Abstract—Hydrogen peroxide (H$_2$O$_2$) is one of the reactive oxygen species (ROS), endogenously produced during metabolism, which acts as a second messenger. In skeletal muscles, hypoxia- or hyperthermia-induced increase in H$_2$O$_2$ might affect synaptic transmission by targeting the most redox-sensitive presynaptic compartment (Giniatullin et al., 2006). However, the effects of H$_2$O$_2$ as a signal molecule have not previously been studied in different patterns of the synaptic activity. Here, using optical and microelectrode recording of synaptic vesicle exocytosis, we studied the use-dependent action of low concentrations of H$_2$O$_2$ and other oxidants in the mouse neuromuscular junction. We found that: (i) H$_2$O$_2$ at low micromole concentrations inhibited both spontaneous and evoked transmitter releases from the motor nerve terminals in a use-dependent manner, (ii) the antioxidant N-acetylcysteine (NAC) eliminated these depressant effects, (iii) the influence of H$_2$O$_2$ was not associated with lipid oxidation suggesting a pure signaling action, (iv) the intracellular oxidant Chloramine-T or (v) the glutathione depletion produced similar to H$_2$O$_2$ depressant effects. Taken together, our data revealed the effective inhibition of neurotransmitter release by ROS, which was proportional to the intensity of synaptic activity at the neuromuscular junction. The combination of various oxidants suggested an intracellular location for redox-sensitive sites responsible for modulation of the synaptic transmission in the skeletal muscle. © 2018 IBRO. Published by Elsevier Ltd. All rights reserved.

Key words: reactive oxygen species, H$_2$O$_2$, synaptic transmission, mouse neuromuscular junction.

INTRODUCTION

Reactive oxygen species (ROS), which are by-products of aerobic metabolism in cells, have typically been described as highly toxic molecules, impairing cell functions due to oxidation of various macromolecules (Bruce-Keller et al., 2010; Lee et al., 2012; Radak et al., 2013). The main source of ROS in cells is the mitochondrial electron transport chain (Kalogeris et al., 2014; Dan et al., 2015; Cobley, 2018; Sidlauskaitė et al., 2018), membrane-bound NADPH oxidases (Haslund-Vinding et al., 2017; Ma et al., 2017), as well as xanthine oxidase (Zuo et al., 2015) and monoamine oxidase (Borroni et al., 2017). However, the growing evidence suggests that ROS serve as signaling molecules producing transient modulatory effects (Oswald et al., 2018). Among other ROS, hydrogen peroxide (H$_2$O$_2$), is a good candidate to serve as a signaling molecule as this compound is stable (Li and Jackson, 2002; Lennicke et al., 2015) while keeping mild oxidizing features. Moreover, H$_2$O$_2$ is stable not only in the intracellular, but also in the extracellular space, which allows this ROS to perform para- and autocrine functions including the regulation of transmitter release and synaptic plasticity (Kamsler and Segal, 2003; Avshalumov et al., 2008; Rice, 2011; Beckhauser et al., 2016).

Previously, in the frog neuromuscular junction, we showed a bidirectional modulation of the transmitter release by different concentrations of H$_2$O$_2$ (Giniatullin and Giniatullin, 2003). While slightly facilitatory at low concentrations in frogs, the high doses of H$_2$O$_2$ produced the typical depressant effect in both amphibian and mammalian muscles (Giniatullin and Giniatullin, 2003; Giniatullin et al., 2006). Notably, H$_2$O$_2$-induced impairment of exocytotic machinery was mediated by oxidation of the t-SNARE protein, which appeared as a synaptic
ROS sensor (Giniatullin et al., 2006). However, in mammals, the effect of low concentrations of ROS was not explored. Moreover, ROS modulation in previous studies was performed at non-physiological low-rate modes of neuronal activity. Finally, the issue of distinguishing temporal modulatory effects from damaging action resulting in membrane lipid peroxidation requires additional testing.

Therefore, in the current study, we applied a multidisciplinary approaches and used the combination of various oxidants to address ROS modulation of transmitter release in the synapse operating with different modes of activity. Our data are consistent with a physiological non-damaging role of low concentrations of H₂O₂ that modulate release machinery in a user-dependent manner.

**EXPERIMENTAL PROCEDURE**

**Ethical approval**

Animal care and experiments were carried out in accordance with the European Communities Council Directive 86/609/EEC approved by the Ethics Committee of Kazan Medical University and the European Directive 2010/63/UE. All efforts were made to minimize animal suffering.

