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Original article

Cadmium desynchronizes neurotransmitter release in the neuromuscular junction: Key role of ROS

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ABSTRACT

Cd^{2+} is one of the most widespread environmental pollutants and its accumulation in central and peripheral nervous systems leads to neurotoxicity as well as aggravation of common neurodegenerative diseases. Mechanism of the Cd^{2+} toxicity is far from being resolved. Here, using microelectrode recordings of postsynaptic responses and fluorescent redox indicators we studied the effect of Cd^{2+} in the submicromolar range on timing of neurotransmitter release and oxidative status in two functionally different compartments of the same frog motor nerve terminal. Cd^{2+} (0.1–1 μM) acting as typical voltage-gated Ca^{2+} channel (VGCC) antagonist decreased neurotransmitter release in both distal and proximal parts of the nerve terminal, but in contrast to the VGCC blockers Cd^{2+} (0.1–0.5 μM) desynchronized the release selectively in the distal region. The latter action of Cd^{2+} was completely prevented by inhibitor of NADPH-oxidase and antioxidants, including mitochondrial specific, as well as redox-sensitive TRPV1 channel blocker. Cd^{2+} markedly increased levels of mitochondrial reactive oxygen species (ROS) in both the distal and proximal compartments of the nerve terminal, which was associated with lipid peroxidation mainly in the distal region. Zn^{2+}, whose transport systems translocate Cd^{2+}, markedly enhanced the effects of Cd^{2+} on both the mitochondrial ROS levels and timing of neurotransmitter release. Furthermore, in the presence of Zn^{2+} ions, Cd^{2+} also desynchronized the neurotransmitter release in the proximal region. Thus, in synapses Cd^{2+} at very low concentrations can increase mitochondrial ROS, lipid peroxidation and disturb the timing of neurotransmitter release via a ROS/TRPV-dependent mechanism. Desynchronization of neurotransmitter release and synaptic oxidative stress could be early events in Cd^{2+} neurotoxicity.

1. Introduction

Cd^{2+} is widely used as a non-selective blocker of voltage-gated calcium channels (VGCCs) [1–4] as well as a potent neurotoxin in model studies [5–7]. Furthermore, Cd^{2+} is one of the most essential environmental pollutants and it accumulates in the tissues, including central and peripheral nervous systems, and contributes to progression of common neurodegenerative disorders, including Alzheimer's and Parkinson's diseases, amyotrophic lateral sclerosis, and multiple sclerosis [8,9]. However, the fundamental pathways for Cd^{2+} neurotoxicity remain poorly understood. Oxidative stress due to mitochondrial dysfunction is considered as one of the leading factors of Cd^{2+}-induced damage of neurons [6,7,9,10].

Cd^{2+} consumption dose- and treatment time-dependently disturbs bioelectrical and higher order functions of the nervous system as well as motor activity [11]. Cd^{2+} profoundly affects expression of essential synaptic proteins and neurotransmission, including cholinergic transmission in the brain and neuromuscular junctions [1,5,9,12,13]. At high concentrations (10–200 μM), Cd^{2+} can modify presynaptic function by suppressing the refilling of synaptic vesicles with neurotransmitter as well as Ca^{2+} entry through VGCCs, which is responsible for triggering both neurotransmitter release and synaptic vesicle endocytosis [1,12,14,15]. Additionally, in a Ca^{2+}-free saline, Cd^{2+} (0.1–1 mM) can increase spontaneous neurotransmitter release leading

Abbreviations: AP, action potential; Cd^{2+}, cadmium; CPZ, capsazepine; CgTx, omega-conotoxin GVIA; EPPs, end plate potentials; m, mean quantal content; NAC, N-acetyl-L-cysteine; NMJ, neuromuscular junctions; ROS, reactive oxygen species; TRPV channels, transient receptor potential vanilloid channels; VGCCs, voltage-gated calcium channels; Zn^{2+}, zinc

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to synaptic vesicle depletion due to blocked endocytosis [13,16]. In the presence of Ca^{2+} ions, Cd^{2+} can also increase the spontaneous release, but in a lesser extent [4,13]. These early studies suggest that presynaptic compartment enriched with Cd^{2+}-binding proteins could be one of the most vulnerable targets for Cd^{2+} toxicity. Interestingly, like Cd^{2+}, reactive oxygen species (ROS) can increase spontaneous neurotransmitter release [17] and suppress evoked quantum release [18–20]. But the ability of Cd^{2+} to modulate ROS levels in the synapses and the role of ROS in synaptic effects of Cd^{2+} have not been studied yet.

The essential aspect of neurotransmission is the timing of the exocytotic neurotransmitter release. Although most synaptic communication is based on synchronous neurotransmitter release in response to the arriving action potential (AP), neurotransmitter is also released asynchronously with a longer, alterable delay following an AP [21]. Desynchronization of neurotransmitter release can affect network activity, efficiency of neurotransmission, release of modulatory neuropeptides as well as cause prolonged inhibition or activation of postsynaptic cells [21–24]. The observations that synchronous and asynchronous release may be differently regulated suggest that these phases of neurotransmitter release can be controlled by independent pathways [21,22,24–26]. Furthermore, in models of neurodegenerative disorders, namely spinal muscular atrophy and Alzheimer's disease, as well as in epileptiform tissues, a shift from synchronous to asynchronous neurotransmitter release occurs [21,27–29]. Theoretically, Cd^{2+} can interrupt synaptic transmission by affecting the timing of neurotransmitter release in response to AP. However, the ability of Cd^{2+} to desynchronize the release still remains unclear.

Frog neuromuscular junction (NMJ) is a classic object for fundamental researches of synaptic transmission, particularly functioning of exocytotic sites [30–33]. The frog motor nerve terminals as part of a large synapse had a prominent proxy-distal gradient of the neurotransmitter release linked with functional differences of the proximal and distal compartments [18,30,34–38]. This organization of the NMJs enables the study of common principals of presynaptic events simultaneously in two different regions of the nerve terminal, characterized by a higher (proxomal) and lower (distal) probability of neurotransmitter release [18,34,38,39]. Additionally, proximal and distal compartments of the frog NMJs utilize specific mechanisms of neurotransmitter release regulation, including ROS-dependent [18,34,40].

