TIME AND DOSE DEPENDENT EFFECTS OF OXIDATIVE STRESS INDUCED BY CUMENE HYDROPEROXIDE IN NEURONAL EXCITABILITY OF RAT MOTOR CORTEX NEURONS

Pardillo-Díaz R[1], Carrascal L[1], Muñoz MF[2], Ayala A[2], Nunez-Abades P[1,3]

[1]*Department of Physiology. School of Pharmacy. University of Seville. Spain.*

[2]*Department of Biochemistry and Molecular Biology. School of Pharmacy. University of Seville. Spain.*

[3] *Correspondence: Pedro Núñez Abades. Departamento de Fisiología.*

Facultad de Farmacia. Universidad de Sevilla. C/ Profesor García González nº2.

41012-Sevilla, España.

e-mail: pnunez@us.es phone:+34-954556130

Abstract

It has been claimed that oxidative stress and the production of reactive oxygen radicals can contribute to neuron degeneration and might be one factor in the development of different neurological diseases. In our study, we have attempted to clarify how oxidative damage induces dose dependent changes in functional membrane properties of neurons by means of whole cell patch clamp techniques in brain slices from young adult rats. Our research demonstrates physiological changes in membrane properties of pyramidal motor cortex neurons exposed to 3 concentrations of cumene hydroperoxide (CH; 1, 10 and 100 μM) during 30 minutes. Results show that oxidative stress induced by CH evokes important changes, in a concentration and time dependent manner, in the neuronal excitability of motor cortex neurons of the rat: i) Low concentration of the drug (1 μM) already blocks inward rectifications (sag) and decreases action potential amplitude and gain, a drug concentration which has no effects on other neuronal populations, ii) 10 μM of CH depresses the excitability of pyramidal motor cortex neurons by decreasing input resistance, amplitude of the action potential, and gain and maximum frequency of the repetitive firing discharge, and iii) 100 μM completely blocks the capability to produce repetitive discharge of action potentials in all cells. Both larger drug concentrations and/or longer times of exposure to CH narrow the current working range. This happens because of the increase in the rheobase, and the reduction of the cancelation current. The effects caused by oxidative stress, including those produced by the level of lipid peroxidation, are practically irreversible and, this, therefore, indicates that neuroprotective agents should be administered at the first symptoms of alterations to membrane properties. In fact, the pre-treatment with melatonin, acting as an antioxidant, prevented the lipid peroxidation and the physiological changes induced by CH. Larger cells (as estimated by their cell capacitance) were also more susceptible to oxidative stress. Our results provide previously unavailable observations that large size and high sensitivity to oxidative stress (even at low concentrations) make pyramidal neurons of the motor cortex, in particular corticofugal neurons, more susceptible to cell death when compared with other neuronal populations. These results could also shed some light on explaining the causes behind diseases such as Amyotrophic Lateral Sclerosis.

Key words: Amyotrophic Lateral Sclerosis, cumene hydroperoxide, lipid peroxidation, neuronal excitability, patch clamp, pyramidal neurons.

INTRODUCTION

Oxidative stress is a pathological condition which implies overproduction of Reactive Oxygen Species (ROS) under conditions in which the cell's antioxidant defense system becomes no longer effective (Heather and Teismann, 2009; Sims-Robinson et al., 2013). ROS are generated as a consequence of an aerobic metabolism determined by mitochondrial respiration (Hool, 2006). To eliminate these ROS, cells develop several protective mechanisms. Among them, enzymes such as superoxide dismutases, catalases and glutathione peroxidases directly transform some ROS into compounds of lower toxicity through the oxidation of antioxidant metabolites: reduced glutathione, thioredoxin, and ascorbic acid (Reynolds et al., 2007; Sha et al., 2013). Low level of ROS participate in cell division and growth regulation, apoptosis regulation, oxidative modifications of biomolecules in extracellular space, protection from pathogen invasion, etc. (Han et al., 2008). High levels of ROS may appear in other situations (such as mitochondrial dysfunction, excitotoxic insult, or inflammation), and cause DNA mutations, ion channel damage, intensification of the lipid peroxidation process, and oxidation of proteins and other biomolecules (Lambert et al., 2004; Ljubisavljevic, 2014; Pitt et al., 2000**)** which lead to impairing cell function. They may also affect different transcription factors, growth factors, kinases phosphatases and cytokines (Arrigo, 1999; Emerit et al., 2004; Tirosh et al., 2000; Valencia and Moran, 2004; Vimard et al., 2011). At the level of the membrane, ROS alter ATP-sensitive K^+ currents, L-type Ca^{2+} currents (Goldhaber and Liu, 1994; Racay et al., 1997) and delayed rectifier K^+ currents (Goldhaber et al., 1989), ions transporters (Kourie, 1998), either through direct oxidation of lipids, or through alterations of cell membrane proteins and intracellular signaling pathways (Hool, 2006; Zhu et al., 2005). ROS can also affect membrane properties including variations in cell cytoskeletal architecture and membrane stiffness, membrane potential, ionic gradients, action potential duration and amplitude, spontaneous activity, and excitability (Jovanovic and Jovanovic, 2013; Nakaya et al., 1992; Nani et al., 2010; Pardillo-Diaz et al., 2015; Sinha et al., 2015).

Lipid peroxidation (LPO) is one of the most commonly studied processes of redox cell signalization disorders where free radicals have a great importance (Ayala et al., 2014). Through different mechanisms, LPO disrupts membrane barrier function, inactivates membrane enzymes, and increases permeability for water, monovalent and divalent ions, and even high molecular weight compounds (Ferretti and Bacchetti, 2011; Ljubisavljevic, 2014; Nam, 2011). Cumene hydroperoxide (CH) is a stable organic oxidizing agent that is known to

penetrate into the inner hydrophobic part of the membrane lipid bilayer, causing extensive peroxidation of lipids (to a much greater extent than hydrogen peroxide) (Jovanovic and Jovanovic, 2013; van den Berg et al., 1992). CH has also been described to be able to react with aminoacids and proteins with multiple effects, such as oxidation of side-chains, backbone fragmentation, dimerization/aggregation, unfolding or conformational changes, enzymatic inactivation, and alterations in cellular handling and turnover of proteins, as singlet oxygen does (Ayala et al., 2014; Davies, 2003; Gracanin et al., 2009). Consequently, CH has been used to inflict oxidative stress in vitro in neurons (Jovanovic and Jovanovic, 2013; Nakaya et al., 1992; Nani et al., 2010; Pardillo-Diaz et al., 2015). Regarding neuronal cell damage, it is widely believed that oxidative stress has a fundamental role in neuronal degeneration and might be one factor in the development of different diseases, such as Amyotrophic Lateral Sclerosis (ALS), Parkinson's, Schizophrenia, Alzheimer's (Andersen, 2004; Cabungcal et al., 2014; Cleveland and Rothstein, 2001; Reynolds et al., 2007) and ageing (Muller et al., 2007). ALS is a progressive neurodegenerative disease that results from the death of the upper motor neurons of the motor cortex, including layer pyramidal neurons, that regulate voluntary control of motor output (Mochizuki et al., 2011). *In vitro* and *in vivo* clinical and preclinical studies show that ALS is characterized by higher levels of oxidative stress biomarkers and by lower levels of antioxidant defense biomarkers in the brain and peripheral tissues (reviewed in Niedzielska et al., 2015). Post-mortem studies on tissue samples from ALS patients support the hypothesis of the oxidative damage in proteins, lipids, and DNA (Bogdanov et al., 2000; Ihara et al., 2005; Smith et al., 1998; Tohgi et al., 1999). Indeed, recent positron emission tomography (PET) imaging data in humans have confirmed that oxidative stress is enhanced in motor cortex in ALS patients compared with controls (Ikawa et al., 2014). Moreover, recent studies have demonstrated extensive early changes to the morphology of motor cortex neurons in SOD1 mice (mouse model of ALS based on oxidative stress), which thus confirms clinically relevant cortical pathophysiology more faithfully than previously thought (Fogarty et al., 2015; Saba et al., 2015). All this evidence leads to the conclusion that oxidative stress may be an important factor associated with the development of neurodegeneration in ALS patients.

In our study, we have attempted to clarify how oxidative stress evokes dose dependent changes in the functional properties of neurons. In previous studies, we have demonstrated that oxidative stress compromises both neuronal excitability and the capability of generating action potentials (Pardillo-Diaz et al., 2015). In order to understand how oxidative stress

modifies neuron cell membrane properties, we have used three concentrations of CH at quantities that do not induce early cell death (Shimura et al., 1985; Vimard et al., 1996 and 2011, Vroegop et al., 1995). We have registered the electrophysiological properties of pyramidal motor cortex neurons in brain slices by means of whole cell recordings. Our study aims to answer important questions such as: i) Does acute oxidative stress induce functional changes in a dose dependent manner? ii) In case such changes were dose dependent, are some membrane properties more sensitive to the drug concentration? iii) Can membrane properties be recovered to resting values after washout of the drug? iv) Can the pre-treatment with antioxidant agents, such as melatonin, prevent the LPO and the physiological changes induced by CH? v) Are alterations in membrane properties dependent of neuronal size? The answers to these questions could be potentially relevant to explain the role oxidative damage may have in neurological disease, such as ALS.

EXPERIMENTAL PROCEDURES

The method has been described in detail in a previous publication by our lab team (Pardillo-Diaz et al., 2015). Briefly, the present study was carried out in strict accordance with the recommendations of the Guide for the Care and Use of Laboratory Animals of the European Community Directive 2003/65 and the Spanish Royal Decree 120/2005. The research protocol was approved by the Animal Ethics Committee of the University of Seville. Wistar rats (20–40 days of age) were deeply anaesthetized with chloral hydrate (4%, Panreac). Brains were quickly removed and placed in ice-cold low-calcium Artificial Cerebro-Spinal Fluid (ACSF). Transverse sections (thickness 300 μm) of the primary motor cortex were cut off on a vibratome (NVLSM1, WPI), placed in an ACSF-filled chamber for 30 min at ~37 °C, and then stored at ~21 °C. The composition of the ACSF (in mM) was as follows: 126 NaCl, 2 KCl, 1.25 Na_2HPO_4, 26 $NaHCO_3$, 10 glucose, 2 $MgCl_2$, and 2 $CaCl_2$. For the low-calcium-ACSF solution, the concentrations were 4 $MgCl_2$ mM and 0.1 $CaCl_2$ mM. Both ACSF and low-calcium-ACSF solutions were bubbled with 95% O_2–5% CO_2 (pH 7.4).

Whole-cell patch-clamp recordings

Slices containing the primary motor cortex were transferred to a recording chamber and superfused at 1–2 $ml \cdot min^{-1}$ with circulating aerated ACSF warmed to 33°±1°C via a feedback-controlled heater (TC 324B; Warner). Neurons were patch-clamped under direct visual control using a Nikon Eclipse FN1 microscope equipped with infrared-differential

interference contrast (IR-DIC) optics, a 40× water immersion objective, and a WAT-902H2 Ultimate Camera. Cortical pyramidal neurons were distinguished by their typical morphology (Stuart et al., 1993) including a large pyramidal-shaped soma and a prominent apical dendrite extending vertically toward the pial surface. Patch pipettes were pulled (PC-10, Narishige, Tokyo, Japan) from borosilicate glass capillaries with filament (inner diameter 0.6, outer diameter 1 mm; Narishige) to a tip resistance of 3–5 MΩ. Patch pipettes were filled with (in mM): 120 K-gluconate, 10 KCl, 10 phosphocreatine disodium salt, 2 MgATP, 0.3 NaGTP, 0.1 EGTA, 10 HEPES, adjusted to pH 7.3 with KOH. The osmolarity of the intracellular solution was 285 mosmol/kg, adjusted with sucrose. Whole-cell recording configuration was obtained using a micromanipulator (MP-225, Sutter) and a patch-clamp amplifier (Multiclamp 700B, Axon Instruments, Molecular Devices, Sunnyvale, CA, USA). Giga seals (>1 GΩ) were always obtained before rupture of the patch and pipette capacitance was compensated for before breaking in, and, in current-clamp mode, the bridge was periodically balanced using the auto-adjust feature. Series resistance was 20 MΩ or less during recording. Current-clamp recordings were low-pass Bessel-filtered at 10 kHz; data were digitized at 2–20 kHz with a Digidata 1550 analog-to-digital converter and acquired using the pCLAMP 10 software (Molecular Devices). Data were stored and subsequently prepared for analysis with the Clampfit 10.4 software (Molecular Devices).