**Innervated diaphragm preparation**

Experiments were performed on the isolated diaphragm muscle of outbred B6/SJL mice of both sexes a body weight of 22–25 g. Mice had free access to food and water and were maintained in a 12-h light/12-h dark cycle. Diaphragm muscle supplied with a phrenic nerve was isolated and then attached to the bottom of a Sylgard-lined chamber, which was superfused at 2 ml/min throughout the experiment with physiological solution containing (in mM): NaCl-120.0, KCl-5.0, CaCl₂-2.0, MgCl₂-1.0, NaH₂PO₄-1.0, NaHCO₃-24.0, glucose-11.0. Solutions were saturated with a 5% CO₂ and 95% O₂ mixture and pH was adjusted to 7.4 with NaOH/HCl. Experiments were performed at 24–25°C which allows the muscle to maintain a stable level of neurotransmitter release for a long period (Glavinović, 1979). In some experiments (electrophysiology), the muscle fibers were cut transversely (‘cut muscles’) to prevent muscle contractions and to simultaneously maintain the physiological level of quantal release (Barstad and Liljeholm, 1968; Glavinović, 1979). In the cut muscles, recordings started after the stabilization of membrane potential approximately 40 min. after the cutting procedure (Lambert et al., 1981; Sokolova et al., 2003). The nicotinic blocker (+)-tubocurarine (3–5 μM) was used to block contractions in experiments using the FM1–43 as the endocytosis marker. One animal was used for each preparation. All drugs were dissolved to the final concentrations in basic solution just before the experiments. H₂O₂ was diluted in the basic solution from 50% stock (Sigma–Aldrich) to obtain the final concentrations (3, 10 or 30 μM).

**Electrophysiological recordings**

Recording of the postsynaptic end-plate currents (EPCs) and miniature EPCs (MEPCs) were performed using the standard two-electrode voltage-clamp technique with intracellular glass microelectrodes (tip diameter ~1 μm, resistance 3–5 MΩ, filled with 2.5 M KCl) using a custom-made low-noise recording amplifier (Timchenko, Kazan, Russia) (voltage-clamp gain × 10,000, band-pass frequency 0–10 kHz) as previously described (Giniatullin et al., 2006; Bernareggi et al., 2018; Mukhutdinova et al., 2018). To prevent muscle contractions we used transverse cutting of muscle fibers (holding potential was kept at ~40 mV). EPCs were elicited by supra-threshold stimulation (0.1-ms duration) of the phrenic nerve via a suction electrode connected to an extracellu-lar stimulator (DS3 Digitimer Ltd., UK). The motor nerve was stimulated by low-frequency (1 stimulus in 20 s, 0.05 Hz) or by high-frequency trains (20 stimuli per 1 s, 20 Hz, for 3 min). The signals were analyzed off-line using PC using a custom made software (Bernareggi et al., 2018). Recordings of MEPCs were performed on uncut muscles with the membrane potentials of fibers maintained at ~60 mV.

**Loading FM1–43**

The endocytosis marker FM1–43 (10 μM, Invitrogen™) was used to estimate the rate of synaptic vesicles exocytosis (Betz and Bewick, 1993). To load the dye, a prolonged rhythmic stimulation of 20 Hz was applied for 3 min following by the 7 min rest period in the presence of the dye. The preparations were then washed with physiological solution for 30 min to decrease the background fluorescence (Petrov et al., 2011). Re-stimulation of the exocytosis (by 20 Hz train) causes a decrease in the nerve terminal fluorescence (“unloading”). The dye-loaded preparations were incubated in physiological solution containing 30 μM H₂O₂ for 20 min. After washing the dye and before applying the oxidant, the intensity of nerve terminal fluorescence was recorded to assess the possible bleaching of the dye by H₂O₂. To record FM1–43 fluorescence intensity we used a 480/10 nm excitation filter and a band pass emission filter of 515–555 nm. Background fluorescence was estimated as mean fluorescence intensity in a 10 × 10 μm² area outside of the nerve terminal. Nerve terminal fluorescence was defined as the average pixel intensity in regions of interest after the background subtraction (Petrov et al., 2008). Fluorescence images were acquired using an Olympus BX51WI microscope with a confocal attachment Disk Speed Unit and UPLAN Sapø 60xw objective. Images were captured with a DP71 (Olympus, Japan) CCD camera, and analyzed using Cell™P (Olympus) and Image Pro software (Media Cybernetics, Bethesda, MD, USA). The fluorescence was calculated in arbitrary units (a.u.) and then converted to percentages. Only nerve terminals on the surface muscle fibers were studied. Samples were not cross-sectioned. For analysis of the kinetics of unloading, the value of the initial fluorescence (before the stimulus train) was taken as 100%.
Assays for hydroperoxides