In the present study we found that Cd^{2+} at very low concentrations (0.1–0.5 μM) markedly desynchronized neurotransmitter release in distal part of the nerve terminal, which was accompanied by an increase in mitochondrial ROS production and lipid peroxidation at the frog NMJs. Inhibitor of NADH-oxidase and antioxidants, including a mitochondria-target antioxidant, as well as a blocker of redox-sensitive TRPV channels effectively prevented the action of Cd^{2+} on the timing of the release. In contrast to Cd^{2+}, blockage of VGCCs with Mg^{2+} or omega-conotoxin GVIA (CgTx) synchronized the neurotransmitter release. Zn^{2+} at the micromolar concentration (acting as pro-oxidant [41]) facilitated the Cd^{2+}-induced desynchronization of neurotransmitter release and mitochondrial ROS production. Thus, we suggest that Cd^{2+} at low concentrations can desynchronize neurotransmitter release in a ROS/TRPV channel dependent manner. This new phenomenon can contribute to Cd^{2+} neurotoxicity especially at early stage of Cd^{2+} accumulation.

2. Methods

2.1. Animals

Experiments were conducted in accordance with the NIH Guide for the Care and Use of Laboratory Animals. Frogs (*Rana ridibunda*) of both genders were collected from lakes during early autumn and kept in the dark at 4 °C in a humidity- and temperature-stable environment in a pool of dechlorinated flowing water. Experiments were carried out in the autumn-winter period. Frogs were euthanized via decapitation and destruction of the brain and spinal cord, then the cutaneous pectoris muscles were quickly excised. All efforts were made to minimize the animal suffering. The experimental protocol met the requirements of the EU Directive 2010/63/EU for animal experiments and was approved by the Ethical Committee of Kazan Medical University.

2.2. Solution and chemicals

Isolated muscle with its nerve was placed in a Sylgard-lined thermostated chamber (volume 5 ml) and superfused (5 ml/min) with physiological saline: 113.0 mM NaCl, 2.5 mM KCl, 4.0 mM $MgCl_2$, 0.3 mM $CaCl_2$, 5 mM HEPES and 1.5 mM $NaHCO_3$. The pH was adjusted to 7.3 with NaOH/HCl and the temperature was kept at 20 ± 0.3°C. $CdCl_2$ at concentration of 0.1, 0.5 and 1 μM was added to the perfusion for 20–25 min. The following chemicals were used: 200 μM apocynin (inhibitor of NADPH oxidase); 2 μM capsazepine/CPZ (TRPV1 channel inhibitor); 800 U/ml catalase from bovine liver (antioxidant enzyme); 5 μM mitoTEMPO (mitochondrial antioxidant); 1 mM N-acetyl-L-cysteine/NAC (antioxidant); 10 nM omega-conotoxin GVIA/CgTx (selective antagonist of N-type VGCCs); 25 μM $ZnSO_4$. All reagents were from Sigma. Apocynin, CPZ, NAC, CgTx, Zn^{2+} were added to the bathing solution 25–30 min before treatment with Cd^{2+} and remained in the perfusion throughout the experiment. Catalase was co-applied with Cd^{2+} in the bathing solution to hydrolyze ROS (specifically, hydrogen peroxide) produced in response to Cd^{2+} application. The muscles were pre-incubated for 2h in mitoTEMPO to allow the mitochondria accumulate this antioxidant.

2.3. Electrophysiological recording

Extracellular microelectrode recording of postsynaptic responses, namely end plate potentials (EPPs) was performed as described in details previously [18]. Briefly, EPPs were evoked by supra-threshold stimulation (0.1-ms duration, 0.5 Hz) of the motor nerve through a suction electrode. Two Ringer-filled extracellular glass micropipettes (2–3 MΩ resistance) were positioned in the proximal (~3–5 μm from the end of the myelinated segment; Electrode #1) and distal region (~90–120 μm; electrode #2) of the nerve terminal. In the proximal region the AP had a typical shape [42], different from the AP in the distal region which serves as an additional criterion for electrode placement. The recorded signals were filtered (a band-pass 0.03–10 kHz) and digitized at 50 kHz. Precise evaluating the time course of the neurotransmitter release from the analysis of the real synaptic delays requires measuring the uni-quantal EPPs [43]. Accordingly, all experiments were performed in the presence of 0.3 mM Ca^{2+} and 4.0 mM Mg^{2+}; and the mean quantal content (m) was calculated from the equation ("method of failures"): $m = \ln N/N_0$, where N is the total number of stimuli and N_0 is the number of synaptic failures [18,43–45]. To obtain reliable EPP measurements, in each experiment up to 2500 stimuli were applied before and after treatment.

Analysis of the real synaptic delays was performed as previously described [18,43]. Briefly, the delays of uni-quantal EPPs were calculated as the time between the peak of the presynaptic Na^+ spike and the time point corresponding to the 20% rise phase of the EPP [18,43]. The stability of electrode position was controlled in each experiment (see in Refs. [18] for details). As characteristic value for dispersion of the synaptic delay P_{90} was calculated (see in Ref. [18,46] for details). Increase or decrease in P_{90} reflects a desynchronization or synchronization of neurotransmitter release, respectively. Delay histograms of the uni-quantal EPPs were plotted with the 0.05-ms bin width [46]. Then, P_{90} parameters for the timing of release were obtained from cumulative curves. The interval between the minimal delay and the time at which 90% of all measured uni-quantal EPPs had occurred were designated as the P_{90} (Fig. 1).

