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Blocking mitochondrial Zn^{2+} accumulation after ischemia reduces mitochondrial dysfunction and neuronal injury.

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Abstract: Zn^{2+} is an important contributor to ischemic brain injury and recent studies support the hypothesis that mitochondria are key sites of its injurious effects. In murine hippocampal slices (both sexes) subjected to oxygen glucose deprivation (OGD), we found that Zn^{2+} accumulation and its entry into mitochondria precedes and contributes to the induction of acute neuronal death. In addition, if the ischemic episode is short (and sublethal), there is ongoing Zn^{2+} accumulation in CA1 mitochondria **after** OGD that may contribute to their delayed dysfunction. Using this slice model of sublethal OGD, we have now examined Zn^{2+} contributions to the progression of changes evoked by OGD and occurring over 4-5 hours. We detected progressive mitochondrial depolarization occurring from ~ 2 hours after ischemia, a large increase in spontaneous synaptic activity between 2-3 hours, and mitochondrial swelling and fragmentation at 4 hours. Blockade of the primary route for Zn^{2+} entry, the mitochondrial Ca^{2+} uniporter (MCU; with ruthenium red, RR) or Zn^{2+} chelation shortly after OGD withdrawal substantially attenuated the mitochondrial depolarization and the changes in synaptic activity. RR also largely reversed the mitochondrial swelling. Finally, using an *in vivo* rat (male) asphyxial cardiac arrest (CA) model of transient global ischemia, we found that ~8 min asphyxia induces considerable injury of CA1 neurons 4 hours later that is associated with strong Zn^{2+} accumulation within many damaged mitochondria. These effects were substantially attenuated by infusion of RR upon reperfusion. Our findings highlight mitochondrial Zn^{2+} accumulation after ischemia as a possible target for neuroprotective therapy.

Significance Statement: Brain ischemia is a leading cause of mortality and long-term disability that still lacks effective treatment. After transient ischemia delayed death of neurons occurs in vulnerable brain regions. There is a critical need to understand mechanisms of this delayed neurodegeneration which can be targeted for neuroprotection. We found progressive and long-lasting mitochondrial Zn^{2+} accumulation to occur in highly vulnerable CA1 neurons after ischemia. Here we demonstrate that this Zn^{2+} accumulation contributes strongly to deleterious events occurring after ischemia including mitochondrial dysfunction, swelling and structural changes. We suggest that this mitochondrial Zn^{2+} entry may constitute a promising target for development of therapeutic interventions to be delivered after termination of an episode of transient global ischemia.

Introduction

Brain ischemia is a leading cause of morbidity and mortality of the aging population for which there are no effective neuroprotective interventions (Hosseini et al., 2020). Hippocampal pyramidal neurons are highly vulnerable to injury. CA3 neurons selectively degenerate after limbic seizures (Ben-Ari et al., 1980a; Ben-Ari et al., 1980b; Tanaka et al., 1988). In contrast, CA1 neurons were found to undergo distinctive delayed degeneration days after transient episodes of ischemia in both humans (Zola-Morgan et al., 1986; Petito et al., 1987) and rodents (Kirino, 1982; Ordy et al., 1993; Sugawara et al., 1999). Considerable evidence has accumulated supporting critical contributions of Zn^{2+} in this injury. While there are large amounts of Zn^{2+} in the brain (>100 μM), the vast majority of it is normally bound or sequestered such that free reactive Zn^{2+} is very low (subnanomolar). However, after ischemia considerable free Zn^{2+} was found to accumulate in vulnerable neuronal populations *in vivo* (Tonder et al., 1990). Further studies established that Zn^{2+} chelation is protective, implicating a Zn^{2+} contribution to the ischemic neurodegeneration (Koh et al., 1996; Calderone et al., 2004). Considerable evidence supports mitochondria as critical targets for the injury promoting effects of Zn^{2+} (Shuttleworth and Weiss, 2011; Ji et al., 2019). Zn^{2+} can quickly enter the mitochondria via the MCU where it was shown to trigger multiple deleterious effects including mitochondrial depolarization, swelling, ROS generation and irreversible inhibition of major mitochondrial enzymes (Saris and Niva, 1994; Sensi et al., 1999; Jiang et al., 2001; Sensi et al., 2003; Gazaryan et al., 2007; Ji and Weiss, 2018; Ji et al., 2020). Indeed, *in vivo* studies found considerable Zn^{2+} accumulation in hippocampal neuronal mitochondria after global brain ischemia which correlates with mitochondrial structural damage (including swelling), activation of large multiconductance channels and release of proapoptotic peptides (Calderone et al., 2004; Bonanni et al., 2006; Yin et al., 2019).

To find effective treatments that can prevent delayed neuronal injury after ischemia, it is crucial to understand the sequence of events leading to irreversible changes. Acute hippocampal slices subjected to OGD provide a valuable model to study such events: they have preserved neuronal networks and Zn^{2+} stores and thereby reproduce some critical aspects of *in vivo* ischemia, while allowing continuous monitoring from single neurons. Our recent studies found that short episodes of OGD (~7-9 min, a few min shorter than needed to evoke acute cell death) trigger delayed and long lasting (at least 1

hour) Zn^{2+} accumulation in mitochondria of CA1, but not in CA3 neurons (Medvedeva et al., 2017). We considered whether this Zn^{2+} accumulation might be responsible for the high susceptibility of CA1 neurons to delayed ischemic degeneration. We further found that the Zn^{2+} accumulates through the MCU (Medvedeva et al., 2017), leading to the hypothesis that the MCU constitutes a valuable therapeutic target for diminishing mitochondrial dysfunction and neuronal injury after a transient ischemic event has occurred.

Current studies, employing the acute hippocampal slice model of ischemia, have further examined the progression of changes over several hours after a short episode (8 min) of OGD. We found that 4-5 hours after OGD, mitochondria of CA1 neurons are markedly depolarized and swollen. Spontaneous synaptic activity, assessed as the frequency of spontaneous postsynaptic currents (SPSCs), initially decreases after OGD, but later quickly and markedly increases (~5 fold), before decreasing again to levels below control. Next we demonstrated that preventing the mitochondrial Zn^{2+} accumulation by MCU inhibition (with RR**)** or by Zn^{2+} chelation after OGD withdrawal substantially attenuated changes in mitochondrial potential ($\Delta\Psi_m$) and synaptic function, and RR largely reversed mitochondrial swelling.

Finally, in test of principle studies using an *in vivo* rat asphyxial CA/resuscitation model, intravenous infusion of RR immediately after resuscitation substantially decreased both the mitochondrial Zn^{2+} accumulation and the mitochondrial and neuronal damage, supporting the possible therapeutic utility of post ischemic treatments targeting mitochondrial Zn^{2+} accumulation through the MCU.

Materials and Methods

Animals.

Animal care and experimental procedures were performed according to a protocol approved by University of California Irvine Animal Care and Use Committee. Efforts were made for minimizing animal number and suffering. Both male and female mice from 129S6/SvEvTac strain (Taconic Biosciences; ~ 4 weeks old) were utilized for *in vitro* experiments, and male Wistar rats (Charles

River Laboratories, Wilmington, MA) weighing 300–350 g (8–12 weeks) were used for *in vivo* CA studies.

Preparation of acute hippocampal slices.

Coronal hippocampal slices were prepared from ~4 weeks old mice as previously described (Medvedeva et al., 2009). Mice were deeply anesthetized with isoflurane and decapitated. The brains were quickly removed and placed in ice-cold solution containing (in mM): 220 sucrose, 3 KCl, 1.25 NaH_2PO_4, 6 $MgSO_4$, 26 $NaHCO_3$, 0.2 $CaCl_2$, 10 Glucose and 0.42 ketamine (pH 7.35, 310 mOsm, equilibrated with 95% O_2 / 5% CO_2). Ketamine was added to preparation solution to decrease NMDA-mediated Ca^{2+} influx caused by cutting trauma. Hippocampal slices (300 μm) were cut using a Leica VT1200 vibratome (Leica, Germany), with vertical vibration of cutting blade adjusted to be <1 μm to decrease injury. After preparation slices were incubated in artificial cerebro-spinal fluid (ACSF) containing (in mM): 126 NaCl, 3 KCl, 1.25 NaH_2PO_4,1 $MgSO_4$, 26 $NaHCO_3$, 2 $CaCl_2$, 10 Glucose (pH 7.35, 310 mOsm, equilibrated with 95% O_2 / 5% CO_2 for 1 hour at 34±0.5ºC, and then kept at room temperature (20-23ºC) in oxygenated ACSF. All experiments were performed at 32±0.5ºC.

OGD in slices and drug administration.

To induce hypoxia-hypoglycemia ACSF was changed to identical solution without glucose and equilibrated with 95% N_2 / 5% CO_2. For sublethal (short) ischemic exposures, OGD was terminated (by restoration of oxygenated ACSF containing glucose) after 8 min (Fig. 1A). The duration of OGD was determined by our previous experiments with the aim to withdraw OGD before Ca^{2+} deregulation occurs in the majority of CA1 neurons, while allowing sufficient time to evoke mitochondrial depolarization (loss of $\Delta\Psi_m$), Zn^{2+} accumulation and delayed (4 hours later) mitochondrial dysfunction (Medvedeva et al., 2009; Medvedeva et al., 2017) (Fig. 3). Since our experiments required use of slices for prolonged periods (up to 7 hours), all measurements in OGD subjected slices are compared with results obtained from control slices at approximately the same time after slice preparation.

RR (10 μM) and N,N,N',N'-tetrakis(2-pyridylmethyl)ethane-1,2-diamine (TPEN, 20 μM) were applied 3 min and immediately after the OGD termination respectively.

