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Effects of copper on viability and functional properties of hippocampal neurons *in vitro*

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ABSTRACT

Copper (Cu^{2+}) is an essential metal presented in the mammalian brain and released from synaptic vesicles following neuronal depolarization. However, the disturbance of Cu^{2+} homeostasis results in neurotoxicity. In our study we performed for the first time a combined functional investigation of cultured hippocampal neurons under Cu^{2+} exposure, its effect on spontaneous spike activity of hippocampal neuronal network cultured on multielectrode array (MEA), and development of long-term potentiation (LTP) in acute hippocampal slices in the presence of Cu^{2+}. Application of 0.2 mM $CuCl_2$ for 24 h reduced viability of cultured neurons to $40 \pm 6\%$, whereas 0.01 mM $CuCl_2$ did not influence significantly on the neuronal survival. However, exposure to the action of 0.01 mM Cu^{2+} resulted in pronounced reduction of network spike activity and abolished LTP induced by high-frequency stimulation of Schaffer's collaterals in CA1 pyramidal neurons of hippocampal slices. Antioxidant Trolox, the hydrosoluble vitamin E analogue, prevented neurotoxic effect and alterations of network activity under Cu^{2+} exposure, but didn't change the impairment of LTP in Cu^{2+}-exposured hippocampal slices. We hypothesized that spontaneous network neuronal activity probably is one of the potential targets of Cu^{2+}-induced neurotoxicity, in which free radicals can be involved. At the same time, it may be suggested that Cu^{2+}-induced alterations of long-lasting trace processes (like LTP) are not mediated by oxidative damage.

1. Introduction

Copper is present throughout the brain, most prominent in the hippocampus, cerebellum and basal ganglia. This transient metal serves as a cofactor for many important enzymes. For example, ceruloplasmin contains approximately 95% of plasma copper and is the key enzyme in handling oxidative stress. It is responsible for the Cu delivery to cells and involved in the dopamine synthesis (Vassiliev et al., 2005). Cu is involved in the essential step in mitochondrial oxidative phosphorylation as a component of cytochrome C oxidase, which catalyzes the final stage of electron transfer onto oxygen. Also, this metal is presented in the superoxide dismutase molecule, the most important antioxidant, which scavenges the superoxide radical. The strict control of Cu homeostasis prevents its excess accumulation in the tissues.

On the other hand, Cu is implicated in the pathogenesis of numerous neurological diseases, including Alzheimer disease, amyotrophic lateral sclerosis, Huntington disease, Parkinson disease (Desai and Kaler, 2008). In addition, the impairment of Cu homeostasis control can lead to development of such serious disorder as hepatocerebral dystrophy (Wilson's disease), followed by such complications as renal tubular dysfunction, liver cirrhosis, the emergence of Kayser-Fleischer rings and damage of different brain structures, most especially thalamus, subthalamic nuclei, brainstem, and frontal cortex (Das and Ray, 2006; Manto, 2014). Moreover, this disorder can initiate epileptic seizures, parkinsonism, cognitive impairment (Lorincz, 2010). For these changes, oxidative stress induced by increased free Cu^{2+} may be responsible (Ranjan et al., 2015). Along with decreasing of neuronal viability, impairment of Cu homeostasis may induce also synaptic alterations (Sadiq et al., 2012; Marchetti et al., 2014). Also Cu toxicity can be caused by exposure to excess Cu resulted from accident, occupational hazard, environmental contamination (Gaetke et al., 2014). The risk group includes workers engaged in the metal treatment and polishing of products from copper, its any solutions

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are extremely toxic to humans. The organism can be reached either through the airways or nutritional route with food.

In view of the neurotoxic properties of Cu and its capacity to impair the neural synaptic activity *in vivo* and *in vitro*, we hypothesized that bioelectric activity of neurons can be one of the major target of Cu-induced neurotoxicity, in which free radicals can be involved. Therefore we performed a combined investigation of cultured hippocampal neurons viability under Cu^{2+} exposure and its effect on spontaneous spike activity of neuronal network cultured on multielectrode array, and on the development of LTP in acute hippocampal slices in the presence of Cu^{2+}. Moreover to prevent Cu^{2+}-induced morphofunctional impairments of hippocampal neurons *in vitro*, we used the antioxidant Trolox that exerted a high neuroprotective potential in nerve cells and tissue cultures (Radesдter et al., 2003; Quintanilla et al., 2005; Isaev et al., 2010).

2. Material and methods

2.1. Primary hippocampal cell culture

Primary cell cultures were prepared from hippocampus of fetal mouse (17–18th gestational day). Cultures were prepared according to the method described by Brewer (Brewer, 1995). The tissue was digested with trypsin/EDTA (0.05/0.02% in PBS; 15 min; 37 °C) followed by mechanical dissociation. Hippocampal cells were seeded in poly-L-lysine-coated 96-well plates (120 000 cells/cm2), grown in serum-free neurobasal medium with B-27 minus AO supplement (Gibco, Germany) and 2 mM L-glutamine. Glutamate (0.025 mM) was added during the first three day *in vitro* (DIV). The cultures were maintained at 36.5 °C in a CO_2-incubator (5% CO_2, 95% air). Starting from DIV 4 the medium was changed by replacing half of the medium twice a week. The cultures were used for the experiments after DIV 9–10.