Drugs and protocols

CH (Sigma–Aldrich, St. Louis, MO, USA) was prepared just prior to experiments from 60 mM stock solutions stored at −80°C. To avoid concerns associated with incomplete drug washout or incomplete recovery to control conditions, and, for all protocols below, only one cortical neuron per slice was recorded.

As a preliminary objective, we wanted to know if the effects demonstrated for CH at 10 µM in our previous study (Pardillo-Díaz et al., 2015) were reversible. In that study, it was found that the application of CH (10 µM) produces noticeable effects in some parameters, such as resistance and rheobase, at minute 5 and 15 (Pardillo-Díaz et al., 2015). For this reason, in this work, we have first tested the effects of the CH washout 5 minutes after administration, and we have compared the electrophysiological parameters obtained in current-clamp mode at the beginning, after 5 min CH application and 15 minutes after the washout. We have secondly measured the same parameters in a similar experiment in which we maintained the CH application for a longer period of 15 minute duration.

A second objective of the present study was to compare the effects of CH at different concentrations: 1 µM (n=30); 10 µM (n=30); and 100 µM (n=30). Data obtained for the 10 µM concentration are those reported in Pardillo-Diaz et al. (2015) and were recorded in the same experimental run as the data reported here for other concentrations. Each neuron was initially superfused with normal ACSF to study their electrophysiological parameters in current-clamp mode under initial condition (time 0). After this, the slice would be superfused with ACSF containing 1, 10 or 100 µM CH for 30 minutes, and voltage responses were acquired at 5, 15 and 30 minutes after CH application. In order to assess if long-lasting recording (30 min) affects the viability of the cells, we have also evaluated the electrophysiological properties of 20 neurons in the absence of the drug, at the same time intervals. Data from these cells were considered as a control group.

In addition, we wanted to find out the sequential effects that different concentrations of CH may have on each neuron. In order to do so, we have applied the 3 different concentrations (1, 10 and 100 µM) to the same cell at 3 moments in time. Electrophysiological parameters were analyzed before drug administration (0 µM), and 10 minutes after the exposure to each of the 3 concentrations.

To determine the level of oxidative stress induced by CH and whether it can be prevented by the use of melatonin, we used a technique to measure hydroperoxide which relies upon the rapid hydroperoxide-mediated oxidation of Fe^{2+} under acidic conditions. Fe^{3+} forms a chromophore with xylenol orange which strongly absorbs at 560 nm (Jiang et al., 1991). To know the level of LPO induced by CH, trimmed brain slices (thickness 300 µm) containing primary motor cortex were placed in artificial ACSF with 1, 10 and 100 µM of CH and they were each incubated for 5, 15 and 30 min. Slices were placed in normal ACSF to stop the chain-reaction evoked by CH and stored in HEPES solution. The slices were homogenized during 10 s using a sonicator (Hielscher UP 100H). Then, samples were centrifuged at 2400 x g and the supernatants were collected. Total protein was determined by the Lowry method. The protocol for LPO measurements was adapted for a microplate reader (Asys UVM 340). 40 µg of protein was incubated with 90 µl of H_2SO_4 for 30 min. After addition of 100 µl FOX reagent (0.5 mM ferrous ammonium sulfate, 0.2 mM xylenol orange and 200 mM sorbitol in 25 mM H_2SO_4) the mixture was incubated at room temperature for 45 min, protected from light. The formation of ferric ions was detected by measuring the resulting colored complex

with xylenol orange. To study if the oxidative stress can be prevented by melatonin the same protocol was used but brain slices were placed in artificial ACSF with 10 μM of CH and 50 μM of melatonin and they were each incubated for 5, 15 and 30 min.

Finally, to investigate if melatonin prevents the physiological changes induced by CH at 10 μM, pyramidal neurons (n=6) were initially superfused with normal ACSF with melatonin (50 μM) to measure electrophysiological parameters in current-clamp mode for the initial condition (time 0). After this, each slice was superfused with ACSF with melatonin (50 μM) and 10 μM CH for 30 minutes, and voltage responses were recorded during this period.

Current-clamp recordings and analysis

All pyramidal neurons included in the analysis showed a stable resting membrane potential of −60 mV or less, an action potential higher than 80 mV, and repetitive firing in response to depolarization pulses of 1 s. The following parameters were recorded and quantified: (1) Cell capacitance. (2) Resting membrane potentials were measured as the difference between the intracellular and extracellular potentials after withdrawing the recording electrode from the cell. (3) Input resistance was determined by passing positive and negative square current steps (500 ms, 1 Hz; with 10 pA increments) and calculated as the slope of the current–voltage plot. When there was evidence of an inward rectification, or sag, the voltage value used for this latter plot was the value achieved at the peak (for details see Carrascal et al., 2006, and 2005). (4) The rheobase was the minimum current injected (square pulse of 100 ms, 1 Hz; 5 pA increments) that generated an action potential in 50% of the cases. (5) Voltage depolarization was the increase in membrane potential required for the cell to reach the spike threshold. To determine spike threshold, the action potential recording was differentiated, with the spike onset taken as the value of the membrane potential at which the first derivative exceeded 10 V s^{-1}. Voltage threshold was calculated by adding depolarization voltage to the resting membrane potential. (6) Spikes were averaged (six sweeps) to measure their characteristics: amplitude and duration (100 μs, 5–20 nA). Amplitude was the voltage increment between the resting level and the spike voltage peak. The value of the duration of the action potential was determined as the width of the spike at its half amplitude (see also Nunez-Abades et al., 1993). (7) The tonic component of firing was measured from the repetitive discharge evoked by depolarizing square current steps (1 s, 0.5 Hz; 10–50 pA increments). The steady-state firing frequency was taken as the average number of spikes during the last 500 ms of the repetitive discharge. The relationship between the steady-state firing frequency and injected

current was plotted (f–I plot) and the slope (*gain*) calculated. Maximum frequency was considered as the highest frequency achieved by the neuron regardless of the current intensity, and the cancelation current was the intensity current in which the neuron would cease to fire.

Statistical analysis

Results were expressed as mean ± standard error of the mean; *n* refers to the number of cells. For statistical calculations, we have used SPSS 22.0 (IBM software, Armonk, NY, USA). Data distribution was first processed with a normality test (Shapiro–Wilk Test). A paired *t*-test was used to check that the mean values (minute 0 and minute 30) were the same when checking cell viability under whole-cell recording configuration. An analysis of variance (ANOVA) was used to compare the means of the electrophysiological parameters for the experiments carried out to check the drug washout effects. Two-way ANOVA was used to analyze the means of the time variable (0, 5, 15 and 30 minutes) and for the concentration variable (0 μM, 1 μM, 10 μM, and 100 μM) for each electrophysiological parameter. ANOVA was again applied to analyze differences of the sequential application of the drug. If there were significant differences, then we used the Bonferroni test to perform pairwise comparisons between groups. Two-way ANOVA was also used to analyze the mean values of LPO at 0, 5, 15 and 30 minutes at the three concentrations previously used in this study (1 μM, 10 μM, and 100 μM). One factor ANOVA was used to determine if there were significant differences between the group treated with CH 10 μM and the group pre-treated with 50 μM melatonin plus 10 μM CH at 0, 5, 15 and 30 minutes. If there were significant differences, Bonferroni correction was used again to perform a comparison between groups. Two groups of data were considered statistically different if $P \leqslant 0.05$. The correlation between variables was measured by the Pearson's correlation coefficient (r).

RESULTS

We have first evaluated the electrophysiological properties of 20 neurons in order to assess whether they had been affected by a whole-cell recording configuration over sessions of at least 30 min duration. As seen in figure 1A, and for a representative neuron, membrane potential remained stable at approximately -74 mV throughout the recording session and the cell responded to a current injection (-100 pA) with similar changes in voltage. Membrane resistance was calculated from these voltage changes and resulted in 210, 205, 209 and 210 MΩ, for minutes 1, 5, 15 and 30 of the recording, respectively (see arrows in Fig. 1A). For all

neurons, mean values obtained at the beginning of the recording session and at minutes 5, 15 and 30 did not show any significant statistical difference on membrane potential, input resistance, amplitude of the action potential, gain or maximum discharge as seen in figures 1-2 and 4.

Cumene hydroperoxide washout effects on resting membrane potential and resistance of pyramidal neurons from the motor cortex

For a representative neuron, figure 1B illustrates the effects of the application of 10 μM CH on membrane potential during 5 minutes and then, during the washout of the drug. The recording shows how depolarization starts at minute 3 reaching its highest value, of approximately 6 mV, at minute 8. Depolarization remained steady until minute 12. Then, membrane potential started a progressive repolarization, reaching to a value similar to the one recorded at rest (-68 mV) at minute 15 (minute 10 of the washout). Membrane potential kept that value until the end of the experiment (minute 30). Repolarization during washout, as described in figure 1B, affected a total of 5 cells. For the other 5 cells (total population n=10), only partial repolarization was observed (<60% of the change).

One of our objectives was to describe CH washout effects on membrane resistance. To accomplish this objective, we applied negative current pulses (-100 pA, 200 ms) at one-minute intervals and recorded voltage response. CH produced a biphasic change on membrane resistance, characterized by a transient increase at minute 5 of drug application which then progressively fell under control values. In Figure 1B, cell resistance shifted from 184 MΩ, in control, to 223 MΩ at minute 5. Later, it progressively diminished until reaching 180 MΩ after recording for 15 minutes (minute 10 of washout, indicated by the third arrow in the figure). The same value remained for a 20 min recording (minute 15 of washout). At minute 30 of the recording, the value registered was 181 MΩ (fourth arrow in the figure). A total of 6/10 cells presented the same result, while the rest showed a membrane resistance with values under the ones registered at the beginning of the experiment. This could be due to a late effect of CH on resistance, and not necessarily to the washout itself.

We also looked into the effect of the washout on the firing repetitive properties. After a CH exposure of 5 minutes, all the cells (n=10) in the study presented a decrease in gain (55.2±12.6 $AP \cdot s^{-1} \cdot nA^{-1}$ vs 36.7±15.5 $AP \cdot s^{-1} \cdot nA^{-1}$). After the washout, 4 cells showed an improvement in the gain and maximum firing frequency (< 40% of the change). Four neurons

did not present any kind of reversibility to the effects of CH and retained a low gain and low maximum frequency. The remaining 2 cells completely lost their ability to repetitively discharge action potentials during the washout. From these experiments, we can conclude that a short exposure to the drug (5 min) allows for a partial recovery from the effects that CH may have over membrane potential, resistance and repetitive discharge properties.

Figure 1C shows, for a representative neuron, how the effect of 15 min CH application is the same to the one described in figure 1B. However, after the washout, the cell did not present any recovery regarding the parameters analyzed in the study. For the neuron depicted, membrane potential depolarized from -73 mV up to -68 mV after 15 minutes of CH application, and that value remained the same until the end of the recording (15 minutes of washout). The same applies for the entire population of neurons. The cell depicted in the figure showed a resistance of 166 MΩ in control, and then augmented to 202 MΩ after 5 minute CH application. There was a decrease to 141 MΩ after 15 minutes. Drug washout did not produce any recovery to the initial values in resistance, on the contrary, resistance continued its decrease up to 112 MΩ at the end of the recording session. This phenomenon was observed for the whole population, with the result of a lower resistance value after the washout which is characteristic of the CH application itself. In terms of the gain and the maximum frequency, only 3 cells partially reverted to original values (30% recovery) during washout, while the remaining 7 retained a decrease in both parameters. Actually, 3 of the latter completely lost their capability of repetitively discharge and did not get recovered during washout. Therefore, continuous exposure to CH (15 min) produced alterations to membrane properties that were hardly reversible.