Evaluating neuronal visual appearance and fluorescent measurements.

To assess changes in cellular morphology slices were placed under the bright field microscope equipped with differential interference contrast (DIC) optics (Olympus, Japan) and photographed. All further evaluations were carried out blindly (using coded images), by an investigator not involved in performing experiments. CA1 neuronal diameters, determined as the maximum width of the cell perpendicular to the apical dendrite, were measured using ImageJ software. Only neurons where we can clearly distinguish the cellular membrane were evaluated.

To study changes in mitochondrial function we evaluated $\Delta\Psi_m$. Slices were bath loaded with the $\Delta\Psi_m$ sensitive indicator, Rhodamine 123 (Rhod123, 26 μM, 30 min, 22-24 ºC). Rhod123 is positively charged and accumulates in negatively charged mitochondria where its fluorescence is quenched. Upon loss of $\Delta\Psi_m$ the indicator is released into the cytoplasm causing increase in fluorescence (Duchen et al., 2003). Rhod123 was excited at 540(25) nm and emitted fluorescence was collected at 605(55) nm. Images were acquired every 15 s. Data are presented as $\Delta F/F_0=(F-F_0)/F_0$ where F is a current fluorescence intensity and F_0 is baseline fluorescence intensity. Carbonyl cyanide-4-(trifluoromethoxy)phenylhydrazone (FCCP, 2 μM) was applied at indicated time points to evoke full loss of $\Delta\Psi_m$ and consequent increase in Rhod123 fluorescence, and, thus, to evaluate the level of $\Delta\Psi_m$ before FCCP application.

Electrophysiological recordings.

Slices were placed in a flow-through chamber (RC-27L chamber with plastic slice anchor, Warner instruments, Hamden, CT), mounted on the stage of an upright microscope (BX51WI Olympus, Japan) and perfused with oxygenated ACSF at 2 ml/min. To evaluate function of synaptic networks we recorded SPSCs from CA1 pyramidal neurons which were patched and held in voltage clamp configuration at -60 mV using the Axopatch 200A amplifier. For evaluation of the resting membrane potential (MP) and action potential (AP) threshold we used current-clamp configuration. Patch pipettes used for voltage clamp (5 MΩ resistance when filled with pipette solution) and current clamp (8-10 MΩ) were pulled from borosilicate glass with filament (WPI) on a Sutter Instruments P-97 micropipette puller. The pipette solution for voltage- and current-clamp recordings contained, in mM: 125 KGluconate, 10 KCl, 3 Mg-ATP, 1 $MgCl_2$, 10 HEPES, pH 7.25 with KOH, 290 mOsm. SPSCs

were recorded from neurons in sham slices (not treated with OGD) and at different time points between 1 and 5 hours after OGD withdrawal. APs were recorded in sham slices and at ~2.5 hours after OGD.

Antibody labeling and confocal microscopy.

Hippocampal slices (300 μm) were subjected to OGD for 8 min, "perfused" in ACSF at 32ºC for 30 min, then incubated at RT for another 3.5 hours and fixed with 4% paraformaldehyde (PFA). RR (10 μM) was added to some slices 3 min after OGD termination for 15 min. Thin (30 μm) sections were cut using a microtome cryostat (ThermoFisher Scientific, Waltham, MA) and stained with primary antibodies against the mitochondrial outer membrane protein TOM20 (1:200, Santa Cruz Biotechnology, Santa Cruz, CA) and secondary anti-rabbit fluorescent antibodies (1:200, DyLight™ 488, Jackson ImmunoResearch, West Grove, PA). To visualize mitochondria the sections were imaged using an inverted stage Olympus FV3000 confocal laser scanning microscope (Olympus, Japan) with high speed resonance scanner, IX3-ZDC2 Z-drift compensator and Olympus 40x silicone oil objective (UPLSAPO40XS, NA = 1.25, WD = 0.3 mm). A 488 nm diode laser was used for excitation and a high-sensitivity cooled GaAsP PMT was used for detection of signal.

An analysis of mitochondria size and shape was performed using ImageJ software as previously described (Medvedeva et al., 2017). Images were adjusted to provide optimal discrimination of mitochondria edges from background. We selected the cells that showed clearly evident mitochondria in the perinuclear regions in a sharp focus, and only the mitochondria that are aligned with their long axes parallel to the nuclear membrane were chosen. Measures of long axes, parallel to, and short axes, perpendicular to nuclear membranes were obtained on all clearly demarcated mitochondria adjacent to and surrounding the nuclear circumference. Average parameters were determined for mitochondria within each independent slice experiment, and presented are average values from 4-5 slices for each condition.

CA/cardiopulmonary resuscitation (CPR) and RR administration.

This study utilizes an asphyxial CA+CPR model similar to previously described (Crouzet et al., 2016; Kang et al., 2017; Crouzet et al., 2020). Rats were calorically restricted to 25% of normal food intake 14 hours prior to CA (Azadian et al., 2020). Shortly before experiment rats were intubated

using a 14-gauge endotracheal tube (B. Braun Melsungen AG, Melsungen, Germany), connected to a TOPO mechanical ventilator (Kent Scientific, Torrington, CT) and isoflurane vaporizer for delivery of 2% isoflurane and 50% O_2 / 50% N_2 gas mixture during surgical preparation. Femoral artery and vein cannulation allowed monitoring of blood pressure and heart rate, and administration of intravenous medication. Invasive arterial blood pressure was measured continuously using a transducer (CWE Inc., Ardmore, PA).

After surgical preparation, CA experiments began with reducing the isoflurane level to 1-1.5% to prepare for anesthesia wash out. The inhaled gas was switched to 100% O_2 for 2 minutes, after which the isoflurane was stopped to wash out anesthesia for 3 minutes. When the isoflurane was stopped, the inlet was also disconnected from oxygen to allow room air to be mechanically delivered to the rat. Additionally, neuromuscular blockade was also initiated at this time with injection of 1 mL of intravenous Vecuronium (2 mg/kg) with 1 mL of heparinized saline. After these 3 minutes of isoflurane washout, asphyxial CA was induced by turning the ventilator off and clamping the ventilator tubing. CA time was defined as systolic blood pressure less than 30 mm Hg and pulse pressure of 10 mm Hg or less. Baseline arterial blood gas (ABG) measurements (Abaxis, Union City, CA) were obtained within 30 min prior to initiation of asphyxia. Duration of asphyxia was 8 min. In the last min of asphyxia, as the ventilator is being reconnected and turned on, epinephrine (0.01 mg/kg) and bicarbonate (140 mg/kg) are given ahead of CPR to stimulate the sympathetic nervous system and manage acidosis, respectively. CPR, (manual sternal compressions at 180-240 per min) was performed after 8 minutes of asphyxia and continued until return of spontaneous circulation (ROSC). Our study used 3 groups of animals: 1) sham treatment group (control, this group was subjected to all experimental procedure w/o asphyxia); 2) rats subjected to CA w/o treatment; and 3) rats subjected to CA with MCU blocker RR administered intravenously at the time of CPR in order to reach the brain with ROSC (Fig. 1B), as we have demonstrated with other drugs administered during the CPR phase (Kang et al., 2017). After obtaining post-CA ABG measurements 10 minutes after ROSC, vessels were decannulated and rats were extubated within the following hour.

Histochemistry and electron microscopy.

Cardiac perfusion and preparation for Timm's labeling. To detect neuronal injury and reactive Zn^{2+} accumulation at the intracellular sites we used a modification of the Timm's sulphide silver method in combination with histochemistry (Danscher and Zimmer, 1978; De Biasi and Bendotti, 1998). Four hours after CPR, animals were anesthetized with isoflurane, and perfused transcardially with 200 ml of phosphate buffered saline (PBS) for 2 min, followed by 500 ml of Millonig's buffer (containing 0.002% $CaCl_2$, 1.6% NaH_2PO_4 and 0.4% NaOH; pH 7.3), also containing 0.2% Na_2S, 4% PFA and 1% glutaraldehyde over the next 20 min to precipitate Zn^{2+}. After perfusion the brains were postfixed using 2% PFA / 2.5% glutaraldehyde / 0.2% Na_2S at 4°C overnight, and 50 µm and 80 µm slices were cut using vibratome.

Timm's labeling. To analyze Zn^{2+} accumulation in mitochondria we performed Timm`s labeling which inserts metallic silver precipitates at sites of labile (loosely bound or reactive) Zn^{2+} accumulation. For Timm's staining 80 µm slices were incubated in the dark in a solution containing 1 part of 1M $AgNO_3$ solution, 20 parts solution containing 2% hydroquinone and 5% citric acid in water, and 100 parts of solution containing 30% gum arabic in water. Development was performed in the dark, was monitored by periodic evaluations under low light, and was terminated by washing in water. Stained slices were placed into PBS and processed for electron microscopy.

Electron microscopy. For Transmission Electron Microscopy (TEM) analysis, we utilized ultrathin sections (~1 µm thickness) prepared largely as previously described (Park et al., 2016) with small modifications. The sections were rinsed in PBS and post-fixed with 1% osmium tetroxide in PBS for 1 hour, then dehydrated in increasing serial dilutions of ethanol (70, 85, 95 and 100%), placed into intermediate solvent propylene oxide (2 times for 10 min), and incubated in 1:1 mixture of propylene oxide/Spurr's resin for 1 hour. Finally slices were embedded in Spurr's resin overnight. Ultrathin sections (~70 nm thickness) were cut using a Leica Ultracut UCT ultramicrotome (Leica, Vienna, Austria) mounted on 150 mesh copper grids, stained with lead citrate and viewed using a JEOL 1400 electron microscope (JEOL, Tokyo, Japan). Images were captured using a Gatan digital camera (Gatan, Pleasanton, CA, USA).