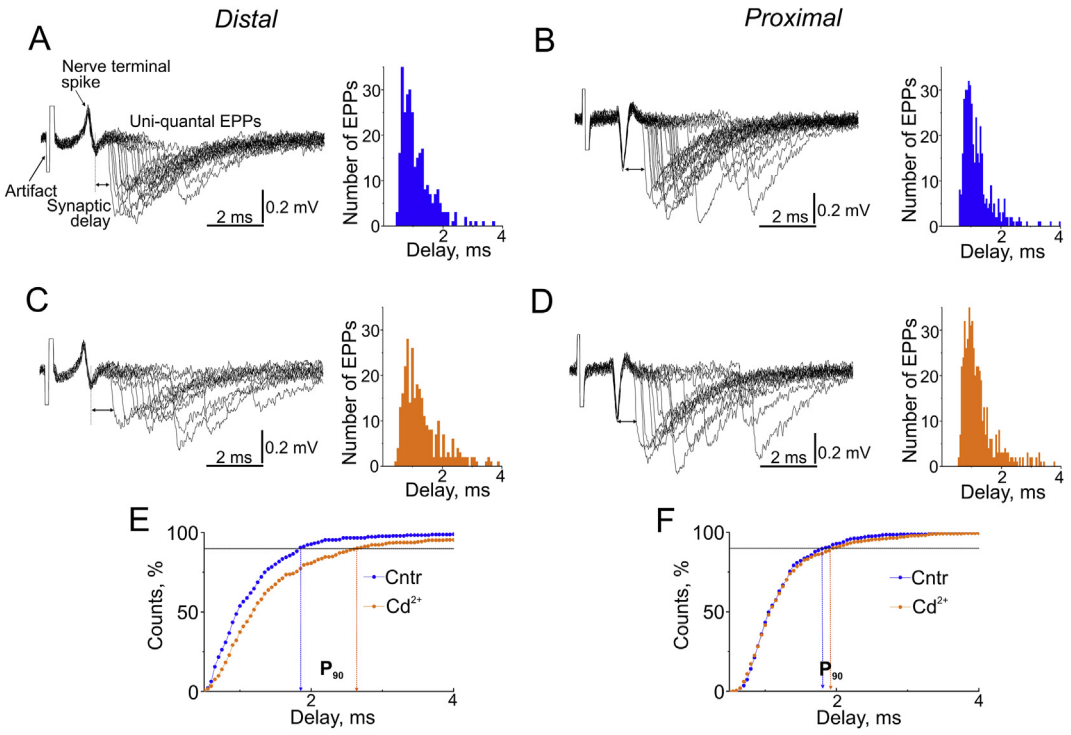

Fig. 1. Estimation of neurotransmitter release timing: P_{90} for quantification of synchrony of the release. Superimposed uni-quantal EPPs recorded in response to 70 stimuli in distal (A, C) and proximal parts (B, D) of the same nerve terminal in control (A, B) and in the presence of 0.5 μM Cd^{2+} (C, D). On A, stimulation artifact, nerve terminal spike and synaptic delay are indicated. Quantal content of the low-quanta EPPs is determined by the method of failures, which is based on calculation of the logarithmic ratio between the number of total stimuli and number of failures (see methods for details). Depressant action of Cd^{2+} on quantum contents displayed as decreased number of EPPs in response to the same number of stimuli (compare the number of EPP traces in A and B *vs* C and D, respectively). In experiments presented in A-D, the quantum content was 0.27/0.34 (A/B) and 0.14/0.18 (C/D) in distal/proximal parts in control and in the presence of Cd^{2+}, respectively. A-D, Right, histogram illustrating distribution of synaptic delay (in ms) in individual experiment; the bin width is 0.05 ms. E, F- Cumulative plot of synaptic delays of uni-quantal EPPs (from A-D) in distal (E) and proximal (F) region of the NMJs. Dashed lines indicate the 90% percentile (P_{90}). P_{90} was estimated in milliseconds (ms). The increase in P_{90} (E) in response to Cd^{2+} application indicates on desynchronization of neurotransmitter release. This corresponds to Cd^{2+}-induced increase in asynchronous release in distal part of NMJs (compare A *vs* C).

2.4. Fluorescent microscopy

Samples were visualized using a BX51WI microscope (Olympus) equipped with spinning disk confocal unit (Olympus), DIC-optics and UPLANSapo 60xw/LumPlanPF 100×w objectives. Images were recorded by CCD-camera DP71 (Olympus) under control of cellSens software (Olympus) and analyzed of-line with ImagePro (Media Cybernetics). MitoSox was used as probe for mitochondrial ROS levels. The indicator is specifically accumulated into the mitochondria and emits a red fluorescence in response to oxidation by ROS, specifically superoxide. The muscles were exposed to 2 μM MitoSox for 10 min and then superfused with a dye-free physiological solution for 30 min. Fluorescence was recorded using a 510/10 nm excitation filter and a 590/20 nm emission filter [47]. To estimate the lipid peroxidation in response to Cd^{2+} application an Image-iT lipid peroxidation kit (Molecular Probes) was used. Upon lipid peroxidation in living cells, fluorescence of BODIPY 581/591C11 reagent (iT-sensor, a main component of the kit) shifts from red (~590 nm) to green (~510 nm), providing a ratiometric indication of lipid peroxidation. Muscles were incubated with the iT-sensor (8 μM) for 30 min, rinsed with a dye-free physiological saline for 30 min and imaged using filters for fluorescein isothiocyanate and Texas Red [17,20]. The ratio of fluorescence intensities at 590 and 510 nm was used to quantify lipid peroxidation. Analysis of the fluorescence was performed in region of interests (25 μm length) in distal and proximal parts of the nerve terminals. Intensity of fluorescence was calculated in arbitrary units, which were then converted into percentage (for MitoSox signal) or ratio between red and green fluorescence (iT-sensor).

2.5. Statistics

Statistical analysis was performed using Origin Pro software. Data represent mean ± SD; where n is the number of independent experiments on individual animals. Statistical significance of the differences between group means was assessed by the Kolmogorov–Smirnov test (P_{90}) or paired (for values before and after drug application) and unpaired (for comparison of two groups) Student's *t*-test assuming a 2-tailed distribution (m, MitoSox and iT-sensor fluorescence). Values of $P < 0.05$ were considered as significant.

3. Results

3.1. Effect of Cd^{2+} on neurotransmitter release: quantum content and timing

Cd^{2+} as a blocker of VGCCs decreased the quantum content of EPP, i.e. evoked neurotransmitter release. Increasing concentrations of Cd^{2+} (from 0.1 to 1 μM) had a more profound depressant effect on the quantum content (Fig. 2A). Similarly, a non-specific Ca^{2+} channel antagonist Mg^{2+} (5 mM) and a selective inhibitor of N-type CgTx (10 nM) suppressed the neurotransmitters release (Fig. 2B) to the similar degree as 0.5 μM Cd^{2+}. VGCC blockers can synchronize synaptic vesicle exocytosis [48], probably since under these conditions only synaptic vesicles located maximally close to the VGCCs fused with the presynaptic membrane. Indeed, Mg^{2+} and CgTx significantly decreased the P_{90} parameter (an indicator of synaptic delay dispersion) suggesting a more synchronous neurotransmitter release (Fig. 2C). Interestingly, in distal part of the nerve terminal Cd^{2+} at low concentrations (0.1 and 0.5 μM)