Dose dependent effects of cumene hydroperoxide on membrane properties of pyramidal neurons from the motor cortex

Dose response effects of CH were studied during 30 minutes on membrane properties, namely membrane potential, input resistance, rheobase and threshold voltage. Figure 2A shows the effects on membrane potential for control and the 3 concentrations (1, 10 and 100 μM). In the cases of control and 1 μM CH, no significant changes were observed for any of the cells along the 30 minute duration of the study. However, for the case of the 10 μM CH concentration, membrane potential significantly depolarized at minute 5, and even though it reached values slightly more depolarized at minute 15 and minute 30, those differences were not statistically

significant when compared with the ones obtained at minute 5. When the concentration of 100 μM CH was applied, depolarization was significant at minute 30 (-68.6±1.3 in initial condition vs -61.9±1.4 at minute 30).

Figure 2B shows the effects on membrane resistance in a pool of neurons in control condition (n=20) and each one of the other 3 populations of neurons were exposed to one of the 3 drug concentrations of the study (n=30 for each one). As seen in the figure, membrane resistance does not change in control condition over time neither when 1 μM CH is applied during the 30 minutes the experiment lasted. However, input resistance showed a biphasic response for the other 2 concentrations, with a significant increase at minute 5. Subsequently, we observed a significant decrease at minutes 15 and 30, respectively, when compared with the initial value. More specifically, input resistance augmented at minute 5 for the 100 μM CH concentration (162.1±9.5 MΩ *vs* 189.2±12.4 MΩ). Later, it diminished at minute 15 and 30 (110.6±9.8 MΩ and 99.7±11.1 MΩ, respectively). Values at minute 30 were slightly lower than the ones obtained at minute 15, but there was not a significant difference between them. The 10 and the 100 μM CH concentrations had bigger (and significant) effects than 1 μM CH at minute 5, but no differences were found between 10 and 100 μM CH.

To confirm that the 10 and 100 μM concentrations produced similar effects on resistance another experiment was carried out (n=10), in which we analyzed the voltage responses to current injections while the neuron was being sequentially exposed to increasing drug concentrations for 10 minutes: 0, 1, 10 and 100 μM of CH. Figure 3 shows, for a representative neuron, that voltage responses were similar before drug application and for the 1 μM CH concentration, both demonstrating that membrane resistance was not modified in this situation. However, in the absence of the drug, the neuron (Fig. 3A) presents a voltage rectification of the membrane potential (known as "sag") approximately at 80 ms, which disappears after 1 μM CH application (Fig. 3B). The resulting voltage responses obtained when the application was of 10 and 100 μM CH were smaller than those obtained in control, and were also accompanied by a membrane depolarization from -75 mV to -66 and -65 mV, respectively. The measure of the membrane resistance for each neuron, before drug application and for the 3 drug concentrations, was calculated as the slope that represents the relation between current intensity and voltage response as depicted in figure 3A-D. For the neuron described in figure 3A-D, resistance was 103 MΩ without CH, 102 MΩ in 1 μM CH.

It dropped to 51 MΩ in 10 μM CH, and to 50 MΩ in 100 μM CH, respectively (Fig. 3E). For the whole population (n=10) resistance was 170.2±6.1 MΩ in control, 165.3±6.7 MΩ in 1 μM CH. It went down again to 135.7±5.7 MΩ in 10 μM CH, and 120.6±11.8 MΩ in 100 μM CH, respectively (Fig. 3F). From this data, we conclude that neurons do not evidence any alteration to membrane potential and resistance in the 1 μM CH concentration in respect to initial values, and that the effects of the 100 μM CH concentration do not vary significantly from the changes produced by the administration of 10 μM CH.

Dose dependent effects of cumene hydroperoxide on rheobase and voltage threshold of pyramidal neurons from the motor cortex

The administration of CH triggered up some changes on the rheobase of pyramidal neurons from the motor cortex. Figure 2C illustrates the effects on the rheobase which depend on the time and the dose of the CH concentration. No significant change could be found on rheobase in control condition and when 1μM CH was applied during 30 minutes. However, when the concentration applied was 10 or 100 μM CH, the rheobase decreased significantly after 5 minute application in comparison with initial condition (for example, for 100 μM the decrement was 193.7±10.6 pA *vs* 149.6±15.7 pA) while it increased significantly if compared with initial condition after 15 and 30 minute application (for 100 μM, the increase was 249.9±41.6 pA and 263.1±35.3 pA). Values at minute 30 were only slightly larger than those obtained at minute 15, and they did not differ significantly. Changes in rheobase were parallel to changes in resistance and, therefore, the voltage threshold remained unaltered during the 30 minute duration for the 3 concentrations (Fig. 2D). As a conclusion, Ohm's Law is observable in the membrane behavior of pyramidal neurons, even under the effect of different concentrations of the drug.

Later on, we analyzed whether the effects of oxidative stress produced by CH on membrane properties would depend on neuronal size: that is to say, whether larger neurons presented a bigger sensitivity to CH or conversely. An estimation of the neuronal size can be inferred from membrane capacitance. Figure 2E-F shows the relation between capacitance and resistance changes of neurons recorded at 100 μM CH, in absolute values (Fig. 2E) and in relative values (Fig. 2F). Data shows a linear relation between capacitance and input resistance change when calculated from absolute values ($P<0.001$; $r>0.69$), and also from relatives values ($P<0.001$; $r>0.71$). Similar results were obtained when 10 μM CH was

administered (not shown). From these figures, we can conclude that pyramidal neurons from the motor cortex that have a larger membrane surface are more likely to suffer alterations in their electrical membrane properties by oxidative stress mediated by CH.

Dose dependent effects of cumene hydroperoxide on amplitude and duration of the action potential of pyramidal neurons from the motor cortex

Figure 4A describes the effects of CH on action potential amplitude in control condition and at the 3 different concentrations. No differences were found in control condition for this parameter while the 1 μM CH concentration produced a gradual decrease on action potential amplitude, which became statistically significant at minute 30. For the 10 and 100 μM concentrations, the mean decrease was more evident, reaching statistical significance at minute 5 as compared to the initial condition, and at minute 30 in comparison with minute 15. For the 100 μM concentration under initial condition, the mean amplitude was 120.1±2.0, going down to 114.8±5.7 mV at minute 5, and even lower to 110.3±3.8 mV (minute 15) and 91.2±3.3 mV (minute 30). The highest difference between initial and final condition for the measure of the amplitude could be seen when the concentration applied was 100 μM. More specifically, for all concentrations, amplitude differences were the following: 8.4±3.9, 18.8±2.3 and 28.9±5.8 mV, for 1, 10 and 100 μM CH respectively. Differences of the effects on the action potential were significant between the 3 concentrations at minute 30.

In figure 4B, we show the effects on the duration of the action potential in control condition and for the 3 concentrations. This parameter became progressively longer after the 10 and 100 μM CH exposure while remaining unaltered in control condition and after the 1 μM CH application. However, statistical significance was already observable at minute 5 in comparison with control condition, and minute 30 in comparison with minute 15 condition, for both 10 μM and 100 μM CH. For example, and for the administration of 100 μM CH, the duration of the action potential was 1.50±0.11 ms (minute 0), 1.82±0.13 ms (minute 5), 1.87±0.28 ms (minute 15), and, finally, 2.31±0.15 ms (minute 30).

Dose dependent effects of cumene hydroperoxide on firing properties of pyramidal neurons from the motor cortex

The pyramidal neurons in the study exhibited a phasic-tonic discharge as a response to sustained current depolarizing pulses, not only in control, but also when the concentration

applied was 1 µM CH (see Fig. 5A, B). Nevertheless, after the administration of 10 µM CH, 20% of the cells (minute 15) and 43% (minute 30) lost their ability to discharge action potentials in a repetitive way. This effect was even greater when the concentration was 100 µM CH affecting 40% (minute 5), 80% (minute 15), and 100% (minute 30) of the cells (Fig. 4C).

Figure 5 A-D shows, for a representative neuron (n=10), the results of another experiment in which we analyzed the repetitive firing properties observed while the neuron was being sequentially exposed to each of 3 different drug concentrations for 10 minutes. Before drug application (0 µM), and for the 1 and 10 µM CH concentrations, the neuron illustrated in figure 5 could discharge along the whole pulse duration (1 s). However, this ability is lost after 10 minutes of being exposed to 100 µM CH, as being depicted in figure 5D. In this figure, we can observe how the cell ceases to discharge before the pulse is over. Also in figure 5A-C, it can be observed how the firing frequency decreases as the concentration increases: 23 $AP.s^{-1}$ in control, 19 $AP.s^{-1}$ for 1 µM CH, and 12 $AP.s^{-1}$ for 10 µM CH. Therefore, this data may suggest that an incremental dose alters the gain of the discharge. Figure 5A-D also shows a decrease of amplitude of the action potential in the train, which becomes more evident for the 100 µM CH concentration.

We then measured the gain, maximum frequency and cancelation current in those cells that retained their repetitive firing properties. Figure 5E represents the relationship between the intensity of the current injected and the firing frequency for the cell depicted in figure 5A-D. In this figure, it can be observed how that relationship is linear everytime low current intensities were applied. However, when intensity increases, accommodation symptoms are appreciated. Figure 5E shows how the neuron starts to accommodate at values close to 500 pA for 0 µM CH, 450 pA for 1 µM CH, and 400 pA for 10 µM CH. This data indicates that the working intensity range decreases as the drug concentration increases. Besides, when we calculated the gain for the neuron shown in figure 5A-E, we could see how this parameter decreased after CH application: 53 $AP{\cdot}s^{-1}{\cdot}nA^{-1}$ (0 µM CH), 46 $AP{\cdot}s^{-1}{\cdot}nA^{-1}$ (1 µM CH), and 26 $AP{\cdot}s^{-1}{\cdot}nA^{-1}$ (10 µM CH). This result is applicable to the rest of the population. Figure 5F shows the mean values of the gain in the different accumulating situations (time and drug concentration): 40.1±5.9 $AP{\cdot}s^{-1}{\cdot}nA^{-1}$ (0 µM CH), 38.3±4.9 $AP{\cdot}s^{-1}{\cdot}nA^{-1}$ (1 µM CH), 27.7±8.6 $AP{\cdot}s^{-1}{\cdot}nA^{-1}$ (10 µM CH) and 15.3±4.1 $AP{\cdot}s^{-1}{\cdot}nA^{-1}$ (100 µM CH). This decrease was

statistically significant for the 10 μM CH and the 100 μM CH concentrations (see Fig. 5F) when compared with initial values and between themselves.

In order to carry out a more detailed analysis of the response effects on the gain and maximum frequency, a pool of 30 neurons were exposed to one of the 3 drug concentrations tested in our study. Figure 4D illustrates how the gain remained unchanged in control condition but progressively decreased after the administration of 1 μM CH, with statistical significance at minute 30. This decrease in gain was already significant at minute 15 for the 10 μM CH, and even earlier (minute 5) for the 100 μM CH concentration. For the highest concentration, the decrease in gain was significant again at minute 15 when compared with minute 5. By minute 30, all cells had already lost their ability to discharge repetitively. Maximum firing frequency progressively decreased with time, as seen in figure 4E, but did not reach significance when 1 μM CH concentration was administered. Significance was achieved (minute 5) for the 10 and the 100 μM CH concentrations. Thus, after 5 minutes of 100 μM CH exposure, the maximum firing frequency decreased from 27.2±3.0 $AP \cdot s^{-1}$ to 17.6±3.2 $AP \cdot s^{-1}$. The decrease in the maximum frequency was significantly lower again at minute 15 and at minute 30, but only after administering 100 μM of CH.