VAF / toluidine blue staining. To assess neuronal injury, after perfusion, tissue fixation and slice preparation as described, 30 µm sections were stained with vanadium acid fuchsin (VAF) or toluidine

blue (TB) largely as previously described (Victorov et al., 2000). Slices were stained with VAF for 1~2 min, washed with PBS, incubated in 0.01% borax solution for 20-30 second, and rinsed in distilled water. Finally, brain slices were cleared by acetate buffer (pH 3.3) for 30 sec and rinsed with distilled water. Another group of slices was stained with 0.025% toluidine blue for 20-30 sec. Stained slices were assessed and photographed using light microscopy. Cell counts of injured vs healthy neurons were carried out blindly by an investigator not involved in performing the experiments.

Reagents.

Rhodamine 123 was obtained from Invitrogen (Carlsbad, CA). N,N,N',N'-Tetrakis(2-pyridylmethyl)ethylenediamine, Ruthenium Red and ketamine were obtained from Sigma (St. Louis, MO). TOM20 antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz, CA) and DyLight™ 488 antibodies were obtained from Jackson ImmunoResearch (West Grove, PA). All other reagents were purchased from ThermoFisher Scientific (Waltham, MA).

Statistics and data analysis.

All comparisons reflect sets of data substantially interleaved in time. Evaluations of mitochondrial and neuronal health and morphology were performed blindly using coded images.

For comparisons between 2 groups, statistical differences were assessed using two sample t-tests. To evaluate differences between more than 2 sets of data with one changing factor (including controls at different time points after slice preparation or controls with/without treatment) we used One-Way ANOVA with Bonferroni test. To evaluate time-dependent changes in $\Delta\Psi_m$ and SPSCs frequency caused by OGD we used Two-Way ANOVA Bonferroni test with interactions where the first factor was time and the second one was OGD. For these tests we used control groups of slices at the same time points after slice preparation as slices from OGD-treated groups that were subjected to identical manipulations but without OGD. All tests described above were performed using Origin 9.1 (OriginLab, Northampton, MA). To evaluate the significance of protection provided by pharmacological interventions (RR or TPEN, assessed as difference between measurements in slices subjected to OGD and control slices, vs difference between OGD+treatment and control+treatment) we used ANOVA linear contrast (coded with R language). The R code used for calculating the contrasts is available upon request.

In vitro experiments. Since our preliminary experiments did not find any differences of investigated parameters between slices of female and male animals, all further analysis included slices from animals of both sexes. We performed 1-2 slice experiments per animal per condition. For electrophysiological recordings 1 neuron was examined per slice. All comparisons were made based on n≥6. For analysis of mitochondrial morphology measured parameters were averaged for each neuron, and only neurons with n≥3 clearly recognizable mitochondria were used, and data averaged per each slice experiment.

In vivo experiments. To analyze neuronal health (using TB or VAF staining) we evaluated 3-5 sections per animal with 50-100 cells examined per each section. To evaluate mitochondrial shape, structural changes and Zn^{2+} accumulation, > 120 mitochondria from ≥ 9 sections (≥ 3/animal) were evaluated per condition. All data obtained in *in vivo* experiments were averaged per each animal.

Results

Dynamic changes of CA1 neuronal visual appearance, mitochondrial potential and synaptic function after OGD.

Our recent observations of prolonged Zn^{2+} accumulation in mitochondria of CA1 neurons after ischemia (Medvedeva et al., 2017; Yin et al., 2019) led to the hypothesis that this Zn^{2+} accumulation could be a key trigger of delayed ischemic injury. Indeed, the delayed injury of CA1 neurons is characterized by mitochondrial swelling and release of cytochrome C (Nakatsuka et al., 1999; Sugawara et al., 1999), effects that are compatible with observations of Zn^{2+} triggered mPTP opening and cytochrome C release (Wudarczyk et al., 1999; Jiang et al., 2001; Calderone et al., 2004; Gazaryan et al., 2007). To test our hypothesis, we developed a hippocampal slice model of "sublethal" ischemia and reperfusion, which does not cause acute cell death, but evokes a sequence of events likely leading to delayed cell injury. Slices were subjected to short (8 min) episodes of OGD (or sham wash in oxygenated media for control) and were monitored over the subsequent 4-5 hours to assess delayed pathological changes. However, since these experiments have prolonged durations (~6 hours), we first sought to confirm neuronal viability for 6-7 hours after slice preparation. Several parameters were evaluated: neuronal visual appearance (using DIC optics), stability of $\Delta\Psi_m$, which reflects

mitochondrial function, and spontaneous synaptic activity, to assess function of synaptic networks. After examination of DIC images of CA1 regions from 2 groups of slices - those at 2-4 hours after preparation and at 6-7 hours - we found no difference in cell appearance and neuronal diameter between groups (Fig. 2A). Of note, ~10% of slices in both groups had mild neuronal swelling probably due to damage from preparation.

$\Delta\Psi_m$ was monitored using the fluorescent indicator, Rhod123. This positively charged indicator is sequestered by negatively charged mitochondria, where its fluorescence is quenched, and the accumulated amount is proportional to $\Delta\Psi_m$. Application of the mitochondrial uncoupler FCCP (2 μM, 5 min), which dissipates the proton gradient across the mitochondrial inner membrane, results in loss of $\Delta\Psi_m$ and consequent release of Rhod123 into the cytosol and a corresponding increase in fluorescence (ΔF). This ΔF is indicative of the $\Delta\Psi_m$ prior to FCCP exposure. We found that untreated slices reliably maintained their $\Delta\Psi_m$ for at least 6-7 hours (Fig. 2B).

To evaluate synaptic function SPSCs were recorded over time from individual CA1 neurons using patch-clamp technique in voltage clamp mode (at -60 mV). We found that SPSCs occured at a stable frequency for up to 7 hours after slice preparation (see Fig. 2C). Note, that in this study we monitored only the inward excitatory synaptic currents since the reversal potential for Cl^- was -61 mV and, thus, the inhibitory outward currents were indistinguishable from noise.

Short OGD caused extensive changes in all monitored parameters. While neurons appeared intact and visually not different from control on images obtained 1, 2 and 3 hours after OGD, this quickly changed from 3 to 4 hours, and by the 4 hour time point most CA1 neurons in all slices became severely swollen (Fig. 3A) with considerably increased diameters (Fig. 4B).

To evaluate dynamic changes in $\Delta\Psi_m$, FCCP (2 μM) was applied to Rhod123 loaded slices 1, 2 and 4-5 hours after OGD termination. Our past studies demonstrated that during OGD $\Delta\Psi_m$ declines rapidly beginning a few min after the start of the OGD episode, but recovers relatively quickly upon reperfusion (Medvedeva et al., 2009; Medvedeva et al., 2017). Here we found that, similar to previous observations, $\Delta\Psi_m$ initially recovered to pre-OGD level. However, 2 hours after OGD we detected a substantial drop of $\Delta\Psi_m$, which persists at 4-5 hours (see Fig. 3B), when experiments were terminated.

306 Since changes in both synaptic structure and function were documented previously in cortex and
307 hippocampus after *in vivo* and *in vitro* ischemia (Jourdain et al., 2002; Crepel et al., 2003; Ai and
308 Baker, 2006; Kovalenko et al., 2006; Radenovic et al., 2011; Neumann et al., 2013), we next sought to
309 determine the dynamic effect of OGD/reperfusion on function of synaptic circuitry in slice. The
310 frequency of SPSCs was evaluated in CA1 pyramidal neurons at multiple time points during 4 hours
311 after OGD. In contrast to the stable SPSC frequency noted in control slices, after short OGD it
312 initially dropped, but later increased to ~5 fold greater than basal rates around 2.5 hours after OGD
313 withdrawal. After peaking between 2 and 3 hours, the SPSC frequency declined again to substantially
314 below baseline level by 4 hours (Fig. 3C).

315 We further questioned whether the abrupt increase in synaptic activity after OGD is facilitated by
316 changes in intrinsic neuronal physiology of CA1 or CA3 neurons. To measure the physiologycal cell
317 properties we evaluated the resting MP and the threshold for AP generation in CA1 and CA3 neurons
318 in control slices and 2.5 hours after OGD. Single APs were elicited by injecting brief supra-threshold
319 depolarizing current pulses into patched neurons. We did not find evidence of differences in MP or
320 AP threshold 2.5 h after OGD compared to control in either CA1 or CA3 neurons. (CA1: MP was -
321 62±0.6 mV, n=9 in control and -61±1.3 mV after OGD, n=9, p=0.6; AP threshold was -54±1.4 mV,
322 n=9 in control and -54±1.7 mV, n=9 after OGD, p=1. CA3: MP in control cells was -62.2±1mV, n=9
323 and -62.4±1 mV after OGD, n=9, p=0.9; AP threshold was -51±1.3 mV in control neurons, n=9 and -
324 51±0.7 after OGD, n=9, p=1; all comparisons by t-test). As the increased SPSC frequency seen in
325 CA1 neurons after OGD did not appear to be explained by increased intrinsic excitability of either
326 CA3 or CA1 neurons, we suggest that the SPSC frequency increase may be best explained as a
327 manifestation of forms of LTP of the CA3-CA1 synapses as has been previously observed to occur
328 after transient brain ischemia (Jourdain et al., 2002; Crepel et al., 2003; Ai and Baker, 2006).

