biomolecules and generate oxidative stress [8–10]. In fact free radicals are crucial components of many intracellular signalling pathways (for a review see [11]), and an exaggerated decrease in their levels could lead to undesired cellular events. Thus, many factors contribute to the varied effectiveness of antioxidants in both human and

animal trials including differences in dose and timing of antioxidant administration.

The present paper explores the effect of two different antioxidants (Trolox and DTT) on pacemaking in Locus coeruleus (LC) neurons. These antioxidants can chelate different reactive species. Both compounds are membrane permeable, so they can easily access intracellular compartments. Trolox is a water-soluble vitamin E analogue with a broad antioxidant spectrum [12], whereas DTT is a reducing agent that acts on thiol (–SH) groups [13]. Here, we demonstrate that LC neurons exhibit three different types of electrophysiological responses to these antioxidants. We believe these responses may represent three neuronal populations that have differential sensitivity to free radicals and are involved in the massive loss of LC neurons observed in pathologies such as Parkinson's disease.

2. Experimental Procedures

2.1. Preparation of Brain Slices. All procedures used in this study were approved by the University of Newcastle Animal Care and Ethics Committee. Brain slices containing the LC were prepared from Swiss mice (P7–12, both sexes) rendered unconscious with Ketamine (100 mg/kg i.p.) according to a previous established protocol [14]. Mice were decapitated, and the brain was rapidly removed and immersed in ice-cold “modified sucrose ringer” containing (in mM): 25 NaHCO₃, 11 Glucose, 235 Sucrose, 2.5 KCl, 1 NaH₂PO₄, 1 MgCl₂, and 2.5 CaCl₂, bubbled with 95% O₂/5% CO₂ [15]. The cerebellum and brain stem were isolated and slices (270–300 μm thick) were cut with a vibrating tissue slicer (Leica VT1000S). Slices were kept in a recovery chamber (containing ACSF) at room temperature and high oxygen for 1.5–2 h before experiments commenced. The slice containing the LC was identified and transferred to a recording bath where it was visualized with an upright microscope (Olympus BX50) and superfused with artificial cerebrospinal fluid (ACSF) containing (in mM): 120 NaCl, 25 NaHCO₃, 11 Glucose, 2.5 KCl, 1 NaH₂PO₄, 1 MgCl₂ and 2.5 CaCl₂, constantly bubbled with 95% O₂/5% CO₂. LC neurons were visualized using infrared video microscopy with differential interference contrast optics and identified according to their large size and location near the ventrolateral border of the fourth ventricle [16]. Most experiments were performed on neurons in slices, whereas our Ca²⁺ imaging and JC-1 experiments used isolated LC neurons.

2.2. Preparation of Fresh Dissociated LC Neurons. Neurons were isolated using an adapted protocol according to [17]. Brain slices were cut using the same protocol described above in “Preparation of brain slices” and allowed to recover for 1 h in the recovery chamber. Slices were then placed in the ACSF-containing recording chamber, and neurons were dissociated and isolated using a custom-made vibrating device. This instrument dissociated neurons by vibrating a fine glass electrode with a sealed tip just above the tissue surface. This method usually provided about 10 healthy neurons per slice. After isolation, neurons were left for 15 min to settle on the

glass bottom of the recording chamber before gentle ACSF perfusion was commenced.

2.3. Electrophysiology. Electrophysiological recordings were made using the whole cell attached voltage clamp or current clamp recording modes (Axopatch-1C amplifier) with data sampled at 100 kHz and low-pass filtered at 5 kHz. Electrodes had resistance ranging from 1.8 to 2.5 MΩ. Neurons were considered to be adequately voltage clamped when no unclamped spikes were observed during voltage ramp application [14, 16].

2.4. Solutions and Pharmacology. To avoid perfusion-associated washout during whole cell recording, we used the same experimental protocol as described previously [16]. In brief, 10 s after intracellular access was obtained, the first voltage protocol was applied, with the treatment perfusion started immediately using a rapid exchange (>95% change over time ~2 s) local-perfusion system [18]. The second voltage protocol was then applied 180 s after commencement of treatment. Total incubation time for voltage clamp experiments was 180 s; the incubation time for other experiments is indicated in the respective figures. Tetrodotoxin (TTX; 1 μM) was added to the perfusate to block voltage-dependent Na⁺ channels when step pulse protocols were applied. The standard internal pipette solution contained (in mM): 135 K methylsulphate, 8 NaCl, 10 HEPES, 2 Mg₂ATP, 0.3 Na₃GTP, 0.1 EGTA, pH: 7.3. The standard external solution used was ACSF. All experiments were carried out at 33 ± 2°C.

2.5. Acquisition and Analysis. Data were acquired using Axograph 4.8 software (ITC-16 interface and a Mac G4 computer) and analysed using Axograph X 1.1.0 software. Input resistance, cell capacitance, and series resistance were measured by the software according to the response to a –5 mV pulse delivered shortly after obtaining the whole cell recording mode. A correction of –8.5 mV, calculated using JPCalc [19], was applied to account for the junction potential between ACSF and K methyl sulphate in the recording pipette. *I-V* plots were constructed from depolarizing pulse protocol recordings. Individual currents were normalised to cell size based on the whole cell capacitance to obtain current density values (pA/pF). Current density values were multiplied by 100 to normalise values to the measured current. Recordings obtained by ramp and depolarizing pulse protocols were filtered at 1 kHz using Axograph X software prior to analysis.

