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In vitro aging promotes endoplasmic reticulum (ER)-mitochondria Ca^{2+} cross talk and loss of store-operated Ca^{2+} entry (SOCE) in rat hippocampal neurons

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ABSTRACT

Aging is associated to cognitive decline and susceptibility to neuron death, two processes related recently to subcellular Ca^{2+} homeostasis. Memory storage relies on mushroom spines stability that depends on store-operated Ca^{2+} entry (SOCE). In addition, Ca^{2+} transfer from endoplasmic reticulum (ER) to mitochondria sustains energy production but mitochondrial Ca^{2+} overload promotes apoptosis. We have addressed whether SOCE and ER-mitochondria Ca^{2+} transfer are influenced by culture time in long-term cultures of rat hippocampal neurons, a model of neuronal aging. We found that short-term cultured neurons show large SOCE, low Ca^{2+} store content and no functional coupling between ER and mitochondria. In contrast, in long-term cultures reflecting aging neurons, SOCE is essentially lost, Stim1 and Orai1 are downregulated, Ca^{2+} stores become overloaded, Ca^{2+} release is enhanced, expression of the mitochondrial Ca^{2+} uniporter (MCU) increases and most Ca^{2+} released from the ER is transferred to mitochondria. These results suggest that neuronal aging is associated to increased ER-mitochondrial cross talking and loss of SOCE. This subcellular Ca^{2+} remodeling might contribute to cognitive decline and susceptibility to neuron cell death in the elderly.

1. Introduction

Aging is associated to cognitive decline and increased risk of neuron cell death related to excitotoxicity and/or neurodegenerative diseases. These processes have been linked to the remodeling of intracellular Ca^{2+} homeostasis including changes in voltage-gated Ca^{2+} channels and glutamate receptors sensitive to *N*-Methyl-D-Aspartate (NMDA). Interestingly, some of these changes are mimicked *in vitro* after long-term culture of rat hippocampal neurons [1,2]. In fact, after several weeks in culture, rat hippocampal neurons display important hallmarks of neuronal aging *in vivo* including accumulation of reactive oxygen species (ROS), lipofuscin granules, heterochromatic foci, activation of the Jun N-terminal protein kinase (pJNK) and p53/p21 pathways, gradual loss of cholesterol, and, as stated above, changes in Ca^{2+} channel density and NMDA receptor expression [1–5]. Accordingly, long-term cultures of hippocampal neurons may provide a suitable model for investigating Ca^{2+} remodeling in aging hippocampal neurons.

In the last few years, it has been reported that memory storage in the hippocampus depends on store-operated Ca^{2+} entry (SOCE) which is required for mushroom spines stability [6]. SOCE is triggered by the release of Ca^{2+} from intracellular stores induced by phospholipase C activation after receptor stimulation [7]. At the molecular level, SOCE is induced by interaction of stromal interaction molecule 1 (Stim1) [8], a Ca^{2+} sensor at the endoplasmic reticulum (ER), and Orai1, a pore forming protein of store-operated channels (SOCs) at the plasma membrane [9,10]. SOCE has been studied in detail in non-excitable cells. However, it is also present in excitable cells including neurons [7,11]. It has been proposed that the Ca^{2+} sensor at ER that triggers SOCE in hippocampal neurons is Stim2 rather than Stim1 [12]. However, this view is controversial and recent results indicate that both Stim proteins are involved in Ca^{2+} homeostasis in neurons. STIM1 mainly activates SOCE, whereas Stim2 regulates resting Ca^{2+} levels in the ER and Ca^{2+} leakage with the additional involvement of Stim1 [13]. Interestingly, recent data suggest that Stim2 is downregulated in aging animals, animal models of AD and AD patients [6] suggesting an important role of Stim2 and SOCE in aging and AD.