329

330 ***Mitochondrial Zn^{2+} uptake after sublethal OGD contributes to delayed changes in CA1 neurons.***

331 To investigate the contribution of prolonged mitochondrial Zn^{2+} accumulation after OGD to
332 observed morphological and functional alterations, we blocked the primary route for Zn^{2+} uptake into
333 mitochondria, the MCU (Malaiyandi et al., 2005; Medvedeva et al., 2017; Ji et al., 2020), with RR (10

μM applied ~3 min after OGD withdrawal for 15 min). Treatment with RR noticeably improved the appearance of CA1 pyramidal neurons 4 hours after OGD (Fig. 4A, compare to Fig. 3A) considerably diminishing their swelling (measured as changes in cell diameters; Fig 4B). RR treatment also markedly attenuated the loss of $\Delta\Psi_m$ 4-5 hours after OGD withdrawal (Fig. 4C, D).

Since the MCU is also the route for Ca^{2+} accumulation into mitochondria, to study whether the observed beneficial effects are caused specifically by Zn^{2+}, in another set of experiments we tested the membrane permeable specific Zn^{2+} chelator TPEN (20 μM, applied immediately after OGD for 20 min). Similar to findings with RR, treatment with TPEN markedly decreased the swelling of CA1 neurons (Fig.4B). Loss of $\Delta\Psi_m$ observed 4-5 hours after the ischemic episode was also considerably attenuated (Fig. 4C, D).

Next we questioned whether alterations in synaptic function observed after OGD are dependent upon mitochondrial Zn^{2+} accumulation. We found that treatments with RR or TPEN reversed OGD evoked changes in synaptic activity, both preventing the aberrant burst of SPSCs at 2-3 hours and reversing the synaptic quieting 4-5 hours after OGD (Fig. 5).

Mitochondrial swelling after OGD in CA1 pyramidal neurons is attenuated by MCU inhibition.

Our recent study demonstrated a strong association between mitochondrial Zn^{2+} accumulation, mitochondrial swelling, and mitochondrial structural changes after transient global ischemia *in a rat model of cardiac arrest* (Yin et al., 2019). Current studies utilized confocal microscopy to investigate effects of mitochondrial Zn^{2+} accumulation after OGD/reperfusion on mitochondrial morphology in acute slices. As above, slices were exposed to sham wash in oxygenated media (control) or to an 8 min episode of OGD and reperfusion, either alone, or with RR applied 3 min after the end of the OGD for 15 min. 4 hours after OGD, slices were fixed with PFA and stained with antibodies for the mitochondrial outer membrane protein, TOM20. Confocal (1000x) images were obtained from the CA1 pyramidal layer (Fig. 6A). Mitochondrial morphological parameters were analyzed as described previously (Medvedeva et al., 2017). We found that OGD caused a noticeable fragmentation and swelling of the mitochondria (see Fig. 6A) with substantial decreases in their lengths, increases in their widths, and considerably decreased length/width (L/W) ratios (control 3.2±0.20, OGD 1.5±0.06,

Fig. 6B). This mitochondrial swelling correlates with the persistent mitochondrial depolarization noted above. We found that application of RR substantially attenuated the observed mitochondrial swelling (Fig. 6A, B, L/W ratio 2.7±0.04).

MCU inhibition decreases CA1 neuronal injury after global ischemia in rats.

Finally, we tested whether inhibiting the MCU **after** an episode of global ischemia improves neuronal recovery *in vivo*. Rats were subjected to sham treatment, to asphyxial cardiac arrest (CA) followed by resuscitation (CPR), or to CA/CPR followed by intravenous injection of RR (2.5 mg/kg) (See Fig. 1). After 4 hours of recovery, rats were perfused, brains isolated and processed for histological analysis (see Methods section).

To evaluate overall injury of CA1 pyramidal neurons, brain sections were stained with toluidine blue (TB) to evaluate neuronal morphology or labelled using a modified vanadium acid fuchsin procedure which identifies acidophilic neurons (VAF staining). Sections were then blindly examined using light microscopy and each identifiable neuron was rated as healthy or injured. Healthy cells in TB stained slices (indicated with yellow arrows, Fig. 7A) displayed distinct non-stained round nuclei surrounded by TB-stained cytoplasm, while injured neurons (shown with red arrows) show TB staining in nuclei, cytoplasm and dendrites. Purple colored VAF labeling in the cytoplasm, which is nominal in healthy neurons, indicates neurons with early moderate injury (shown with red arrows, Fig. 7B). We found that in comparison to sham treatment, global ischemia caused distinct injury to CA1 pyramidal neurons, similar to that observed in our recent study (Yin et al., 2019). Treatment with RR substantially preserved neuronal morphology and considerably decreased injury (Fig. 7).

Cardiac arrest induced mitochondrial structural disruption and Zn^{2+} accumulation are attenuated by RR.

To investigate effects of global brain ischemia on mitochondria, further studies utilized transmission electron microscopy (TEM) techniques to examine mitochondrial ultrastructure. Since somata of CA1 neurons can be identified by their characteristic nuclei, to examine mitochondria in these neurons we obtained high-magnification TEM images adjacent to nuclei showing mitochondria

in perinuclear and proximal dendritic regions. A majority of mitochondria in slices from sham treated animals appeared intact and healthy. They were usually elongated with visible distinct cristae structure and an intact double membrane (Fig 8A, left). A minority of mitochondria (~30%) had mild injury, with rounder shape, suggesting moderate swelling, and some disruption of their cristae structure (this injury probably was caused by the brain perfusion procedure). After ischemia the number of injured mitochondria increased greatly, and similar to our previous observations (Yin et al., 2019), a large number (~75%) showed varying degrees of damage 4 hours after CA/CPR (Fig 8A middle, B). However, blocking the MCU with RR immediately after CPR substantially decreased the mitochondrial structural damage (Fig. 8A right, B).

Since our prior study (Yin et al., 2019) found that mitochondrial injury after ischemia strongly correlates with persistent excessive mitochondrial Zn^{2+} accumulation, we also analyzed Zn^{2+} buildup in mitochondria in the current study. As in our previous study, reactive Zn^{2+} was visualized by Timm's sulfide silver labeling. This stain appears to be quite specific for Zn^{2+}, and was found to be virtually absent in mice lacking the vesicular Zn^{2+} transporter, ZnT3 (Cole et al., 1999). Similar to our recent study, most (>90%) damaged mitochondria (Fig. 8C, left),and only a small subset of healthy appearing mitochondria (Fig. 8C, right) in all 3 conditions contained Zn^{2+} deposits (visible as electron dense spots). However even visually healthy appearing mitochondria from ischemic animals contained Zn^{2+} in a greater proportion (~30% vs ~9% in sham treated animals; Fig. 8C, right). Thus, treatment with RR after ischemia markedly diminished Zn^{2+} accumulation in all mitochondria (both intact and damaged) (ischemia 79.8±1.7%; sham 36.4±3%, $p<0.01$ vs ischemia; ischemia+RR 40.7±4.1%, $p<0.01$ vs ischemia One-Way ANOVA) and decreased mitochondrial injury (Fig. 8).

Discussion

As noted, we have found progressive and long-lasting Zn^{2+} accumulation in mitochondria of CA1 pyramidal neurons to occur after ischemia (Medvedeva et al., 2017; Yin et al., 2019). Present studies are the first to examine effects of this mitochondrial Zn^{2+} accumulation on mitochondrial function and synaptic activity in CA1 neurons in slice over several hours after an ischemic episode. We found that a short (sublethal) episode of OGD causes distinct changes in spontaneous synaptic activity and

evokes delayed and persistent loss of $\Delta\Psi_m$ in CA1 hippocampal neurons. These changes seems to be specifically catalyzed by Zn^{2+} entry into mitochondria via the MCU, since they are markedly attenuated by either MCU blockade or Zn^{2+} chelation after the ischemic episode. Furthermore, the short OGD episodes lead to considerable delayed swelling and fragmentation of mitochondria, which is also largely prevented by post-ischemic MCU inhibition. Finally, in early test of principle studies in an *in vivo* rat CA model of ischemia, we found that administration of an MCU inhibitor after ischemia appears to diminish mitochondrial Zn^{2+} accumulation, mitochondrial disruption, and delayed neuronal injury (Fig. 9).

Role of Zn^{2+} - mitochondria interactions in ischemic injury.

Multiple lines of evidence support a critical contribution of Zn^{2+} to ischemic injury. Specifically, large amounts of free Zn^{2+} were found to accumulate after ischemia in some vulnerable groups of neurons including hippocampal CA1 neurons (Tonder et al., 1990; Koh et al., 1996). Furthermore, Zn^{2+} chelators were protective in both *in vitro* and *in vivo* ischemic models (Koh et al., 1996; Yin et al., 2002; Calderone et al., 2004; Medvedeva et al., 2009; Medvedeva and Weiss, 2014).

While there is considerable Zn^{2+} in the brain, the majority of it is normally bound or sequestered, such that free Zn^{2+} is very low (subnanomolar). One important Zn^{2+} pool comprises Zn^{2+} sequestered in synaptic vesicles, from which it is released upon strong synaptic stimulation, and can enter neurons through certain Ca^{2+} permeable channels (Weiss et al., 2000; Shuttleworth and Weiss, 2011). Another important Zn^{2+} pool is that bound to cytosolic buffering proteins (in neurons largely comprising metallothionein-III; MT-III) from which it is released upon metabolic perturbations (oxidative stress and acidosis) as occur during ischemia/reperfusion (Maret and Vallee, 1998; Jiang et al., 2000; Maret, 2011). Zn^{2+} mobilization from this pool appears to contribute considerably to ischemic injury in CA1 neurons (Lee et al., 2003; Medvedeva et al., 2017; Ji et al., 2019). Our recent findings using short ischemia in hippocampal slice suggest that Zn^{2+}, which is progressively released from MT-III during ischemia/reperfusion, permeates the MCU, accumulating in CA1 mitochondria (Medvedeva et al., 2017).