Concentrations of hydroperoxides were measured by ferrous oxidation in Xylenol Orange (FOX1). This method is highly sensitive and consists of peroxide-mediated oxidation of ferrous ions in an acidic medium containing the dye xylenol orange, which binds the resulting ferric ions to produce a blue–purple complex with an absorbance maximum of between 540 and 580 nm. The FOX1 reagent was prepared as described by Wolff (Wolff, 1994) with slight modifications (Giniatullin et al., 2005). The peroxide content of samples was determined with reference to a calibration curve obtained with known concentrations of H$_2$O$_2$ and expressed as micromoles of peroxide per gram of tissue.

ROS imaging

ROS in the muscle were also detected with CM–H$_2$DCFDA which is a chloromethyl derivative of H$_2$DCFDA, useful as an indicator for ROS in cells (Invitrogen™) (Opara et al., 2016; Wu et al., 2018). In comparison with H$_2$DCFDA oxidation of the CM variant induces formation of fluorescent CM–DCF, which displays a lower passive leakage from the cell (Forkink et al., 2010). This dye freely permeates the cells, and becomes trapped inside after hydrolysis to carboxy-dichlorodihydro fluorescein, which upon oxidation in live cells, fluorescence of the MCB–GSH adduct in selected areas of the muscles (up to 15 sites per muscle) was carried out only once, in order to prevent the dye bleaching.

GSH measurement

To determine the level of intracellular glutathione in the control, neuromuscular drugs were incubated in physiological solution for 90 min, and placed in a solution containing monochlorobimane (MCB; Invitrogen™) at 25 °C for 40 min, or until a steady state had been reached before images were acquired for quantitation (Keelan et al., 2001; Majumder et al., 2015). MCB was prepared as a 20 mM stock solution in DMSO and used at a final concentration of 200 μM. The muscles were then washed with physiological solution, and images of the fluorescence of the MCB–GSH adduct were acquired using the fast CCD imaging system as described using excitation at 380 nm and emission at >400 nm. At the end of the L-BSO treatment (90 min) the muscles were loaded with 200-μM MCB (40 min) and after wash out MCB (30 min), MCB–GSH fluorescence data were collected as described above. Registration of fluorescence intensity of the MCB–GSH adduct in selected areas of the muscles (up to 15 sites per muscle) was carried out only once, in order to prevent the dye bleaching.

Imaging lipid peroxidation

To evaluate the lipid peroxidation induced by H$_2$O$_2$ we used the Image-iT Lipid Peroxidation Kit (Invitrogen™). The main component of this kit, the BODIPY 581/591 C11 reagent is a sensitive fluorescent reporter for lipid peroxidation. Upon oxidation in live cells, fluorescence of the phenylbutadiene segment of the fluorophore shifts from red to green, allowing a ratiometric evaluation of lipid peroxidation. Diaphragm muscles were incubated with 10 μM Lipid Peroxidation Sensor for 30 min at room temperature, rinsed 3° with normal physiological solution for 30 min (Giniatullin et al., 2015), and visualized with the Olympus BX51 fluorescent microscope with DSU (Olympus, Center Valley, PA, USA), combined with the fast CCD camera DP72 (Olympus), with a LUM PlanFI 60°/0.90 W objective. Fluorescence intensity was recorded over the surface of the muscle fibers (Murrant and Reid, 2001). Our microscope field allowed visualization of an area containing three to four muscle fibers. Muscle illumination was limited to 21 frames at 2- to 3 min intervals (exposure time 300 ms) for 15 min to minimize photo-oxidation of CM–H$_2$DCFDA. This protocol allowed to prevent non-specific changes in baseline CM–H$_2$DCFDA fluorescence and, thus, to overcome the limitations (Kalyanaraman et al., 2012) of using DCF-based dyes. Fluorescence intensity (excitation 475 nm; emission 525 nm), was measured over three to five distinct muscle areas within the imaged field. The intensity of fluorescence was quantified using image analysis software (Morphostar, Paris) or Metafluor software (Metafluor Imaging Series 6.0, Universal Imaging Corporation, USA) and relative change in fluorescence from baseline (dF/F$_0$ × 100%) was calculated as previously described (Khiroug et al., 1998).