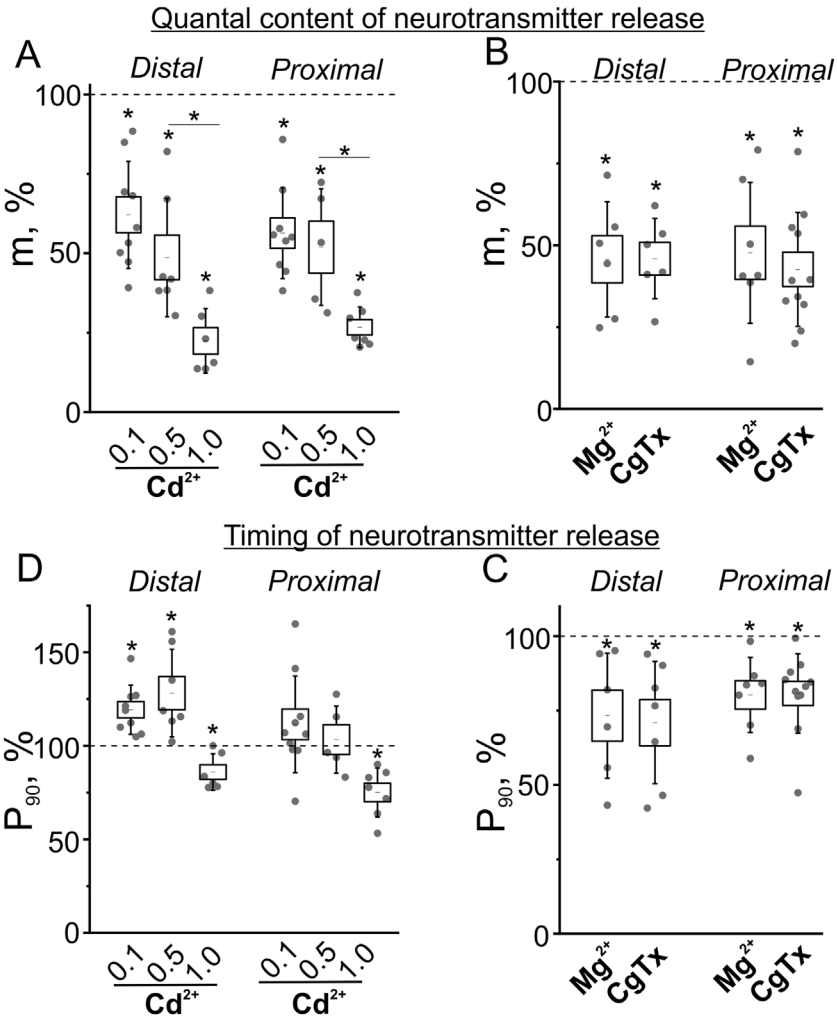

Fig. 2. Influence of Cd^{2+} and VGCC blockers on quantal content (m) and timing (P_{90}) of neurotransmitter release. A, B – the effects of the chemicals on quantal content in distal and proximal parts of the NMJs. D, C – the effects on P_{90} (an indicator of synaptic delay dispersion and synchrony of neurotransmitter release). Data are represented as gray circles – values in individual animals, box range – SEM, whiskers – SD. Y-axis shows percentage changes, relative to the value before onset of Cd^{2+} (A,D) and Mg^{2+} or CgTx (B,C) application. The dashed line denotes the value prior (100%) to the drug application. *P < 0.05 compared to value prior to drug administration.

desynchronized the exocytotic release (Fig. 1) and only at higher concentration (1 μM) Cd^{2+} slightly increased synchronous neurotransmitter release in both distal and proximal regions (Fig. 2D). In proximal part of the nerve terminals Cd^{2+} at low concentrations (0.1 and 0.5 μM) had no influence on P_{90} (Fig. 2D). Thus, Cd^{2+} suppresses neurotransmitter release and can bi-directionally affect the synchrony of the quantum release in the NMJs. In contrast to other Ca^{2+} channel blockers, Cd^{2+} (0.1–0.5 μM) promotes asynchronous acetylcholine exocytosis in response to AP. This action of 0.1–0.5 Cd^{2+} is clearly manifested in distal parts of NMJs. In the further experiments we mainly used 0.5 μM Cd^{2+}. Note that in the presence of CgTx, the effect of 0.5 μM Cd^{2+} on the quantum content was fully expressed in both distal and proximal part of the NMJs, suggesting that different types of VGCCs can be involved in the depressant action of Cd^{2+} (S. Fig. 1).

The absolute value of the quantum content in the control was 0.23 ± 0.096 (n = 9) and 0.30 ± 0.12 (n = 10) in distal and proximal parts of the nerve terminal, respectively. In control, P_{90} was 1.35 ± 0.46 ms (n = 9) and 1.16 ± 0.67 ms (n = 10) in distal and proximal segments of the nerve terminal, respectively. These data are in consistent with previous studies [18,34,38,39] indicating less quantum content of neurotransmitter release in distal vs proximal parts of the frog nerve terminal.

3.2. Dependence of Cd^{2+} effects on ROS

Cd^{2+} can provoke oxidative stress in many cell types leading to cell death [6,7,9,10,49,50]. We tested the ability of various antioxidants and NADPH oxidase inhibitor (apocynin) to modulate the effect of Cd^{2+}. All these compounds, namely ROS chelator NAC, H_2O_2 decomposing enzyme catalase, mitochondrial ROS chelator mitoTEMPO and apocynin, completely prevented the desynchronization of neurotransmitter release induced by Cd^{2+} in distal compartment of the nerve terminal (Fig. 3B). Antioxidant NAC and inhibitor of NADPH oxidase apocynin itself had no influence on P_{90} in distal and proximal parts of nerve terminals (Fig. 3A). Interestingly, that antioxidants and apocynin cannot suppress the negative action of Cd^{2+} on evoked quantum release (Fig. 3D). NAC alone did not modify the quantum content in distal region, but slightly decreased it in proximal part of the NMJs (Fig. 3C). Apocynin itself decreased the evoked neurotransmitter release in both parts of the NMJs and enhanced the depressant effect of Cd^{2+} in distal region (Fig. 3C and D). Similarly, in the presence of mitoTEMPO, Cd^{2+} profoundly suppressed the neurotransmitter release in distal part (Fig. 3D). Catalase had no statistical significant influence on effect of Cd^{2+} on quantum release (Fig. 3D). Note that NAC did not modulate effect of 0.1 μM Cd^{2+} on the quantum content, while completely prevented Cd^{2+} -induced desynchronization of exocytotic events in distal compartment of the NMJs (S. Fig. 2).