445 It is apparent that mitochondria are key players in ischemic neuronal injury and were proposed as
446 important targets for treatment (Liu and Murphy, 2009; Sims and Muyderman, 2010). Changes in
447 brain mitochondrial structure after transient global ischemia have been detected early (during first few
448 hours) (Solenski et al., 2002; Bonanni et al., 2006; Yin et al., 2019) with more severe damage hours
449 and days later (Colbourne et al., 1999).

450 While the precise mechanisms through which Zn^{2+} promotes neurodegeneration are not defined,
451 multiple studies have highlighted mitochondria as a target for Zn^{2+}. Zn^{2+} is taken up into mitochondria
452 via the MCU (Saris and Niva, 1994; Malaiyandi et al., 2005; Clausen et al., 2013; Medvedeva and
453 Weiss, 2014; Ji et al., 2020), and affects their function with much greater potency than Ca^{2+}, causing
454 mitochondrial depolarization, ROS generation, and potent induction of swelling, probably due to
455 activation of the mitochondrial permeability transition pore (mPTP) (Sensi et al., 1999; Sensi et al.,
456 2000; Jiang et al., 2001; Ji and Weiss, 2018). Indeed, studies on isolated mitochondria have
457 demonstrated potent mPTP activation after Zn^{2+} entry through the MCU (Jiang et al., 2001; Gazaryan
458 et al., 2007; Ji et al., 2019). Although mechanisms of these effects are incompletely understood, Zn^{2+}
459 was found to induce irreversible inhibition of several mitochondrial enzymes of energy production
460 and antioxidant defense, likely contributing to both mitochondrial ROS production and mPTP
461 induction (Gazaryan et al., 2002; Gazaryan et al., 2007). The mPTP induction, in turn, triggers release
462 of apoptotic mediators including cytochrome C and apoptosis inducing factor (AIF) (Jiang et al.,
463 2001) that contribute to activation of downstream apoptotic injury pathways (Bernardi, 1999;
464 Crompton, 1999; Fatokun et al., 2014).

465 Notably, mitochondria seem to be critically involved in the delayed selective degeneration of CA1
466 pyramidal neurons after transient ischemia. These neurons show mitochondrial swelling with release
467 of cytochrome C into the cytoplasm beginning within hours of ischemia, followed by caspase-3
468 activation, and with neurodegeneration and associated prominent DNA fragmentation, occurring over
469 the next days (Antonawich, 1999; Nakatsuka et al., 1999; Ouyang et al., 1999; Sugawara et al., 1999).
470 In addition, treatment with the Zn^{2+} chelator, Ca-EDTA decreased cytochrome C release in CA1
471 neurons after ischemia (Calderone et al., 2004) supporting a Zn^{2+} contribution to the activation of this
472 apoptotic pathway. Our recent study using the slice OGD model of ischemia found that mitochondrial

Zn^{2+} uptake appears to contribute to ROS production during ischemia (Medvedeva and Weiss, 2014). Present findings that Zn^{2+} accumulation into CA1 mitochondria after short ischemia contributes to delayed loss of $\Delta\Psi_m$, mitochondrial swelling, and neuronal injury support the hypothesis that prolonged Zn^{2+} accumulation in CA1 neuronal mitochondria after ischemia represents a critical and targetable event contributing to their delayed degeneration.

Alterations in synaptic function after OGD.

Alterations in synaptic structure and function have been observed hours and days after an ischemic event. These include remodeling of synaptic networks and increases in amplitude of evoked postsynaptic potentials in the CA1 area, with postsynaptic LTP detected in CA3-CA1 synapses in hippocampal slices (Jourdain et al., 2002; Crepel et al., 2003; Ai and Baker, 2006; Neumann et al., 2013). Structural changes at both presynaptic and postsynaptic levels that evolve after ischemia include postsynaptic spine swelling, decreased synaptic spine density, depletion of pre-synaptic vesicle pools, and decreased numbers of mitochondria in CA1 presynaptic terminals (Kovalenko et al., 2006; Radenovic et al., 2011; Neumann et al., 2013).

These observations are compatible with our findings of alterations in synaptic activity, with increases in frequency of excitatory SPSCs recorded in CA1 neurons 2-3 hours after OGD followed by progressive quieting of synaptic function. We found that these alterations depend upon Zn^{2+} accumulation into mitochondria, since they were largely prevented by MCU inhibition or Zn^{2+} chelation. It is thus likely that mitochondrial damage and dysfunction catalyzed by Zn^{2+} accumulation contributes to observed changes in synaptic activity.

Zn^{2+} uptake through the MCU: a target for neuroprotective treatment.

Despite being a leading cause of death and disability, treatment of brain ischemia is largely limited to restoration of blood flow. Multiple past studies have proposed that massive neuronal Ca^{2+} loading via NMDA channels constitutes a promising target for neuroprotection. But glutamate triggered Ca^{2+} overload occurs primarily during acute ischemia. However, since ischemia cannot be predicted, the main opportunity for intervention is after ischemia has been terminated and blood flow

restored. If ischemia ends before acute cell death has occurred, neurons initially recover, but often die hours or days later. Targeting NMDA receptors after an episode of ischemia did not help in preventing this delayed cell death (Ikonomidou and Turski, 2002; Hoyte et al., 2004). Thus, other mechanisms, occurring after an acute ischemic episode, need to be targeted. Zn^{2+} accumulation in mitochondria seems to be such a mechanism.

While routes for Zn^{2+} translocation into mitochondria are not completely defined, the MCU appears to be a major route for substantial rapid mitochondrial Zn^{2+} uptake (Malaiyandi et al., 2005; Medvedeva and Weiss, 2014; Ji et al., 2020). Indeed, our recent study found that blocking the MCU prevents the long lasting mitochondrial Zn^{2+} uptake in CA1 neurons occurring after ischemia (Medvedeva et al., 2017). Present studies demonstrate that inhibiting the MCU after ischemia has occurred greatly diminishes deleterious changes evoked by ischemia/reperfusion in hippocampal slices, and decreases neuronal and mitochondrial injury after CA in animals. Furthermore, Zn^{2+} chelation (also administered after OGD in slice) had similar effects, supporting the contention that protection is due to preventing Zn^{2+} accumulation in mitochondria. Thus, the occurrence of long-lasting postischemic mitochondrial Zn^{2+} accumulation in vulnerable CA1 hippocampal pyramidal neurons, but not in more resistant CA3 neurons, together with observation of beneficial effects of MCU inhibition or Zn^{2+} chelation after ischemia provides support to the idea that mitochondrial Zn^{2+} accumulation is a critical event occurring after ischemia which leads to delayed neuronal injury. Furthermore, this mechanism can be targeted for neuroprotection and thus may constitute a promising target for development of therapeutic interventions to be delivered after the ischemia has already occurred.

References

Ai J, Baker A (2006) Long-term potentiation of evoked presynaptic response at CA3-CA1 synapses by transient oxygen-glucose deprivation in rat brain slices. Experimental brain research 169:126-129.

Antonawich FJ (1999) Translocation of cytochrome c following transient global ischemia in the gerbil. Neurosci Lett 274:123-126.

Azadian M, Tian G, Bazrafkan A, Maki N, Rafi M, Chetty N, Desai M, Otarola I, Aguirre F, Zaher SM, Khan A, Suri Y, Wang M, Lopour BA, Steward O, Akbari Y (2020) Overnight Caloric Restriction Prior to Cardiac Arrest and Resuscitation Leads to Improved Survival and Neurological Outcome in a Rodent Model. Frontiers in neuroscience 14:609670.

Ben-Ari Y, Tremblay E, Ottersen OP (1980a) Injections of kainic acid into the amygdaloid complex of the rat: an electrographic, clinical and histological study in relation to the pathology of epilepsy. Neuroscience 5:515-528.

Ben-Ari Y, Tremblay E, Ottersen OP, Meldrum BS (1980b) The role of epileptic activity in hippocampal and "remote" cerebral lesions induced by kainic acid. Brain Res 191:79-97.

Bernardi P (1999) Mitochondrial transport of cations: channels, exchangers, and permeability transition. Physiol Rev 79:1127-1155.

Bonanni L, Chachar M, Jover-Mengual T, Li H, Jones A, Yokota H, Ofengeim D, Flannery RJ, Miyawaki T, Cho CH, Polster BM, Pypaert M, Hardwick JM, Sensi SL, Zukin RS, Jonas EA (2006) Zinc-dependent multi-conductance channel activity in mitochondria isolated from ischemic brain. J Neurosci 26:6851-6862.

Calderone A, Jover T, Mashiko T, Noh KM, Tanaka H, Bennett MV, Zukin RS (2004) Late calcium EDTA rescues hippocampal CA1 neurons from global ischemia-induced death. J Neurosci 24:9903-9913.

Clausen A, McClanahan T, Ji SG, Weiss JH (2013) Mechanisms of Rapid Reactive Oxygen Species Generation in response to Cytosolic Ca2+ or Zn2+ Loads in Cortical Neurons. Plos One 8:e83347.

Colbourne F, Sutherland GR, Auer RN (1999) Electron microscopic evidence against apoptosis as the mechanism of neuronal death in global ischemia. J Neurosci 19:4200-4210.

Cole TB, Wenzel HJ, Kafer KE, Schwartzkroin PA, Palmiter RD (1999) Elimination of zinc from synaptic vesicles in the intact mouse brain by disruption of the ZnT3 gene. Proc Natl Acad Sci U S A 96:1716-1721.

Crepel V, Epsztein J, Ben-Ari Y (2003) Ischemia induces short- and long-term remodeling of synaptic activity in the hippocampus. Journal of cellular and molecular medicine 7:401-407.