In some experiments with CM–H$_2$DCFDA postsynaptic acetylcholine receptors were labeled by exposing the preparation to rhodamine-conjugated α-bungarotoxin (100 ng/ml α-Btx, Invitrogen™) to help identify the synaptic region for detection of CM–H$_2$DCFDA. α-Btx was added to the external solution after CM–H$_2$DCFDA loading. Fluorescence of α-Btx was excited by light of 555/15 nm wavelength and emission was detected using a band-pass filter of 610–650 nm.

Measurements of extracellular H$_2$O$_2$

The extracellular H$_2$O$_2$ was measured with the Amplex® Red Hydrogen Peroxide/Peroxidase Assay Kit (Life Technologies) containing a sensitive Amplex® Red reagent (10-acetyl-3,7-dihydroxyphenoxazine) following the manufacturer’s instructions (Giniatullin et al., 2015). The baseline level of H$_2$O$_2$ (Ctrl) in the bath containing neuromuscular preparation was determined after the incubation of the sample for 90 min in physiological solution (total volume 400 μl), containing 100 μM Amplex Red and 0.2 U/ml HRP. Then, the preparation was embedded for 90 min into a new solution, containing
1 mM or 3 mM l-BSO (in addition to Amplex Red and HRP). The background fluorescence, determined in a control solution containing no H$_2$O$_2$ has been subtracted from each value (Odnoshivkina et al., 2015).

Statistical analysis
Mean amplitudes, rise times (from 10% to 90% of the peak amplitude), decay time constants (Tau) of EPC and MEPC and the inter-event intervals, were analyzed using Origin Pro 9.2 software (Microcal Software, Northampton, MA, USA). The data are presented as mean ± S.E.M where n is the number of independent experiments on different animals, with statistical significance assessed by the Student’s paired t-test. A P value of less than 0.05 was accepted as indicative of a statistically significant difference.

**RESULTS**

**H$_2$O$_2$ depresses transmitter release at low and high stimulation frequencies**
First, we tested the action of low concentrations of H$_2$O$_2$ on EPCs elicited by low-frequency (0.05 Hz) stimulation of the motor nerve. In control conditions, the amplitude of EPCs was 103 ± 12nA (n = 9). Fig. 1A–C shows that after 20-min exposure to 3, 10, and 30 μM H$_2$O$_2$, the amplitudes of EPC were 101 ± 3% (n = 6; p > 0.05), 85 ± 2% (n = 6; p < 0.05) and 60 ± 3% (n = 7; p < 0.05), respectively. This depressant effect of H$_2$O$_2$ was partially reversible after 15–20 min of H$_2$O$_2$ washout. In control conditions, MEPCs occurred at a mean frequency of 1.35 ± 0.5 s$^{-1}$ whereas the amplitude of MEPCs was 1.8 ± 0.15nA (n = 6). Like evoked release, the frequency of MEPCs was dose-dependently reduced by 3, 10 and 30 μM H$_2$O$_2$ to 92 ± 5% (n = 6; p > 0.05), 75 ± 4% (n = 6; p < 0.05), 62 ± 3% (n = 6; p < 0.05) of the pre-H$_2$O$_2$ baseline, respectively (Fig. 1D). The amplitude of MEPCs was not changed even with 30 μM H$_2$O$_2$ (1.9 ± 0.15nA in control vs. 1.8 ± 0.25nA in H$_2$O$_2$ n = 6, p > 0.05, Fig. 1B). Furthermore, H$_2$O$_2$ did not change the decay time of MEPCs (0.82 ± 0.05 ms in control vs. 0.87 ± 0.07 ms in 30 μM H$_2$O$_2$; n = 6; p > 0.05). Taken together, these data indicated a presynaptic-specific effect of H$_2$O$_2$.

Stimulation of the motor nerve at frequencies of less than 1 Hz, which is far from the much higher native activity of the motoneurones, releases only a small number of synaptic vesicles belonging mainly to the readily releasable pool. Stimulation at higher frequencies, in particular 20 Hz, allows more accurate simulation of conditions in vivo (Funk and Parkis, 2002; Slater, 2015). In this mode of activity, the synaptic efficiency is determined by the intensity of traffic of undocked synaptic vesicles to the active zone and the rate of vesicle recycling (Petrov et al., 2008). Therefore, next, we tested the action of 3–30 μM H$_2$O$_2$ in conditions of high-frequency (20 Hz for 3 min) stimulation. In response to this pattern of stimulation, in control, the EPCs initially declined by 1 min to 67 ± 3% (n = 9) and then remained unchanged during the next period (3 min) of stimulation (63 ± 4%, n = 9; Fig. 2A, B). After application of 3, 10, 30 μM H$_2$O$_2$, the EPC amplitudes reduced to 59 ± 4%, 54 ± 5% and 40 ± 3% (n = 6) by 3 min of stimulation versus the control level before onset of the stimulation, respectively (Fig. 2A, B). The more pronounced effect at high frequency suggested that H$_2$O$_2$ is able to reduce the quantal secretion proportionally to the activity of the motor nerve.