Accordingly, the desynchronizing quantum release action of Cd^{2+} can be linked with an elevation of ROS production. In the case of quantum content regulation, the role of ROS is complex, and assumably ROS can partially attenuate Cd^{2+}-induced decrease in neurotransmitter release in distal compartment, since NADPH-oxidase inhibitor as well as mitochondrial antioxidant enhanced the Cd^{2+} effect on the quantum content in this part. Furthermore, the observation that mitoTEMPO (but

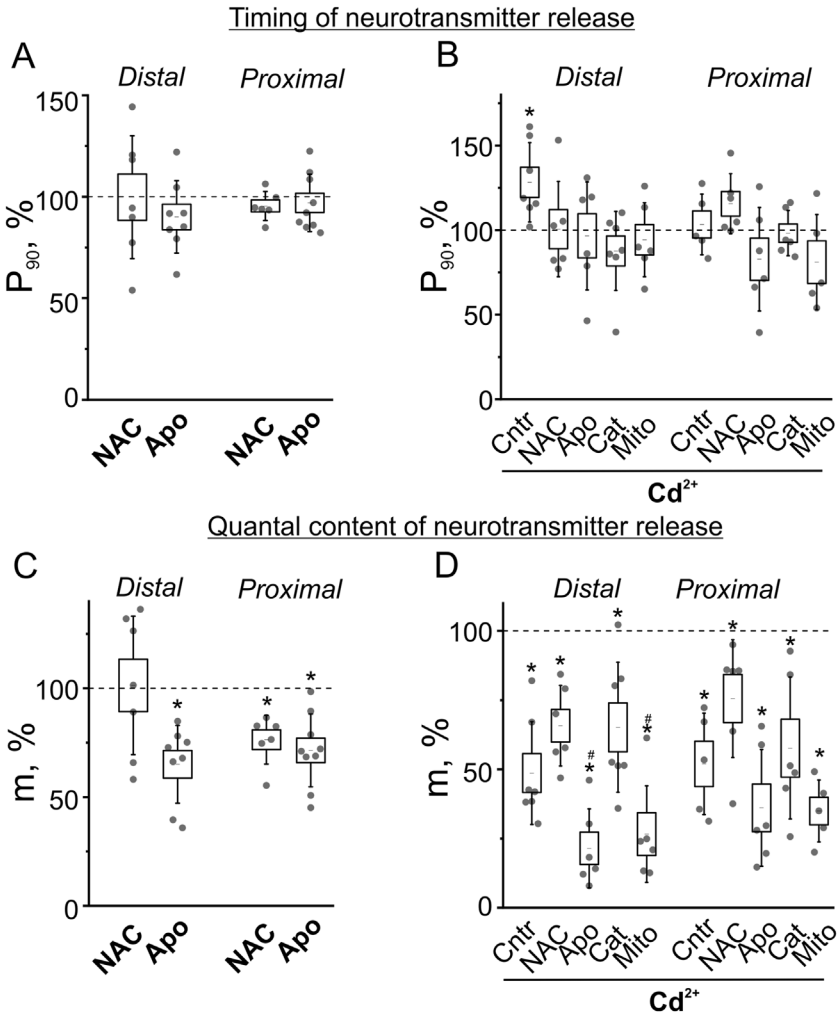

Fig. 3. Role of ROS in the effects of Cd^{2+} on timing (P_{90}) and quantal content (m) of neurotransmitter release. A, B – the effects of antioxidants itself (A) and Cd^{2+} on background of pretreatment with the antioxidants (B) on P_{90} (an indicator of synchrony of neurotransmitter release) in distal and proximal parts of the NMJs. C, D – the effects on quantal content of neurotransmitter release. Results on B and D point on the action of Cd^{2+} when the preparations were pre-exposed with the antioxidants and the measured parameters (P_{90} or m) were on steady-state levels prior to Cd^{2+} administration. Cntr – the effect 0.5 μM Cd^{2+} itself (from Fig. 2). Data are represented as gray circles – values in individual animals, box range – SEM, whiskers – SD. Y-axis shows percentage changes, relative to the value before onset of antioxidant (A, C) or Cd^{2+} (B, D) application. The dashed line denotes the value prior (100%) to the antioxidant (A, C) or Cd^{2+} (B, D) application. *P < 0.05, compared to 100%; #P < 0.05, compared to the effect of Cd^{2+} in control (Cntr).

not catalase) markedly enhanced the negative action of Cd^{2+} on quantum content in distal part of the NMJs suggests that this action of Cd^{2+} could be mainly dependent on superoxide rather than on H_2O_2.

3.3. Cd^{2+}-induced ROS overproduction and lipid peroxidation

One of the main sources of ROS in neurons are mitochondria. Indeed, Cd^{2+} markedly increased MitoSox fluorescence (an indicator of mitochondrial ROS production) in proximal (more markedly) and distal parts of the nerve terminals (Fig. 4A). There is no visible difference in amount of MitoSox-positive spots (probably, mitochondria) between proximal and distal parts (S. Fig. 3A). Note that MitoSox fluorescence was co-localized with MitoTracker signal in NMJ (S. Fig. 3B). Furthermore, application of Cd^{2+} led to an increase in the ratio of oxidized 510 nm (green)/reduced 590 nm (red) fluorescence of iT-sensor, indicating on membrane lipid peroxidation (Fig. 4B). This increase was significantly more profound in the distal parts of NMJs suggesting a high vulnerability of the distal compartment to oxidative damage. Thus, Cd^{2+} can cause an increased ROS production which is more marked in proximal *vs* distal part; but Cd^{2+}-induced lipid peroxidation is greater in distal *vs* proximal compartment. The latter suggests a less antioxidant capacity of the distal part of NMJs. This is consistent with the observation that NADPH-oxidase inhibitor apocynin and antioxidant mitoTEMPO modulated the effect of Cd^{2+} on the quantum content statistically significant in distal (but not proximal) part (Fig. 3D).

3.4. Effect of TRPV channel blocker capsazepine (CPZ)

Previously, we found a relatively high immunoexpression of TRPV1 channels at the frog NMJs and their sensitivity to ROS [17]. Theoretically, these channels can contribute to regulation of synaptic vesicle exocytosis and timing of neurotransmitter release [17,47,51–53]. Inhibition of TRPV1 with CPZ itself slightly decreased the quantum release in both proximal and distal compartments (Fig. 5A). CPZ increased the depressant effect of Cd^{2+} on neurotransmitter release in the distal part (Fig. 5A). Note that the same influence on the Cd^{2+}-mediated change in neurotransmitter release had apocynin and mitoTEMPO. More importantly, CPZ itself had no influence on the P_{90} (i.e. synchronicity of quantum release), but completely prevented Cd^{2+}-induced desynchronization of neurotransmitter release (Fig. 5B). Accordingly, similarly to antioxidants and NADPH-oxidase inhibitor, CPZ can rescue distal parts of NMJs from Cd^{2+}-induced disturbance of neurotransmitter release timing.

