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Oxidative stress induced by cumene hydroperoxide produces synaptic depression and transient hyperexcitability in rat primary motor cortex neurons

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ABSTRACT

Pyramidal neurons of the motor cortex are selectively degenerated in Amyotrophic Lateral Sclerosis (ALS). The mechanisms underlying neuronal death in ALS are not well established. In the absence of useful biomarkers, the early increased neuronal excitability seems to be the unique characteristic of ALS. Lipid peroxidation caused by oxidative stress has been postulated as one of the possible mechanisms involved in degeneration motor cortex pyramidal neurons. This paper examines the effect of lipid peroxidation on layer V pyramidal neurons induced by cumene hydroperoxide (CH) in brain slices from wild type rats. CH induces a synaptic depression of pyramidal neurons in a time dependent manner, already observable on GABAergic synaptic transmission after 5 min application of the drug. Altogether, our whole-cell patch-clamp recording data suggest that the functional changes induced by CH upon pyramidal neurons are due to pre- and postsynaptic mechanisms. CH did not alter mEPSCs or mIPSCs, but decreased the frequency, amplitude, and decay rate of spontaneous EPSCs and IPSCs. These effects may be explained by a presynaptic mechanism causing a decrease in action potential-dependent neurotransmitter release. Additionally, CH induced a postsynaptic inward current that underlies a membrane depolarization. Depressing the input flow from the inhibitory premotor interneurons causes a transient hyperexcitability (higher resistance and lower rheobase) in pyramidal neurons of the motor cortex by presumably altering a tonic inhibitory current. These findings, which resemble relevant cortical pathophysiology of ALS, point to oxidative stress, presumably by lipid peroxidation, as an important contributor to the causes underlying this disease.

1. Introduction

Amyotrophic Lateral Sclerosis (ALS) is a fatal disease that results from degeneration of both the lower and upper motor neurons, including the motor cortex layer V of pyramidal neurons that regulate voluntary control of motor output (Mochizuki et al., 2011). Upper motor neuron degeneration causes spasticity, hyperreflexia and compromises motor control, while degeneration of lower motoneurons is characterized by muscular weakness and cell death (Boillée et al., 2006; Kim et al., 2014). In a small percentage of cases, the origin of the ALS has a genetic component, but 90% of cases are sporadic, and the initial cause is still unknown. Motoneuron degeneration in sporadic ALS may be the consequence of a combination of mechanisms, including excitotoxicity that triggers mitochondrial dysfunctions and deregulation of Ca^{2+} homeostasis, aberrant protein aggregation, neuroinflammation, altered ionic channels and excitability, and environmental toxicity (Cleveland and Rothstein, 2001; Foran and Trotti, 2009; Grosskreutz et al., 2007; Guatteo et al., 2007; Martorana et al., 2012; Philips and Robberecht, 2011; Pieri et al., 2013, 2009; Saba et al., 2016; Van Den Bosch et al., 2006; Yin et al., 2017; Zona et al., 2006). It is also widely believed that oxidative stress has a major role in the development of such a disease (Niedzielska et al., 2016; Poppe et al., 2014; Reynolds et al., 2007). In fact, a clinical study demonstrated that the magnitude of oxidative stress correlated well with clinical severity in patients with ALS (Ikawa et al., 2015). Furthermore, postmortem studies also reveal extensive damage of lipids, proteins, and DNA due to oxidative stress (Bogdanov et al., 2000). In addition, some of the above mentioned alterations may also be linked to an excessive activation of glutamate receptors that induce an excitotoxicity cascade (Spalloni et al., 2013). Studies with transcranial magnetic stimulation have demonstrated cortical hyperexcitability in patients with ALS, which were detected before the onset of symptoms of the disease (Vucic et al., 2011, 2008). From these findings, it has been proposed that motoneurons degeneration in ALS may be the result of cortical hyperexcitability, which would induce modifications of glutamatergic activity in cortical neurons.

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Therefore, increased release of glutamate from presynaptic terminals could induce excitotoxicity in upper and lower motoneurons (King et al., 2016; Maekawa et al., 2004). However, there is also evidence that cortical inhibition is disrupted in ALS (Geevasinga et al., 2014). Therefore, more accurate characterization is needed to determine whether there is an increase of excitation or a disruption of inhibition to explain the cortical hyperexcitability, and their contribution to motoneuron excitotoxicity in ALS.

