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Age-dependent action of reactive oxygen species on transmitter release in mammalian neuromuscular junctions

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ABSTRACT

Reactive oxygen species (ROS) are implicated in aging, but the neurobiological mechanisms of ROS action are not fully understood. Using electrophysiological techniques and biochemical assays, we studied the age-dependent effect of hydrogen peroxide (H_2O_2) on acetylcholine release in rat diaphragm neuromuscular junctions. H_2O_2 significantly inhibited both spontaneous (measured as frequency of miniature end-plate potentials) and evoked (amplitude of end-plate potentials) transmitter release in adult rats. The inhibitory effect of H_2O_2 was much stronger in old rats, whereas in newborns tested during the first postnatal week, H_2O_2 did not affect spontaneous release from nerve endings and potentiated end-plate potentials. Proteinkinase C activation or intracellular Ca^{2+} elevation restored redox sensitivity of miniature end-plate potentials in newborns. The resistance of neonates to H_2O_2 inhibition was associated with higher catalase and glutathione peroxidase activities in skeletal muscle. In contrast, the activities of these enzymes were downregulated in old rats. Our data indicate that the vulnerability of transmitter release to oxidative damage strongly correlates with aging and might be used as an early indicator of senescence.

1. Introduction

It has been well documented that in physiological state, reactive oxygen species (ROS) play a key role in physiological cell signaling, but increased level of ROS production (and/or decreased antioxidant and repair systems activity) leading to increased oxidative stress are implicated in the development of different pathologies (Droge, 2002; Rhee, 2006; Sena and Chandel, 2012). The nervous system is highly vulnerable to oxidative stress and redox imbalance, which contributes to neuronal apoptosis and cognitive decline resulting in a number of neurodegenerative disorders including Parkinson's disease (Sanders and Timothy Greenamyre, 2013; Zuo and Motherwell, 2013), Alzheimer's disease (Jomova et al., 2010; Yan et al., 2013), and amyotrophic lateral sclerosis (ALS; D'Amico et al., 2013; Naumenko et al., 2011; Shi et al., 2010). Aging is associated with increased oxidative damage and failure of antioxidant defense, which result in higher incidence of a wide range of the oxidative stress-induced neurodegenerative processes (Balaban et al., 2005; Jackson and McArdle, 2011; Radak et al., 2013; Salminen and Paul, 2014). Nevertheless, there is limited information on the impact of ROS on the function of synapses, key elements of the nervous system. In particular, there are no studies which address the developmental aspect of ROS action on synaptic transmission. We have shown earlier that hydrogen peroxide (H_2O_2) caused a strong inhibition of synaptic transmission in adult mice. This effect was likely mediated by synaptosomal-associated protein 25, one of the presynaptic soluble N-ethylmaleimide-sensitive factor activating protein receptor proteins (Giniatullin et al., 2006). The neuromuscular junction is a classical model to study synaptic processes, performing reliable recordings from young, adult, and old animals. In the present study, we investigated the action of ROS on synaptic transmission at different stages of life

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(from newborns to old animals) at the neuromuscular junction. We show here a strong inhibition of transmitter release by ROS in adult and old animals with almost full resistance to ROS on spontaneous release along with the enhancement of evoked release in neonates.

2. Materials and methods

2.1. Preparation and solutions

Experiments were carried out on rat phrenic nerve—diaphragm and soleus muscle in vitro preparations from newborn (P1–P7; P0 was a day of birth), adult (P35–6 months), and old (24–30 months) rats at room temperature (~20 °C–22 °C) as these muscles show clear age-dependent changes (Brown et al., 1992; Greising et al., 2013; Imagita et al., 2009). All experiments were performed in accordance with the European Community Council Directive for the humane treatment of laboratory animals of September 22, 2010 (2010/63/EEC), and the experimental protocol was approved by the Animal Care and Use Committee of Kazan State Medical University and University "G. d'Annunzio" of Chieti-Pescara. The left diaphragm muscle was isolated together with the phrenic nerve and placed thoracic side up into the experimental chamber (2.5 mL). To prevent muscle contractions and preserve a physiologically high level of transmitter release in experiments with stimulation of the motor nerve, we used transverse cutting of muscle fibers (Glavinovic, 1979). The muscle was slightly stretched, and muscle fibers were carefully cut across their length, about 5 mm on each side of the main nerve branch. Before recording, the cut muscle was rinsed for at least 40 minutes with a basic physiological solution. The cutting procedure does not produce significant changes in cable properties and enables long-lasting stable recording of multiquantal synaptic currents (Glavinovic, 1979; Sokolova et al., 2003). Recordings of spontaneous transmitter release were performed on uncut diaphragm muscles. The basic physiological solution contained (mM): NaCl 120, KCl 5, $CaCl_2$ 2, $MgCl_2$ 1, glucose 11, $NaHPO_4$ 1, $NaHCO_3$ 24, permanently bubbled with a mixture of 95% O_2 and 5% CO_2 (starting usually 1 hour before the experiment), pH 7.3–7.4. K^+-evoked transmitter release was measured after muscles had been incubated for 10–15 minutes with solutions containing high K^+ concentrations (15 mM or 30 mM) where the increase of KCl was accompanied by the proportional reduction of NaCl.

H_2O_2 was diluted in the basic solution from 30% stock (Sigma) to obtain a concentration of 300 μM. Phorbol 12-myristate 13-acetate (PMA) and chelerythrine (both from Sigma) were prepared from 10 mM stock solutions in dimethylsulfoxide (DMSO; Tocris, Ellisville, MO, USA). The working concentration of PMA was 0.5 μM, chelerythrine—5 μM. All drugs were dissolved to the final concentrations in basic solution just before the experiments and were applied to a muscles maintained in the chamber via a superfusion system (rate ~2 mL/min) for 20–40 minutes until their full effect achievement. DMSO concentrations in applied solutions never exceeded 0.1% of the total volume. This amount of DMSO does not affect transmitter release.

2.2. Electrophysiology

Recording of evoked postsynaptic multiquantal end-plate potentials (EPPs) and spontaneous miniature end-plate potentials (MEPPs) was performed using standard glass microelectrodes (resistance 8–15 MΩ when filled with 3 M KCl). For adult and old animals, only muscle fibers with a resting membrane potential more negative than −30 mV and −60 mV were considered for evoked and spontaneous transmitter release measurement, respectively. In the cut muscles, recording started after the stabilization of membrane potential approximately 40 minutes after the cutting procedure. MEPPs at stable baseline conditions were recorded for 10–15 minutes in control and 10–15 minutes after the maximal drug effect was reached (20–40 minutes after application) in the same cells and then averaged to obtain the mean values. EPPs (elicited every 5–10 seconds by a single supramaximal phrenic nerve stimulation using a suction electrode) were collected from several individual synapses (during 2–5 minutes in every synapse) of each diaphragm before and after drug application. In newborn animals, muscle fiber membrane potentials values were lower than in adults (between −35 and −55 mV in intact muscles, and ~−22 mV in cut muscles). As the instability of membrane potential in newborns precluded long-lasting recording of synaptic events from a single fiber, we, therefore, recorded both evoked EPPs and spontaneous MEPPs from 5–12 individual synapses (for 2–5 minutes in every synapse) of each diaphragm, before and after 20–40 minutes of drug application. Only fibers in which the membrane potential dropped by less than 5 mV during the recording time were taken into consideration.