Crompton M (1999) The mitochondrial permeability transition pore and its role in cell death. The Biochemical journal 341 (Pt 2):233-249.

Crouzet C, Wilson RH, Lee D, Bazrafkan A, Tromberg BJ, Akbari Y, Choi B (2020) Dissociation of Cerebral Blood Flow and Femoral Artery Blood Pressure Pulsatility After Cardiac Arrest and Resuscitation in a Rodent Model: Implications for Neurological Recovery. Journal of the American Heart Association 9:e012691.

Crouzet C, Wilson RH, Bazrafkan A, Farahabadi MH, Lee D, Alcocer J, Tromberg BJ, Choi B, Akbari Y (2016) Cerebral blood flow is decoupled from blood pressure and linked to EEG bursting after resuscitation from cardiac arrest. Biomedical optics express 7:4660-4673.

Danscher G, Zimmer J (1978) An improved Timm sulphide silver method for light and electron microscopic localization of heavy metals in biological tissues. Histochemistry 55:27-40.

De Biasi S, Bendotti C (1998) A simplified procedure for the physical development of the sulphide silver method to reveal synaptic zinc in combination with immunocytochemistry at light and electron microscopy. Journal of neuroscience methods 79:87-96.

Duchen MR, Surin A, Jacobson J (2003) Imaging mitochondrial function in intact cells. Methods in enzymology 361:353-389.

Fatokun AA, Dawson VL, Dawson TM (2014) Parthanatos: mitochondrial-linked mechanisms and therapeutic opportunities. British journal of pharmacology 171:2000-2016.

582 Gazaryan IG, Krasinskaya IP, Kristal BS, Brown AM (2007) Zinc irreversibly damages major
583 enzymes of energy production and antioxidant defense prior to mitochondrial permeability
584 transition. J Biol Chem 282:24373-24380.
585 Gazaryan IG, Krasnikov BF, Ashby GA, Thorneley RN, Kristal BS, Brown AM (2002) Zinc is a
586 potent inhibitor of thiol oxidoreductase activity and stimulates reactive oxygen species
587 production by lipoamide dehydrogenase. J Biol Chem 277:10064-10072.
588 Hosseini M, Wilson RH, Crouzet C, Amirhekmat A, Wei KS, Akbari Y (2020) Resuscitating the
589 Globally Ischemic Brain: TTM and Beyond. Neurotherapeutics : the journal of the American
590 Society for Experimental NeuroTherapeutics 17:539-562.
591 Hoyte L, Barber PA, Buchan AM, Hill MD (2004) The rise and fall of NMDA antagonists for
592 ischemic stroke. Current molecular medicine 4:131-136.
593 Ikonomidou C, Turski L (2002) Why did NMDA receptor antagonists fail clinical trials for stroke and
594 traumatic brain injury? The Lancet Neurology 1:383-386.
595 Ji SG, Weiss JH (2018) Zn(2+)-induced disruption of neuronal mitochondrial function: Synergism
596 with Ca(2+), critical dependence upon cytosolic Zn(2+) buffering, and contributions to
597 neuronal injury. Exp Neurol 302:181-195.
598 Ji SG, Medvedeva YV, Weiss JH (2020) Zn(2+) entry through the mitochondrial calcium uniporter is
599 a critical contributor to mitochondrial dysfunction and neurodegeneration. Exp Neurol
600 325:113161.
601 Ji SG, Medvedeva YV, Wang HL, Yin HZ, Weiss JH (2019) Mitochondrial Zn(2+) Accumulation: A
602 Potential Trigger of Hippocampal Ischemic Injury. The Neuroscientist : a review journal
603 bringing neurobiology, neurology and psychiatry 25:126-138.
604 Jiang D, Sullivan PG, Sensi SL, Steward O, Weiss JH (2001) Zn(2+) induces permeability transition
605 pore opening and release of pro-apoptotic peptides from neuronal mitochondria. J Biol Chem
606 276:47524-47529.
607 Jiang LJ, Vasak M, Vallee BL, Maret W (2000) Zinc transfer potentials of the alpha - and beta-
608 clusters of metallothionein are affected by domain interactions in the whole molecule. Proc
609 Natl Acad Sci U S A 97:2503-2508.
610 Jourdain P, Nikonenko I, Alberi S, Muller D (2002) Remodeling of hippocampal synaptic networks
611 by a brief anoxia-hypoglycemia. J Neurosci 22:3108-3116.
612 Kang YJ, Tian G, Bazrafkan A, Farahabadi MH, Azadian M, Abbasi H, Shamaoun BE, Steward O,
613 Akbari Y (2017) Recovery from Coma Post-Cardiac Arrest Is Dependent on the Orexin
614 Pathway. Journal of neurotrauma 34:2823-2832.
615 Kirino T (1982) Delayed neuronal death in the gerbil hippocampus following ischemia. Brain
616 research 239:57-69.
617 Koh JY, Suh SW, Gwag BJ, He YY, Hsu CY, Choi DW (1996) The role of zinc in selective neuronal
618 death after transient global cerebral ischemia. Science 272:1013-1016.
619 Kovalenko T, Osadchenko I, Nikonenko A, Lushnikova I, Voronin K, Nikonenko I, Muller D, Skibo
620 G (2006) Ischemia-induced modifications in hippocampal CA1 stratum radiatum excitatory
621 synapses. Hippocampus 16:814-825.
622 Lee JY, Kim JH, Palmiter RD, Koh JY (2003) Zinc released from metallothionein-iii may contribute
623 to hippocampal CA1 and thalamic neuronal death following acute brain injury. Exp Neurol
624 184:337-347.
625 Liu RR, Murphy TH (2009) Reversible cyclosporin A-sensitive mitochondrial depolarization occurs
626 within minutes of stroke onset in mouse somatosensory cortex in vivo: a two-photon imaging
627 study. J Biol Chem 284:36109-36117.
628 Malaiyandi LM, Vergun O, Dineley KE, Reynolds IJ (2005) Direct visualization of mitochondrial
629 zinc accumulation reveals uniporter-dependent and -independent transport mechanisms. J
630 Neurochem 93:1242-1250.
631 Maret W (2011) Redox biochemistry of mammalian metallothioneins. Journal of biological inorganic
632 chemistry : JBIC : a publication of the Society of Biological Inorganic Chemistry 16:1079-
633 1086.
634 Maret W, Vallee BL (1998) Thiolate ligands in metallothionein confer redox activity on zinc clusters.
635 Proc Natl Acad Sci U S A 95:3478-3482.

636 Medvedeva YV, Weiss JH (2014) Intramitochondrial Zn(2+) accumulation via the Ca(2+) uniporter
637 contributes to acute ischemic neurodegeneration. Neurobiology of disease 68:137-144.
638 Medvedeva YV, Lin B, Shuttleworth CW, Weiss JH (2009) Intracellular Zn2+ accumulation
639 contributes to synaptic failure, mitochondrial depolarization, and cell death in an acute slice
640 oxygen-glucose deprivation model of ischemia. J Neurosci 29:1105-1114.
641 Medvedeva YV, Ji SG, Yin HZ, Weiss JH (2017) Differential Vulnerability of CA1 versus CA3
642 Pyramidal Neurons After Ischemia: Possible Relationship to Sources of Zn2+ Accumulation
643 and Its Entry into and Prolonged Effects on Mitochondria. J Neurosci 37:726-737.
644 Nakatsuka H, Ohta S, Tanaka J, Toku K, Kumon Y, Maeda N, Sakanaka M, Sakaki S (1999) Release
645 of cytochrome c from mitochondria to cytosol in gerbil hippocampal CA1 neurons after
646 transient forebrain ischemia. Brain Res 849:216-219.
647 Neumann JT, Cohan CH, Dave KR, Wright CB, Perez-Pinzon MA (2013) Global cerebral ischemia:
648 synaptic and cognitive dysfunction. Current drug targets 14:20-35.
649 Ordy JM, Wengenack TM, Bialobok P, Coleman PD, Rodier P, Baggs RB, Dunlap WP, Kates B
650 (1993) Selective vulnerability and early progression of hippocampal CA1 pyramidal cell
651 degeneration and GFAP-positive astrocyte reactivity in the rat four-vessel occlusion model of
652 transient global ischemia. Experimental neurology 119:128-139.
653 Ouyang YB, Tan Y, Comb M, Liu CL, Martone ME, Siesjo BK, Hu BR (1999) Survival- and death-
654 promoting events after transient cerebral ischemia: phosphorylation of Akt, release of
655 cytochrome C and Activation of caspase-like proteases. Journal of cerebral blood flow and
656 metabolism : official journal of the International Society of Cerebral Blood Flow and
657 Metabolism 19:1126-1135.
658 Park J, Trinh VN, Sears-Kraxberger I, Li KW, Steward O, Luo ZD (2016) Synaptic ultrastructure
659 changes in trigeminocervical complex posttrigeminal nerve injury. J Comp Neurol 524:309-
660 322.
661 Petito CK, Feldmann E, Pulsinelli WA, Plum F (1987) Delayed hippocampal damage in humans
662 following cardiorespiratory arrest. Neurology 37:1281-1286.
663 Radenovic L, Korenic A, Maleeva G, Osadchenko I, Kovalenko T, Skibo G (2011) Comparative
664 ultrastructural analysis of mitochondria in the CA1 and CA3 hippocampal pyramidal cells
665 following global ischemia in Mongolian gerbils. Anatomical record 294:1057-1065.
666 Saris NE, Niva K (1994) Is Zn2+ transported by the mitochondrial calcium uniporter? FEBS Lett
667 356:195-198.
668 Sensi SL, Yin HZ, Weiss JH (2000) AMPA/kainate receptor-triggered Zn2+ entry into cortical
669 neurons induces mitochondrial Zn2+ uptake and persistent mitochondrial dysfunction. The
670 European journal of neuroscience 12:3813-3818.
671 Sensi SL, Yin HZ, Carriedo SG, Rao SS, Weiss JH (1999) Preferential Zn2+ influx through Ca2+-
672 permeable AMPA/kainate channels triggers prolonged mitochondrial superoxide production.
673 Proc Natl Acad Sci U S A 96:2414-2419.
674 Sensi SL, Ton-That D, Sullivan PG, Jonas EA, Gee KR, Kaczmarek LK, Weiss JH (2003) Modulation
675 of mitochondrial function by endogenous Zn2+ pools. Proceedings of the National Academy
676 of Sciences of the United States of America 100:6157-6162.
677 Shuttleworth CW, Weiss JH (2011) Zinc: new clues to diverse roles in brain ischemia. Trends
678 Pharmacol Sci 32:480-486.
679 Sims NR, Muyderman H (2010) Mitochondria, oxidative metabolism and cell death in stroke.
680 Biochimica et biophysica acta 1802:80-91.
681 Solenski NJ, diPierro CG, Trimmer PA, Kwan AL, Helm GA (2002) Ultrastructural changes of
682 neuronal mitochondria after transient and permanent cerebral ischemia. Stroke 33:816-824.
683 Sugawara T, Fujimura M, Morita-Fujimura Y, Kawase M, Chan PH (1999) Mitochondrial release of
684 cytochrome c corresponds to the selective vulnerability of hippocampal CA1 neurons in rats
685 after transient global cerebral ischemia. The Journal of neuroscience : the official journal of
686 the Society for Neuroscience 19:RC39.
687 Tanaka S, Kondo S, Tanaka T, Yonemasu Y (1988) Long-term observation of rats after unilateral
688 intra-amygdaloid injection of kainic acid. Brain research 463:163-167.