We have previously demonstrated that 10 μM of CH evokes lipid peroxidation in a time dependent manner (Pardillo-Díaz et al., 2016). Lipid peroxidation caused by oxidative stress can be described generally as a process under which oxidants such as free radicals affect lipids containing carbon-carbon double bonds. Lipid peroxidation modifies membrane barrier properties increasing the permeability for water and ions, and to a lesser extent, high molecular weight compounds (Ferretti and Bacchetti, 2011; Nam, 2011). The organic oxidizing agent, cumene hydroperoxide (CH), has been used to inflict oxidative stress in in vivo studies (Muñoz et al., 2017). This agent penetrates the membrane lipid bilayer causing not only peroxidation of lipids, but also reacting with aminoacids, and proteins, as singlet oxygen does (reviewed in Ayala et al., 2014). In previous in vitro work from our laboratory, we have also demonstrated that oxidative stress, induced by CH, evokes dose and time dependent changes in the functional properties of pyramidal neurons from the motor cortex, compromising both neuronal excitability, and the capability of generating action potentials (Pardillo-Díaz et al., 2016, 2015). Specifically, resting membrane potential of pyramidal cells of the motor cortex under CH exposure become progressively depolarized with no changes in voltage threshold. Furthermore, membrane resistance shows a biphasic change on membrane resistance, increasing after 5 min of drug application and then it started to decrease, even under control values, after longer periods of exposure. At the same time, changes in membrane resistance produce compensatory variations in the rheobase that lead to a transient increase in excitability. In some neurons, long exposure to the drug caused loss of their ability to discharge repetitively action potentials. However, most of the neurons maintain their repetitive discharge even though their maximum frequency and gain decreased. Furthermore, cancelation of the repetitive firing discharge took place at intensities that decreased with larger drug concentration and/or longer time of exposure to CH, which resulted in a narrower working range. These changes are thought to be mainly caused by changes in the intrinsic membrane properties of the pyramidal cells (Pardillo-Díaz et al., 2016, 2015). However, in hypoglossal motoneurons, Nani and colleagues have shown that oxidative stress also affects synaptic transmission that is accompanied by a significant diminution in the frequency of spontaneous postsynaptic currents due to changes in the release of several neurotransmitters (Nani et al., 2010). Additionally, it also has been shown that hydrogen peroxide application decreases synaptic inhibition in cortical neurons (Frantseva et al., 1998). Therefore, we propose that some of the reported changes in membrane resistance and rheobase could be the result of modifications in the release of neurotransmitters on pyramidal neurons. As a consequence, further studies are needed to evaluate the influence of synaptic transmission on pyramidal cells from the motor cortex under CH exposure.

To that end, we obtained electrophysiological recordings from pyramidal motor cortex neurons in brain slices to examine the effects of lipid peroxidation on synaptic and membrane properties. Our study aims to address the following questions: i) Does oxidative stress affect synaptic transmission? ii) Are some synaptic inputs to pyramidal neurons more sensitive than others to the exposure to CH. iii) Is synaptic transmission recoverable after washout of the drug? iv) Are the reported changes in membrane resistance and rheobase due to the result of modification in the release of neurotransmitters on pyramidal neurons? v) Does alteration of inhibitory premotor synaptic inputs underlie the hyperexcitability of cortical neurons? The answers to these questions could be potentially relevant to understand the role of lipid peroxidation in some neurological diseases such as ALS.

2. Material and methods

2.1. Animals and slice preparations

All procedures were conducted 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. Every effort was made to minimize the number of animals used and their suffering. Wistar rats (20–40 days of age) of both sexes were deeply anaesthetized with chloral hydrate (4%, Panreac) and decapitated. Brains were quickly removed and placed in ice-cold artificial cerebrospinal fluid (ACSF). This cutting solution was a low-calcium-ACSF. Transverse 300-μm-thick slices that included the primary motor cortex were cut on a vibratome (NVLSM1, WPI) kept at 35° for 30 min in an ACSF-filled chamber after slicing, and then stored at ~21 °C in the same solution until use. The composition of the ACSF (in mM) was as follows: 126 NaCl, 2 KCl, 1.25 NaH_2PO_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).

2.2. Whole-cell patch clamp recordings

Slices containing the primary motor cortex were transferred to a recording chamber and superfused at 1–2 ml·min^{-1} with recirculating aerated ACSF warmed to 33° ± 1 °C via a feedback-controlled heater (TC 324B; Warner). Neurons were whole cell patch voltage clamped under visual guidance 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 including a rhomboidal cell body, a prominent apical dendrite extending vertically towards the surface and basal dendrites radiating out from the base of the soma (Fig. 1A–B). Patch pipettes were pulled (PC-10, Narishige) from borosilicate glass capillary with filament (inner diameter 0.6, outer diameter 1 mm; Narishige). For voltage-clamp experiments, patch electrodes had 3–4 MΩ resistance, whereas for current-clamp experiments they had 5–6 MΩ resistance. Patch pipettes were filled with intracellular solution containing (in mM): 125 MeSO4·Cs, 10 KCl, 10 HEPES, 0.5 EGTA, 2 ATP-Mg, 0.3 GTP-Na2 (pH 7.2 with CsOH; 285 ± 5 mOsm) for voltage-clamp experiments and MeSO4·Cs, that minimized the leak current of the recorded cell, was used to enable larger changes in voltage threshold. For current-clamp experiments, patch pipettes contained (in mM): 120 K-gluconate, 10 KCl, 10 phosphocreatine disodium salt, 2 MgATP, 0.3 NaGTP, 0.1 ethyleneglycol-bis (2-aminoethylether)-N,N,N′,N′-tetra acetic acid (EGTA), 10 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), adjusted to pH 7.3 with KOH. The osmolarity of 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). Giga seals (>1 GΩ) were always obtained before rupture of the patch and pipette capacitance was compensated for before breaking in. In current-clamp mode, the bridge was periodically balanced using the auto-adjust feature. Throughout voltage-clamp recordings, the whole-cell capacitance and series resistance were measured and resistances were compensated by 70%. Recordings were discontinued if the series resistance increased by >50% or exceeded 20 MΩ.

2.3. Drugs and general protocol

All drugs were prepared just prior to experiments from stock solutions stored at −20 °C. The following drugs were used: 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX, 50 μM), d-amino-phosphonovalerate