2.2. Assay of cell viability

The MTT assay (3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide) was used to quantitatively assess the viability of the cells (Isaev et al., 2004). The percentage of viable cells was quantified after incubation with 0.5 mg/ml MTT for 30 min. The viability of untreated control cultures was set to 100%, and that of treated cells were expressed as a percentage of formazan absorbance compared with that in control cultures. Each value was derived from 3 independent experiments at least.

2.3. Registration of spike spontaneous activity of hippocampal neuronal network in vitro

Hippocampal cells were plated with the final density of about 1600–2000 cells/mm^2 on MEAs pretreated with adhesion promoting molecules of polyethyleneimine (Sigma P3143). All recordings were performed after at least 10 days *in vitro* to ensure that the neuronal networks had stable spontaneous bioelectric activity (Mukhina et al., 2009). Extracellular potentials were collected by 64 planar indium tin-oxide (ITO) platinum black electrodes integrated into the MED64 system (60ThinMEA200/30iR-ITO-gr, Alpha MED Science, Japan). The MED probe had 8×8 (64) electrode arrays with $50\,\mu m \times 50\,\mu m$ micro-electrodes spaced by 150 μm. Data was recorded simultaneously in 64 channels at a sampling rate of 20 kHz/channel. The spiking activity characteristics data were normalized to their baseline level activity.

2.4. Registration of LTP in hippocampal slices

Registration of LTP in hippocampal slices has been performed according to Kapay et al. (Kapay et al., 2011). Experiments were carried out on 80–110 g male Wistar rats. 400 nm thick transverse slices from hippocampus were maintained at room temperature in an incubation chamber with modified Ringer solution of the following composition (mM): NaCl, 124; KCl, 3; $CaCl_2$, 2.5; $MgSO_4$, 2.5; Na_2HPO_4, 1.25; $NaHCO_3$, 26; D-glucose, 10, continuously bubbled with 5%CO_2, 95%O_2. After 1.5 h incubation, slices were transferred into immersion recording chamber perfused with the same solution at a 2 ml/min flow rate, 29–30 °C. Extracellular recordings were made from the pyramidal layer of the CA1 region using glass micropipettes filled with 1.5 M NaCl. To elicit synaptic responses, Schaffer's collateral/commissural afferents in stratum radiatum were stimulated through bipolar glass electrodes filled with the perfusion medium. A focal response was caused by stimulation of Schaffer's collaterals by single rectangular impulses (duration 0.1 ms, interval 1/15 s) with an intensity that was able to elicit a 50% response of the pyramidal neuron population (popspike, PS). LTP of PS was induced using a train of high-frequency stimulation (HFS) (100 Hz, 1 s) with pulses of the same current strength, applied via the same stimulating electrodes. In a number of experiments, hippocampal slices were preincubated with 0.1 mM Trolox (30 min, modified Ringer solution) then during the recording some of the slices were exposed to 0.01 mM $CuCl_2$ (20 min, modified Ringer solution).

All experimental protocols were approved by the Animal Ethics Committees of the Research Center of Neurology (the Protocol Registration number 2-5/16) and accordance with Council Directive 2010/63EU of the European Parliament and the Council of 22 September 2010 on the protection of animals used for scientific purposes

2.5. Pharmacological treatment

$CuCl_2$ (0.01–0.5 mM) and Trolox (0.1 mM) were supplemented directly to the culture medium.

2.6. Statistics

The one-way ANOVA with Neuman–Keuls and Bonferroni post-test was used for statistical analysis. Levels of $p < 0.05$ were considered as statistically significant. The results are represented as means ± SEM. All data were obtained from 3 to 4 independent experiments.

3. Results

3.1. Viability of hippocampal neurons under Cu^{2+} exposure

Application of $CuCl_2$ (0.05–0.5 mM) lead to occurrence of pyknotic nuclei. Their formation and increasing number well indicate cell death. $CuCl_2$ for 24 h pronounced dose-dependent neuronal death (Fig. 1). The antioxidant Trolox partially protected neurons from neurotoxic effect (Fig. 2). 0.1 mM $CuCl_2$ did not significantly change the viability of hippocampal neurons.

3.2. Cu^{2+}-induced impairment of spontaneous network activity

Cultured neurons on MEAs formed dense networks (Fig. 3A) and became spontaneous bioelectrical activity on 4st-5th day *in vitro*. The extracellular recordings were performed between DIV 10–12. In the base line recordings (on DIV 10) the mean spike frequency was 11.1 ± 0.9 Hz (100%) and after 24 h this characteristic value was 98 ± 3%. The 24 h 0.01 mM Cu^{2+} incubation decreases this parameter to 69 ± 5% (Fig. 3C) and 48 h 0.01 mM Cu^{2+} incubation to 56 ± 5% (Fig. 3D). The mean spike frequency in control cultures was 93 ± 4% in this time (Fig. 3D). Antioxidant