Each synaptic event was visually inspected to prevent noise disturbance of the analysis. Both EPPs and MEPPs were amplified using custom-made low-noise amplifier, digitized at 50 kHz, stored on a PC, and analyzed off-line to calculate mean amplitudes and interevent intervals (for MEPPs) using Origin 9.0 software (OriginLab Corp).

2.3. Assays for hydroperoxides

Concentrations of hydroperoxides were measured by ferrous oxidation in xylenol orange (FOX1; Deiana et al., 1999; Giniatullin et al., 2005; Jiang et al., 1992; Wolff, 1994). 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 (1994) with slight modifications: 50 mM xylenol orange, 500 μm ammonium ferrous sulphate, 200 mM D-sorbitol, and 50 mM sulphuric acid. All reagents were of at least analytic grade. The calibration curve was made using diluted 30% H_2O_2 (Ultra Pure grade). Diaphragm muscle samples were fixed in liquid nitrogen, then homogenized in cold (−20 °C) acetone and centrifuged for 10 minutes at 12,000 g. The supernatant was mixed with an equal volume of the FOX1 reagent. The reaction mixtures were incubated at room temperature for 5 minutes to enable the reaction to reach a stable end point. After the reaction was completed, the tissue extract was centrifuged for 3 minutes at 12,000 g, and the supernatant was assayed spectrophotometrically (absorbance at 560 nm). Solutions containing pure acetone mixed with an equal volume of FOX1 reagent were used as the blanks. The FOX1 reagent was made up 1 day before the analysis and kept overnight at 4 °C in the dark. All procedures were performed under dimmed light. Each measurement was made at least 3 times and then averaged. The peroxide content of samples was determined with reference to a calibration curve obtained with known concentrations of H_2O_2 and expressed as micromoles of peroxide per gram of tissue.

2.4. Antioxidant enzyme activity

The activities of various enzymes, including catalase, Se-dependent glutathione peroxidase 1 (GPX1), superoxide dismutase type 1 (SOD1), glutathione reductase (GSR), and glutathione S-transferase (μ + p) (GST μ + p), were measured in the cytosolic fractions of diaphragm muscle preparations from newborn (P7), adult (4 months), and old (24 months) rats in vitro at room

temperature (~20 °C–22 °C). Muscle samples were washed with phosphate-buffered saline, cleaned of connective tissue and kept in Krebs solution bubbled for 20 minutes with a mixture of O_2 (95%) and CO_2 (5%). After treatment, all samples were homogenized in homogenization buffer 1:10 w/v (Na-phosphate buffer 20 mM pH 7.0, pepstatin 1 Mg/mL) in a Waring Blendor (max speed) 4 × 15 seconds, pause 15 seconds, on ice. Homogenates were centrifuged at 100,000*g*, 1 hour at 4 °C. Protein content was determined according using Bradford assay (Bradford, 1976). Enzyme activities were measured spectrophotometrically by continuous registration at room temperature in accordance to Fulle et al. (2005).

Cytosolic catalase activity was determined by measuring the decrease in absorbance due to H_2O_2 consumption ($\varepsilon = -0.04\ mM^{-1}\ cm^{-1}$) at 240 nm in accordance to Fulle et al. (2005). The final reaction mixture (1 mL) contained 100 mM Na-phosphate buffer, pH 7.0, 12 mM H_2O_2, and 30–80 μg samples.

To measure SOD1 also known as Cu/Zn superoxide dismutase (cytosolic) activity, the assay mixture contained in a final volume of 1 mL was 20 mM Na_2CO_3 buffer pH 10.0, l M cytochrome c, 1 mM xanthine, and 15 mU mL^{-1} xanthine oxidase. As the activity of xanthine oxidase varies, the amount used was the one that produced a rate of cytochrome c reduction resulting in a change in A_{415} of 0.025/min without any added SOD1. One unit of SOD1 was defined as the activity that inhibits the rate of cytochrome c reduction by 50%.

GST μ + p activity was determined by using 1-chloro-2-4-dinitrobenzene as substrate. The assay was performed at 340 nm ($\varepsilon = 9.6\ mM^{-1}\ cm^{-1}$), and a final volume of 1 mL contained 100 mM Na-phosphate buffer pH 6.5, 1 mM 1-chloro-2-4-dinitrobenzene, 1 mM reduced glutathione (GSH), and 30–80 μg sample.

GSR activity was determined by the rate of decrease in absorbance, induced by oxidation of nicotinamide adenine dinucleotide phosphate (NADPH), at 340 nm ($\varepsilon = -6.22\ mM^{-1}\ cm^{-1}$). The assay mixture contained in a final volume of 1 mL was 100 mM Na phosphate buffer pH 7.0, 1 mM glutathione disulphide, 60 μM NADPH, and 50–160 μg sample.

Cytosolic GPX1 activity was measured following the formation of glutathione disulphide by a coupled enzyme system with GSR. Oxidation of NADPH was recorded at 340 nm ($\varepsilon = -6.22\ mM^{-1}\ cm^{-1}$). The Se-dependent as well as the sum of the Se-dependent and Se-independent activity (also having GST activity) were determined by using H_2O_2 and cumene hydroperoxide, respectively, as substrates. A final volume of 1 mL contained 100 mM Na-phosphate buffer pH 7.5, 1 mM ethylenediaminetetraacetic acid (EDTA), (1 mM NaN_3 only for H_2O_2 assay), 2 mM GSH, 1 U GSR, 0.24 mM NADPH, and 30–80 μg sample.

2.5. Statistical analysis

The data are presented as the mean ± standard error of the mean, with statistical significance assessed by the Student *t* test (for normally distributed data) or the Mann-Whitney test (for not normally distributed data). N corresponds to the number of animals. *p*-values <0.05 were considered statistically significant. Statistical analysis of antioxidant activities was performed by one-way analysis of variance with Dunnett's post-test, using GraphPad Prism, version 6.00, for Windows (GraphPad Software, San-Diego CA, USA, www.graphpad.com).