Further evidence of the narrowing of the working intensity range, after the administration of CH, was observed when analyzing the cancelation current (Fig. 4F). This figure illustrates how the cancelation of repetitive discharge occurs at lower intensities at doses of 10 and 100 μM CH, but is unaffected in control condition and at 1 μM CH. In experiments in which the 100 μM CH concentration was tested, the average value for the population in the initial condition was 650.3±48.4 pA. Intensity values fell progressively to 599.3±56.5 pA at minute 5, and 503.7±49.9 pA at minute 15, at which level statistical significance was reached. After that, it dropped to a value of zero with the complete loss of the ability to discharge repetitively for all neurons at minute 30. Once again, the 10 and 100 μM CH concentrations had larger and more significant effects than the 1 μM and among themselves with respect to repetitive firing properties (gain, maximum frequency and cancelation current) at minute 30.

Prevention of lipid peroxidation and membrane excitability changes caused by cumene hydroperoxide by melatonin

Figure 6A illustrates LPO expressed in percentage as the increase when compared with the control situation, as a result of the exposure of the brain slices to 0 (control), 1, 10 and 100

μM of CH during 0, 5, 15 and 30 min. As seen in figure 6A, CH evokes LPO in a dose and time dependent manner. Longer time or higher dose caused a progressive increase in LPO, significant at 5 min at the three concentrations of CH tested in respect to the control. However, the increased lipid peroxide level plateaued in min 15 and 30. Furthermore, it is also observable in the fact that the level of LPO with CH 1 μM in min 30 is similar to the average value found after 1 μM CH exposure in min 5. Although the increment in LPO is dose dependent, mainly at shorter times, those increments are not proportional to the concentration, i.e., increments in LPO found with 100 μM CH are bigger but not statistically different than those found with 10 μM CH, but they are statistically different when compared 10 vs 1 μM CH.

No effect was observed in LPO in cells treated with ACSF with 50 μM melatonin when compared with cells treated with ACSF alone. As seen in figure 6B, melatonin prevents LPO induced by 10 μM CH. No significant differences in LPO were found between tissues treated with melatonin alone (control of melatonin) and tissues treated with melatonin and 10 μM CH at 5, 15 or at 30 min although a moderate increase of LPO was found at 30 min. Irrespective of the time period applied (5, 15 and 30 min) the differences found between tissues treated with ACSF containing 10 μM of CH and tissues treated with ACSF containing 10 μM of CH and melatonin 50 μM, were always significant.

As melatonin prevents the LPO induced by CH, we wondered if pre-treatment with this antioxidant could act as a neuroprotector and could prevent the membrane changes induced by 10 μM CH. Figure 6C illustrates the effects of 50 μM melatonin and 10 μM CH application on membrane potential during 30 minutes. As seen in this figure, no changes on membrane potential or input resistance were produced for the whole period. The same response was found in 4 of the 6 neurons studied and no changes in membrane potential, input resistance or firing properties were found. However, 2 of the 6 cells showed gain decrease, around minute 30.

DISCUSSION

In the present work, we have studied early changes in membrane properties of pyramidal motor cortex neurons exposed to 3 concentrations of CH (1, 10 and 100 μM). Our data indicate that CH depresses the excitability of pyramidal motor cortex neurons decreasing

input resistance, amplitude of the action potential, and gain and maximum frequency of the repetitive firing discharge. These effects were time and dose dependent. Reversibility to alterations produced by CH to membrane properties is inversely proportional to exposure time: the longer the exposure, the lower the reversibility. Among all the membrane properties analyzed, the ability to repetitively discharge action potentials was the property most affected by the administration of the drug. Our data also suggest that large neurons of the motor cortex have more sensitivity to the oxidant. Finally, we have shown that pre-treatment with melatonin, an antioxidant agent, prevents the effects induced by CH on membrane properties. The antioxidant benefits of melatonin have been extensively described in the literature (Reiter et al., 2000; Tan et al., 2007).

Methodological considerations

Oxidative stress is caused when the production of ROS exceeds the antioxidant capacity of the tissues (Heather and Teismann, 2009; Ljubisavljevic, 2014; Sims-Robinson et al., 2013). In neuronal cell damage, it is believed that this high level of ROS makes a large contribution to neuron degeneration and might be one factor in the development of different neurological diseases, such as ALS, Parkinson's and Alzheimer's (Andersen, 2004; Reynolds et al., 2007). Our research study has considered pyramidal neurons of the layer V of the primary motor cortex. Although there are several pathophysiological mechanisms that trigger motor neuron vulnerability in ALS, oxidative stress has been described to be an important factor in the case of pyramidal neurons of the motor cortex (Cleveland and Rothstein, 2001; Kim et al., 2014). Our results demonstrate that CH is an organic oxidant agent that induces lipidic peroxidation on the cell membrane which, consequently, results in cell alterations (Ayala et al., 2014; Nakaya et al., 1992; Vimard et al., 2011). Indeed, we have demonstrated that the effects of CH on membrane properties are due to oxidative stress because melatonin prevents the electrophysiological effects of CH. In Cai et al. (2009), malondialdehyde, a typical intermediate of lipid peroxidation, gradually damaged hippocampal neurons following a 3h exposure at concentrations of 1, 10, 100 and 1,000 μM. According to these authors, malondialdehyde may have biochemical effects on hippocampal neurons that are time and dose dependent. A similar conclusion was reached in works that studied the dose response effects of CH toxicity on cultured cells (Shimura et al., 1985; Vimard et al., 1996 and 2011, Vroegop et al., 1995). Those studies proved that cell mortality rate is low (less than 5%) when CH is applied in concentrations under 1,000 μM, and with administration time under 30 minutes. However, they also report how mortality rate reached approximately 70% when

administration times are longer (60-180 minutes) or when drug concentrations are larger. On the basis of this evidence, we decided to use 3 CH concentrations (1, 10 and 100 μM) and 30 min administration time, which guarantee a low mortality rate, in order to study the dose and time dependent effects of CH. We have analyzed several electrophysiological parameters during drug administration with data being recorded at 5, 15 and 30 minutes to identify alterations to membrane properties. Prior to that, we checked that the neurons in the motor cortex keep their membrane properties stable, under our recording conditions, for a duration of 30 minutes, as previously demonstrated with other pools of neurons (Nani et al., 2010). Therefore, any alteration to the physiological parameters analyzed can be considered to be the effect of the drug administration to the cells and not to the recording conditions.

Alteration of membrane properties by oxidative stress

No effects on membrane potential or resistance were observed in our study in control condition and when CH was administered at 1 μM. At that concentration, it seems that neurons are able to block the oxidative effects on the physiological properties of the membrane, although a significant level of LPO was found. A reason to explain this could be that pyramidal neurons have an efficient scavenging enzyme system that reacts rapidly with CH at a 1 μM concentration, as proposed by Jovanovic and Jovanovic (2013) when attempting to explain the effects that oxidative stress has on the electrophysiological properties of Retzius neurons of the leech. The values of LPO we found at the 1 μM concentration were under 15% of increment in respect to those registered for control. These small values are similar to those found during ageing in the hypothalamus-hypophysis, (Arguelles et al., 2011). It seems that those levels of LPO are not high enough to alter membrane properties. Contrary to the results obtained at the 1 μM concentration, our exposure of cortical neurons to CH concentrations of 10 and 100 μM already produced changes in membrane potential, membrane resistance and rheobase as a result of higher levels of LPO. An unexpected finding was that the 2 concentrations caused changes of equal magnitude in these parameters. This finding may reflect that values of LPO found when 100 μM was administered were only slightly higher, but not significant, than those obtained with 10 μM. Our results suggest that oxidative stress affected the channels and/or conductances that determine passive membrane properties (Frantseva et al., 1998; Nakaya et al., 1992; Nani et al., 2010; Pardillo-Diaz et al., 2015) along a very narrow concentration range of 1-10 μM, which resembles an “all-or-nothing” response. In any case, conductances that are affected by oxidative stress would not be those involved in determining the voltage threshold. In our

study, regardless of the CH dose used, voltage threshold remained unchanged, as occurs in neurons of the hippocampus exposed to peroxide (Pellmar, 1987).

Exposure of leech neurons to CH (0.25, 1, and 1.5 μM) extended the duration of the action potentials in a dose dependent manner (Jovanovic and Jovanovic, 2013). For example, the concentration of 0.25 μM did not significantly change the action potential within 20 min, but it was significant with 1 μM. Moreover, a higher concentration (1.5 μM) of CH caused an extreme change in the shape and duration of the action potential. In our study, we observed that the action potential diminished, while its duration increased in a progressive way along the time of exposure to CH and in a concentration dependent manner. When the CH concentration was of 1 μM, the effects on the amplitude of the action potential were evident at 30 minute drug administration. However, with the 10 or 100 μM CH concentrations, effects were already significant at minute 5, showing the highest alterations in amplitude with the 100 μM CH concentration. It has been proposed that oxidative stress could be affecting conductances that participate in the repolarization of the action potential and the Na^+ ionic gradients (Angelova and Muller, 2006; Jovanovic and Jovanovic, 2013; Nakaya et al., 1992; Pardillo-Díaz et al., 2015). The capability of maintaining repetitive firing of the action potentials is probably the most dramatic effect produced by CH over the electrical properties of the membrane. Our results are similar to those obtained by Jovanovic and Jovanovic (2013) in which CH (1.5 μM, 20 min) produced a complete cancelation of firing properties in leech neurons. However, they do not coincide with the study by Nani et al. (2010) in which their H_2O_2 oxidative model (1 μM, 30 min) caused an increase in the firing of the hypoglossal motoneurons of rats. This discrepancy might be due to the fact that H_2O_2 can only exert the effects achieved by CH at concentrations 10 times higher (Vimard et al., 2011). Regardless of which channels and/or conductances are underlying action potential and repetitive firing (Pardillo Diaz et al., 2015), in our study we have demonstrated that they are dramatically affected by oxidative stress in a concentration dependent manner, being a low concentration of CH (100 μM) high enough to completely block firing discharge.

Functional considerations

We can conclude that oxidative stress induced by CH evokes important changes, in a concentration and time dependent manner, in the neuronal excitability of rat motor cortex neurons as demonstrated in modified hippocampal cells (Oh et al., 2012). Low concentration

of the drug (1 μM), but only with a long exposure, already blocks the Ih current underlying sag (Robinson and Siegelbaum, 2003) and may alter conductances involved in the action potential, and firing properties, at a drug concentration which has no biochemical, physiological or morphological effects in other neuronal populations (Jovanovic and Jovanovic, 2013; Sinha et al., 2015; Vimard et al., 2011). A concentration of 10 μM of CH produced changes in membrane potential, membrane resistance and rheobase as previously demonstrated by Pardillo-Díaz et al. (2015) in a time dependent manner. Finally, 100 μM completely blocks the ability to produce repetitive discharge of action potentials with long term exposure. Brief exposure (15 minutes or less) also narrow the current working range because of an increase in the rheobase and a decrease of the cancelation current. Then, we can conclude that larger concentrations of CH have similar consequences as those evoked by smaller concentrations at longer exposure times.

