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**TITLE:** Rapid fragmentation of the endoplasmic reticulum in cortical neurons of the mouse brain in situ following cardiac arrest

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**ABSTRACT**

Neuronal endoplasmic reticulum (ER), continuous from soma to dendritic spines, undergoes rapid fragmentation in response to NMDA receptor stimulation in hippocampal slices and neuronal primary cultures. Here, we show that ER fragments in the mouse brain following cardiac arrest-induced brain ischemia. The ER structure was assessed *in vivo* in cortical pyramidal neurons in transgenic mice expressing ER-targeted GFP using 2-photon laser scanning microscopy with fluorescence recovery after photobleaching (FRAP). ER fragmentation occurred 1-2 min after cardiac arrest and once induced, fragmentation was rapid (<15 sec). We propose that acute ER fragmentation may be a protective response against severe ischemic stress.

**KEYWORDS**

Endoplasmic reticulum, ischemia, cardiac arrest, fragmentation, calcium, *in vivo*, 2-photon

## INTRODUCTION

The endoplasmic reticulum (ER) is involved in biosynthesis, protein quality control, cellular stress responses and Ca<sup>2+</sup> homeostasis. Neuronal ER is a key regulator of intracellular Ca<sup>2+</sup> signaling, acting as a sink and store of releasable Ca<sup>2+</sup>, thereby important for synaptic transmission and plasticity (Verkhratsky 2005). Three-dimensional reconstructions depict the neuronal ER as a complex, continuous network of interconnected cisternae and tubules extending from soma to apical dendrites (Terasaki *et al* 1994). The continuous ER lumen permits an efficient diffusion and equilibration of ions from the soma to the dendrites (Choi *et al* 2006; Dayel *et al* 1999) and ER is often referred to as neuron-within-a-neuron where ER Ca<sup>2+</sup> channels transmit a signal along its membrane through different neuronal compartments (Berridge 1998).

We have previously shown that extracellular Ca<sup>2+</sup> influx triggers a rapid and reversible loss of ER continuity (fragmentation) in hippocampal slices or cultured neurons when transiently stimulated by NMDAR agonists or high potassium concentrations (Kucharz *et al* 2009; Kucharz *et al* in press) It is well established that during stroke, brain trauma and epilepsy neurons are transiently or permanently overloaded with Ca<sup>2+</sup> that could be detrimental (Lau and Tymianski 2010). Therefore we asked whether ER dynamics could be monitored in the living brain, and if rapid ER fragmentation occurs in a condition of neuronal Ca<sup>2+</sup> overload.

We utilized *in vivo* 2-photon imaging techniques using transgenic mice expressing ER-targeted Enhanced Green Fluorescent Protein (EGFP) to assess ER dynamics in the brain of the anesthetized mouse. Using the global ischemia paradigm we demonstrate for the first time the ER structure in the living mouse brain, and validate the occurrence of rapid ER fragmentation *in situ*.

## MATERIALS AND METHODS

### **Generation of Thy1-EGFP-ER transgenic mice**

Transgenic C57BL/6 mice were generated to express ER lumen-targeted soluble EGFP (EGFP-ER hereafter called GreenER) under Thy1 promoter. The transgene construct was created using EGFP gene containing calreticulin ER-targeting sequence and KDEL ER-retention sequence (gift from Thomas Oertner; Friedrich Miescher Institute) cloned into pThy-1 vector (gift from Joshua R. Sanes Washington University). We assessed the fluorescence signal and the cellular expression patterns among three generated transgenic lines and chose the Thy1-EGFP-ER-7b line for further studies.