2.6. Measurement of Cytosolic Ca²⁺ and Mitochondrial Membrane Potential (Ψ_m). Relative intracellular [Ca²⁺] or Ψ_m was measured in freshly dissociated LC neurons. After isolation (Methods), LC neurons were incubated in ACSF at room temperature containing the fluophores 10 μM Oregon green/AM for 45 min or 5 μg/mL JC-1 for 1 h [20]. The recording chamber containing the isolated and loaded neurons was then placed on the stage of a Nikon TE200 inverted microscope connected to a BIORAD 1000 confocal scanner system. The isolated neurons were viewed with a 60X

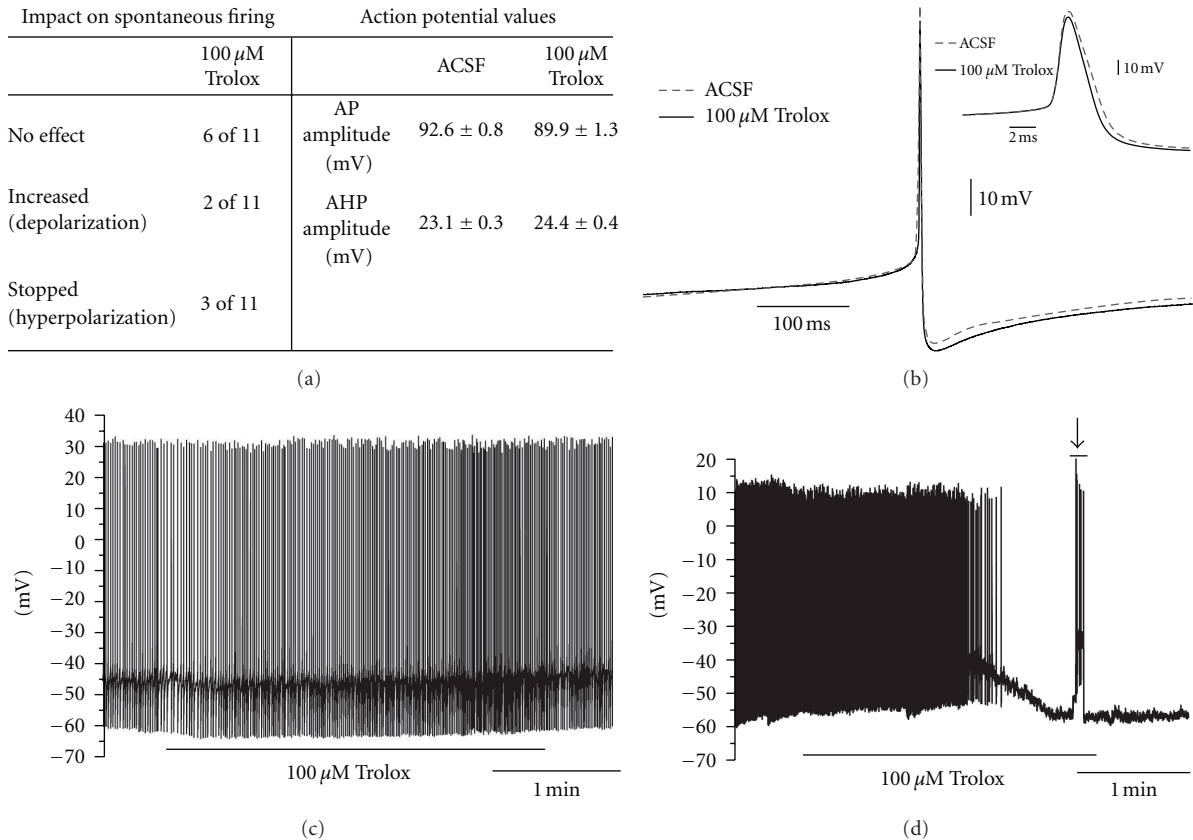


FIGURE 1: Impact of Trolox treatment on pacemaker activity in LC neurons. (a) Table demonstrating the effect of 100 μ M Trolox on the spontaneous firing of LC neurons and comparison of values obtained for action potentials (APs) before (ACSF) and 180 s after 100 μ M Trolox treatment ($n = 11$ for spontaneous firing and $n = 23$ for AP comparison). (b) Comparison of the waveshape of averaged APs before (ACSF) and 180 s after application of 100 μ M Trolox in ACSF ($n = 23$). Hyperpolarizing group was excluded from comparison demonstrated in a and b due to lack of APs at 180 s treatment. (c) and (d) Recordings demonstrating opposite effects induced by 100 μ M Trolox which in a small number of cases depolarized neurons and increased firing (c) or hyperpolarized neurons which led to abolition of AP firing (d) ($n = 11$). AP firing recommenced in response to depolarization induced by current injection (d, Arrow).

water immersion objective. ACSF was perfused for at least 10 min before experiments commenced. The Oregon green fluophore was excited using a 488 nm 20 mW Argon laser with intensity set to 3% and recordings made using a 522 nm emission filter. JC-1 experiments were made using the Argon laser set to 1% intensity with a filter combination of 488 nm for excitation and 522 nm for emission to record the green fluorescence and 514 nm for excitation and 585 nm for emission to record the red fluorescence. Relative fluorescence plots were analysed offline using ImageJ software.

2.7. Statistics. All data are presented as mean \pm SEM. GraphPad Prism 4.02 was used to prepare graphics. Statistical analysis was performed with SPSS version 17.0 using one-way ANOVA.

3. Results

3.1. Effect of Trolox and DTT on Spontaneous Firing and Voltage-Dependent Currents of LC Neurons. We first examined the effects of antioxidants on spontaneous firing in LC

neurons. Application of 100 μ M Trolox had no significant effect on 6 of 11 neurons (Figure 1(a)). Curiously, under the same conditions, 2 neurons increased their firing rate (Figures 1(a) and 1(c)) and 3 neurons exhibited strong hyperpolarization, which abolished spontaneous firing. In such cases firing could be reinstated by repolarizing the membrane potential via current injection (Figures 1(a) and 1(d)). Comparison of averaged action potentials (APs) from test (100 μ M Trolox) and control (ACSF) neurons showed that there were no differences in AP shape after 180 s of treatment (Figures 1(a) and 1(b)). The electrophysiological properties of neurons that did not respond and those that hyperpolarized were compared to examine whether the hyperpolarizing neurons were damaged. This comparison showed there were no differences in resting membrane potential, input resistance, firing frequency, and after hyperpolarization amplitude. This indicates that the hyperpolarizing neurons were not simply damaged or unhealthy neurons (See Supplementary Table in Supplementary Material available online at doi:10.1155/2012/820285).