3. Results

3.1. Age-dependent action of H_2O_2 on spontaneous quantal release

In general, we tested how the relatively stable ROS, H_2O_2 can affect spontaneous transmitter release in neonatal, adult, and old synapses. Starting with neonatal animals, we performed a systematic analysis of ROS action for every day of the first postnatal week. Fig. 1 shows a typical example, that in neuromuscular junctions obtained from a newborn rat, H_2O_2 at concentrations as high as 300 μM was unable to cause any significant effect on spontaneous acetylcholine (ACh) release. Particularly, at P3, after application of H_2O_2, MEPPs frequency was not significantly changed (decreased by 9.6 ± 19.5%; n = 6, $p = 0.282$; Fig. 1A). Similar insensitivity was observed for each day of the neonatal period from P1 to P6 (Fig. 2A and B). These results indicated a lack of sensitivity of spontaneous transmitter release to oxidative stress mimicked by H_2O_2 application during the first week of the life (Fig. 2A and B).

Data with neonates were in sharp contrast to data obtained from adult (Fig. 1B) or old (Fig. 1C) animals. Thus, in adult rats, 300 μM H_2O_2 reduced MEPPs frequency by 58.0 ± 6.2% (n = 7, $p < 0.001$; Fig. 2A and

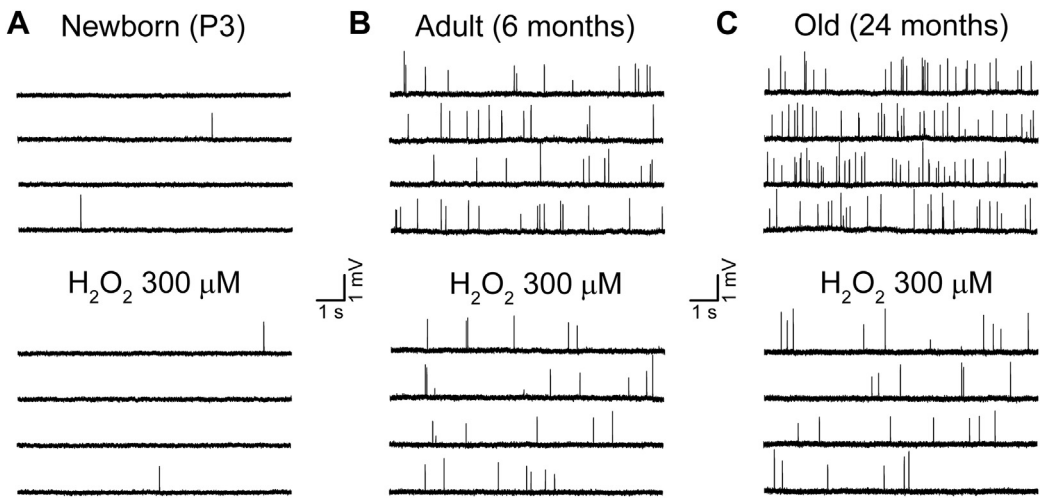

Fig. 1. H_2O_2 action on spontaneous quantal ACh release at neuromuscular junctions of newborn, adult and old rat diaphragms. (A) Native MEPPs in control (upper traces) and after 20 minutes of H_2O_2 (300 μM) application (bottom traces) at the newborn (P3) rat neuromuscular junction; (B) Native MEPPs in control and after 20 minutes of H_2O_2 application at the adult (6 months) rat neuromuscular junction; (C) Native MEPPs in control and after 20 minutes of H_2O_2 application at the old (24 months) rat neuromuscular junction; Represented recordings were obtained from individual rat diaphragm for each age group. Abbreviations: ACh, acetylcholine; H_2O_2, hydrogen peroxide; MEPPs, miniature end-plate potentials.

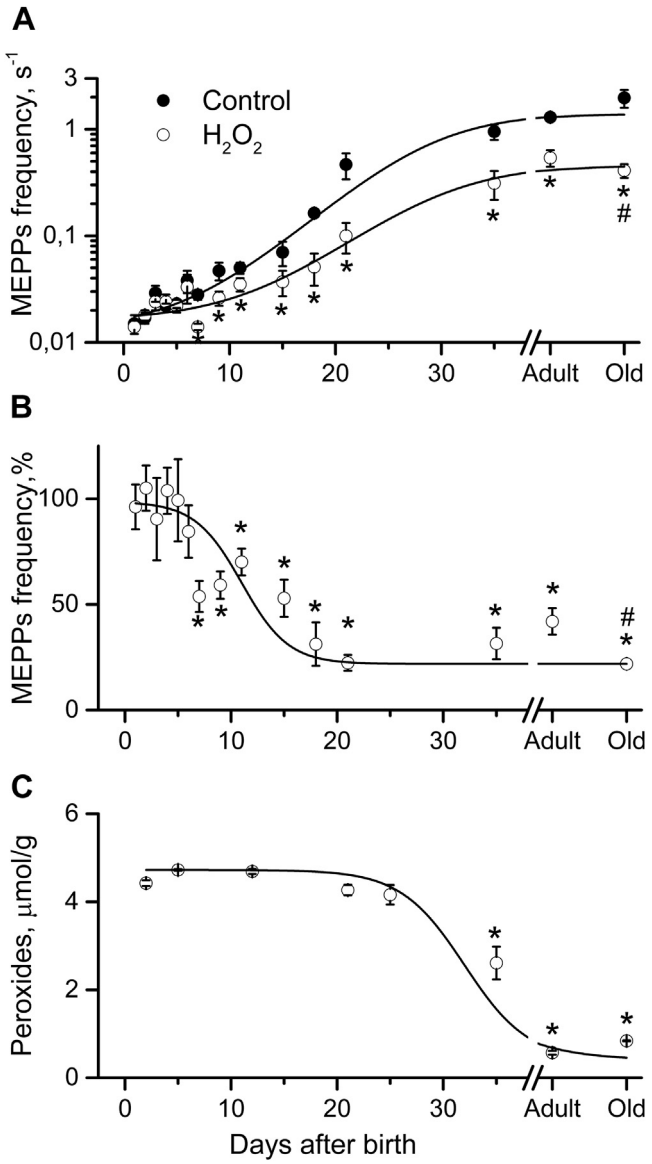

Fig. 2. Development of H_2O_2 inhibitory action on spontaneous transmitter release and age-dependent alterations in hydroperoxides content in rat diaphragm. (A) Changes of MEPPs frequency (seconds^{-1}) in control and under H_2O_2 action at rat neuromuscular junctions during the animal life (●, control, ○, H_2O_2 action; n = 3–8 animals in each case). Significant differences from control values are shown by asterisks. Significant difference between adult and old rats is shown by hash; (B) Development of MEPPs frequency depression by 300 μM H_2O_2 during the postnatal life (MEPPs frequency showed as % from control level before H_2O_2 application; n = 3–8 animals in each age group). Significant differences from control are shown by asterisks. Significant difference between adult and old rats is shown by hash; (C) The level of peroxides in diaphragm muscles measured by FOX1 assay (n = 6 muscle preparations at each age group). Abbreviations: FOX1, ferrous oxidation in xylenol orange; H_2O_2, hydrogen peroxide; MEPPs, miniature end-plate potentials.