### **Surgery**

All animal procedures were approved by the Malmö/Lund ethical committee. The surgery was according to Anthony Holtmaat and colleagues (Holtmaat *et al* 2009). Thy1-EGFP-ER mice were anesthetized (3.5% isoflurane in N<sub>2</sub>O/O<sub>2</sub> (30:70)). During all steps of surgery and imaging, the temperature of animal was monitored by a rectal thermistor probe (Linton Instrumentation, UK) and maintained at 37°C. The head was mounted onto a stereotactic frame, the eyes lubricated with Viscotears (Novartis, Switzerland), and the animals were treated with Temgesic (0.1 mg/kg, Schering-Plough, Germany). Isoflurane was decreased to 1.8-2%, the scalp and periosteum removed, and Marcaine (2.5 mg/ml, bupivacain, AstraZeneca, UK) applied to the scar, then covered with tissue adhesive (Vetbond, 3M). Animals were treated with Rimadyl (5 mg/kg carprofen, Pfizer) and Dexafort (2 mg/kg, dexamethasone, Intervet, Netherlands). The craniotomy (4 mm diameter) was performed 2 mm posterior to bregma and 2 mm right lateral to midline. To prevent excessive heating, the skull was flushed every 5 minutes with cold saline. The bone flap was removed and the intact dura was covered with a wetted gelatin sponge (Spongostan®, Ferrosan, Denmark). Low melting agarose (Promega) was applied to the brain surface and a sterile 5 mm diameter

coverglass (Karl Hecht GmbH & Co KG “Assistent”, Germany) positioned on top of the cranial opening with dental cement. A custom-made titanium bar was attached in order to stabilize the head during the imaging procedures.

For induction of cardiac arrest (CA), a femoral vein catheter was inserted. Thereafter, the animal was mounted to the imaging stage insert by the titanium bar. The animal was spontaneously breathing 1.8-2% isoflurane in N<sub>2</sub>O/O<sub>2</sub> (30:70) gas mixture during imaging. CA was induced by i.v. injection of 0.5 ml 2 M KCl.

### ***In vivo* imaging**

The *in vivo* imaging was performed with a multiphoton Zeiss LSM 7MP upright laser scanning microscope using W-Plan Apochromat 20x/1.0 DIC Vis-IR water immersion objective (Carl Zeiss, Germany). EGFP was excited with Mai Tai DeepSee Ti:Sapphire pulse laser (Spectra-Physics, USA) tuned to 900 nm. For initial ER morphology analysis Non-Descanned Detector was used and the fluorescence was collected in range of 500–550 nm. For detailed and deep tissue analysis we utilized Gallium-Arsenide-Phosphide (GaAsP) detector. The 8-bit color depth Z-stack images were projected using ZEN 2010 (Carl Zeiss, Germany) imaging software with maximum intensity projection to assess ER continuity or semi-transparent intensity projection to assess the details of ER morphology.

### **FRAP analysis**

To assess the ER continuity, fluorescence recovery after photobleaching (FRAP) was used. From each mouse, 10 random dendrites with typical ER morphology were chosen. The GreenER signal was collected using GaAsP detector from a 2 μm x 2 μm selected region of interest (ROI) on a dendrite for 10 scanning cycles, bleached for 15 cycles with 900 nm Mai

Tai laser and the FRAP was recorded for 90 cycles. The parameters used for collecting FRAP were optimized for each animal used in the study. The data was normalized to the initial signal fluorescence intensity = 1.

## RESULTS

### **The morphology of ER *in vivo* prior to cardiac arrest**

The GreenER was expressed under control of Thy1 promoter in a subset of cortical neurons. The morphology of ER defined by GreenER signal did not differ from that observed in our previous studies in primary neuronal cultures and hippocampal slice cultures (Kucharz *et al* 2009). The ER appeared continuous with no abnormalities in morphology such as blebbing or vacuolization (Figure 1a). For our studies, we selected structures in dendrites with typical ER morphology expressing GreenER in molecular layer I or external granular layer II.

### **ER fission in the mouse brain**

The analysis of ER morphology showed that under basal conditions in the anesthetized mouse, the dendritic ER in cortical neurons is continuous. The continuity of ER was verified by Z-series of image scans (Figure 1b; Supplementary Video 1). To assess the ER structural changes during ischemia, an intravenous bolus injection of 0.5 ml of 2 M KCl via the femoral vein catheter was performed, which resulted in immediate CA. Within the first 1-2 after CA, the ER remained continuous. ER fragmentation occurred 1-2 minutes after injecting KCl, and loss of ER continuity was rapid, occurring within seconds (Figure 1c; Supplementary Video 2). The ER appeared as a string of small, disconnected beaded structures (Figure 1d; Supplementary Video 3). ER fission after CA was a global processes occurring in essentially all neurons expressing GreenER (Figure 1e).