Subcellular Ca^{2+} homeostasis is critical for neuron cell damage induced by different insults including excitotoxicity and neurodegeneration. For example, activation of NMDA receptors promotes mitochondrial Ca^{2+} overload and cell death in long-term cultured hippocampal neurons [4,5]. Likewise, oligomers of the amyloid β peptide (Aβ), the most likely neurotoxin in AD, promote Ca^{2+} entry, mitochondrial Ca^{2+} overload and neuron cell death in

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cerebellar granule cells [14]. Both Aβ and NMDA promote Ca^{2+} influx but they also mobilize Ca^{2+} from stores. Specifically, Aβ mobilizes ER Ca^{2+} *via* IP_3 dependent and independent mechanisms [15]. Aβ and NMDA receptor activation cause mitochondrial dysfunction involving ER Ca^{2+} release [16]. These findings suggest a relevant role for Ca^{2+} transfer from ER to mitochondria in neuron cell death. The role of Ca^{2+} transfer from ER to mitochondria in aging has not been addressed. In fact, mitochondria Ca^{2+} handling has remained elusive due to the constrains of monitoring accurately mitochondrial Ca^{2+} concentration ($[Ca^{2+}]_{mit}$) in individual neurons. However, bioluminescence imaging of targeted probes like aequorin has revealed the presence of subpopulations of mitochondria able to sense high Ca^{2+} domains (Ca^{2+} microdomains) near Ca^{2+} channels at the plasma membrane [17,18] and the ER [19,20]. More recent data indicates that Ca^{2+} transfer from ER to mitochondria depends on the formation of close contacts sites between ER and mitochondria [21] and specialized structures named mitochondria-associated membranes (MAMs) [22,23]. Ca^{2+} transfer from the ER to mitochondria is involved in cell survival as well. Absence of this transfer results in enhanced phosphorylation of pyruvate dehydrogenase and AMP kinase (AMPK) activation, promoting prosurvival macroautophagy. Thus, constitutive $InsP_3R$ dependent Ca^{2+} release to mitochondria is an essential cellular process required for efficient mitochondrial respiration and maintenance of bioenergetics [24]. Whether this transfer is affected by aging has not been addressed. Inasmuch as mitochondrial potential ($\Delta\Psi$), the main driving force for mitochondrial Ca^{2+} uptake, decreases with age [4,25], it is possible that aging may influence Ca^{2+} transfer from ER to mitochondria. Here we have used long-term cultures of rat hippocampal neurons to address whether SOCE and ER–mitochondria Ca^{2+} cross talk are influenced by *in vitro* aging.

2. Methods

2.1. Animals and reagents

Wistar rat pups were obtained from the Valladolid University animal facility. All animals were handled according to ethical standards approved by the animal housing facility of the Valladolid University Medical School (Valladolid, Spain) in agreement with the European Convention 123/Council of Europe and Directive 86/609/EEC. Fura2/AM, TMRM, coelenterazine and lipofectamine® 2000 are from Invitrogen (Barcelona, Spain). Fetal bovine serum (FBS) is from Lonza (Barcelona, Spain). Horse serum, neurobasal medium, HBSS medium, B27, L-glutamine and gentamicin are from Gibco (Barcelona, Spain). Papain solution is from Worthington (Lakewood, NJ). The poly-D-lysine and Annexin V are from BD (Madrid, Spain). DNase I and antibody against mitochondrial calcium uniporter (MCU) are from Sigma (Madrid, Spain). Antibodies against Stim1 and Orai1 are from Alomone (Jerusalem, Israel). Antibody against βIII tubulin is from Covance (Princeton, NJ, USA). Other reagents and chemicals were obtained either from Sigma or Merck. Plasmids for mitochondria-targeted aequorin fused to GFP are a kind gift from Prof. P. Brulet (CNRS, France).

2.2. Primary hippocampal neuron culture

Hippocampal neurons were prepared from Wistar rat pups under sterile conditions as reported by Brewer et al. [26] with further modifications by Perez-Otaño et al. [27]. Briefly, newborn rat pups were decapitated and, after brain removal, meninges were discarded and hippocampi were separated from cortex. Hippocampal tissue was cut in small pieces, transferred to papain solution (20 u/ml) and incubated at 37 °C for 30 min. After 15 min, DNase I (50 μg/ml) was added. Tissue pieces were washed with Neurobasal Medium and cell suspension was obtained using a fire-polished pipette in the same medium supplemented with 10% FBS. Cells were centrifuged at 160*g* for 5 min and pellet was suspended in Neurobasal medium. Hippocampal cells were plated onto poly-D-lysine-coated, 12 mm diameter glass coverslips at 30×10^3 cells/dish (plating density, 169 cells/mm^2), and grown in Neurobasal medium supplemented with L-glutamine (2 mM), gentamicin (1 μg/ml), 2% B27 and 10% FBS, and maintained in a humidified incubator at 37 °C with 5% CO_2 without further media exchange. Cells were cultured for 4–8 days *in vitro* (DIV) for resembling "young" neurons or 15–21 DIV for mimicking "aged" neurons before experiments. Other details have been reported in detail elsewhere [4,28].