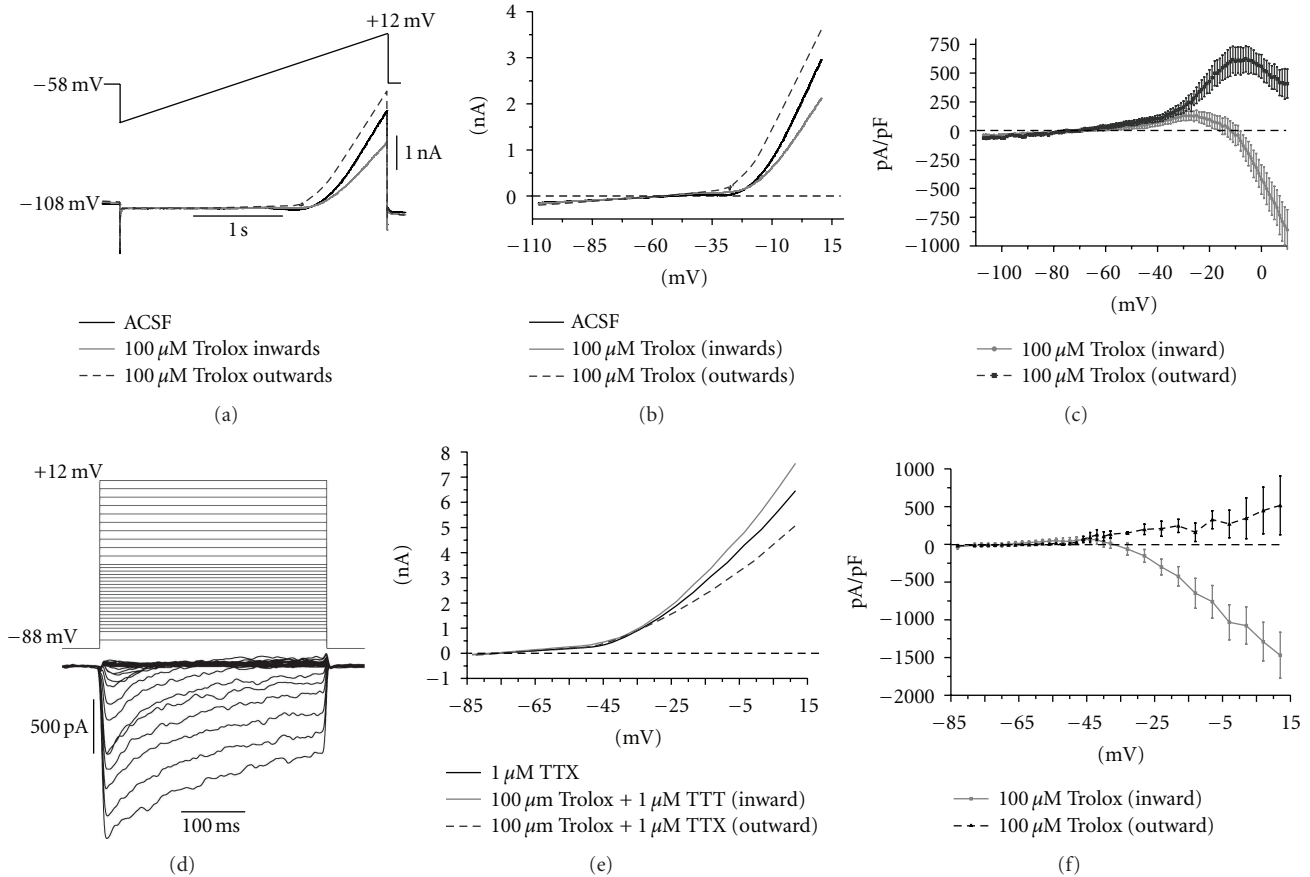


FIGURE 2: Effect of Trolox treatment in voltage-dependent currents evoked by ramps and pulses. (a) Representative recording of the currents evoked by depolarizing ramps showing the current evoked in ACSF (control solution; solid black line) and the inward (solid light grey) and outward (dashed grey) currents evoked at 180 s after 100 μM Trolox application ($n = 14$). (b) I - V plot for the currents presented in (a). (c) Mean differential (i.e., Trolox-control) with records obtained 180 s after rapid application of Trolox showing the inward (solid light grey, 8 of 14 neurons) and outward (dashed grey, 6 of 14 neurons) currents. (d), (e), and (f) were performed in 1 μM TTX background. (d) Representative depolarizing pulse-evoked differential currents obtained by taking the currents in test solution (i.e., 100 μM Trolox + 1 μM TTX) and subtracting the corresponding currents in control (i.e., 1 μM TTX) 180 s after Trolox application. (e) Averaged I - V plot showing the currents evoked by pulses for control (1 μM TTX—solid black) and the inward (solid light grey) and outward (dashed grey) currents after 180 s of 100 μM Trolox application. (f) Mean differential I - V plots (i.e., Trolox—TTX) inward (solid light grey, 6 of 8 neurons) and outward (dashed grey, 2 of 8 neurons) after 180 s application of 100 μM Trolox. Graph shows mean \pm SEM with $n = 8$ for (d), (e) and (f).