**Assessing ER fragmentation by FRAP analysis**

To confirm the ER fragmentation and compartmentalization, we carried out FRAP analysis on dendritic ER of cortical neurons. Due to the secondary effects related to phototoxic damage occurring during high power multiphoton bleaching step and high frame rate of time-lapse recording of signal recovery, the parameters for collecting the FRAP signal (i.e. the time interval between scans, bleaching laser power and iterations) were adjusted separately for each mouse used in study.

The dendritic signal from GreenER prior to CA displayed high degree of FRAP, reaching above 67% of initial signal strength, confirming the continuous structure of ER (Figure 2). Subsequently, CA was induced and 2 minutes thereafter the continuity of ER lumen was assessed by FRAP from neurons in the same brain area, as prior to CA. In all analyzed animals the neurons exhibited loss of FRAP (Figure 2), confirming the reduction in ER lumen continuity. A similar loss of FRAP was seen in additional two investigated animals (data not shown).

**DISCUSSION**

Multiphoton *in vivo* imaging of neurons has become increasingly useful to study dynamic events in the ischemic brain such as spine morphology and Ca<sup>2+</sup> homeostasis (Misgeld and Kerschensteiner 2006). Here, for the first time, we demonstrate the *in vivo* imaging of the neuronal ER and its dynamic structural changes in pyramidal cortical neurons in mice using 2-photon laser scanning microscopy (2PLSM). The gross morphology of the dendrites in the living mouse was similar to that *in vitro*. Hence, under basal conditions, the neuronal ER lumen was continuous, displaying high degree of FRAP, as in cultured neurons and hippocampal slice cultures (Kucharz *et al* 2009, Kucharz *et al* in press).

The ER in primary cultures and organotypic slices undergoes rapid and reversible fragmentation upon NMDAR stimulation while inhibition of NMDAR or removal of extracellular  $\text{Ca}^{2+}$  abolishes ER fragmentation. (Kucharz *et al* 2009; Kucharz *et al* in press). Cardiac arrest, results in an immediate loss of cerebral blood flow, followed by depletion of tissue glucose, ATP, PCr, and subsequently depolarization of neuronal plasma membranes (Hansen 1985). The shortage of energy supply leads to inhibition of  $\text{Ca}^{2+}$  extrusion from neuronal cytosol and increase in intracellular  $\text{Ca}^{2+}$  levels (Silver and Erecinska 1992). Noteworthy, the depolarization of cell membranes and intracellular  $\text{Ca}^{2+}$  increase is rapid and occurs 1-2 minutes after cessation of blood flow to the brain (Hansen 1985; Xie *et al* 1995). In our study, using potassium-induced CA we validated the occurrence of ER fragmentation *in vivo* in ischemic conditions. During the first 1-2 minutes after CA the ER morphology appeared intact. Subsequently, ER fragmented rapidly (<15 s) which corresponded to the time at which  $\text{Ca}^{2+}$  enters brain cells (Silver and Erecinska 1992). This result strongly suggests that ER fragmentation during CA is regulated by the same mechanisms as *in vitro*, i.e.  $\text{Ca}^{2+}$  entry to the cell (Kucharz *et al* 2009; Kucharz *et al* in press, Subramanian and Meyer 1997). The rapid reduction in ER continuity in the brain was validated by loss of FRAP, and corresponded to the results from *in vitro* studies (Kucharz *et al* 2009; Kucharz *et al* in press). The ER fragmentation was not restricted to selected neurons, but was a global process, observed in all cortical neurons. Although we speculate, that the influx of  $\text{Ca}^{2+}$  into neurons during ischemic conditions is responsible for the rapid fragmentation of ER, the injected potassium levels in the blood could potentially enter the brain causing ER fragmentation. This is unlikely, since the potassium must then cross the blood-brain barrier and enter the brain during the few cardiac strokes expected after KCl injection. If this occurred, an instantaneous fragmentation would be expected. Instead, ER fragmentation occurs more than one minute after the injection and coincides with the well-described effect of anoxic depolarization

implicated in previous studies on ER structural rearrangements in the brain (Banno and Kohno 1996).