Present data demonstrate that effects induced by a brief exposure to CH (5 min) are partially reversible, while those produced by a long-term exposure to CH (15 min) are practically irreversible. Membrane potential and resistance presented higher reversibility to the effects of CH. Similarly to our results, H_2O_2 induced a large depolarizing shift in membrane potential that was reversible in almost half of the thalamic neurons tested (Frantseva et al., 1998). Also the change observed in input resistance could be reversed in some cases, but not in frequency depression of spontaneous postsynaptic currents induced by H_2O_2 in hypoglossal motoneurons (Nani et al., 2010). The reversibility of some of the oxidative stress-mediated effects is difficult to explain (Pellmar, 1987). Oxygen radicals produced by CH might directly attack ion channel proteins, or LPO caused by CH might indirectly inhibit ion channel functions by altering the membrane lipid milieu surrounding the channel protein (Nakaya et al., 1992). Effects reported to be reversible may be mediated by modified lipid-protein interactions rather than by irreversible protein oxidation (Frantseva et al., 1998). Then, our results may suggest that CH effects on resting membrane potential and membrane resistance are, at least in part, a consequence of the observed LPO and, for that reason, their modifications could be partially reversed with a prompt washout of the drug. This work demonstrates that CH induces LPO in pyramidal neurons and that it can be prevented by the use of melatonin. Besides, melatonin also partially or totally prevents the physiological changes that occur in cortical neurons under CH conditions. The antioxidant benefits of melatonin have been extensively described. It has been demonstrated that melatonin has direct scavenging actions against the free radicals and related products (Reiter et al., 2000; Tan et

al., 2007), acts indirectly by inducing antioxidant enzymes (Reiter et al., 2000), increases the activities of the major antioxidant enzymes (Reiter, 2000) and stimulates glutathione synthesis (Barlow et al., 1995). Melatonin has also been shown to reduce the accumulation of the major products of LPO when membranes are exposed to radical-generating agents (Reiter, 2000) and to preserve, *in vivo* and *in vitro*, protein synthesis under oxidative stress condition (Argüelles et al., 2012). Then, we cannot discard in our study that other mechanisms underlying membrane changes induced by CH, apart from LPO, such as protein oxidation, can also be prevented by melatonin. However, since 50 μM melatonin has not totally prevented the effects of long time exposition to CH in some cells, additional trials with melatonin alone (a higher dose) and in combination with other drugs, such as thiol-reducing agents like N-acetylcysteine or β-mercaptoethanol (Cabungcal et al., 2014; Vimard et al., 2011), or other anti-oxidant substances, such as vitamin C (Chang et al., 2008), are needed to clarify its potential benefit. Nevertheless, we propose that neuroprotective agents should be administered before membrane properties of neurons start to change, given the low reversibility of the effects caused by oxidative stress when this is maintained in time.

In motoneurons, cell capacitance has been used as an estimation for neuronal size (Viana et al., 1994). In other words, larger neurons would present a higher capacitance. Our results provide the first experimental evidence that neurons mostly affected by oxidative stress are the largest ones, those with the highest values of capacitance. Pyramidal neurons of the motor cortex are rather large in comparison with neurons from other layers of the motor cortex and from other brain nuclei (Oswald et al., 2013). Furthermore, regarding pyramidal neurons from the motor cortex, as from other cortical areas, it has been demonstrated that morphological parameters revealed clear differences between large soma, thick shafted, large tufted corticofugal neurons and small soma, slender shafted, small tufted commissural neurons (Chagnac-Amitai et al., 1990; Mason and Larkman, 1990; Oswald et al., 2013). We propose that corticofugal neurons could be the most affected by oxidative stress in our study. If we considered oxidative stress to be the main cause of neuronal death in neurodegenerative pathologies (Andersen, 2004; Cabungcal et al., 2014; Cleveland and Rothstein, 2001; Niedzielska et al., 2015; Parakh et al., 2013; Reynolds et al., 2007), our findings (large size and high sensitivity to oxidative stress even a low concentration) would serve to explain how pyramidal neurons of the motor cortex, in particular corticofugal neurons, also have the highest death rate levels in diseases such as ALS.

ACKNOWLEDGEMENT

This work was partially supported by ERDF (European Regional Development Fund) UNSE10-1E-0949. Mr. Ricardo Pardillo-Diaz obtained a research fellowship funded by CACOF. We sincerely thank The Centre of Research Technology and Innovation of the University of Seville (CITIUS) for their technical support, and Ms. Rosa María Andrade García for her technical assistance. Gratitude to Professor Juan Ramón Lacalle Remigio is expressed for his advice on statistical analysis.

REFERENCES

Andersen JK (2004) Oxidative stress in neurodegeneration: cause or consequence? Nat Med 10 Suppl:S18-25.

Angelova P,Muller W (2006) Oxidative modulation of the transient potassium current IA by intracellular arachidonic acid in rat CA1 pyramidal neurons. Eur J Neurosci 23:2375-2384.

Argüelles S, Cano M, Machado A, Ayala A (2011) Effect of aging and oxidative stress on elongation factor-2 in hypothalamus and hypophysis. Mech Ageing Dev 132:55-64.

Argüelles S, Muñoz MF, Cano M, Machado A, Ayala A (2012) In vitro and in vivo protection by melatonin against the decline of elongation factor-2 caused by lipid peroxidation: preservation of protein synthesis. J Pineal Res 53:1-10.

Arrigo AP (1999) Gene expression and the thiol redox state. Free Radic Biol Med 27:936-944.

Ayala A, Munoz MF,Arguelles S (2014) Lipid peroxidation: production, metabolism, and signaling mechanisms of malondialdehyde and 4-hydroxy-2-nonenal. Oxid Med Cell Longev 2014:360438.

Bogdanov M, Brown RH, Matson W, Smart R, Hayden D, O'Donnell H, Flint Beal M, Cudkowicz M (2000) Increased oxidative damage to DNA in ALS patients. Free Radic Biol Med 29:652-8.

Cai J, Chen J, He H, Yin Z, Zhu Z, Yin D (2009) Carbonyl stress: malondialdehyde induces damage on rat hippocampal neurons by disturbance of Ca2+ homeostasis. Cell Biol Toxicol 25:435–445.

Barlow-Walden LR, Reiter RJ, Abe M, Pablos M, Menendez-Pelaez A, Chen LD, Poeggeler B (1995) Melatonin stimulates brain glutathione peroxidase activity. Neurochem Int 26:497-502.

Cabungcal JH, Counotte DS, Lewis EM, Tejeda HA, Piantadosi P, Pollock C, Calhoon GG, Sullivan EM, Presgraves E, Kil J, Hong LE, Cuenod M, Do KQ, O'Donnell P (2014) Juvenile antioxidant treatment prevents adult deficits in a developmental model of schizophrenia. Neuron 83:1073-1084.

Carrascal L, Nieto-Gonzalez JL, Cameron WE, Torres B,Nunez-Abades PA (2005) Changes during the postnatal development in physiological and anatomical characteristics of rat motoneurons studied in vitro. Brain Res Brain Res Rev 49:377-387.

Carrascal L, Nieto-Gonzalez JL, Nunez-Abades P,Torres B (2006) Temporal sequence of changes in electrophysiological properties of oculomotor motoneurons during postnatal development. Neuroscience 140:1223-1237.

Chagnac-Amitai Y, Luhmann HJ, Prince DA (1990) Burst generating and regular spiking layer 5 pyramidal neurons of rat neocortex have different morphological features. J Comp Neurol 296:598-613.

Chang HM, Huang YL, Lan CT, Wu UI, Hu ME,Youn SC (2008) Melatonin preserves superoxide dismutase activity in hypoglossal motoneurons of adult rats following peripheral nerve injury. J Pineal Res 44:172-180.

Cleveland DW, Rothstein JD (2001) From Charcot to Lou Gehrig: deciphering selective motor neuron death in ALS. Nat Rev Neurosci 2:806-819.

Davies MJ (2003) Singlet oxygen-mediated damage to proteins and its consequences. Biochem Biophys Res Commun 305:761-770.

Emerit J, Edeas M,Bricaire F (2004) Neurodegenerative diseases and oxidative stress. Biomed Pharmacother 58:39-46.

Ferretti G, Bacchetti T (2011) Peroxidation of lipoproteins in multiple sclerosis. J Neurol Sci 311:92-97.

Fogarty MJ, Noakes PG, Bellingham MC (2015) Motor cortex layer V pyramidal neurons exhibit dendritic regression, spine loss, and increased synaptic excitation in the presymptomatic hSOD1(G93A) mouse model of amyotrophic lateral sclerosis. J Neurosci 35:643-7.

Frantseva MV, Perez Velazquez JL,Carlen PL (1998) Changes in membrane and synaptic properties of thalamocortical circuitry caused by hydrogen peroxide. J Neurophysiol 80:1317-1326.

Goldhaber JI, Ji S, Lamp ST,Weiss JN (1989) Effects of exogenous free radicals on electromechanical function and metabolism in isolated rabbit and guinea pig ventricle. Implications for ischemia and reperfusion injury. J Clin Invest 83:1800-1809.

Goldhaber JI,Liu E (1994) Excitation-contraction coupling in single guinea-pig ventricular myocytes exposed to hydrogen peroxide. J Physiol 477 (Pt 1):135-147.

Gracanin M, Hawkins CL, Pattison DI,Davies MJ (2009) Singlet-oxygen-mediated amino acid and protein oxidation: formation of tryptophan peroxides and decomposition products. Free Radic Biol Med 47:92-102.

Han MH, Hwang SI, Roy DB, Lundgren DH, Price JV, Ousman SS, Fernald GH, Gerlitz B, Robinson WH, Baranzini SE, Grinnell BW, Raine CS, Sobel RA, Han DK, Steinman L (2008) Proteomic analysis of active multiple sclerosis lesions reveals therapeutic targets. Nature 451:1076–1081.

Heather LM, Teismann P (2009) Glutathione—a review on its role and significance in Parkinson's disease. FASEB J 23:3263– 3272.

Hool LC (2006) Reactive oxygen species in cardiac signalling: from mitochondria to plasma membrane ion channels. Clin Exp Pharmacol Physiol 33:146-151.

Ihara Y, Nobukuni K, Takata H, Hayabara T (2005) Oxidative stress and metal content in blood and cerebrospinal fluid of amyotrophic lateral sclerosis patients with and without a Cu, Znsuperoxide dismutase mutation. Neurol Res 27:105–108.

Ikawa M, Okazawa H, Tsujikawa T, Muramatsu T, Kishitani T, Kamisawa T, Matsunaga A, Yamamura O, Mori T, Hamano T, Kiyono Y, Nakamoto Y, Yoneda M (2014) Increased cerebral oxidative stress in amyotrophic lateral sclerosis: a 62CU-ATSM pet study. Neurology 84:2033-9.

Jiang ZY, Woollard AC, Wolff SP (1991) Lipid hydroperoxide measurement by oxidation of Fe2þ in the presence of xylenol orange: comparison with the TBA assay and an iodometric method. Lipids 26:853–856.

Jovanovic Z, Jovanovic S (2013) Comparison of the effects of cumene hydroperoxide and hydrogen peroxide on Retzius nerve cells of the leech Haemopis sanguisuga. Exp Anim 62:9-17.

Kim C, Lee HC,Sung JJ (2014) Amyotrophic lateral sclerosis - cell based therapy and novel therapeutic development. Exp Neurobiol 23:207-214.

Kourie JI (1998) Interaction of reactive oxygen species with ion transport mechanisms. Am J Physiol 275:C1-24.

Lambert AJ, Portero-Otin M, Pamplona R, Merry BJ (2004) Effect of ageing and caloric restriction on specific markers of protein oxidative damage and membrane peroxidizability in rat liver mitochondria. Mech Ageing Dev 125:529-538.

Ljubisavljevic S (2014) Oxidative Stress and Neurobiology of Demyelination. Mol Neurobiol. doi: 10.1007/s12035-014-9041-x.

Mason A, Larkman A (1990) Correlations between morphology and electrophysiology of pyramidal neurons in slices of rat visual cortex. II. Electrophysiology. J Neurosci 10:1415-1428.

Mochizuki Y, Mizutani T, Shimizu T, Kawata A (2011) Proportional neuronal loss between the primary motor and sensory cortex in amyotrophic lateral sclerosis. Neurosci Lett 503:73-5.

Muller FL, Lustgarten MS, Jang Y, Richardson A,Van Remmen H (2007) Trends in oxidative aging theories. Free Radic Biol Med 43:477-503.

Nakaya H, Takeda Y, Tohse N,Kanno M (1992) Mechanism of the membrane depolarization induced by oxidative stress in guinea-pig ventricular cells. J Mol Cell Cardiol 24:523-534.

Nam TG (2011) Lipid peroxidation and its toxicological implications. Toxicol Res 27:1–6.

Nani F, Cifra A,Nistri A (2010) Transient oxidative stress evokes early changes in the functional properties of neonatal rat hypoglossal motoneurons in vitro. Eur J Neurosci 31:951-966.