Voltage-dependent currents were first investigated using depolarizing ramps (slope of 40 mV/s) that were designed to mimic the natural depolarization during the interspike interval (see [16]). In these experiments ($n = 14$ LC neurons) Trolox induced a heterogeneous response (Figure 2(a)) with 8 neurons presenting a differential outward current followed by an inward current at depolarized potentials. In contrast, the remaining 6 neurons only exhibited a differential outward current (Figures 2(b) and 2(c)). The second protocol applied voltage steps designed to rapidly activate/deactivate a broad range of ionic channels. When this protocol was applied to neurons held at a hyperpolarized potential of -88 mV, similar results were observed, but in this case, more polarized towards the inward current (6 of 8 neurons, Figures 2(d) to 2(f)). Importantly, the step pulse protocols only generated either an outward or inward current (Figure 2(f)), versus the presence of both currents for ramp protocols

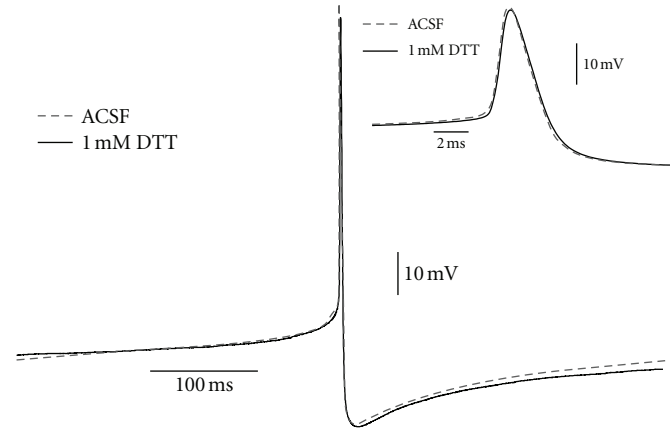
(Figure 2(c)). These results indicate that a mix of different voltage-dependent channels are directly modulated by Trolox and/or by reactive species chelated by this antioxidant, and this effect is heterogeneous within the population of LC neurons.

Dithiothreitol (DTT; 1 mM) also produced varied effects on spontaneous firing activity of 19 LC neurons (Figure 3(a)). AP firing was not significantly altered in 10 neurons and was abolished in 4 due to marked hyperpolarization (Figure 3(c)), and 3 neurons ceased AP firing with no apparent hyperpolarization (Figures 3(a) and 3(d)). A small portion of the neurons (2/19) increased their AP firing rate after depolarization (2 of 19; Figure 3(a)). AP comparison demonstrated no differences in AP shape (Figure 3(b)) as was the case for Trolox.

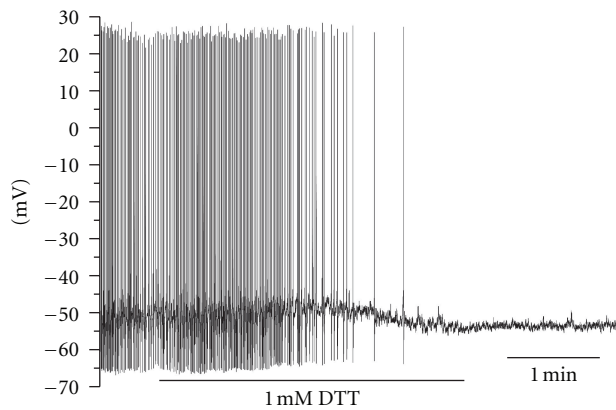
The voltage-dependent current responses that were observed after DTT application were more heterogeneous than

Impact on spontaneous firing		Action potential values		
	1 mM DTT		ACSF	1 mM DTT
No effect	10 of 19	AP amplitude (mV)	95 ± 1.2	95.9 ± 1
Increased (depolarization)	2 of 19	AHP amplitude (mV)	24.9 ± 0.3	26.2 ± 0.2
Stopped (hyperpolarization)	4 of 12			
Stopped (no hyperpolarization)	3 of 12			

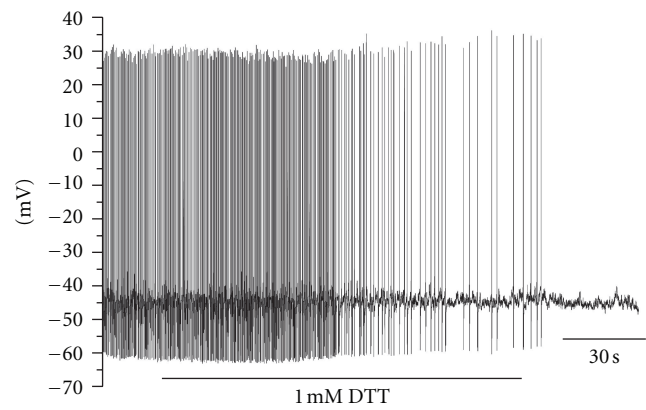
(a)



(b)



(c)



(d)

FIGURE 3: Impact of DTT treatment in the pacemaker process of LC neurons. (a) Table demonstrating the effect of 1 mM DTT in the spontaneous firing of LC neurons and comparison of values obtained for AP before (ACSF) and 180 s after 1 mM DTT treatment ($n = 19$ for spontaneous firing and $n = 28$ for AP comparison). (b) Averaged APs demonstrating comparison before (ACSF) and after 180 s after 1 mM DTT treatment ($n = 28$). Hyperpolarizing group was excluded from comparison demonstrated in (a) and (b) due to lack of APs at 180 s treatment. (c) and (d), Example for recordings demonstrating the two minor effects induced by 1 mM DTT where neurons hyperpolarized and ceased firing (c) and stopped firing without hyperpolarization (d) ($n = 19$).

those for Trolox application. Application of depolarizing ramps to 9 LC neurons indicated that 7 exhibited a large inward current at depolarized potentials with minimal if any initial outward current, and 2 neurons exhibited only outward currents (Figures 4(a) to 4(c)). Voltage step protocols applied to further 10 LC neurons produced the same characteristic depolarization-induced responses with 5 exhibiting inward and 5 exhibiting outward differential currents (Figures 4(d) to 4(f)). The size of the currents induced by both voltage ramp and step protocols during DTT treatment was larger than the currents induced by Trolox treatment (compare Figures 2(c) and 2(f) with Figures 4(c) and 4(f)).