3.5. Interplay between Cd^{2+} and Zn^{2+} in effects on quantum content and timing

Evidently, the depressant effect of Cd^{2+} on neurotransmitter release is mainly linked with its ability to block the voltage gated Ca^{2+} channels [1,2]. However, how Cd^{2+} can affect ROS production and timing of neurotransmitter release remain unknown. One possibility is that Cd^{2+} can penetrate into the neurons *via* transport system(s) which is (are) also responsible for Zn^{2+} translocation, because of similar size and charge of the cations [9]. Note that Zn^{2+} is important for synaptic

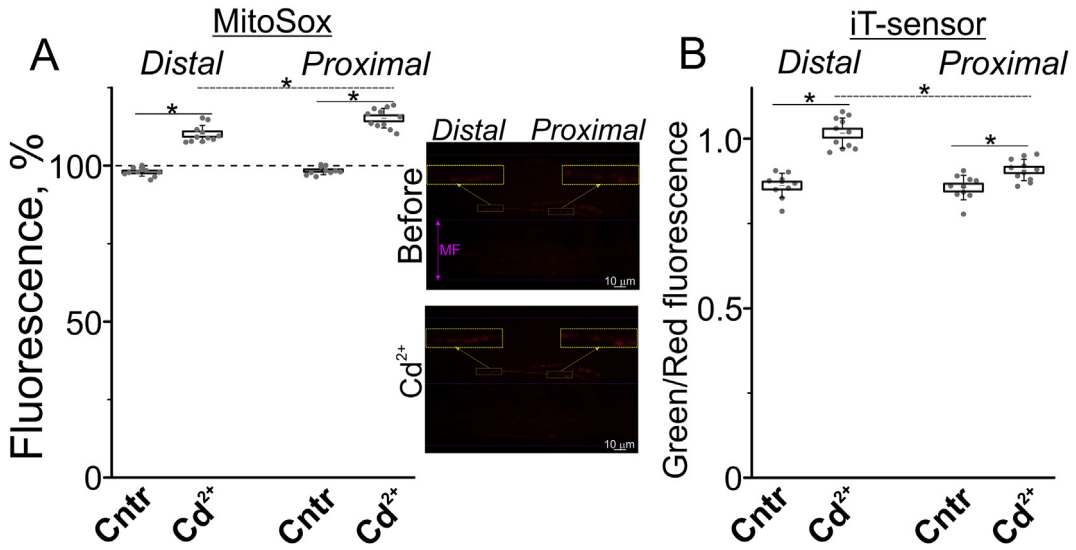

Fig. 4. Effect of Cd^{2+} on mitochondrial ROS production and lipid peroxidation. Changes in MitoSox fluorescence (marker of mitochondrial ROS levels) or ratio of green/reed fluorescence of iT-sensor (probe for lipid peroxidation) in distal and proximal parts of NMJs. The muscles were exposed to physiological saline (control; Cntr) or that containing 0.5 μM Cd^{2+}. Data are represented as gray circles – values in individual animals, box range – SEM, whiskers – SD. A, right – representative fluorescence images before and after administration of Cd^{2+}. Yellow-colored rectangles denote proximal and distal regions that are shown at three times higher magnification; magenta dashed lines indicate borders of muscle fiber (MF). Scale bar − 10 μm. A, Y-axis shows percentage changes in red fluorescence, relative to the value before onset (denoted with dashed line) of Cd^{2+} application. B, Y-axis – ratio of fluorescence in green and red channels. *P < 0.05, differences between Cntr and Cd^{2+} groups or action of Cd^{2+} in distal *vs* proximal part.

plasticity *via* modulation of neurotransmitter release as well as mitochondria functioning [5,41,54–56]. We discovered that 25 μM Zn^{2+} profoundly decreased the quantum release in both the distal and proximal parts of the NMJs (Fig. 6A). Furthermore, Zn^{2+} strongly desynchronized the neurotransmitter release in distal and proximal compartments (Fig. 6B). Thus, Cd^{2+} and Zn^{2+} seem to share some similar properties, namely the ability to depress and desynchronize the neurotransmitter release. Surprisingly, in the presence of Zn^{2+}, Cd^{2+} completely preserved the depressant effect on neurotransmitter release (Fig. 6A), but its desynchronizing action was greatly potentiated and expressed both in proximal and distal compartments (Fig. 6B). Similarly, Zn^{2+} markedly potentiated Cd^{2+}-induced in ROS production in both distal and proximal parts of the NMJs (Fig. 6C). Thus, the effect of Cd^{2+} on the quantum release is Zn^{2+}-independent, while desynchronization of transmitter release and increase in ROS levels in response to Cd^{2+} can be augmented by Zn^{2+}. This suggests that ROS production is a key factor for the regulation of neurotransmitter release timing.

4. Discussion

The fact that Cd^{2+} decreases quantum neurotransmitter release acting as a non-selective VGCC blocker is well established [1–3]. Here, for the first time we found that at extremely low concentrations (0.1–0.5 μM), Cd^{2+} can desynchronize neurotransmitter release, and this effect is opposite to the action of other VGCC antagonists or Cd^{2+} itself at a higher concentration (1 μM). Indeed, N-type VGCC blocker CgTx as well as non-specific antagonist Mg^{2+} synchronized neurotransmitter release, despite reducing the quantum content of the release. Suggesting that when VGCCs are blocked, only the closest to VGCCs primed synaptic vesicles undergo exocytosis in a fast (synchronous) manner [21,24,57,58].

Recent studies suggest that synaptic vesicles, responsible for synchronous and asynchronous neurotransmitter release in response to arrival AP, belong to different functional vesicle pools (in review [21,24]). However, there is also a view that synchronous and