The cardiac arrest experiments are terminal, however, in the transient middle-cerebral artery occlusion (MCAO) experimental model of stroke, the infarct core with irreversibly injured tissue is surrounded by penumbra area with cells that have the potential to recover and exhibit trains of transient membrane depolarizations. A transient ER fragmentation in these conditions may contribute to neuroprotection by limiting the diffusion of Ca<sup>2+</sup> within ER, as proposed previously (Kucharz *et al* 2009; Kucharz *et al* in press). Whether the reversible ER fission occurs in penumbra and if it contributes to cell survival are of subject of future research.

#### **DISCLOSURE/CONFLICT OF INTEREST**

The authors declare no conflict of interests.

The supplementary videos are available at the JCBFM website.

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## FIGURE LEGENDS

**Figure 1. Rapid dendrite ER fragmentation in cortical neurons following the cardiac arrest.** (A) The projected Z-stack of dendrites of typical pyramidal neuron expressing GreenER in outer layer of the cortex in Thy1-EGFP-ER-7b transgenic mouse line. The arrow indicates a blood vessel. Scale bar = 100  $\mu\text{m}$ . (B) The projected Z-stack of image scans show that dendritic ER in *in vivo* in its basal state is continuous. Scale bar = 20  $\mu\text{m}$ . (C) Cessation of blood flow results in rapid reduction in ER continuity occurring over a period of 13 seconds starting one minute after cardiac arrest. Scale bar = 20  $\mu\text{m}$ . (D) Z-stack of image scans shows ER in its fragmented state, 3 minutes after cardiac arrest. Scale bar = 20  $\mu\text{m}$ . (E) The projected Z-stack of images of the fragmented state of dendritic ER in neurons of outer layer of cerebral cortex (dimensions of projected area: X=900  $\mu\text{m}$  x Y=450  $\mu\text{m}$  x Z=120  $\mu\text{m}$ ) following the cardiac arrest. Scale bar = 100  $\mu\text{m}$ .

**Figure 2. FRAP analysis of dendritic ER fragmentation in cortical neurons following the cardiac arrest.** Normalized average FRAP signal over time in dendrites of neurons in a mouse subjected to cardiac arrest. The graphs show FRAP signal recovery before (blue), and loss of FRAP (red) after the 2 minutes of cardiac arrest. Photobleaching was performed in time between the arrows. Time = 0 was set to when photobleaching ends and fluorescence starts to recover. The recovery of FRAP signal from dendritic ER before the cardiac arrest indicates ER continuity, while loss of FRAP demonstrates a fragmented structure of ER. Error bars are SEM, n = 12 neurons.

**Supplementary Video 1. 3D reconstruction of continuous ER *in vivo*.**

The 3D reconstruction of continuous ER structure in dendrite in pyramidal neurons of superficial cortex layer before the cardiac arrest.

**Supplementary Video 2. Timelapse recording of ER fragmentation *in vivo*.**

The timelapse recording shows a rapid ER fission occurring after the first minute of cardiac arrest. The timer starts at 60 seconds after cardiac arrest. The ER fragmented rapidly, and the first signs of ER fission can be observed at 18th second of recording while at 31st second ER displays no detectable signs of continuity. The images were collected at 0.22 Hz, the movie is encoded to 20 frames per second. Scale bar = 20  $\mu\text{m}$ .

**Supplementary Video 3. 3D reconstruction of fragmented ER *in vivo*.**

The 3D reconstruction of fragmented ER morphology in dendrite in pyramidal neurons of superficial cortex layer 2 minutes after the cardiac arrest.