2.3. Fluorescence imaging of cytosolic free Ca^{2+} concentration

Hippocampal cells were cultured for 4–8 DIV or 15–21 DIV and washed in standard external medium (SEM) containing (in mM) NaCl 145, KCl 5, $CaCl_2$ 1, $MgCl_2$ 1, glucose 10 and Hepes/NaOH 10 (pH 7.4). Cells were incubated in the SEM containing fura2/AM (4 μM) for 60 min at room temperature in the dark. Then coverslips were placed on the perfusion chamber of a Zeiss Axiovert 100 TV inverted microscope, perfused continuously with pre-warmed (37 °C) SEM and epi-illuminated alternately at 340 and 380 nm light using a filter wheel. Light emitted at 520 nm was filtered with the dichroic mirror and recorded every 5 s with a Hamamatsu ER camera (Hamamatsu Photonics France). Pixel by pixel ratios of consecutive frames were captured and $[Ca^{2+}]_{cyt}$ values from regions of interest (ROIs) corresponding to individual neurons were averaged and expressed as the ratio of fluorescence emission following excitation at 340 and 380 nm as reported in detail previously [4,14]. For calculations of cytosolic $[Ca^{2+}]$ the Grynkiewicz equation was used: $[Ca^{2+}] = Kd * (R - Rmin) / (Rmax - R) * F380max/F380min$ [29] where Kd is the dissociation constant of fura2 (224 nM), R is the observed fluorescence ratio at both wavelengths (F340/F380); Rmin is the minimum ratio value (in absence of Ca^{2+}); Rmax is the maximum ratio value (when Fura-2 is saturated by Ca^{2+}) and F380max/F380min is a scaling factor (fluorescence intensity at 380 nm excitation in the absence of Ca^{2+} and at Ca^{2+} saturation). We used the following values for our imaging setup: Rmax = 1.4; Rmin = 0.1, respectively. F380max/F380min = 2.7. For best comparison both ratios and calculated cytosolic $[Ca^{2+}]$ are shown.

For measurements of SOCE, fura2-loaded cells were treated with the sarcoplasmic and endoplasmic reticulum Ca^{2+} ATPase (SERCA) pump blocker thapsigargin (Tg, 1 μM) for 10 min in SEM devoid of extracellular Ca^{2+} before imaging. Then cells were subjected to fluorescence imaging and stimulated with 5 mM Ca^{2+} to monitor the SOCE-dependent rise in $[Ca^{2+}]_{cyt}$. SOCE recordings were made in the presence of tetrodotoxin (TTX) to prevent activation of voltage-gated Ca^{2+} channels by connected neurons. For estimation of Ca^{2+} store content, we measured the rise in $[Ca^{2+}]_{cyt}$ induced by low concentrations of the Ca^{2+} ionophore ionomycin (400 nM) added in the absence of extracellular Ca^{2+}. Ionomycin releases Ca^{2+} stored by making holes in endomembranes. Accordingly, the rise in $[Ca^{2+}]_{cyt}$ depends solely on the amount of stored Ca^{2+} and not on expression of G-protein coupled receptors and or IP_3 receptors. This procedure has been used extensively to estimate Ca^{2+} store content [11].

For quantification of rises in $[Ca^{2+}]_{cyt}$, the maximum rise in ratio was computed for responsive cells. In addition, we calculated the fraction of responsive cells. Finally, we calculated the product of the maximum rise in ratio by the fraction of responsive cells in order to have an estimation of the increase in $[Ca^{2+}]$ in response to a given agonist in the whole cell population. This procedure has been described in the same cells in detail previously [4].

2.4. Bioluminescence imaging of mitochondrial free Ca^{2+} concentration

Cultured neurons were transfected with the mitGA and mutated mitGA plasmids using lipofectamine® 2000. These plasmids contain wild type or mutated, low affinity aequorin targeted to mitochondria