**N-Acetylcysteine (NAC) has preventive effect**
Next, we tested the action of H$_2$O$_2$ in the presence of NAC, a typical scavenger of ROS. This antioxidant has controversial mechanisms of action but effectively inhibits H$_2$O$_2$-induced effects in different ex vivo samples, including neuromuscular junctions (Petrov et al., 2014; Shahripour et al., 2014; Odnoshivkina et al., 2015; Okamoto et al., 2016; Wu et al., 2018). The application of 1 mM NAC increased the amplitude of EPCs evoked by 0.05-Hz stimulation to 132 ± 7% (n = 6; p < 0.05). Subsequent application of 30 μM H$_2$O$_2$ did not change the amplitude of EPCs (107 ± 4% of amplitude in the presence of NAC; n = 5; p > 0.05). Thus, the ROS scavenger, NAC, abolished the inhibitory effects of H$_2$O$_2$, indicating that this effect was indeed produced by ROS.

**H$_2$O$_2$ slows down FM1–43 unloading indicating reduced exocytosis**
As an independent approach to explore the action of H$_2$O$_2$ on endo/exocytosis we labeled the nerve terminals with the marker FM1–43. In control conditions, stimulation of the motor nerve at 20 Hz decreased the fluorescence level of FM1–43 in pre-loaded nerve terminals (indicating intensity of exocytosis) to 69 ± 2% (n = 7 mice, p < 0.01) and 48 ± 3% (p < 0.001) after 2 and 10 min of stimulation, respectively (Fig. 3A, B). Consistent with the electrophysiological approach, H$_2$O$_2$ (30 μM) significantly reduced the rate of FM1–43 unloading. Thus, the fluorescence decreased to 86 ± 2% (n = 7 mice, p < 0.05) and 76 ± 3% (p = 0.001) after 2 and 10 min of stimulation, respectively (Fig. 3A, B). These data indicated that H$_2$O$_2$ suppressed the synaptic vesicle exocytosis during intense stimulation. Using a previously described approach (Betz and Bewick, 1993; Kasimov et al., 2016), we estimated the time of synaptic vesicle recycling during the prolonged high-frequency activity by comparing a cumulative EPC amplitude curve (from Fig. 2) and inverted-FM1–43 loss curve (from Fig. 3B). The apparent divergence of the curves was ~40–50 s of 20 Hz stimulation in control (Fig. 3C) and ~70–80 s in H$_2$O$_2$-treated neuromuscular junctions (Fig. 3D). This suggested that H$_2$O$_2$ suppressed the recycling of synaptic vesicles.

**Fast inhibition by H$_2$O$_2$ is not associated with lipid peroxidation**
In order to distinguish ‘physiological’ action from damaging action of the concentrations of H$_2$O$_2$ used, we tested the appearance of the long-living lipid peroxidation products during application of H$_2$O$_2$. In control, the prevailing
fluorescent signal was detected in the red vs green channel (high 590/510 nm ratio) indicating no or a low level of lipid peroxidation in the resting muscle (Giniatullin et al., 2015). Application of H$_2$O$_2$ (30 μM) did not change this ratio significantly (Fig. 4). Only much higher, non-physiological concentration of H$_2$O$_2$ as high as 1 mM was needed to reduce the 590/510 ratio to 71 ± 5% of the control (n = 4; p = 0.004, paired t-test, Fig. 4). These data suggested that the depressant action of low concentrations of H$_2$O$_2$ on transmitter release detected in short-lasting functional tests was not associated with an induction of lipid peroxidation.

Differential effects of the pro-oxidants Chloramine-T and DTNB

To explore the location of the main ROS-sensitive cellular targets, we used the oxidant Chloramine-T which is able to penetrate the cell membrane and 5,5-dithiobis-2-nitrobenzoic acid (DTNB) which cannot pass through the cell membrane. Chloramine-T (200 μM), like H$_2$O$_2$, reduced the amplitudes of the EPCs, at a low-frequency stimulation, to 75 ± 2% (n = 6; p < 0.05; Fig. 5A). Even more pronounced depression was observed during high-frequency stimulation (to 21 ± 9%; n = 6; p < 0.05; Fig. 5B). The subsequent recovery of EPC after simulation was to 55–60% of the level prior to stimulation (n = 5, p < 0.02 compared with control).