Tonder N, Johansen FF, Frederickson CJ, Zimmer J, Diemer NH (1990) Possible role of zinc in the selective degeneration of dentate hilar neurons after cerebral ischemia in the adult rat. Neurosci Lett 109:247-252.

Victorov IV, Prass K, Dirnagl U (2000) Improved selective, simple, and contrast staining of acidophilic neurons with vanadium acid fuchsin. Brain research Brain research protocols 5:135-139.

Weiss JH, Sensi SL, Koh JY (2000) Zn(2+): a novel ionic mediator of neural injury in brain disease. Trends Pharmacol Sci 21:395-401.

Wudarczyk J, Debska G, Lenartowicz E (1999) Zinc as an inducer of the membrane permeability transition in rat liver mitochondria. Archives of biochemistry and biophysics 363:1-8.

Yin HZ, Sensi SL, Ogoshi F, Weiss JH (2002) Blockade of Ca2+-permeable AMPA/kainate channels decreases oxygen-glucose deprivation-induced Zn2+ accumulation and neuronal loss in hippocampal pyramidal neurons. J Neurosci 22:1273-1279.

Yin HZ, Wang HL, Ji SG, Medvedeva YV, Tian G, Bazrafkan AK, Maki NZ, Akbari Y, Weiss JH (2019) Rapid Intramitochondrial Zn2+ Accumulation in CA1 Hippocampal Pyramidal Neurons After Transient Global Ischemia: A Possible Contributor to Mitochondrial Disruption and Cell Death. Journal of neuropathology and experimental neurology 78:655-664.

Zola-Morgan S, Squire LR, Amaral DG (1986) Human amnesia and the medial temporal region: enduring memory impairment following a bilateral lesion limited to field CA1 of the hippocampus. J Neurosci 6:2950-2967.

Figure legends

Figure 1. Experimental procedure. A - *OGD in brain slices.* Schematic show steps of experiment modeling ischemia/reperfusion in acute brain slices. **B -** *In vivo CA/CPR experimental procedure.* Schematic represents steps of an experiment modeling CA evoked transient global ischemia in rats. RR was administered to the CA+RR group at the time of CPR. All animals were cannulated, intubated and attached to ventilator before CA or sham procedure. Chest compressions were performed on sham animals to mimic CPR.

Figure 2. Visual neuronal appearance, mitochondrial potential and spontaneous synaptic activity does not change during 7 hours after slice preparation.

A *Left, middle* - Bright field images of CA1 region of representative slices at indicated time after slice preparation. *Right* - Bar graphs show average diameter of CA1 neuronal somata. Note that no differences were found between pyramidal neuron diameters at 2-4 h (28 slices evaluated, 19.1±0.6 μm) and at 6-7 hrs (7 slices, 19.2±0.5 μm, p=0.9, t-test). **B** - *Assessment of* $\Delta\Psi_m$. Brain slices were loaded with Rhod123, and FCCP (2 μM) was applied after 10-20 min of baseline (BL) recording. *Upper* - pseudocolor Rhod123 fluorescent images of slices before (BL) and after FCCP application. *Lower* - Traces represent Rhod123 $\Delta F/F_0$ changes. Arrows show the time points at which fluorescent images were taken. Bar graph *(right)* indicate average Rhod123 $\Delta F/F_0$ (±SE) after FCCP application 2-4 hours (81.1±12%, n=11) and 6-7 hours (78±4.5%, n=11, p=0.72, t-test) after slice preparation. **C** - *Spontaneous postsynaptic currents (SPSCs) recorded from CA1 neurons***.** *Left* – representative recordings of SPSCs 2.5 hours *(upper)* and 6 hours *(lower)* after slice preparation. *Right* – bar graph show average SPSCs frequency (±SE) (44.2±6.7 min^{-1}, n=10 in 2-4 hour group, and 39.7±3.6 min^{-1}, n=8 at 6-7 hour after slice preparation, p=0.59, t-test)

Figure 3. Short OGD causes delayed neuronal swelling, persistent mitochondrial dysfunction and changes in spontaneous synaptic activity in CA1 neurons.

A - *Bright field images of CA1 neurons in control slice, and in slices 1 and 4 hours after OGD termination*. Note similar appearance of neurons in control slice and 1 hour after OGD. After longer

period (4 hours) neurons were markedly swollen (average diameter of CA1 neurons was 32.4±1.0, n=8 slices 4 hours after OGD vs 19.2±0.5 μm, n=34 slices in control, p<0.001, t-test). **B -** $\Delta\Psi_m$ *changes after OGD in CA1*. Slices were subjected to sham treatment or to OGD and later loaded with Rhod123. FCCP (2 μM) was applied to sham treated slices (control) or at indicated times after OGD to evoke loss of $\Delta\Psi_m$. *Upper* - Fluorescent images were taken before (BL) and after FCCP application (arrows indicate time points at which fluorescent images were taken). Traces represent Rhod123 $\Delta F/F_0$ changes in the same slices (black - control, magenta - 1 hour and blue - 4 hours after OGD withdrawal). *Lower* - Bar graph show average Rhod123 $\Delta F/F_0$ changes (±SE) in control slices and after OGD at indicated times. (Control 79.5±6.3%, n=22 evaluated slices; 1 hour 81.2±7.2%, n=10; 2 hours 44.6±4.2%, n=13; and 4 hours after OGD 37.7±4.8%, n=9). Note the significant decrease in Rhod123 $\Delta F/F_0$ 2 and 4 h after OGD (p<0.01 compared to 1 h after OGD or corresponding control, Two-Way ANOVA Bonferroni test). * - p<0.01 compared to control. While we used 3 separate control groups for statistical analyses, we found no difference between the controls (p=0.93, One-Way ANOVA), thus the bar graph shows an average fluorescence value of all control slices. **C -** *SPSCs recordings from CA1 neurons. Upper* - Traces demonstrate SPSCs recorded from representative neurons in control slices and 2.5 hours after OGD withdrawal. *Lower* – bar graph show average frequency of SPSCs (±SE) in CA1 neurons in control (42.2±4.0, n=18, bar represents average data for all control slices, as there were no differences between controls at different time points, p=0.86 , One-Way ANOVA) and at indicated time points after OGD (24.2±3.4 min^{-1} after 2 hours, n=11; 214.3±62.6 min^{-1} at 2.5 hours, n=8; 97.6±28.2 min^{-1} at 3 hours, n=7; and 23.3±6.8 min^{-1} at 4 hours, n=10). Note the abrupt increase in SPSCs frequency at 2.5 h after OGD (p<0.001, compared to other time points or control, Two-Way ANOVA Bonferroni test). * - p<0.001 vs control.

Figure 4. MCU inhibition or Zn^{2+} chelation <u>after</u> an OGD episode attenuates delayed neuronal swelling and mitochondrial depolarization.