B). In old rats, H_2O_2 decreased spontaneous release at neuromuscular junctions even more: by 78.2 ± 2.0% ($p = 0.009$; n = 5; Fig. 2A and B). This effect was significantly ($p = 0.023$) higher than the release inhibition in adults. Consistent with our previous studies (Giniatullin and Giniatullin, 2003; Giniatullin et al., 2006), 300 μM H_2O_2 did not change the amplitude of MEPPs at any age (Fig. 1A–C) indicating its pure presynaptic action. Thus, presynaptic ACh release machinery was greatly affected by H_2O_2 in adult and old rats, but the latter were even more sensitive to H_2O_2.

We explored in more detail this unusual resistance to oxidative stress on spontaneous release in newborn animal ACh release apparatus. Like others (Carlson, 1992; Dennis et al., 1981; Diamond and Miledi, 1962), we found that background spontaneous quantal ACh release at immature neuromuscular junctions was very weak compared to mature synapses. During the first postnatal week including P7, MEPPs frequency was very low and did not change notably, varying in the range of 0.015–0.038 seconds^{-1} (n = 3–7 animals for each age, Fig. 2A). On subsequent days, it began to increase gradually in some synapses, showing individual differences in synaptic transmission development. Then, after 2 weeks of postnatal life, MEPPs frequency rose considerably faster, reaching almost adult level by 35–40 days after birth (MEPPs frequency at P35 was 0.95 ± 0.16 seconds^{-1}, n = 3; Fig. 2A). As mentioned previously, the inhibitory effect of 300 μM H_2O_2 on spontaneous quantal release was absent during the first week of life, appearing only at P7 (Fig. 2A and B). Thus, at P7, H_2O_2 inhibited MEPPs frequency from 0.028 ± 0.003 to 0.014 ± 0.001 second^{-1} (n = 6, $p = 0.006$). In the subsequent days, the depressant action of H_2O_2 was progressively intensified (Fig. 2A and B). Notably, control MEPPs frequency still did not differ significantly between P7 and younger (P3–P6) animals (Fig. 2A). Thus, a low level of spontaneous release could not be the leading reason for the absence of H_2O_2 inhibitory effect during the first week after birth.

To test whether the age-dependent sensitivity to ROS was restricted only to the diaphragm, or was more widely presented, we performed similar experiments on the rat soleus muscle. In neuromuscular junctions of adult rat soleus muscles, 300 μM H_2O_2 significantly inhibited spontaneous release from 1.82 ± 0.11 seconds^{-1} (n = 5) to 0.90 ± 0.10 seconds^{-1} (n = 5; $p < 0.001$). Similar to the neonatal diaphragm, the synapses of the soleus muscles taken from P6 rats were resistant to H_2O_2 (0.026 ± 0.004 seconds^{-1} in control versus 0.025 ± 0.003 seconds^{-1} in H_2O_2, n = 3, $p = 0.508$, not shown).

These results revealed strong age-dependent inhibitory action of H_2O_2 on spontaneous transmitter release, demonstrating its insensitivity to ROS during the first week after birth and also showed that this phenomenon is not restricted to a defined muscle type.

3.2. Developmental changes in ROS levels in diaphragm muscle

Skeletal muscle fibers are considered to be an essential source of endogenous ROS (Powers and Jackson, 2008). In mammalian skeletal muscles, ROS are produced in resting conditions (Reid et al., 1992; Zuo et al., 2014), and their production can be increased several fold during exercise (Jackson, 2011; Murrant and Reid, 2001; Radak et al., 2013; Vasilaki et al., 2006). In our study, using the FOX1 assay (Deiana et al., 1999; Giniatullin et al., 2005; Jiang et al., 1992; Wolff, 1994), we observed that in diaphragm of neonatal rats (P2–P5), the level of endogenous peroxides was higher than in adult or old rat muscles (Fig. 2C). Even at P12 and P21 when synaptic transmission became sensitive to H_2O_2, the level of endogenous peroxides was almost as high as in newborns and significantly higher than in adults (n = 6 muscles; $p = 0.02$). The level of peroxides started to decline after 3 postnatal weeks and became significantly lower than in newborns at P35. The peroxide levels in the diaphragm of old animals did not differ significantly from adults (Fig. 2C).

3.3. Action of H_2O_2 on evoked release

Like spontaneous release, Ca^{2+}-dependent evoked ACh release has distinct properties in early development than in adults (Dennis et al., 1981; Santafe et al., 2001; Sugiura and Ko, 1997). Indeed, in our experiments, EPPs recorded from neonatal diaphragm muscle fibers had smaller amplitudes and slower time course than in adult

neuromuscular junctions (Fig. 3A–C). In P3–P5 rats, 300 μM H_2O_2 enhanced the amplitude of EPPs evoked by motor nerve stimulation (Fig. 3A, C). Thus, the amplitude of EPPs was 6.4 ± 0.35 mV (n = 6) in control, and 9.1 ± 0.5 mV after 20 minutes application of 300 μM H_2O_2 (n = 6, $p = 0.003$; Fig. 3A, C). In sharp contrast, in adult (P35) animals, the amplitude of EPPs was significantly decreased by the same concentration of H_2O_2 from 62.5 ± 3.5 mV (n = 4), to 29.5 ± 2.5 mV (n = 4, $p = 0.004$; Fig. 3B and C).

These results indicated that the releasing machinery underlying Ca^{2+}-dependent evoked transmitter release in newborn animals was stimulated by ROS, but this effect changed to a depressant action of ROS during the maturation of neuromuscular synapses.

3.4. Redox sensitization of newborn rat synapses by persistent depolarization

To test whether the resistance of spontaneous release to ROS, in newborn synapses with not fully established Ca^{2+} coupling to the release machinery can be overcome via the promotion of Ca^{2+} influx, we exposed neuromuscular junctions to solutions containing high depolarizing concentrations of potassium. K^+-evoked depolarization provides enhanced Ca^{2+} influx via voltage-gated Ca^{2+} channels resulting in increased MEPPs frequency (Rosato Siri and Uchitel, 1999; Van der Kloot et al., 1997). As this persistent Ca^{2+} overload could affect the redox balance of the terminal (Feissner et al., 2009), we expected that it might change also the effect of H_2O_2.