Like H$_2$O$_2$, Chloramine-T did not cause changes in the amplitude and time parameters of MEPCs, but reduced the frequency of spontaneous release from 1.54 ± 0.4 s$^{-1}$ to 0.98 ± 0.4 s$^{-1}$ (n = 6 different diaphragm muscles, p < 0.05). In contrast to Chloramine-T, DTNB (300 μM) did not decrease EPCs evoked by low-frequency stimulation (109 ± 3%; n = 6; p < 0.05; Fig. 5A). DTNB also did not change the time-course of EPCs under high-frequency stimulation in comparison to the control (Fig. 5C). Finally, DTNB did not change the amplitude and time parameters of MEPCs, and had no effect on the frequency of spontaneous neurotransmitter release (1.52 ± 0.5 s$^{-1}$ vs 1.62 ± 0.3 s$^{-1}$ in control; n = 6 diaphragm muscles, p < 0.05). Taken together, these results indicate that the cell impermeable oxidant DTNB changed neither of the tested parameters of synaptic transmission.

L-BSO depresses transmitter release at high frequencies of stimulation

It is well established that buthionine–sulfoximine (L-BSO) can enhance the level of endogenous ROS by reducing the major intracellular antioxidant glutathione (Chi et al., 2007; Sato et al., 2013; Salvi et al., 2016). To test whether this pro-oxidant in our conditions indeed increased the general level of endogenous ROS we used the FOX method (Wolff, 1994; Shakirzyanova et al., 2016). In control, the total level of hydroperoxides was 2.3 ± 0.3 μm/g (n = 6). L-BSO (1 mM and 3 mM applied for 90 min) increased the level of hydroperoxides to 7.4 ± 1.2 and 10.5 ± 1.3 μm/g, respectively (n = 6; p < 0.05; Fig. 6A). Thus, the treatment with L-BSO indeed increased the total number of hydroperoxides in the muscle tissue. Thus, in the next set of experiments the synaptic action of on EPCs was evaluated. After applica-

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**Fig. 1.** Effect of the H$_2$O$_2$ on evoked and spontaneous neurotransmitter release. (A) Representative EPCs during low-frequency (0.05 Hz) stimulation under control conditions (Ctrl), 20 min after treatment with different concentrations of H$_2$O$_2$, and 15 min after washout. (B) Representative MEPCs in Ctrl and 20 min after treatment with 30 μM H$_2$O$_2$. (C, D) Histograms showing the action of different concentrations of H$_2$O$_2$ on peak EPC amplitude (C) and (D) MEPC frequency. Mean ± SEM, n = 6–9, *p < 0.05.
treatment with different concentration of H$_2$O$_2$. Every hundredth amplitudes during high-frequency stimulation in Ctrl and 20 min after

\[ n \pm 4\% \text{ and } 125 \pm 5\% \], respectively (Fig. 6 B, C). The fluorescent signal in control, without

Likewise, H$_2$O$_2$ (10$\mu$M) increased fluorescence to 108 ± 3% ($n = 4$; $p < 0.05$) and 131 ± 3% ($n = 4$; $p < 0.05$), respectively (Fig. 6C). It should be noted that when we used 3 mM L-BSO, there was an increase in the fluorescence of muscle fibers including synaptic regions (Fig. 6B).

The action of L-BSO as the glutathione depleting agent was confirmed with MCB known as a marker of the glutathione level (Keelan et al., 2001; Majumder et al., 2015). Indeed, after exposure of the muscle to 1 mM and 3 mM L-BSO for 90 min, the fluorescence level of MCB was lowered to 68 ± 6% and 56 ± 4%, respectively ($n = 5$; $p < 0.05$, Fig. 6D).

Next, we estimated the level extracellular H$_2$O$_2$ using the Amplex Red Hydrogen Peroxide/Peroxidase Assay Kit (Giniatullin et al., 2015; Odnoshivkina et al., 2015). L-BSO (1 mM and 3 mM) also increased the level of extracellular H$_2$O$_2$ to 2.75 ± 0.03 and 5.52 ± 0.04 $\mu$M, respectively (Fig. 6F). It was equal to 0.85 ± 0.05 $\mu$M ($n = 4$) in the control conditions (Fig. 6F). Thus, the elevated level of endogenous ROS reproduced the synaptic effects of exogenously applied H$_2$O$_2$ at low concentrations of H$_2$O$_2$.