Niedzielska E, Smaga I, Gawlik M, Moniczewski A, Stankowicz P, Pera J, Filip M (2015) Oxidative Stress in Neurodegenerative Diseases. Mol Neurobiol [Epub ahead of print].

Nunez-Abades PA, Spielmann JM, Barrionuevo G,Cameron WE (1993) In vitro electrophysiology of developing genioglossal motoneurons in the rat. J Neurophysiol 70:1401-1411.

Oh J M, Choi E-K, Carp R I, Kim Y-S (2012) Oxidative stress impairs autophagic flux in prion protein-deficient hippocampal cells. Autophagy 8:10, 1448–1461.

Oswald MJ, Tantirigama ML, Sonntag I, Hughes SM,Empson RM (2013) Diversity of layer 5 projection neurons in the mouse motor cortex. Front Cell Neurosci 7:174.

Parakh S, Spencer DM, Halloran MA, Soo KY, Atkin JD (2013) Redox regulation in amyotrophic lateral sclerosis. Oxid Med Cell Longev 2013:408681. doi: 10.1155/2013/408681

Pardillo-Díaz R, Carrascal L, Ayala A, Nunez-Abades P (2015) Oxidative stress induced by cumene hydroperoxide evokes changes in neuronal excitability of rat motor cortex neurons. Neuroscience 289:85-98.

Pellmar TC (1987) Peroxide alters neuronal excitability in the CA1 region of guinea-pig hippocampus in vitro. Neuroscience 23:447-456.

Pitt D, Werner P, Raine CS (2000) Glutamate excitotoxicity in a model of multiple sclerosis. Nature Med 6:67–70.

Racay P, Kaplan P, Mezesova V,Lehotsky J (1997) Lipid peroxidation both inhibits Ca(2+)-ATPase and increases Ca2+ permeability of endoplasmic reticulum membrane. Biochem Mol Biol Int 41:647-655.

Reiter RJ (2000) Melatonin: lowering the high price of free radicals. News Physiol Sci 15:246-250.

Reiter RJ, Tan DX, Osuna C, Gitto E (2000) Actions of melatonin in the reduction of oxidative stress. A review. J Biomed Sci 7:444-458.

Reynolds A, Laurie C, Mosley RL,Gendelman HE (2007) Oxidative stress and the pathogenesis of neurodegenerative disorders. Int Rev Neurobiol 82:297-325.

Robinson RB,Siegelbaum SA (2003) Hyperpolarization-activated cation currents: from molecules to physiological function. Annu Rev Physiol 65:453-480.

Saba L, Viscomi MT, Caioli S, Pignataro A, Bisicchia E, Pieri M, Molinari M, Ammassari-Teule M, Zona C (2015) Altered Functionality, Morphology, and Vesicular Glutamate Transporter Expression of Cortical Motor Neurons from a Presymptomatic Mouse Model of Amyotrophic Lateral Sclerosis. Cereb Cortex [Epub ahead of print].

Sha W, Martins AM, Laubenbacher R, Mendes P,Shulaev V (2013) The genome-wide early temporal response of Saccharomyces cerevisiae to oxidative stress induced by cumene hydroperoxide. PLoS One 8:e74939.

Shimura J, Shimura F, Hosoya N (1985) Functional disability of rat splenocytes provoked to lipid peroxidation by cumene hydroperoxide. Biochim Biophys Acta 845:43-7.

Sims-Robinson C1, Hur J, Hayes JM, Dauch JR, Keller PJ, Brooks SV, Feldman EL (2013) The role of oxidative stress in nervous system aging. PLoS One 8:e68011. doi: 10.1371/journal.pone.0068011.

Sinha A, Chu TTT, Dao M, Chandramohanadas R (2015) Single-cell evaluation of red blood cell bio-mechanical and nano-structural alterations upon chemically induced oxidative stress. Sci. Reports. 5: 9768. doi: 10.1038/srep09768.

Smith RG, Henry YK, Mattson MP, Appel SH (1998) Presence of 4-hydroxynonenal in cerebrospinal fluid of patients with sporadic amyotrophic lateral sclerosis. Ann Neurol 44:696–699.

Stuart GJ, Dodt HU, Sakmann B (1993) Patch-clamp recordings from the soma and dendrites of neurons in brain slices using infrared video microscopy. Pflugers Arch 423:511-518.

Tan DX, Manchester LC, Terron MP, Flores LJ, Reiter RJ (2007) One molecule, many derivatives: a never-ending interaction of melatonin with reactive oxygen and nitrogen species? J Pineal Res 42:28-42.

Tirosh O, Sen CK, Roy S, Packer L (2000) Cellular and mitochondrial changes in glutamate-induced HT4 neuronal cell death. Neuroscience 97:531-541.

Tohgi H, Abe T, Yamizaki K, Murata T, Ishizaki E, Isobe C (1999) Remarkable increase in cerebrospinal fluid 3-nitrotyrosine in patients with sporadic amyotrophic lateral sclerosis. Ann Neurol 46:129–131.

Valencia A, Moran J (2004) Reactive oxygen species induce different cell death mechanisms in cultured neurons. Free Radic Biol Med 36:1112-1125.

van den Berg JJ1, Op den Kamp JA, Lubin BH, Roelofsen B, Kuypers FA (1992) Kinetics and site specificity of hydroperoxide-induced oxidative damage in red blood cells. Free Radic Biol Med 12:487-498.

Viana F, Bayliss DA, Berger AJ (1994) Postnatal changes in rat hypoglossal motoneuron membrane properties. Neuroscience 59:131-48.

Vimard F, Nouvelot A, Duval D (1996) Cytotoxic effects of an oxidative stress on neuronal-like pheochromocytoma cells (PC12). Biochem Pharmacol 51:1389-95.

Vimard F, Saucet M, Nicole O, Feuilloley M,Duval D (2011) Toxicity induced by cumene hydroperoxide in PC12 cells: protective role of thiol donors. J Biochem Mol Toxicol 25:205-215.

Vroegop SM, Decker DE, Buxser SE (1995) Localization of damage induced by reactive oxygen species in cultured cells. Free Radic Biol Med 18:141-51.

Zhu D, Tan KS, Zhang X, Sun AY, Sun GY, Lee JC (2005) Hydrogen peroxide alters membrane and cytoskeleton properties and increases intercellular connections in astrocytes. J Cell Sci 118:3695-3703.

FIGURE LEGENDS

Figure 1. Cumene hydroperoxide (CH) washout effects on membrane potential of pyramidal neurons from the motor cortex. A. Electrophysiological recording showing that membrane potential and voltage response to negative current pulses of -100 pA remains stable for 30 minutes in the control condition. **B.** CH washout 5 minutes after drug administration (10 μM). **C.** CH washout 15 minutes after drug administration (10 μM).

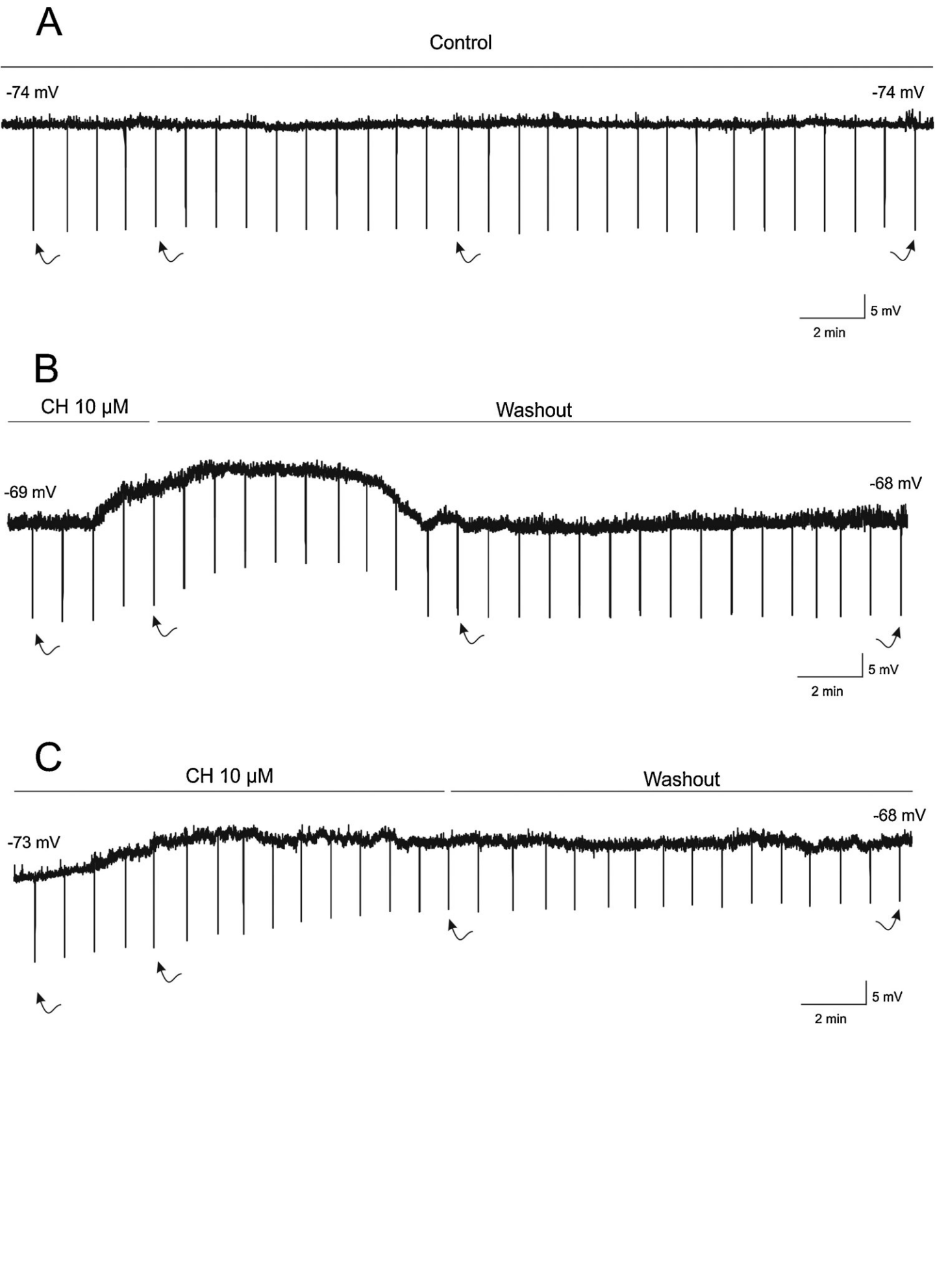
Figure 2. Dose, time and size dependent effects of cumene hydroperoxide (CH) of pyramidal neurons from the motor cortex. A-D, Plots showing dose and time dependent effects of CH on membrane potential (A), input resistance (B), rheobase (C) and voltage threshold (D). These plots illustrate the mean values ± standard error at 0, 5, 15 and 30 minutes in control condition (green; n=20) and after the exposure to CH 1 μM (blue; n=30), 10 μM (red; n=30) and 100 μM (black; n=30). The asterisk indicates statistical significance in relation to the control situation and a cross represents statistical significance between correlative measures. **E-F.** Relationship between capacitance and input resistance change in absolute (E) and in relative (F) values after 30 minutes to CH 100 μM.