First, we found that KCl as high as 15 mM (3 times more than normal) did not affect spontaneous release at P1–P6. Similarly, H_2O_2 (300 μM) applied in the presence of 15 mM KCl to P3–P5 synapses did not change MEPPs frequency (not shown). Application of 15 mM KCl increased MEPPs frequency significantly only at P7 (from 0.028 ± 0.003 seconds^{-1} to 0.048 ± 0.005 seconds^{-1}, n = 3, $p = 0.001$), a time point when H_2O_2 was also effective. The effect of 15 mM KCl on spontaneous release was intensified in subsequent days closely correlating with the development of inhibitory H_2O_2 action. Like the effect of H_2O_2 on spontaneous release, 15 mM KCl action on MEPPs frequency reached almost adult level by P35–P40 (MEPPs frequency was increased to 16.26 ± 1.56 seconds^{-1} at P35 [n = 3] while to 19.14 ± 2.99 seconds^{-1} at adult synapses [n = 3]).

In contrast, 30 mM KCl-containing solution dramatically increased MEPPs frequency already at P2 (from 0.017 ± 0.002 to 0.93 ± 0.18 seconds^{-1}; n = 4; $p < 0.001$; Fig. 4A and B). This pretreatment also promoted the inhibitory action of 300 μM H_2O_2 on spontaneous transmitter release (depression by 42.5 ± 10.3%; n = 4, $p = 0.021$; Fig. 4A and B). These data indicate that high persistent Ca^{2+} influx can sensitize nerve terminals in newborns to reveal the normally invisible inhibitory action of H_2O_2.

3.5. Redox sensitization of newborn synapses with proteinkinase C activation

Presynaptic proteinkinase C (PKC; including Ca^{2+}-dependent isoforms) contributes to control of transmitter release from presynaptic terminals (D'Angelo et al., 1992; Oancea and Meyer, 1998; Santafe et al., 2005, 2006). The activation of PKC could also sensitize transmitter releasing machinery to the depressant action of H_2O_2 (Giniatullin and Giniatullin, 2003; Shibukawa et al., 2003). To test whether synapses of newborn animals could be similarly sensitized by PKC activation, we pretreated P3–P4 diaphragm muscles with the PKC activator PMA (0.5 μM). Indeed, PMA unmasked the depressant action of 300 μM H_2O_2 on spontaneous release in neonates (depression by 37.7 ± 7.2%, n = 8; $p < 0.001$; Fig. 5A and B). Consistent with this, in P3–P4 newborns, pretreatment with PMA also promoted the depressant action of H_2O_2 on multiquantal EPPs (depression by 62.3 ± 3.8%, n = 6; $p < 0.01$, not shown).

At P3–P4, the depressant action of H_2O_2 on spontaneous release in the presence of PMA was abolished by the PKC inhibitor chelerythrine (5 μM, MEPPs frequency decreased by 6.2 ± 12.8%; n = 8; $p = 0.400$; Fig. 5B). Similarly, at P3–P5, chelerythrine abolished the depressant action of H_2O_2 on evoked ACh release (EPPs amplitude

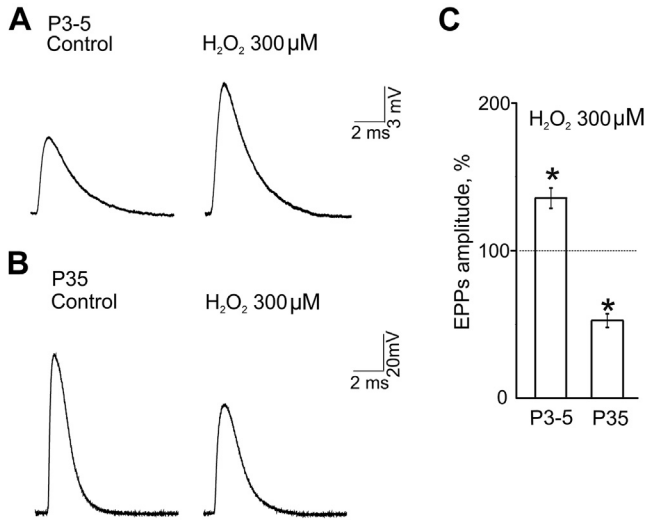

Fig. 3. H_2O_2 action on Ca^{2+}-dependent stimulus-evoked quantal ACh release from motor nerve endings at newborn and adult neuromuscular junctions. Native EPPs of newborn (P3–P5) rats (A) and adult (P35) rats (B) before and after 20 minutes H_2O_2 (300 μM) application (summarized several EPPs). (C) Averaged EPPs amplitude under H_2O_2 action at newborn (P3–P5) and adult (P35) neuromuscular junctions (EPPs amplitude shown as % from control level before H_2O_2 application). Significant difference from control is shown by asterisk. Abbreviations: ACh, acetylcholine; EPPs, end-plate potentials; H_2O_2, hydrogen peroxide.

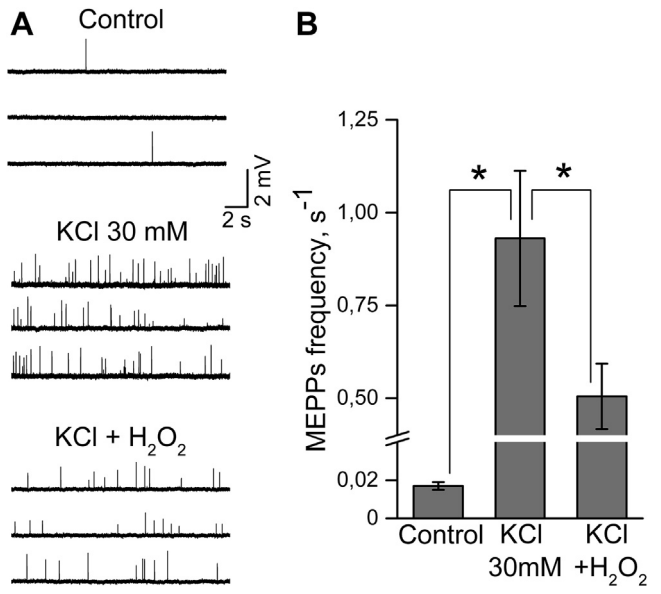

Fig. 4. Action of 30 mM KCl-containing solution on spontaneous ACh release and H_2O_2 effect at neonatal neuromuscular junctions. (A) Native MEPPs at P2 neuromuscular junctions in normal basic solution (upper traces), in the presence of 30 mM KCl in perfusion solution (central traces) and after 300 μM H_2O_2 (300 μM) application (bottom traces); (B) Averaged MEPPs frequency (seconds^{-1}) at P2 rats neuromuscular junctions in control, after 10 minutes perfusion with 30 mM KCl-containing solution and after H_2O_2 addition to this modified solution. Significant differences from control are shown by asterisks. Abbreviations: ACh, acetylcholine; H_2O_2, hydrogen peroxide; MEPPs, miniature end-plate potentials.