**DISCUSSION**

Here we have shown that relatively low, ‘physiological’ concentrations of H$_2$O$_2$ inhibited both spontaneous and evoked ACh release from the motor nerve terminals, without signs of oxidative modifications of membrane lipids. This inhibitory effect was observed already at low levels of activity but synaptic depression was further amplified in a use-dependent manner during high-frequency stimulation of the motor nerve. The antioxidant NAC eliminated these effects. The action of H$_2$O$_2$ on transmitter release was reproduced by the L-BSO, depleting glutathione and by the membrane penetrating oxidant Chloramine-T suggesting an the intracellular location of the ROS-sensitive cellular targets.

**Action of low concentrations of H$_2$O$_2$ on synaptic transmission**

Today, studies using exogenous H$_2$O$_2$ are sometimes criticized because of the assertion that exogenous addition of H$_2$O$_2$ is always non-physiological (Forman, 2007). Our previous studies in frog muscle indicated the bi-directional actions of H$_2$O$_2$ on synaptic transmission, namely, its enhancement by low doses and depression at high concentrations of H$_2$O$_2$ (Giniatullin and Giniatullin, 2003). In several subsequent studies we confirmed the inhibitory action of high concentrations of H$_2$O$_2$ on synaptic transmissions also in the mammalian synapses (Giniatullin et al., 2006; Shakirzyanova et al., 2016). However, the action of low ‘physiological’ concentrations of H$_2$O$_2$ had not been not tested in mammalian synapses so far. Here, we report that concentrations as low as 10–30 $\mu$M H$_2$O$_2$ significantly inhibited synaptic transmission in the adult murine muscle synapses. Moreover, this inhibition was use-dependent, being more pronounced when the motor nerve was repeatedly stimulated by ‘physiological’ patterns of activity (Crago and Makowski, 2012). This approach mimicked the native patterns of synapse activity which is important for inter-
interpreting the significance of the identified effects of these concentrations of H$_2$O$_2$.

Unlike in frog muscle, we did not observe the enhancement of synaptic transmission either by 3 or 10 µM H$_2$O$_2$. One of reasons for this could be linked with the level of PKC which largely determines the probability of synaptic facilitation (Giniatullin and Giniatullin, 2003). On the other hand, we did observe the enhancement of synaptic transmission even by high concentrations of H$_2$O$_2$ in newborn rats (Shakirzyanova et al., 2016) suggesting the role of other developmental factors. The other reason could be the level of endogenous ROS, which in diaphragmatic muscle is in the range of 10 to 100 nM (Jackson, 2011), and this tonic signaling may sensitize the redox-sensitive mechanisms. Finally, the direction and intensity of the depressant effect can be determined by the level of anti-oxidants, in particular, the level of the main antioxidant, glutathione. It should be noted that the use of L-BSO in the current study to reduce the level of the endogenous glutathione, led to a pronounced inhibition of synaptic transmission comparable with the action of H$_2$O$_2$.

**Location of possible targets for H$_2$O$_2$**

We previously showed that in the neuromuscular synapse the motor nerve terminals are most sensitive to exogenous H$_2$O$_2$ (Giniatullin et al., 2003). Moreover, we identified SNAP25, belonging to proteins of the SNARE complex, as the main ROS-sensitive ‘sensor’ within the exocytotic machinery (Giniatullin et al., 2006). At least, it was the most sensitive to oxidative modification under the effect of high concentrations of H$_2$O$_2$. All these data are consistent with view that the disturbances in the assembly of the SNARE complex, playing a key role in the exocytosis of synaptic vesicles in cold-blooded and warm-blooded animals (Söllner et al., 1993; Schiavo et al., 2000) is underlies the depressant action of ROS.

In line with specific signaling by H$_2$O$_2$ at low concentrations in relatively short exposure times, we did not find damaging action of ROS. Thus, the concentrations of H$_2$O$_2$ showing the depressant effect on EPCs did not produce nonspecific oxidation of membrane lipids, indicating the selectivity of synaptic modulation by low physiological concentrations of H$_2$O$_2$. H$_2$O$_2$ has shown the ability to induce lipid peroxidation followed by phospholipase C activation and calcium signaling (Domijan et al., 2014). Phospholipase C activation following increase in intracellular Ca$^{2+}$ should theoretically increasing the frequency of spontaneous release, which is proportional to resting Ca$^{2+}$ levels in nerve terminals. We did not see an increase in spontaneous release. In contrast, 30 µM H$_2$O$_2$ led to decrease in MEPC frequency. It also suggests that H$_2$O$_2$ does not influence resting nerve terminal Ca$^{2+}$ levels and in line with Domijan et al. (2014) does not modify lipid peroxidation.