3.2. Effect of Trolox and DTT Cotreatment with the Mitochondrial Protonophore CCCP during Ramp-Elicited Currents. We next investigated a role of mitochondria in the antioxidant actions as these organelles are the main source of reactive oxygen species in neurons and also play an important role in

Ca^{2+} buffering [21]. To do this, we perturbed mitochondrial metabolism using the mitochondrial protonophore carbonyl cyanide *m*-chlorophenylhydrazone (CCCP) without and with cotreatment with Trolox or DTT.

Ramp protocols were used as they can reveal voltage-dependent currents, including those that flow during the interspike interval [16]. CCCP ($1 \mu\text{M}$) induced an initial differential outward (up to ~ -20 mV) followed by an inward current during the ramp depolarization (Figure 5). Cotreatment with Trolox ($100 \mu\text{M}$) caused a small reduction in the CCCP-induced differential outward current between membrane potentials ~ -40 mV to ~ -30 mV (Figure 5(a)). DTT cotreatment in contrast had a more profound impact on the CCCP-induced differential outward current. It partially reversed the outward current and reduced it to less than half at its peak (Figure 5(b)). DTT co-treatment also shifted the reversal potential from ~ 80 mV to ~ -60 mV, suggesting recruitment of different channels and/or modulation of the channel's selectivity to ions. These results