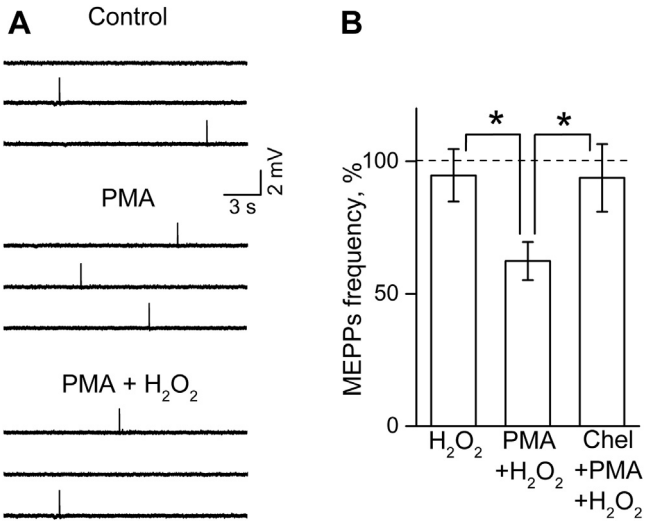

Fig. 5. Role of PKC in H_2O_2 inhibitory action on quantal ACh release at newborn and adult neuromuscular junctions. (A) Native MEPPs at P3 rat diaphragms in normal basic solution (upper traces), in the presence of the PKC activator PMA (0.5 μM, central traces) and after H_2O_2 (300 μM) application (bottom traces) in these conditions; (B) Averaged MEPPs frequency ($seconds^{-1}$) at newborn rats (P3–P5) neuromuscular junctions (% from initial level) under H_2O_2 (300 μM) action in control, and under H_2O_2 action in the presence of PMA and PMA together with chelerythrine (5 μM). Significant difference from control is shown by asterisk. Abbreviations: ACh, acetylcholine; H_2O_2, hydrogen peroxide; MEPPs, miniature end-plate potentials; PKC, proteinkinase C; PMA, phorbol 12-myristate 13-acetate.

declined by 2.5 ± 12.8% change, n = 6; $p = 0.508$, not shown) which was promoted by PMA. All these results indicated that the initial lack of H_2O_2 inhibition in newborns could be reverted by the activation of PKC.

Thus, PKC activation increased the susceptibility of transmitter releasing machinery of newborn neuromuscular junctions to the inhibitory action of H_2O_2.

3.6. Age-dependent changes in antioxidant enzymes

The variable age-dependent action of H_2O_2 found in our study might be caused by an altered protective capacity of antioxidant enzymes, which normally quickly destroy ROS. To address this issue, we examined the enzymatic activities of catalase, GST, SOD1, GPX1, and GSR in muscles from newborn (P7), adult (4 months), and old (24 months) rats. Fig. 6 shows that the level of SOD1 and GSR did not change in all examined ages (Fig. 6A and B). The strongest age-dependent effects were observed with catalase and GPX1. Thus, catalase levels (presented as μmol/min/mg protein) were significantly decreased from 54.80 ± 7.32 in newborns (n = 15), to 21.76 ± 2.44 in adults (n = 4) and to 14.21 ± 1.09 in old animals (n = 3; $p < 0.001$ for all cases; Fig. 6C). Similarly, GPX1 levels were 7.26 ± 1.72 (n = 15), 3.54 ± 0.65 (n = 4), and 2.00 ± 0.40 (n = 3) nmol/min/mg protein in newborns, adults, and old rats, respectively ($p < 0.05$ for all cases; Fig. 6D). In contrast, the activity of GST was significantly increased in old rats (Fig. 6E). This developmentally shifted redox balance of key antioxidant enzymes could also be an important contributor to the vulnerability of nerve terminals to high ROS level.

4. Discussion

This study provides a comprehensive, life-long scale analysis of H_2O_2 action on motor nerve terminals in mammalian muscles. One of our main findings is the increased vulnerability of nerve terminals to the inhibitory effect of ROS, which is proportional to aging. These results are consistent with the generally accepted importance of ROS's role in aging processes. We also show for the first time a paradoxical (but tunable) resistance of spontaneous transmitter secretion to ROS and even enhancement of evoked ACh release by H_2O_2 in newborns. Our data imply that the inhibitory action of ROS may contribute to compromised neuromuscular transmission in the elderly, which could be a reason for muscle weakness, fatigue, and denervation-induced atrophy, especially during extended episodes of activity associated with generation of endogenous ROS.

4.1. Age-dependent changes in vulnerability to ROS

In the present study, we compared the inhibitory action of the relatively mild oxidant H_2O_2 on quantal transmitter release at synapses of newborn, adult, and old rats. Previous observations established that the presynaptic rather than the postsynaptic part of the neuromuscular junction was selectively sensitive to the inhibitory action of ROS (Giniatullin and Giniatullin, 2003; Giniatullin et al., 2006). In this study, we show that H_2O_2 dramatically decreased transmitter release at the neuromuscular junctions of adult and senescent rats. In sharp contrast, H_2O_2 did not change spontaneous release and even enhanced neuromuscular transmission in newborns.

Interestingly, synaptic transmission in diaphragm muscle was functional even in P0 rats (evident from regular breathing involving the diaphragm). In such synapses, the rate of spontaneous release was low, along with small amplitude of evoked EPPs. Consistent with data from others (Dennis et al., 1981; Diamond and Miledi, 1962), we observed a progressive growth of MEPPs frequency and EPPs amplitude during the first 3 weeks of life, achieving the adult's level by the end of first month. Being resistant to exogenous H_2O_2 during the first week, spontaneous release is largely suppressed by H_2O_2 in subsequent days. By the end of the third postnatal week, the inhibitory effect of ROS on spontaneous transmitter release was comparable to the strong depressant action of H_2O_2 in adults. This resistance of spontaneous transmitter release of newborns to ROS was not limited to the diaphragm but was observed also in soleus muscle suggesting that it is a general phenomenon.