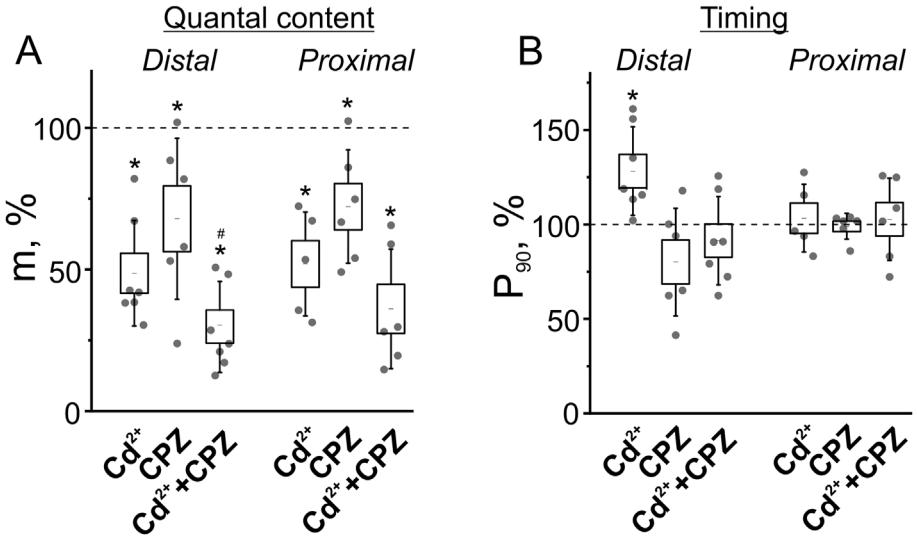

Fig. 5. TRPV1 channels in the effects of Cd^{2+} on quantal content (m) and timing (P_{90}) of neurotransmitter release. A, B – the effect 0.5 μM Cd^{2+} itself (from Fig. 2), blocker of TRPV1 channels (CPZ) itself and Cd^{2+} on background of pretreatment with CPZ (Cd^{2+} + CPZ) on quantal content (A) and synchrony (B) of neurotransmitter release in distal and proximal parts of the NMJ. "Cd^{2+} + CPZ" group indicates on the action of Cd^{2+} when the muscles were pre-exposed with the CPZ and the estimated parameters (P_{90} or m) were on steady-state levels prior to Cd^{2+} application. Data are represented as gray circles – values in individual animals, box range – SEM, whiskers – SD. Y-axis shows percentage changes, relative to the value before onset of Cd^{2+} ("Cd^{2+}" and "Cd^{2+} + CPZ" groups) or CPZ ("CPZ" group) application. The dashed line denotes the value prior (100%) to the drug application. *P < 0.05, compared to 100%; #P < 0.05, compared to the effect of Cd^{2+} itself.

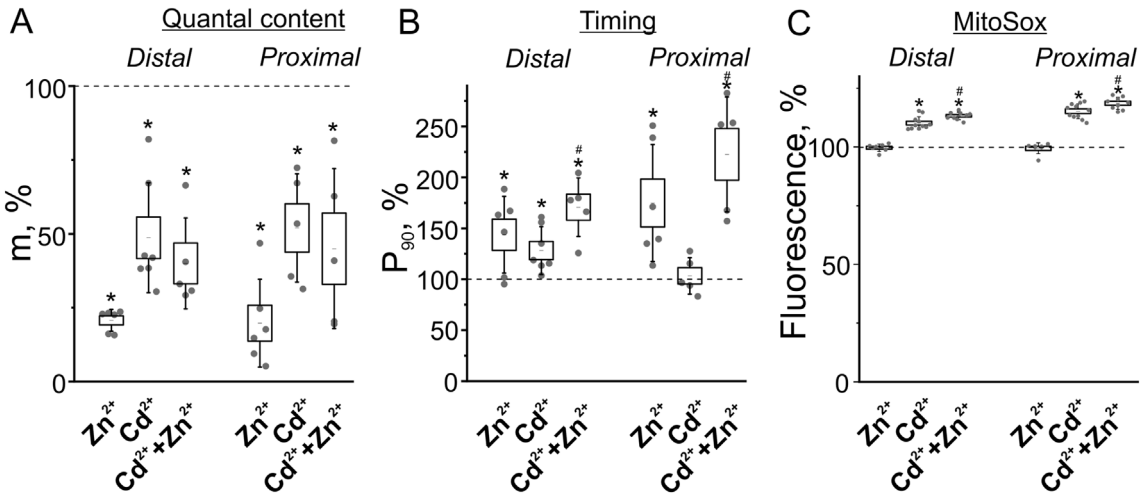

Fig. 6. Zn^{2+} modulates the effects of Cd^{2+} in the NMJ. A, B- quantal content (m) and timing (P_{90}) of neurotransmitter release in distal and proximal parts of NMJs. C - MitoSox fluorescence (indicator of mitochondrial ROS levels). A-C, shown effects of Zn^{2+} and Cd^{2+} itself (from Fig. 2) as well as Cd^{2+} in muscles pretreated with Zn^{2+}. In the latter case, the measured parameters (m, P_{90}, MitoSox fluorescence) were on steady-state levels before Cd^{2+} addition. Data are represented as gray circles – values in individual animals, box range – SEM, whiskers – SD. Y-axis shows percentage changes, relative to the value before onset of Zn^{2+} ("Zn^{2+}" group) or Cd^{2+} ("Cd^{2+}" and "$Cd^{2+}+Zn^{2+}$" groups) application. The dashed line denotes the value of 100%. *P < 0.05 - compared to 100%; #P < 0.05 - compared to "Cd^{2+}" group.

asynchronous release occurs from the same synaptic vesicle pool [59] and unique characteristic of exocytotic sites, expression of Ca^{2+} sensor proteins or specific regulation of exocytosis determine the balance between the synchronous and asynchronous release [60–62]. Our previous [18,63] and present data suggest that ROS can be an important switch for these types of exocytotic events. Here we revealed that Cd^{2+}-induced ROS production markedly desynchronized the neurotransmitter release in distal part of the nerve terminal, which is a more vulnerable to lipid peroxidation. Different antioxidants and NADPH-oxidase inhibitor effectively blocked the desynchronizing action of Cd^{2+}. Augmentation of Cd^{2+}-mediated ROS production by Zn^{2+} caused a profound increase in asynchronous release (desynchronization) in either distal and proximal compartments of the NMJs. Accordingly, intoxication with Cd^{2+} causing oxidation stress can desynchronize neurotransmitter release thereby disturbing the normal neuronal network activity and synaptic efficiency.

The mechanism of Cd^{2+}/ROS dependent desynchronization could rely on TRPV1 channels. Relatively dense expression of TRPV1 channels was found in NMJs as well as in somato- and viscerosensory afferents, solitary tract nucleus in the brain stem and caudal hypothalamus [17,51]. Probably, TRPV1 channels can control release of a discrete vesicle population and are sensitive to numerous stimuli, including ROS [17,47,51–53]. Indeed, ROS via activation of TRPV1 channels can trigger sensory axon degeneration [52] as well as enhancement of spontaneous exocytosis in central and peripheral nerve terminals [17,53]. Activity of TRPV1 channels contribute significantly to asynchronous glutamate release in the solitary tract afferents and pharmacological inhibition of TRPV1 suppressed by 50% of the the asynchronous release [64]. Consistent with this, TRPV1 knockout led to lacked asynchronous release in part of these afferents [65]. In the present study, the Cd^{2+}-mediated desynchronization of neurotransmitter release was prevented by TRPV1 channel antagonist. Probably, that Cd^{2+} provokes ROS overproduction which, in turn, can facilitate TRPV1 channel activation causing a shift of neurotransmitter release mode to asynchronous.