Furthermore, the results of testing other oxidants were also consistent with intracellular location of redox-sensitive sites. Thus, the experiments with the membrane-penetrating Chloramine-T, (like H$_2$O$_2$ causing oxidation of cysteine residues and methionine of the protein macromolecules) and DTNB, which also leads to oxidation of cysteine residues but does not penetrate through the cell membrane (Schlief et al., 1996; Susankova et al., 2006; Schink et al., 2016), suggesting that the H$_2$O$_2$ targets are located inside the nerve terminals.
More profound EPC amplitude depression during high-frequency activation and data obtained with nerve terminals pre-loaded with the endocytosis marker FM1–43 also showed that H$_2$O$_2$ inhibits exocytosis along with mobilization of synaptic vesicles to the active zone (Reid et al., 1999; Petrov et al., 2008). Thus, in control experiments the mean synaptic vesicle recycling time was equal to about 40–50 s whereas after application of H$_2$O$_2$, this time amounted to 70–80 s suggesting the reduced reuse of synaptic vesicles. These data may indicate that some protein targets define the intensity of synaptic vesicle mobilization and/or their endocytosis is also sensitive to oxidative modifications by the action of exogenous H$_2$O$_2$.

**Physiological implication**

There is increasing evidence that ROS are involved in a number of different physiological processes, acting as fast signaling molecules (Miki and Funato 2012; Petrov et al., 2014; Weidinger and Kozlov, 2015; Odnoshivkina et al., 2015; Giniatullin et al., 2005; Borquez et al., 2016; Sies et al., 2017). H$_2$O$_2$ is one of the major redox metabolites involved in various signaling and regulatory processes (Marinho et al., 2014) along with Ca$^{2+}$ and ATP (Cordeiro and Jacinto (2013); Van der Vliet and Janssen-Heininger, 2014). The relatively high stability of H$_2$O$_2$ supports its signaling role (Miller et al., 2010; Rice, 2011), including the regulation of neurotransmitter release and synaptic plasticity (Avshalumov et al., 2003, 2008; Accardi et al., 2014; Giniatullin et al., 2015; Weidinger and Kozlov, 2015; Calvo and Beltrán González, 2016).

The main breathing muscle – the diaphragm with its unique set of different types of muscle fibers and constant contractile activity, might serve as source of ROS (H$_2$O$_2$), especially in stress conditions (Nethery et al., 2000; Zuo et al., 2012). The total amount of H$_2$O$_2$ in plasma can approach to 1–5 mM (Forman et al., 2016), which is more than 100 times higher than the amount of H$_2$O$_2$ inside the cells (Sies et al., 2017). Notably, the level of H$_2$O$_2$ production in the muscle depends on the contractile activity (Jackson, 2011; Mailloux, 2015) and our data are consistent with this view suggesting higher sensitivity of secretion to H$_2$O$_2$ at high-rate activation patterns (more activity, more depression).

The amount of endogenous ROS in the cell is determined by the activity of antioxidant systems of cells such as superoxide dismutase (SOD), glutathione...
Depletion of intracellular glutathione (GSH) induced ROS generation. (A) Level of peroxides on untreated muscles (Ctrl) and on muscles treated with different concentrations of the L-BSO (90 min exposure) measured by FOX assay. (B) MCB labeling of GSH in vitro. Fluorescence intensity of loaded MCB neuromuscular preparations in Ctrl and after preliminary incubation in the physiological solution containing L-BSO: (C) The action of L-BSO on the level of extracellular H$_2$O$_2$ detected with the Amplex® Red assay; (D) Fluorescent images of nerve terminals dual-labeled with rhodamine-conjugated α-bungarotoxin (α-Btx) and CM–H$_2$DCFDA (CM–DCF). Top, control nerve terminals kept in the physiological solution, bottom – nerve terminals 90 min incubated in 3 mM L-BSO. Scale bar = 15 μm; (E) Average fluorescence of CM–H$_2$DCF from the Ctrl muscles and after exposure to different concentrations of the L-BSO or H$_2$O$_2$. Increased CM–H$_2$DCF fluorescence indicated an increased level of endogenous ROS. Mean ± SEM. *p < 0.05.

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**CONFLICT OF INTEREST**

The authors report no conflict of interest.

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