It has been shown earlier that active zones density of mice and rats nerve endings significantly decrease with the onset of senescence (Chen et al., 2012). On the other hand, Ca^{2+} deregulation in aging synapses could be associated with increased levels of intraterminal Ca^{2+}, which can accelerate spontaneous transmitter release. As we did not find lowering of MEPPs frequency in old animals, we can suggest that the latter is prevailing in elderly, shaping the final level of spontaneous release.

We used relatively high concentration of H_2O_2, based on our previous experience (Giniatullin and Giniatullin, 2003; Giniatullin et al., 2006) as we considered 300 μM concentration of H_2O_2 for extracellular application as the efficient and safe dose. Although these concentrations could hardly be considered as physiological, it has been shown that in some pathological states in brain tissues the level of endogenous H_2O_2 could be as high as 100 μM (Hyslop et al., 1995). Most importantly, H_2O_2 has a high instability; it is rapidly decomposed by GPX, catalase, and peroxiredoxins and interacts with endogenous thiols. Particularly because of the distribution of GPXs and peroxiredoxins throughout the cell, exogenous H_2O_2 degraded very quickly and within a few molecular diameters (Forman, 2007). In addition, according to some reports, H_2O_2 has a limited ability to diffuse into the cell, altogether suggesting that the intracellular sustained concentrations of ROS at the presynaptic target are expected to be less than those initially applied to extracellular compartment (Mishina et al., 2011; Rice, 2011). On the other

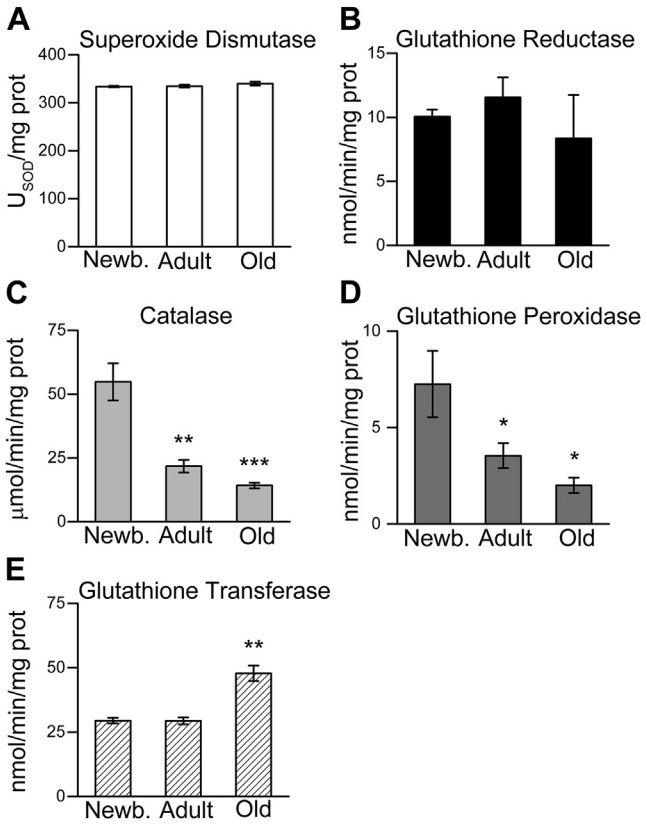

Fig. 6. The antioxidant enzyme activities measured in newborn, adult, and old rat diaphragm muscles. Activities of enzymes, (A) superoxide dismutase 1 (SOD1), (B) glutathione reductase (GSR), (C) catalase, (D) Se-dependent glutathione peroxidase 1 (GPX1), and (E) glutathione S-transferase (GST), were measured in cytosolic fraction of diaphragm muscles derived from newborn (P7), adult (4 months), and old (24 months) rats, following spectrophotometrically determined enzyme kinetics at room (20 °C–22 °C) temperature. Significant difference from control is shown by asterisks. Note the significant differences of catalase and GPX1 activities at newborn muscles compared to adult and old muscles, and at old muscles compared to adult ones.

hand, the impact of exogenous H_2O_2 on redox regulation is mediated through the modification of the redox status of the ROS sensitive molecules and activation of redox-sensitive transcription factors (Forman, 2007; Radak et al., 2013). It is worth noting that even with the acutely applied H_2O_2 (which was the same dose for all age groups of rats), spontaneous transmitter release in newborns was still insensitive to this ROS challenge.

Surprisingly, evoked ACh release was potentiated by 300 µM H_2O_2 in newborn neuromuscular junctions, while being depressed by ROS in adult synapses. Such PKC-dependent enhancement of evoked synaptic currents was observed by us previously in frog muscle with low concentrations of H_2O_2 (Giniatullin and Giniatullin, 2003). One possible reason for the ROS-potentiated release in neonatal rats could be the disrupted calcium clearance from nerve terminals. This ROS effect can be mediated by oxidation of the key thiol groups in proteins such as ryanodine/IP3 receptors or compromised mitochondria responsible for Ca^{2+} clearance in nerve terminals (Barrett et al., 2014; Csordás and Hajnóczky, 2009).

Apart from variable susceptibility to ROS, there are other age-dependent changes in the function and morphology of skeletal muscles. Thus, aging is associated with reduced volume of muscle fibers, loss of motor neurons, disruption of postsynaptic ACh receptor clusters, local denervation, and reinnervation of muscle fibers (Delbono, 2003; Deschenes, 2011). All these changes, along with highly ROS sensitive transmitter releasing machinery, are potential contributors to the vulnerable phenotype of senescent muscle. Thus, in line with the generally accepted crucial role of ROS in aging (Schriner et al., 2005), our data emphasize that vulnerability to ROS-induced dysfunction in old rats is an important indicator of aging.

4.2. Potential mechanisms of resistance to ROS inhibitory action in newborns

One potential mechanism for neonatal resistance of spontaneous transmitter release to ROS could be an immature phenotype underlying Ca^{2+} mechanism controlling transmitter release in newborns. Thus, Breugelmans and Bazzy (1997) hypothesized that the low transmitter release in the newborn diaphragm is the consequence of low levels of Ca^{2+} influx into the nerve terminal as well as differences in the structural arrangement of the vesicular exocytosis complex at early postnatal ages compared to mature muscles (Breugelmans and Bazzy, 1997). Consistent with this assumption, exposure to 15 mM KCl in neonates neither had any significant effect on spontaneous release nor made terminals susceptible to ROS. However, stronger depolarization induced by 30 mM KCl not only facilitated spontaneous release but also largely promoted the inhibitory effect of H_2O_2 even in P2 rats. Thus, the lack of inhibitory action of H_2O_2 on spontaneous release was unmasked in newborns in conditions associated with persistent Ca^{2+} influx. Interestingly, the transient increase in presynaptic Ca^{2+} during motor nerve stimulation and generation of the EPP was associated with potentiation of EPPs by ROS. These data revealed a distinct role of tonic and phasic action of Ca^{2+} for ROS vulnerability in newborns.