A - *Representative bright field images of CA1 neurons 4 hours after the short OGD episode in slices treated with RR or TPEN.* **B** – *OGD induced neuronal swelling was attenuated by RR or TPEN.* Bar graph shows average diameter of CA1 neurons in control and 4 hours after OGD with or without

784 treatments (control 19.2±0.5 μm, n=34 slices; OGD 32.4±1.0, n=8 slices, p<0.001 vs control;
785 OGD+RR 23.7±1.1, n=17 slices, p<0.001 vs OGD alone or control; OGD+TPEN 24.6±1.2, n=19
786 slices, p<0.001 vs OGD alone or control, One-Way ANOVA Bonferroni test). * - p<0.001 compared
787 to control, # - p<0.001 vs OGD. As we didn`t find difference in cell diameter between control non-
788 treated and TPEN or RR treated slices (p=0.7, One-Way ANOVA), the control bar represents grouped
789 data. **C** - *OGD evoked changes in $\Delta\Psi_m$ were attenuated by RR or TPEN. Upper* - fluorescent images
790 of Rhod123 loaded slices recorded before (BL) and after FCCP application (2 μM) in control slices
791 and 4 hours after OGD episode**.** Left panel - slices, both control and subjected to OGD, were treated
792 with RR (10 μM) as described. Right panel shows slices treated with TPEN (20 μM). *Lower* - traces
793 below demonstrate FCCP evoked Rhod123 $\Delta F/F_0$ changes; black traces show changes in control
794 slices, blue traces – in slices subjected to OGD, 4 hours after OGD episode. Arrows indicate time
795 points at which fluorescent images were taken. **D** - Bar graphs show average Rhod123 $\Delta F/F_0$ increase
796 (±SE) after FCCP application (Upper, no treatment: control 79.5±6.3%, n=22, OGD 37.7±4.8%, n=9,
797 p<0.001. Middle, treated with RR: control+RR 74.3±8.1%, n=12; OGD+RR 61.8±4.8%, n=12, p=0.2.
798 Lower, TPEN: control+TPEN 74.2±6.3%, n=12; OGD+TPEN 65.8±6.7%, n=12, p=0.37, t-test), *
799 represents p<0.001 vs control. Both treatments provide considerably better preserved mitochondrial
800 potential compared to OGD alone (p<0.01 for both TPEN and RR. The significance of effect of
801 treatment on OGD-evoked loss of $\Delta\Psi_m$ was assessed with ANOVA linear contrast).
802
803 **Figure 5. MCU inhibition or Zn^{2+} chelation attenuates changes in synaptic activity after**
804 **sublethal OGD.**
805 **Left** - *Treatment with either RR or TPEN shortly after OGD withdrawal reversed increase in SPSCs*
806 *frequency observed 2.5 hours after OGD* (SPSCs frequency after OGD without treatment was
807 214.3±62.6 min^{-1}, n=8 vs 42.2±4.0, n=18 in control, p<0.001; after OGD+RR - 36.5±8.2 min^{-1}, n=7 vs
808 46.8±6.6, n=9 in control+RR, p=0.35; after OGD+TPEN - 43.9±17.1 min^{-1}, n=7 vs 49.5±11.6, n=9 in
809 control+TPEN, p=0.78, t-test). * - p<0.001 compared to control. **Right** - *Treatment with RR or TPEN*
810 *prevents silencing of SPSCs 4 hours after OGD* (SPSC frequency after OGD was: 17.3±3.6 min^{-1}, n=9
811 vs 42.2±4.0, n=18 in control, p<0.001; after OGD+RR - 51.6±18.3 min^{-1}, n=6 vs 46.8±6.6, n=9 in

control+RR, p=0.78; after OGD+TPEN - 58±7.5 min^{-1}, n=8 vs 49.5±11.6, n=9 in control+TPEN, p=0.56, t-test). * - p<0.001 compared to control. Bar graphs represent average frequency of SPSCs (±SE). Notably both treatments largely eliminated the OGD-evoked increase in SPSC frequency at 2.5 hours (p<0.01 for RR and p<0.01 for TPEN compared to OGD without treatment) and the decrease in SPSC frequency at 4 hours after OGD (p<0.05 for both RR and TPEN, significance of differences were evaluated using ANOVA linear contrast).

Figure 6. Mitochondrial swelling after OGD in CA1 pyramidal neurons is attenuated by MCU blockade. Brain slices were subjected to sham wash in oxygenated medium (control) or to 8 min OGD either alone or with RR (10 µM, applied 3 min after OGD withdrawal for 15 min). 4 hours after OGD slices were fixed (with 4% PFA), and stained with TOM-20 antibody.

A - *Appearance of mitochondria in CA1 neurons.* Representative confocal images show TOM-20 labeled mitochondria. Bar – 10 µm. Enlarged images *(lower panel)* of regions indicated on upper panel show representative mitochondria at high magnifications. Note that OGD caused fragmentation and a "rounding up" of mitochondria, with decrease in length and increase in width; and that this change was attenuated by delayed treatment with RR. **B -** *Quantitative measurements. Left* - Bar graphs show average mitochondrial measurements (length and width; obtained using ImageJ software) of independently treated hippocampal slices after the indicated treatment. Each bar comprises mean from n ≥ 4 slices for each condition (>100 mitochondria were measured per condition from 34 neurons in control, 48 neurons after OGD and 51 neuron in slices treated with OGD followed by RR). Length (in µm): control 2.1±0.1; OGD 1.4±0.01; OGD+RR 1.9±0.01. Width (in µm): control 0.7±0.02; OGD 1.0±0.03; OGD+RR 0.7±0.01. *Right* - Bar graphs represent mean Length/Width (**L/W**) ratios observed after each treatment (based on the same data; control 3.2±0.2; OGD 1.5±0.06; OGD+RR 2.7±0.04). * indicates p<0.01 vs OGD alone, One-Way ANOVA.

Figure 7. MCU inhibition attenuates neuronal injury in CA1 pyramidal neurons induced by transient global ischemia. Rats were subjected to sham treatment, transient global ischemia induced by CA (8 min), or CA followed by intravenous treatment with RR (2.5 mg/kg). After 4 hours of

recovery, rats were euthanized, and brain tissue collected for histological examination. To assess injury, slices were stained with toluidine blue (TB) or subjected to a modified acid fuchsin labeling procedure (VAF). **A -** *Representative images of TB stained slices.* CA1 regions of hippocampal slices were photographed under the light microscope (Bar - 50 μm). While most neurons appear intact (shown with yellow arrow) in sham slices, note the substantial numbers of TB stained neurons after ischemia (red arrows). Further note that injury was largely attenuated in neurons from animals treated with RR. **B -** *Representative images of VAF stained slices*. Note that majority of neurons appear healthy (yellow arrows) in slices from sham treated animals, but after ischemia most neurons are injured (red arrows) and have VAF labelling in cytoplasm. Note that injury was significantly reversed in animals treated with RR. **C -** *Quantitative assessment: left - TB stain, right - VAF labeling.* All neurons were blindly analyzed and rated as intact or injured, and the percentages of CA1 neurons determined to be injured within each animal were calculated. Note that the number of injured cells was far greater in the CA1 zone of animals subjected to ischemia than after sham treatment, and that RR administration greatly diminished neuronal injury. Bars represent mean (±SE) from 3 independent animals (with ≥ 3 sections analyzed for each animal). Number of TB stained injured neurons: sham treatment 27.9±2.8%; ischemia 95.4±2.9%; ischemia+RR 39.7±3.9%. Number of VAF stained injured neurons: sham treatment 25.3±4.2%; ischemia 69.7±5.7%; ischemia+RR 28.7±6.2%. * indicates $p<0.01$ vs ischemia, One-Way ANOVA.

Figure 8. MCU inhibition decreases mitochondrial structural damage and Zn^{2+} accumulation evoked by ischemia/reperfusion. Rats were subjected to sham treatment, ischemia induced by cardiac arrest (CA) or to ischemia followed by intravenous injection of ruthenium red (RR, 2.5 mg/kg). Four hours later, rats were sacrificed, brains fixed with 4% PFA, and subjected to Timm's staining. **A -** *Appearance of mitochondria from rats subjected to indicated treatment.* Representative EM images show healthy mitochondria with normal morphology, intact intramitochondrial crista structures and membranes in sham-treated animals while mitochondria from CA rats are swollen, fragmented, have rounded morphology and disrupted membranes. Note clearly visible increased numbers of healthy mitochondria with improved morphology and membrane structure in rats treated

with RR after CA. Also note that most of the damaged mitochondria show Zn^{2+} (visualized by Timm`s stain as electron dense spots).

B, C - *Quantitative measurements.* **B:** Bar graphs show average numbers (as percent of total mitochondria ± SE) of injured mitochondria (sham 30.4±2.4%; ischemia 75.2±1.3%; ischemia+RR 31.7±0.8%). **C:** Bar graphs indicate percent of mitochondria that exhibited substantial amounts of zinc staining (*Left*, number of injured mitochondria containing Zn^{2+}, as percentage of injured mitochondria: sham 98.2±0.4%; ischemia 95.5±1.7%; ischemia+RR 90.4±6.3%). Right, number of healthy mitochondria with Zn^{2+}, as percentage of healthy mitochondria: sham treatment 9.5±4.6%; ischemia 32.3±12.3%; ischemia+RR 17.5±1%). Each bar comprises measurements from 3 animals per treatment condition. Bars represent mean (±SE) from 3 independent animals (with >100 mitochondria counted per each condition) * indicates $p<0.01$ vs ischemia, One-Way ANOVA.

Figure 9. Mitochondrial Zn^{2+} uptake through the MCU after ischemia is a target for neuroprotection. Sequence of events occurring during ischemia/reperfusion: **(1)** During early OGD Zn^{2+} enters into the cell and is released from MT-III. Zn^{2+} accumulates in mitochondria which start to depolarize; **(2)** Mitochondria abruptly depolarize releasing Zn^{2+} into cytosol; **(3)** with reperfusion mitochondria repolarize and reuptake the Zn^{2+}. *This stage is targeted to prevent the Zn^{2+} uptake and resulting injury.* **(4)** Zn^{2+} stays in CA1 (but not CA3) mitochondria for prolonged period of time. Blue trace reflects changes in cytoplasmic [Zn^{2+}] measured with Zn^{2+} sensitive indicator FluoZin-3. Representative images on the right show EM microphotograph of CA1 mitochondria and bright field images of TB labeled CA1 neurons 4 hours after global ischemia *(upper)* and 4 hours after ischemia+RR *(lower)* in rat.

A. Mouse hippocampal slice studies (*in vitro*)

B. Rat cardiac arrest studies (*in vivo*)