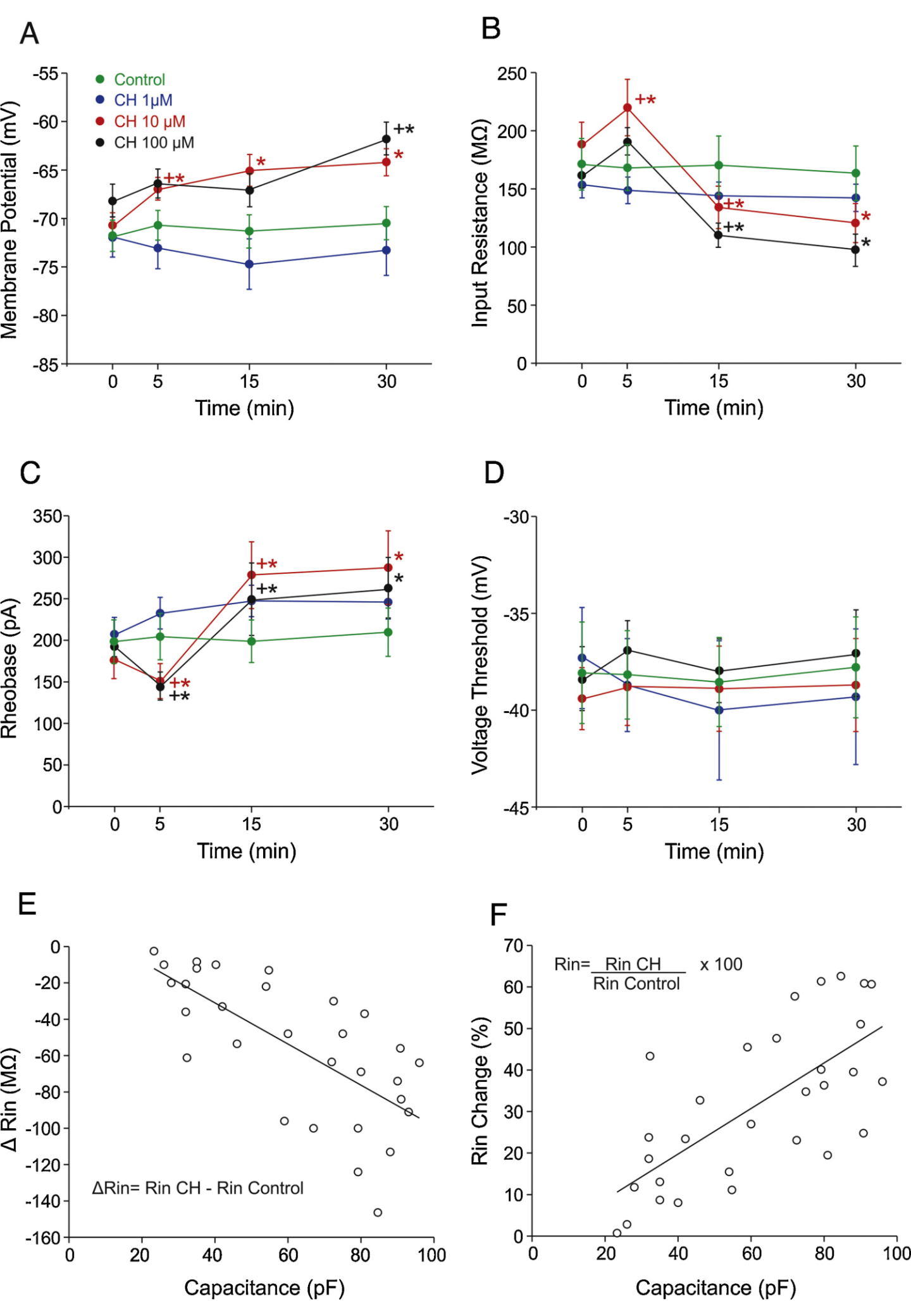
Figure 3. Effects of sequential application of increasing doses of cumene hydroperoxide (CH) on membrane resistance of pyramidal neurons from the motor cortex. A-D. Recordings showing, in the same cell, membrane potential responses to current steps of 10 pA without CH administration (A), after 10 minutes of 1 μM CH administration (B), after 10 more minutes of 10 μM CH administration (C) and after 10 more minutes of 100 μM CH application (D). The black arrow in A indicates the presence of sag. **E.** Relation between current and voltage response for the cell represented in A-D. **F.** Histogram illustrating the mean values of membrane resistance in control condition and the other three situations for the whole population (n=10).

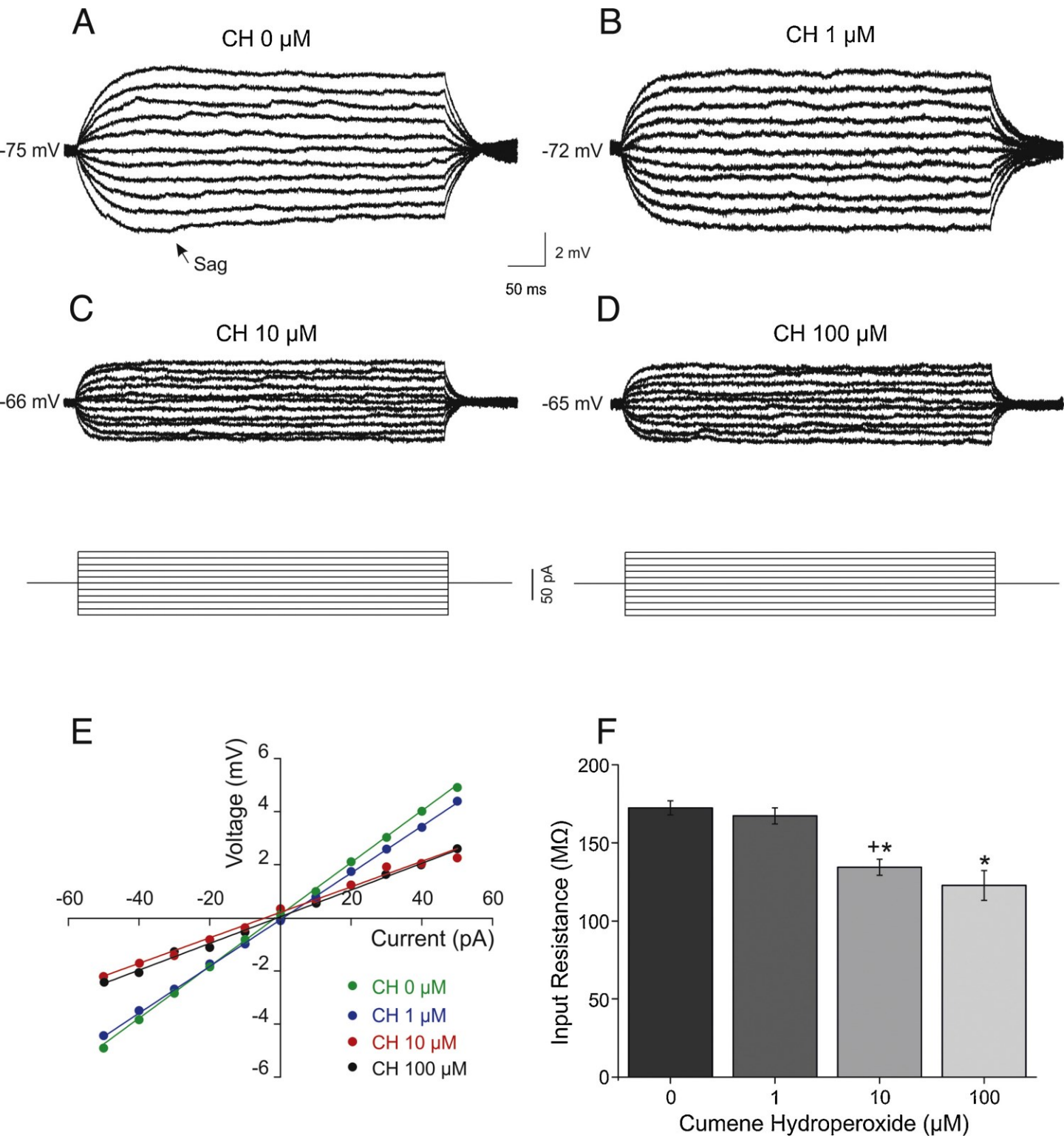
Figure 4. Dose and time dependent effects of cumene hydroperoxide (CH) on action potential amplitude (A), action potential duration (B), firing repetitive properties (C), gain (D), maximum frequency (E) and cancelation current (F) of pyramidal neurons from the motor cortex. These plots illustrate the mean values ± standard error at 0, 5, 15 and 30 minutes in control condition (green; n=20) and after the exposure to CH 1 μM (blue; n=30), 10 μM (red; n=30) and 100 μM (black; n=30). The asterisk indicates statistical significance in relation to the control situation and a cross represents statistical significance between correlative measures.

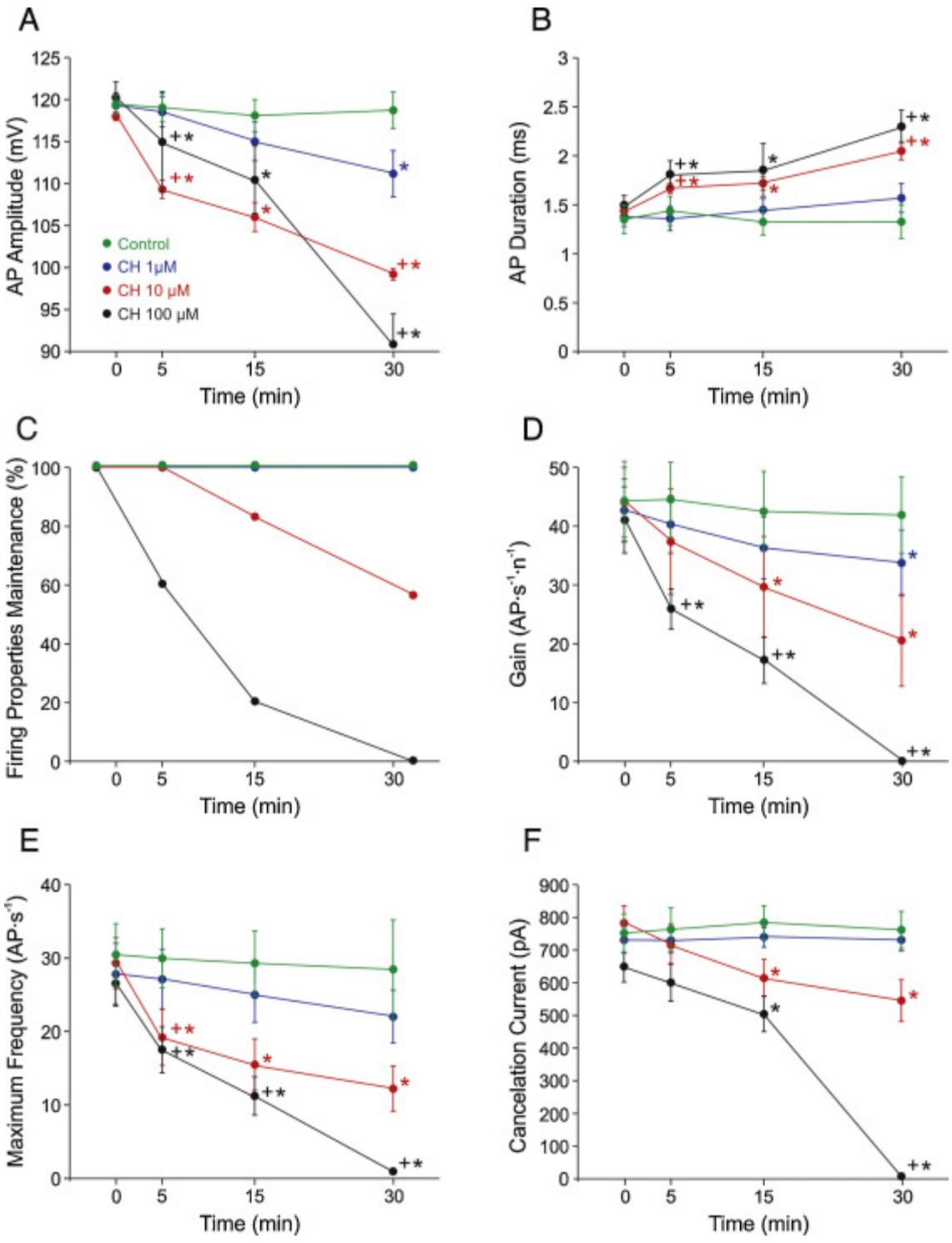
Figure 5. Effects of sequential application of increasing doses of cumene hydroperoxide (CH) on firing repetitive properties of pyramidal neurons from the motor cortex. A-D. Recordings showing, in the same cell, voltage response to depolarizing current pulses of 1 s duration and 500 pA intensity without CH administration (A), after 10 minutes of 1 μM CH administration (B), after 10 more minutes of 10 μM CH administration (C) and after 10 more minutes of 100 μM CH application (D). **E.** Relation between current and firing frequency for the cell represented in A-D. **F.** Histogram illustrating the mean values of gain in control condition and the other three situations for the population that kept their firing properties during 30 minutes (n=6).