Many PKC subtypes are known to be Ca^{2+}-sensitive (Barclay et al., 2005; Hongpaisan et al., 2004; Santafe et al., 2005, 2006). In addition, our previous study showed the involvement of PKC in the inhibitory action of H_2O_2 (Giniatullin and Giniatullin, 2003). Therefore, next we tested if activation of PKC can help, like elevated Ca^{2+}, to identify the inhibitory action of H_2O_2 in newborns. Indeed, when PKC was activated by PMA, H_2O_2 significantly depressed both spontaneous release and evoked EPPs at neonates. Consistent with a conditioning role of PKC, this effect was abolished by the PKC inhibitor chelerythrine. Moreover, activation of PKC with PMA further enhanced the inhibitory action of H_2O_2 at P35 (not shown). Taken together, the data are consistent with the critical role of Ca^{2+} ions and PKC in transmitter release modulation by ROS at newborn neuromuscular junction in early animal life.

Interestingly, resistance of spontaneous transmitter release to the inhibitory action of exogenous ROS at newborn synapses was observed along with the high level of endogenous ROS. Thus, we show here that in the diaphragms of neonatal rats, the level of endogenous peroxides is much higher than later during maturation. The latter is consistent with the previous observation, that in the diaphragm of newborn lambs, the level of endogenous ROS was higher than in later developmental stages (Song and Pillow, 2013). However, this is in contrast to previous reports which emphasize those ROS level increases in adult and aged muscles, contributing to the aging process (Bejma and Ji, 1999; Palomero and Jackson, 2010; Salminen and Paul, 2014). One potential mechanism how enhanced endogenous ROS in newborn muscle coexist with unusual vulnerability to exogenous ROS could be the adaptation of the presynaptic inhibitory redox mechanisms. The molecular mechanism of such adaptation could be “desensitization” of ROS responsive sensors. However, in this case, evoked release should be also insensitive to ROS. In fact, we observed an enhancement of evoked release by

H_2O_2 in newborns suggesting that the releasing machinery is not "desensitized" by endogenous ROS.

Endogenous compounds such as extracellular adenosine, which collaborates with ACh in the control of transmitter release (Santafe et al., 2015) and plays a role in aging (Pousinha et al., 2012), could also contribute to the regulation of ROS sensitivity. Thus, it has been shown earlier, that adenosine A_1 and A_{2A} receptors at the rat diaphragm neuromuscular junction modulate release in opposite manner (Correia-de-Sá et al., 1996). The magnitude of the excitatory effects of endogenously released adenosine on neuromuscular transmission prevails in young age and tends to decrease with aging, disappearing in old rats, where only A_1 receptor-mediated inhibition was evident (Pousinha et al., 2012). On the other hand, we found that ROS potentiate adenosine effects in the processes of ACh quantal release synchronization in frog neuromuscular junctions (Tsentsevitsky et al., 2013). However, unlike the timing of release, the inhibitory action of adenosine on the release probability was redox independent. More studies are needed to explore the role of adenosine in this interesting phenomenon.

4.3. Age-dependent antioxidant mechanisms

Another reason for the absence of the inhibitory effect of H_2O_2 in newborn neuromuscular junctions may be the high activity of antioxidant enzymes (Khan and Black, 2003; Musaro et al., 2010). Song and Pillow (2013) observed an increased level of peroxidases eliminating ROS in newborn lamb diaphragms. Consistent with this, in our study, we show that the activities of the main antioxidant enzymes catalase and GPX1, H_2O_2, and organic peroxides scavengers (only GPX1) were significantly higher in newborn (P7) diaphragms compared to adult or old animals. However, it is clear from our data that even comparatively high activities of antioxidant enzymes do not compensate for the increased level of endogenous peroxides in neonatal muscles.

The lower levels of catalase and GPX1 in adults suggest a less-efficient detoxification of H_2O_2 and could potentially contribute to a higher vulnerability of terminals to H_2O_2. Consistent with this, an even lower level of activity of these enzymes was detected in old animals in accordance with the remarkable depressant effect of ROS at this age. Increased GST was observed in muscles of old animals. The partial protective role of subclasses of GST against lipid peroxidation and its toxic end products is likely insufficient to provide an efficient antioxidant defense.

4.4. Functional implications

It is currently believed that increased oxidative stress and the accumulation of oxidative damage products in the body tissues occur during aging (Buonocore et al., 2012; Marseglia et al., 2014). In contrast to this complex explanation of aging, our study suggests a simple mechanistic model where age-dependent susceptibility of nerve terminals to ROS corresponds to the expected time profile and the phenotype of the aging process. Although not providing a novel theory of aging, these data suggest that neuronal ROS sensitivity measured via transmitter release might be an early biomarker of aging. We showed previously that nerve terminals and the transmitter releasing machinery in particular are the primary targets of the inhibitory action of ROS (Giniatullin and Giniatullin, 2003; Giniatullin et al., 2006). Here, we found that during maturation and aging, the nerve terminals become progressively more sensitive to the inhibitory effect of ROS which could explain the well known muscle weakness in elderly. Similarly, the muscle fatigue based on the presynaptic mechanism might also underlie the premature aging syndrome (Marseglia et al., 2014) and probably contribute to the neurodegenerative diseases associated with motoneuron pathologies such as ALS (Naumenko et al., 2011). In our recent study, we show that modeling ALS with increased level of the endogenous aminoacid homocysteine provided exactly the same effect as aging in the present study by enhancing the sensitivity of nerve terminals to the inhibitory action of ROS (Bukharaeva et al., 2015). The stimulatory action of ROS on evoked release in neonates may indicate an activity-dependent contribution of ROS to the maturation of neuromuscular transmission and consistent with the current concept of redox regulation endogenous ROS play important signaling and regulatory roles, and their effect depends on the cellular and extracellular environment (Patel and Rice, 2012).

4.5. Conclusion

In conclusion, we show, in this study, the progressive inhibitory action of the diffusible and relatively mild oxidant H_2O_2 on transmitter release in a wide time window from early postnatal life to senescence. In newborn animals, spontaneous release from nerve endings is resistant to the inhibitory action of ROS, whereas depression was exceptionally strong in elderly animals. Age-dependent vulnerability to ROS is likely based on variable intracellular signaling including PKC and the profile of certain antioxidant enzymes such as the gradually decreasing activity of catalase and GPX1. Our results offer promise for the development of new strategies against muscle weakness in elderly, especially those based on the presynaptic mechanisms but also against neurodegenerative diseases associated with motoneuron pathologies.

Disclosure statement

The authors have no actual or potential conflicts of interest.

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