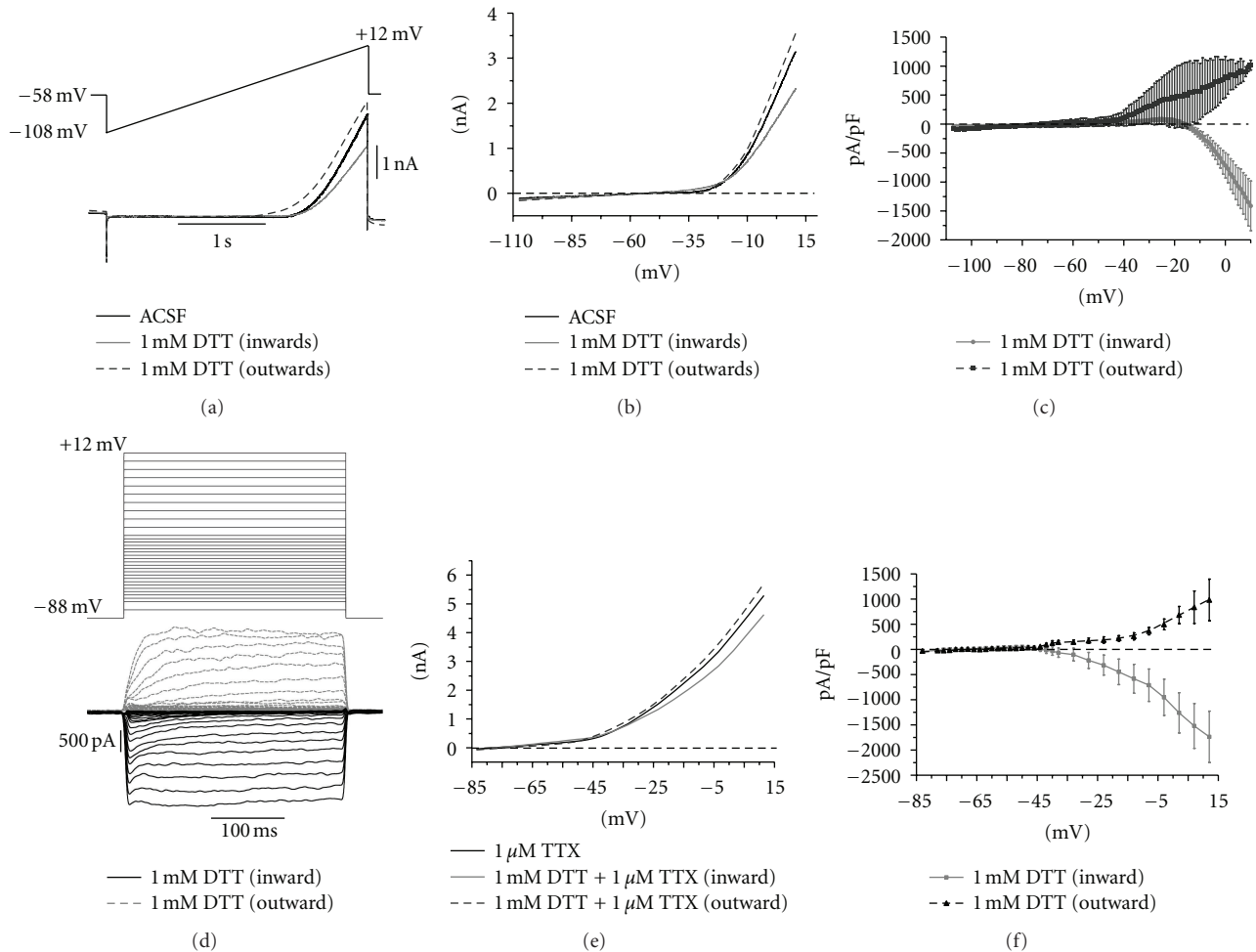


FIGURE 4: Effect of DTT treatment on voltage-dependent currents evoked by ramps and pulses. (a) Representative recording of the currents evoked by depolarizing ramps showing the current evoked in ACSF (control solution; solid black line) and the inward (solid light grey) and outward (dashed grey) currents evoked at 180 s after 1 mM DTT application ($n = 9$). (b) I - V plot for the currents presented in a. (c) Mean differential (i.e., DTT-control) with records obtained 180 s after rapid application of DTT showing the inward (solid light grey, 7 of 9 neurons) and outward (dashed grey, 2 of 9 neurons) currents. (d), (e), and (f) were performed in 1 μM TTX background. (d) Representative depolarizing pulse-evoked differential currents obtained by taking the currents in test solution (i.e., 1 mM DTT + 1 μM TTX) and subtracting the corresponding currents in control (i.e., 1 μM TTX) 180 s after DTT application. (e) Averaged I - V plot showing the currents evoked by pulses for control (1 μM TTX—solid black) and the inward (solid light grey) and outward (dashed grey) currents after 180 s of 1 mM DTT application. (f) Mean differential I - V plots (i.e., DTT—TTX) inward (solid light grey, 5 of 10 neurons) and outward (dashed grey, 5 of 10 neurons) after 180 s application of 1 mM DTT. Graph shows mean \pm SEM with $n = 10$ for (d), (e), and (f).

suggest that the antioxidants Trolox and DTT can affect mitochondrial function either directly or by altering existing neuronal free radicals.

3.3. Impact of Trolox and DTT Treatment on Cytosolic Ca^{2+} and Mitochondrial Membrane Potential under Control Conditions and When Mitochondrial Function Is Impaired. Due to the heterogeneous nature of the responses produced by the two antioxidant treatments, we next investigated if the activation of different currents was related to the cytosolic Ca^{2+} concentration ($[Ca^{2+}]_c$) in both control conditions and those where mitochondrial function was impaired. Figure 6(a) demonstrates that under control conditions,

neither Trolox or DTT treatments were able to increase $[Ca^{2+}]_c$, suggesting that Ca^{2+} -activated pathways are not involved in the induction/modulation of ionic currents by these antioxidants. When CCCP was added to impair mitochondrial function, Trolox (100 μM) caused a further enhancement of the CCCP-induced increase in $[Ca^{2+}]_c$ (Figure 6(b)). In contrast, cotreatment with DTT (1 mM) had no impact on the CCCP-induced increase in $[Ca^{2+}]_c$ (Figure 6(b)). The consequences of the Trolox-induced increase in $[Ca^{2+}]_c$ are unclear given that Trolox was less effective than DTT in modulating CCCP-related voltage dependent currents (Figure 5(a)).

Given that mitochondrial function is generally dependent on mitochondrial membrane potential (Ψ_m) [22], we

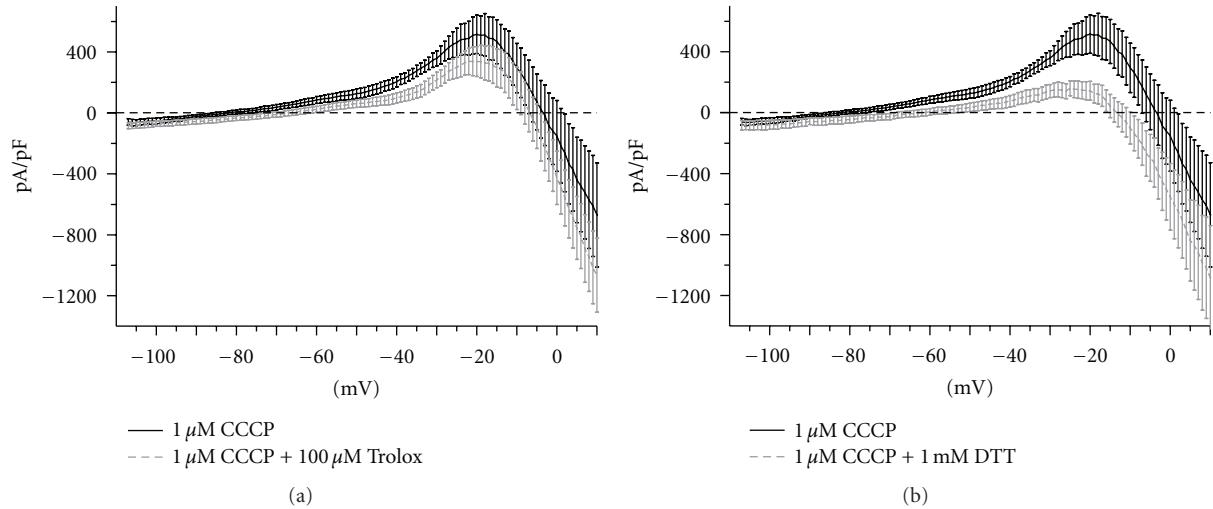


FIGURE 5: Effect of Trolox and DTT treatment on ramp-evoked currents when mitochondrial membrane potential was impaired with CCCP. (a) and (b), Mean differential $I-V$ plots (i.e., Antioxidants-control) demonstrating the impact of $100 \mu\text{M}$ Trolox (a) and 1mM DTT (b) cotreatments with $1 \mu\text{M}$ CCCP. Graph shows mean \pm SEM with $n = 13$ for (a) and $n = 6$ for (b).

investigated whether antioxidant treatments were able to modulate Ψ_m . Application of CCCP ($1 \mu\text{M}$) reduced Ψ_m (Figure 6(c)). Trolox ($100 \mu\text{M}$) or DTT (1mM) also reduced Ψ_m to an extent that was indistinguishable from that produced by impairing mitochondrial function with CCCP alone (Figure 6(c)). Application of the antioxidants together with CCCP appeared to be synergistic as it caused a profound decrease Ψ_m , with the effect of being most pronounced for DTT where a $\sim 70\%$ reduction in Ψ_m occurred (Figure 6(c)). Importantly, fluorescence levels partially recovered to the same levels after 10 min of washout for both antioxidants in control and/or impaired conditions (Figure 6(c)). Taken together, these results suggest that mitochondria could be involved in the currents induced by Trolox and DTT treatments, but such actions are unlikely to be dependent on $[\text{Ca}^{2+}]_c$.