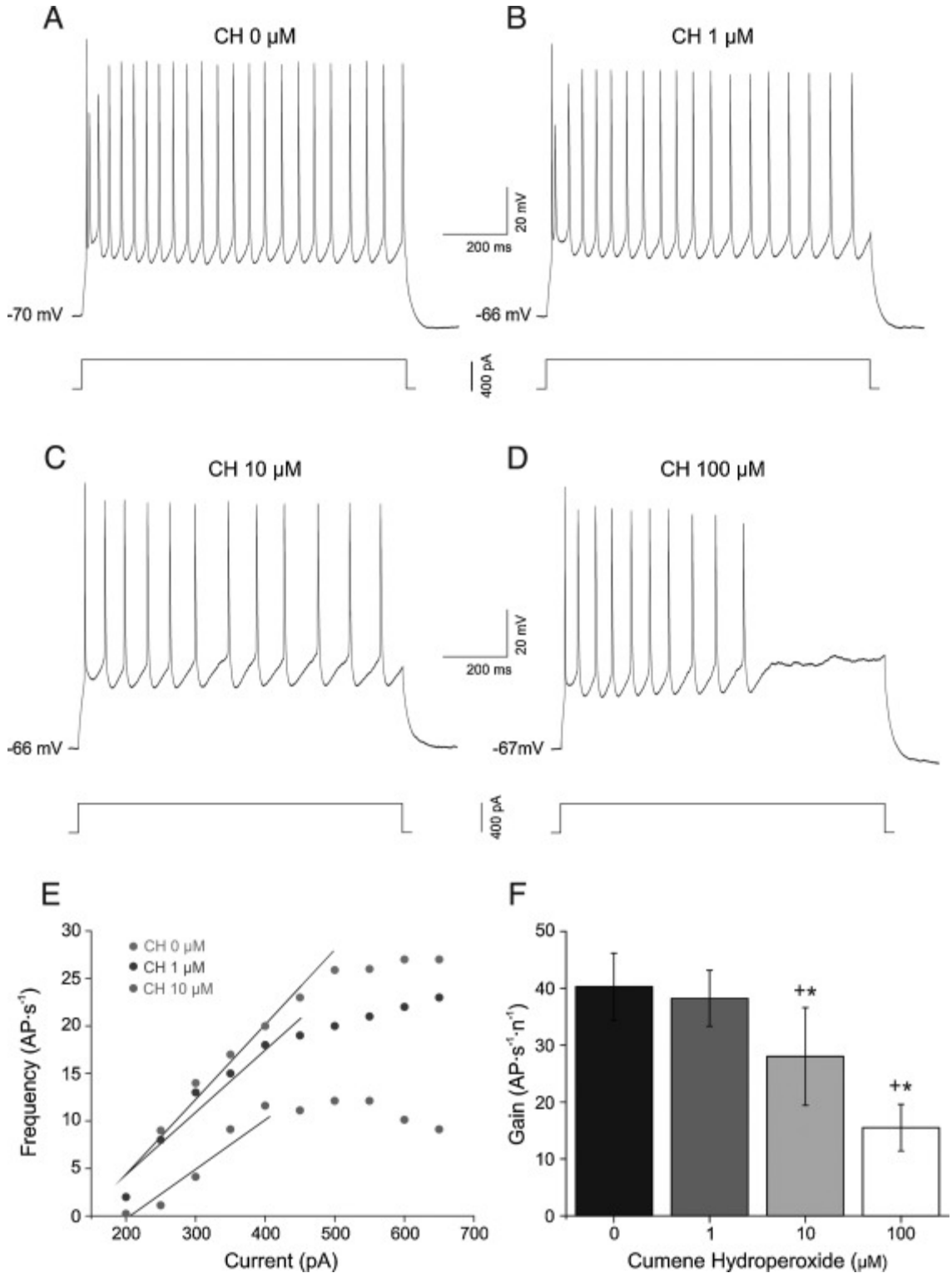
Figure 6. Prevention by melatonin of lipid peroxidation (LPO) induced by cumene hydroperoxide (CH) on pyramidal neurons from the motor cortex. A. Graphic showing LPO values, expressed in %, at 0, 5, 15 and 30 minutes after the exposure of CH 1µM (blue; n=6), 10 µM (red; n=6) and 100 µM (black; n=6). **B.** Plot showing LPO values, expressed in %, at 0, 5, 15 and 30 minutes after the exposure of CH 10 µM (n=6) and CH 10 µM plus melatonin 50 µM (n=6). **C.** Electrophysiological recording showing that membrane potential and voltage response to negative current pulses of -100 pA and 500 ms remain stable along 30 minutes exposition to CH 10 µM + 50 µM melatonin. Arrows indicate voltage response at 5, 15 and 30 minutes. The letter ”a” indicates statistical significance in relation to control situation, “b” represents statistical significance between a concentration and the precedent one for the same period of time, and “c” shows statistical significance between 10 µM CH application and 10 µM CH + 50 µM melatonin for the same period of time.

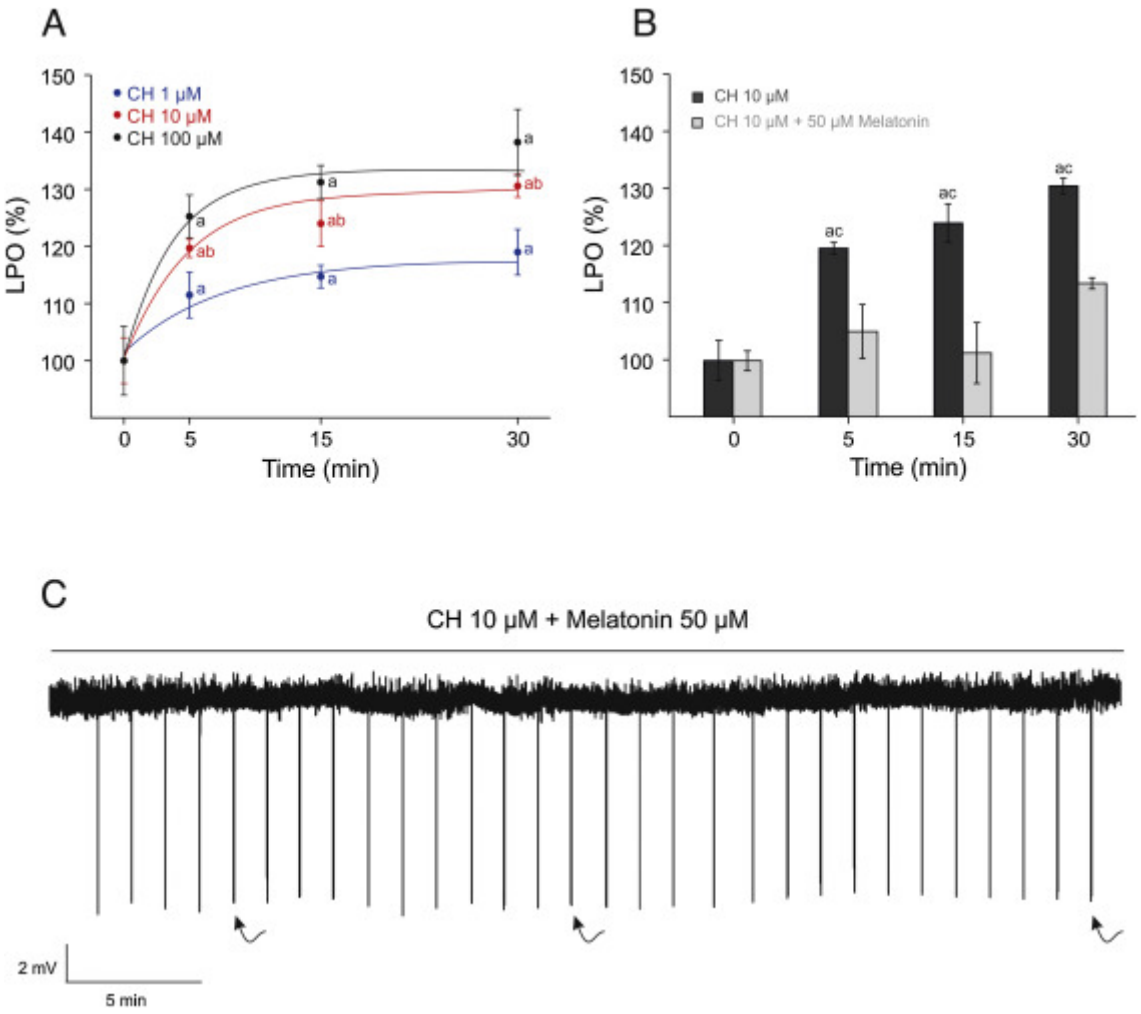

A
Control
-74 mV
-74 mV
5 mV
2 min
B
CH 10 µM
Washout
-69 mV
-68 mV
5 mV
2 min
C
CH 10 µM
Washout
-73 mV
-68 mV
5 mV
2 min


A
Control
CH 1μM
CH 10 μM
CH 100 μM
Membrane Potential (mV)
-55
-60
-65
-70
-75
-80
-85
0
5
15
30
Time (min)
B
Input Resistance (MΩ)
250
200
150
100
50
0
Time (min)
C
Rheobase (pA)
350
300
250
200
150
100
50
0
Time (min)
D
Voltage Threshold (mV)
-30
-35
-40
-45
Time (min)
E
Δ Rin (MΩ)
0
-20
-40
-60
-80
-100
-120
-140
-160
ΔRin= Rin CH - Rin Control
Capacitance (pF)
F
Rin= Rin CH / Rin Control x 100
Rin Change (%)
70
60
50
40
30
20
10
0
Capacitance (pF)


A
CH 0 µM
-75 mV
Sag
B
CH 1 µM
-72 mV
2 mV
50 ms
C
CH 10 µM
-66 mV
D
CH 100 µM
-65 mV
50 pA
E
Voltage (mV)
Current (pA)
CH 0 µM
CH 1 µM
CH 10 µM
CH 100 µM
F
Input Resistance (MΩ)
Cumene Hydroperoxide (µM)


A
AP Amplitude (mV)
125
120
115
110
105
100
95
90
Control
CH 1μM
CH 10 μM
CH 100 μM
0
5
15
30
Time (min)
B
AP Duration (ms)
3
2.5
2
1.5
1
0.5
0
Time (min)
C
Firing Properties Maintenance (%)
100
80
60
40
20
0
Time (min)
D
Gain (AP·s⁻¹·n⁻¹)
50
40
30
20
10
0
Time (min)
E
Maximum Frequency (AP·s⁻¹)
40
30
20
10
0
Time (min)
F
Cancelation Current (pA)
900
800
700
600
500
400
300
200
100
0
Time (min)


A
CH 0 µM
-70 mV
B
CH 1 µM
-66 mV
20 mV
200 ms
400 pA
C
CH 10 µM
-66 mV
D
CH 100 µM
-67mV
E
CH 0 µM
CH 1 µM
CH 10 µM
Frequency (AP·s-1)
Current (pA)
F
Gain (AP·s-1·n-1)
Cumene Hydroperoxide (µM)


A
150
140
130
120
110
100
LPO (%)
CH 1 µM
CH 10 µM
CH 100 µM
a
ab
a
a
a
ab
a
0
5
15
30
Time (min)
B
150
140
130
120
110
100
LPO (%)
CH 10 µM
CH 10 µM + 50 µM Melatonin
ac
ac
ac
0
5
15
30
Time (min)

C
CH 10 µM + Melatonin 50 µM
2 mV
5 min