Cd^{2+} is redox inter metal and unable to directly generate ROS via Fenton reaction [66]. However, Cd^{2+} can induce oxidative stress in numerous cell types by inhibiting mitochondrial electron transport chain [10]. The increased ROS production can subsequently inactivate protein phosphatases (2A and 5) and AMP-activated protein kinase as well as disturb the mitochondrial integrity, leading to caspase-3 activation [6,67,68]. Ultimately, these events contribute to neuronal apoptosis. Additionally, it has been found that acute application of Cd^{2+} can quickly increase ROS levels in a NADPH oxidase-dependent manner in hepatocytes, HepG2 and endothelial cells [69–71]. Furthermore, Cd^{2+} can increase vasoconstrictor activity via increasing ROS production by NADPH oxidase [72]. In SH-SY5Y cells, Cd^{2+} induced the generation of ROS by upregulating the expression of NADPH oxidase 2 and its regulatory proteins [73]. Accordingly, Cd^{2+} can induce ROS production *via* both mitochondria and NADPH-oxidases dependent manners.

In our experiments we tried to identify the source of ROS that is most sensitive to Cd^{2+}. Surprisingly, inhibitor of NADPH oxidase, extracellular antioxidant enzyme catalase as well as mitochondrial-specific antioxidant prevented the effect of Cd^{2+} on timing of neurotransmitter release. It is difficult to identify what is the primary and secondary source, because of positive feedbacks between ROS production from different sources, particularly mitochondria and NADPH oxidases [74,75]. In many cell types, including neurons, Cd^{2+} toxicity is linked with induction of ROS overproduction by mitochondria [6,7,9,10,49,50]. We revealed that Cd^{2+} increased mitochondrial ROS levels in the nerve terminal which was associated with lipid peroxidation of the synaptic membranes. We suggest that the mitochondria are the primary presynaptic targets for Cd^{2+} and an elevation of mitochondrial ROS can lead to desynchronization of neurotransmitter release and even affect a probability of neurotransmitter release [20]. These changes in presynaptic function may be early events in manifestation of mitochondrial dysfunction as well as Cd^{2+} poisoning in nervous system.

Other important results of the present study are related with interaction of Cd^{2+} and Zn^{2+}. Initially, we supposed that Zn^{2+} can block the effects of Cd^{2+} on ROS production and timing of neurotransmitter release, because Zn^{2+} can compete with Cd^{2+} for using transport system responsible to the cation translocation into the cytoplasm [9]. Zn^{2+} acting in a Zn-transporter 3 dependent manner can protect hippocampal cells in culture from Cd^{2+} -induced neurotoxicity [5]. Surprisingly, Zn^{2+} itself had similar to Cd^{2+} effects and augmented Cd^{2+}-induced desynchronization of the neurotransmitter release as well as mitochondrial ROS production. Zn^{2+} has dual action and can function as an antioxidant and prooxidant [76]. Both deficiency and excess of Zn^{2+} provoke cellular oxidative stress [76,77]. Observed synergetic role of Cd^{2+} and Zn^{2+} may reflect that Zn^{2+} acting as a prooxidant, can

inhibit the mitochondrial electron transport chain, thus giving rise to ROS production [41,56,76,78]. Zn^{2+}-induced mitochondrial ROS production may contribute to neuronal cell death at the early stage after ischemic brain injury [79]. Additionally, Zn^{2+} can promote ROS production by inducing NADPH oxidases via a protein kinase C-dependent manner [80]. Interestingly, elevations of both Cd^{2+} and Zn^{2+} can aggravate neurodegenerative diseases, particularly Alzheimer's disease [8,9,81], which are associated with desynchronization of neurotransmitter release as well as oxidative stress [21,27,55]. The fact that Cd^{2+} and Zn^{2+} might act synergistically raises the possibility that presynaptic terminals containing the highest levels of Zn^{2+}, especially in the synaptic vesicles [82,83], could be the most vulnerable to Cd^{2+} neurotoxicity. Note that content of Zn^{2+} transporters was reduced in AP3-deficient neurons [84], suggesting that population of synaptic vesicles formed by endocytosis in AP-3 dependent manner (via endosomes) preferentially contains high levels of Zn^{2+} and its transporters [85]. In the absence of neuronal AP-3, asynchronous release was profoundly attenuated in hippocampal neurons [26]. Accordingly, synaptic vesicle pool responsible for the asynchronous neurotransmitter release could be a primary target for consequences of overload with Zn^{2+} and (or) Cd^{2+}.

We revealed that Cd^{2+} can desynchronize the neurotransmitter release only in distal compartment of the nerve terminal; at the same time Cd^{2+}, like other VGCC blockers decreased the quantum content in both distal and proximal parts (in the same degree). Although the action of Cd^{2+} on timing of neurotransmitter release was dependent on ROS, the enhancement of ROS production in response to Cd^{2+} was expressed in both distal and proximal regions of the NMJ. Only if Cd^{2+}-induced ROS upregulation was enhanced by Zn^{2+}, Cd^{2+} increased a dispersion of exocytotic events in the distal and proximal compartments. These results suggest that distal part is more sensitive to Cd^{2+}/ROS-mediated desynchronization. One possible reason is a decreased antioxidant capacity of the distal nerve terminal compartment. This is consistent with the observation that Cd^{2+} caused a more marked sign of lipid peroxidation in this region.

5. Conclusions

Collectively, the present work discovered that Cd^{2+} at low concentrations desynchronizes neurotransmitter release and increases mitochondrial ROS levels at the frog NMJ. The mechanism of Cd^{2+}-induced disruption of the neurotransmitter release timing is likely linked with an increase in ROS production and involved the TRPV1 channels. Antioxidants and inhibitor of NADPH oxidase as well as TRPV1 blocker prevented Cd^{2+}-induced desynchronization of release, whereas Zn^{2+} augmented both the release desynchronization and mitochondrial ROS production in response to Cd^{2+}. These changes in the neurotransmitter release and synaptic ROS levels can underlie Cd^{2+} neurotoxicity and ability of Cd^{2+} to aggravate neurodegenerative diseases. The latter are frequently associated with desynchronization of neurotransmitter release and oxidative damage of synaptic contacts. Further studies are required to establish the effects of Cd^{2+} on neurotransmitter release timing and redox homeostasis in central synapses.

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Declaration of competing interest

We declare no competing interests.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.freeradbiomed.2020.05.017.

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