4. Discussion

Many neuronal populations in the CNS are spontaneously active, including nuclei such as the LC and Substantia nigra (SN) [23, 24]. Such sustained AP firing is tightly controlled and, given that nuclei such as the LC project widely over the brain [25], has major implications for brain function.

Free radicals and oxidative stress are known to be involved in the initiation and/or progression of a variety of neurological conditions, such as Alzheimer's and Parkinson's diseases [26–28], and disturbances in mitochondrial metabolism seem to be a key event in the occurrence of such pathologies [29]. Here, we demonstrate that antioxidant treatment of LC neurons, a neuronal population that is suggested to be involved in progression of many neurological diseases [30, 31], produced a heterogeneous response on the pacemaking activity in these neurons. Our results indicate that within the LC there is a subpopulation of neurons, which are more sensitive to free radical content

and consequent modulation of the pacemaker activity. This opens the possibility that this neuronal subpopulation is particularly vulnerable and likely to undergo apoptosis or necrosis during the onset of specific neurological diseases.

The LC is the largest concentration of noradrenergic neurons in the brain, and it projects to and releases noradrenaline across a multitude of brain regions [32, 33]. LC neuron involvement in neurological pathologies, especially Parkinson's disease (PD), has been proposed because of the high levels of LC neuron degeneration observed in PD patients and because the LC plays a fundamental role in the early stages of the disease [31, 34]. Further support for this hypothesis is provided by the fact that lesions to LC neurons increase the sensitivity of SN neurons to a range of insults [35, 36] and that simultaneous partial destruction of SN and LC neurons slows recovery in animal models of Parkinson's disease [30]. Furthermore, pharmacological stimulation of LC neurons imparts some resistance to SN neurons in animal models; Parkinson's disease [37] and genetically modified animals with increased noradrenergic innervation appear to be relatively immune to insults on SN neurons [38]. Therefore neuronal death in the LC may be a primary factor in the onset of PD with the consequent loss of noradrenergic signalling contributing to the loss of dopaminergic neurons in the SN. The fact that oxidative stress is one of the main causes of neuronal death in PD [39] makes the finding that there are subpopulations of LC neurons that are highly sensitive to free radicals of considerable interest.

Our results indicate that both Trolox and DTT induce heterogeneous responses in LC neuronal pacemaking (Figures 1–4), suggesting a differential sensitivity to antioxidants (or free radical content) in neuronal subpopulations within the LC. The majority of the neurons exhibited no detectable alteration in their firing rates (Figures 1(a) and 3(a)). However, Trolox and DTT revealed two other responses and by

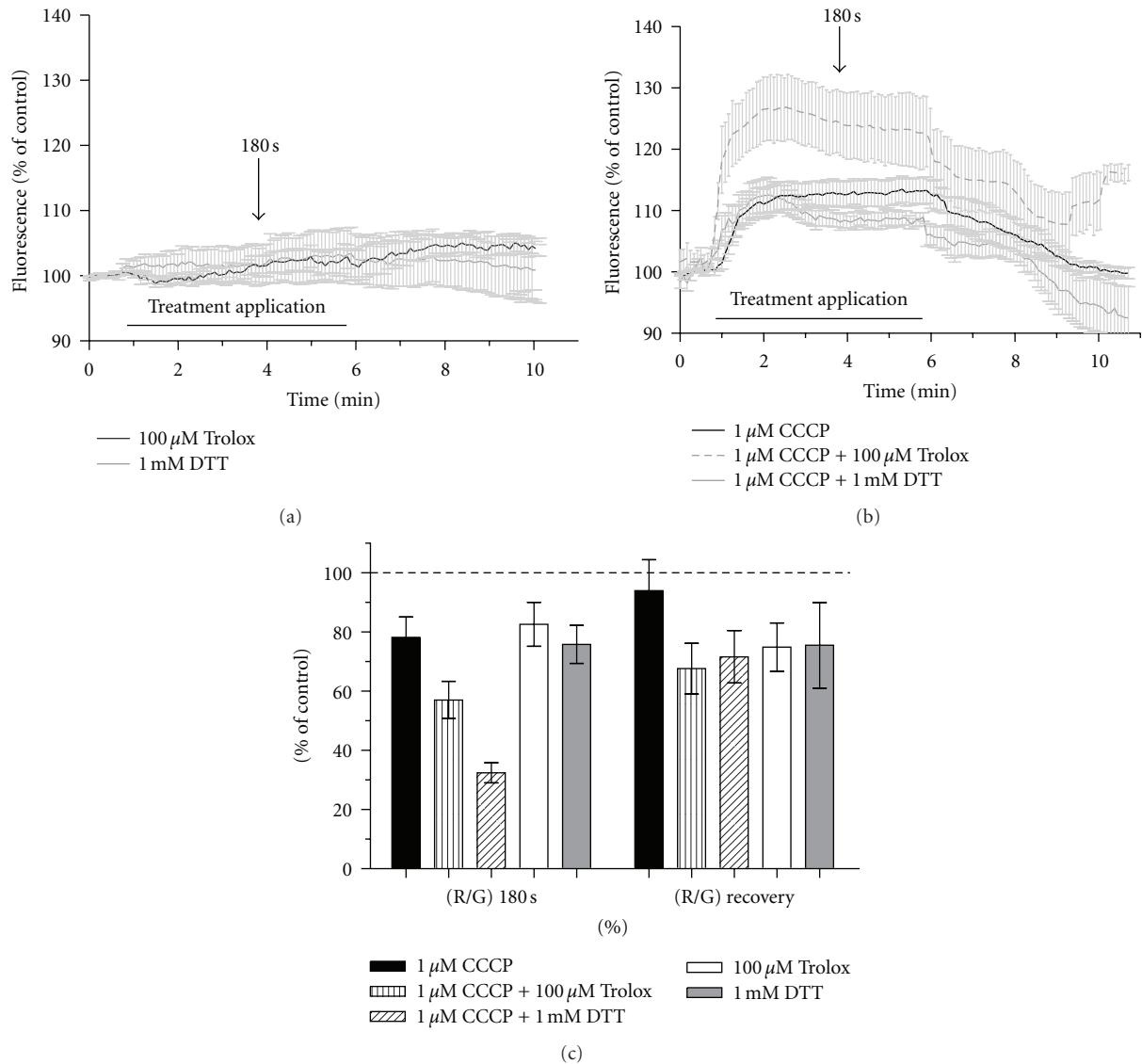


FIGURE 6: Effect of Trolox and DTT treatment in $[Ca^{2+}]_c$ and Ψ_m in control and impaired conditions. (a) Graph demonstrating that both antioxidants did not impact on $[Ca^{2+}]_c$ in control conditions ($n = 5$ for both). (b) Graph demonstrating that 100 μM Trolox co-treatment with 1 μM CCCP increased $[Ca^{2+}]_c$ and 1 mM DDT did not effect the normal CCCP-induced increase in $[Ca^{2+}]_c$ ($n = 6$ for Trolox $n = 5$ for DTT cotreatments, and $n = 7$ for CCCP by itself). (c) JC-1 fluorescence demonstrating the impact of Trolox and DTT in Ψ_m in control and impaired (1 μM CCCP cotreatment) conditions ($n = 8$ for Trolox and $n = 9$ for DTT by themselves; $n = 12$ for Trolox and $n = 10$ for DTT cotreatments; $n = 9$ for CCCP by itself). All graphs show mean \pm SEM.

inference neuronal subpopulations: the first featured hyperpolarization and slowing down or cessation of pacemaking activity, whereas the second caused depolarization and an increase in firing rate (Figures 1 and 3). These observations are surprising given that LC neurons have generally been considered to be fairly homogeneous with respect to their electrophysiological properties, and previous results from our group detected no electrophysiological differences in the neuronal population on which our experiments were made [14, 16, 40]. Voltage clamp experiments confirmed the heterogeneity we observed in our experiments on LC neurons, as application of the antioxidants also revealed two types of responses when ramps or pulses were applied

(Figures 2 and 4). Another important finding came from our Ca^{2+} imaging experiments where, in contrast, application of antioxidants produced no change in $[Ca^{2+}]_c$ (Figure 6(a)). This suggests that the effects we observed were caused by changes in the neuronal free radical content and not by changes in $[Ca^{2+}]_c$. Trolox treatment had a small impact on voltage-dependent currents (Figure 5(a)) however DTT treatment had a profound impact. DTT partially reversed the currents induced by CCCP coapplication (Figure 5(b)) and this effect was unlikely to be mediated by $[Ca^{2+}]_c$ (Figure 6(b)). A role for mitochondria was indicated as the antioxidants altered Ψ_m when applied alone or combined with CCCP (Figure 6(c)).

The finding of two neuronal subpopulations within LC that are responsive to antioxidant treatment may be of considerable importance for our understanding of the environment and physiological conditions that lead to development of neurological diseases. It is difficult to delineate which subpopulation will be most adversely affected by changes in free radical content. The subpopulation that responded to antioxidants with hyperpolarization will be protected by antioxidants, as pacemaking and hence firing rate would slow down or stop, thus saving energy and accelerating recovery time in cases of hypoxia [41]. Abolition of firing would also prevent activation of voltage-dependent Ca^{2+} channels and the subsequent increase in $[\text{Ca}^{2+}]_c$. This would slow activity in all Ca^{2+} -dependent pathways.

Control of $[\text{Ca}^{2+}]_c$ is of fundamental importance given apoptotic and/or necrotic pathways can be initiated by uncontrolled increases in $[\text{Ca}^{2+}]_c$ [42, 43]. In contrast, the neuronal subpopulation where antioxidants produced depolarization and elevated firing rates would be stressed by such treatment. We do not know the mechanism whereby some cells respond with inward or outward currents following antioxidant application. However, if these actions were due to altering free radical content, then “mopping up” free radicals may not necessarily protect cells. Given that specific neurological pathologies have been associated with an increase in free radicals, then it highlights the fact that antioxidant treatments may need to be carefully evaluated.

In summary, our findings indicate that there is a small neuronal subpopulation within LC that responds to antioxidants, and, on the basis that this is caused by altering free radicals, members of this subpopulation may be more subject to apoptosis or necrosis. We believe that the results presented here represent an important step for our understanding of how free radicals contribute to neuropathologies (such as LC loss in Parkinson’s diseases) and suggest that antioxidant therapies must be carefully evaluated before administration.

Abbreviations

ACSF:	Artificial cerebrospinal fluid
AP:	Action potential
$[\text{Ca}^{2+}]_c$:	Cytosolic Ca^{2+} concentration
CCCP:	Carbonyl cyanide m-chlorophenyl hydrazine
CNS:	Central nervous system
DTT:	Dithiothreitol
LC:	Locus coeruleus
PD:	Parkinson’s disease
TTX:	Tetrodotoxin
Vm:	Membrane potential
Ψm :	Mitochondrial membrane potential.

Acknowledgments

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